Organización estructural de la diversidad genética y caracterización etofuncional en la Raza Asnal Andaluza

Structural organization of genetic diversity and ethofunctional characterization in the Andalusian donkey breed

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Doctorado Recursos Naturales y Gestión Sostenible

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Departamento de Genética

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Facultad de Veterinaria

Departamento de Genética

PROGRAMA DE DOCTORADO EN RECURSOS NATURALES Y GESTIÓN SOSTENIBLE

"Organización Estructural de la Diversidad Genética y Caracterización Etofuncional en la Raza Asnal Andaluza"

"Structural organization of genetic diversity and ethofunctional characterization in the Andalusian donkey breed"

MEMORIA PARA OPTAR AL GRADO DE DOCTOR PRESENTADA POR

Francisco Javier Navas González

DIRECTORES

Juan Vicente Delgado Bermejo

Jordi Jordana Vidal

En Córdoba, a 07 de junio de 2019

TÍTULO DE LA TESIS:

"Organización Estructural de la Diversidad Genética y Caracterización Etofuncional en la Raza Asnal Andaluza"

DOCTORANDO/A:

Francisco Javier Navas González

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

Ambos directores somos profesionales de una muy larga experiencia en la formación de doctorado, y tenemos que destacar aquí que el trabajo realizado por el doctorando, Francisco Javier Navas González, rebasa de forma extraordinaria todo lo que hemos vivido hasta el momento.

Lo que empezó como una tesis menor dedicada a una especie con un interés funcional actual muy limitado, con escasa implantación en el sector y muy poco interés científico general; debido a la abnegación y la pasión del candidato, fue tomando unas proporciones sorprendentes, colocándose como la tesis más espectacular que hemos dirigido en nuestra carrera.

Para documentar estas palabras basta hacer un breve recorrido cuantitativo. La tesis ha generado nueve artículos, seis de ellos ya publicados en revistas de primer cuartil (uno en primer decil), y tres sometidos a revista de igual categoría. Además, una colaboración externa de los contenidos de la tesis dio lugar a un artículo en segundo cuartil colateral.

Además, la tesis ha dado lugar a tres artículos en revistas internacionales no indexadas, pero de gran prestigio en el sector.

Además, el perfil funcional de la tesis doctoral ha dado lugar a una enciclopedia de tres tomos, en inglés y en castellano, compilada, editada y traducida por el propio candidato, estando ya en la calle el primero de ellos como Ebook financiado por el servicio de publicaciones de la Universidad de Córdoba (UCoPress); el segundo ya está en prensa y el tercero en edición. Esta enciclopedia se ha desarrollado con base en una Red Internacional (The Worldwide Donkey Breeds Project), creada, desarrollada y moderada por el doctorando, que sigue en plena actividad con representantes en todos los continentes.

Avances de estos resultados se han presentado en diversos eventos internacionales en forma de catorce comunicaciones; 10 de ellas como poster y 5 como orales, de las cuales, tres han sido ponencias invitadas en eventos de la Universidad de Davis (California, USA), con financiación de esa universidad de todos los gastos del candidato.

Así mismo el doctorando no ha olvidado la transferencia al sector de sus resultados, llevándolo a cabo con la publicación de un artículo de divulgación y el desarrollo de una entrevista de prensa. Promoviendo además un contrato de colaboración entre la UCO y la Asociación de criadores de la gran raza Asnal Andaluza (UGRA) y una red nacional de técnicos españoles trabajando con las razas asnales autóctonas de nuestro país.

Con todo lo expuesto, no nos queda más que reconocer nuestro orgullo por haber dirigido esta gran tesis doctoral y nuestro agradecimiento al candidato, antes de hacer constar nuestro acuerdo unánime sobre la madurez de la tesis y del candidato para proceder a su defensa.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 15 de Abril de 2019

Firma del/de los director/es

Fdo.Juan Vicente Delgado Bermejo

Fdo.: Jordi Jordana Vidal

Tesis Doctoral

Departamento de Genética

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MEMORIA PARA OPTAR AL GRADO DE DOCTOR POR LA UNIVERSIDAD DE CÓRDOBA

PRESENTADA POR

Francisco Javier Navas González

Vº Bº DIRECTORES

Juan Vicente Delgado Bermejo *Jordi Jordana Vidal*

Córdoba, a 5 junio de 2019

A mis abuelos

Agradecimientos

Cuando empecé pensaba que lo mío y el doctorado no era nada más que una extraña casualidad. Sin embargo, sabiendo lo que sé hoy, pienso que inevitablemente, aquella casualidad tenía que ocurrir.

Si bien echando la vista atrás, aún recuerdo como los últimos días del último curso de licenciatura, de camino al tren pensaba: "venga vamos…un día menos", confieso que todo lo que mi licenciatura, máster y doctorado han supuesto para mi vida no ha hecho otra cosa que proporcionarme felicidad.

Desde aquellos días hace hoy como unos nueve años, y es que no pilla a nadie por sorpresa que sea un chico de tomarme mi tiempo para mis cosas, así que supongo la tesis no podía ser menos. Esto podría verse como todo un logro si tenemos en cuenta mi conocida pero frecuentemente no reconocida impaciencia.

Hoy, son muchas las personas que me vienen a la mente y a las que querría expresar mi gratitud. Todos vosotros habéis sido imprescindibles para el desarrollo y conclusión de este proyecto que tanto bueno me ha traído y que sin vosotros no habría sido posible.

En primer lugar, me gustaría agradecerte a ti, Juanvi, mi director, padre científico y posiblemente el mayor caso de hipocondría no diagnosticada del mundo. Como poder expresar en unas cuantas palabras cuanto he aprendido y aprendo cada día. Siempre estas al pie del cañón. Eres un estímulo creativo constante, un ejemplo a seguir y una de las personas con las que más me gusta discutir (y no digo debatir, porque el monumento en forma de ladrillo del despacho describe suficientemente los méritos que he hecho para ganarme la atribución que por cabezón me he ganado). Tienes una forma de entender la vida y de enfocar la vocación veterinaria que contamina a cualquiera que se te pone cerca. La defensa y dedicación que prestas a los recursos genéticos animales como parte del trasfondo de nuestra cultura me hacen estar orgulloso no sólo de la persona con la que comparto despacho sino del gran amigo que te considero. Cuando sea mayor, yo quiero ser como tú.

Jordi, mi otro director, aún recuerdo lo intimidado que estaba el primer día que te envié un artículo para que me revisaras y es que quizás, seas una de las personas a las que más he admirado y admiro. Muchas gracias por estar ahí, por atender mis dudas y mis comentarios incluso cuando te he acribillado con trabajos y trabajos que hacían perder la cuenta. Además. gracias por transmitirme el amor hacia los burros a través de todo tu trabajo. Es precisamente tu visión la que en parte hace que yo trabaje con esta especie.

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Cecilio, entre las personas que he tenido el placer de conocer gracias a mi trabajo en el departamento y el desarrollo de esta tesis, tú seas quizás una de las personas que más admiración me despierta. Quizás seas la persona que tiene un conocimiento más amplio de nuestra profesión que conozco y es sorprendente como puedes ayudar a localizar hasta las más recónditas referencias bibliográficas en segundos. Muchas gracias por orientarme y aconsejarme bien siempre. Ha sido un honor para mí participar contigo en este y en los espero muchos proyectos que tenemos por delante.

Hay dos personas sin las que esta tesis no habría salido adelante y sois vosotros. Ander y Gabriela, este proyecto debería llevar un letrero que dijera que ningún vasco ni ninguna chilena fueron dañados durante la realización de esta tesis y es que no han sido pocas las veces que os he puesto en peligro; desde muerte por arrastre de burro hasta electrocución por pastor eléctrico. Nunca bajáis los brazos ni me habéis dicho que no cuando he necesitado vuestra ayuda. Me habéis inspirado para conseguir muchas metas que sin vuestra compañía dudo hubiera podido alcanzar. Gracias amigos por ser trabajadores incansables, piezas clave en mi vida y en los proyectos que hemos conseguido y que nos quedan por conseguir. No me dejéis nunca, me tenéis para lo que necesitéis.

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por alto en estos agradecimientos es la acogida y el cariño con la que me recibisteis en vuestra familia. Vosotros sois sin duda el espíritu de lo que Archivos de Zootecnia significa. Cuando abrí la puerta del despacho de Gustavo la primera vez, estaba dando de manera inconsciente entonces, quizás uno de los pasos más importantes de mi vida hasta ahora. Mi trabajo en la revista ha impulsado en gran medida el desarrollo de esta tesis y me ha dado acceso a unos conocimientos tan útiles que no podría pagar. No sólo me brindasteis la posibilidad de evolucionar, de aprender de la mejor de las formas sino también de convertirme en lo que soy hoy. Gracias por confiar en mí.

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amarillo si no a comprender que muchas veces, ser tan poco racionales como somos los dos puede ser una ventaja, sobre todo si va ligado a no camuflar como nos sentimos. Muchas gracias por ser mi chica de guardia y estar ahí para todo.

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Muchas gracias

Índice

Compendio de publicaciones

La Tesis Doctoral titulada "Organización Estructural de la Diversidad Genética y Caracterización Etofuncional en la Raza Asnal Andaluza", llevada a cabo por el doctorando Francisco Javier Navas González, DVMZ, relativa al proyecto de tesis registrado bajo el mismo nombre y aprobado por primera vez por la Comisión Académica del Programa de Doctorado en extinción en Recursos Naturales y Gestión Sostenible (RD 1393/2007, Plan 2007) en septiembre de y por segunda vez en el Programa de Doctorado en Recursos Naturales y Gestión Sostenible (RD99/2011, Plan 2011) en septiembre de 2018.

La presente Tesis ha sido realizada siguiendo la modalidad de "*Compendio de publicaciones*". Las referencias completas de los artículos y libros que constituyen el cuerpo de la Tesis Doctoral son las siguientes:

1. **Navas, F.J.**; Jordana, J.; León, J.M.; Barba, C.; Delgado, J.V. A model to infer the demographic structure evolution of endangered donkey populations. *animal* **2017**, *11*, 2129-2138.

2. **Navas González, F.J.**; Jordana Vidal, J.; León Jurado, J.M..; McLean, A.K.; Pizarro Inostroza, G.; Delgado Bermejo, J.V. Genetic parameter estimation and implementation of the genetic evaluation for gaits in a breeding program for assisted-therapy in donkeys. *Veterinary research communications* 2018, *42*, 101-110.

3. **Navas González, F.J.**; Jordana Vidal, J.; Camacho Vallejo, M.E.; León Jurado, J.M.; de la Haba Giraldo, M.R.; Barba Capote, C.; Delgado Bermejo, J.V. Risk factor meta-analysis and Bayesian estimation of genetic parameters and breeding values for hypersensibility to cutaneous habronematidosis in donkeys. *Veterinary Parasitology* **2018**, *252*, 9-16.

4. **Navas González, F.J.**; Jordana Vidal, J.; Pizarro Inostroza, G.; Arando Arbulu, A.; Delgado Bermejo, J.V. Can Donkey Behavior and Cognition Be Used to Trace Back, Explain, or Forecast Moon Cycle and Weather Events? *Animals* **2018**, *8*, 215.

5. **Navas, F.J.**; Jordana, J.; León, J.M.; Arando, A.; Pizarro, G.; McLean, A.K.; Delgado, J.V. Measuring and modeling for the assessment of the genetic background behind cognitive processes in donkeys. *Research in Veterinary Science* **2017**, *113*, 105-114.

6. **Navas González, F.J.**; Jordana Vidal, J.; León Jurado, J.M.; Arando Arbulu, A.; McLean, A.K.; Delgado Bermejo, J.V. Genetic parameter and breeding value estimation of donkeys' problem-focused coping styles. *Behavioural Processes* **2018**, *153*, 66-76.

7. **Navas González, F.J.**; Jordana Vidal, J.; León Jurado, J.M.; McLean, A.K.; Delgado Bermejo, J.V. Non-parametric analysis of the noncognitive determinants of response type and response intensity, mood and learning in donkeys. Submitted to *Journal of Veterinary Behavior: Clinical Applications and Research.*

8. **Navas González, F.J.**; Jordana Vidal, J.; León Jurado, J.M.; McLean, A.K.; Delgado Bermejo, J.V. Dumb or smart asses? Donkey's cognitive capabilities *(Equus asinus)* share the heritability and variation patterns of human's cognitive capabilities *(Homo sapiens)*. Submitted to *Journal of Veterinary Behavior: Clinical Applications and Research.*

9. **Navas González, F.J.**; Jordana Vidal, J.; McLean, A.K.; León Jurado, J.M.; Barba, C.J.; Arando, A.; Delgado Bermejo, J.V. Modeling for the Inheritance of Endangered Equid Multiple Births and Fertility: Determining Risk Factors and Genetic Parameters in Donkeys *(Equus asinus)*. Submitted to *Research in Veterinary Science.*

Summary

Tools allowing to understand the evolution of donkey populations in time, the future trends that these populations describe, and the factors conditioning such trends, become invaluably critical when aiming at preserving and later recovering such populations from their endangerment status. Basing on the characteristic lack of information regarding the genealogical background of donkey populations and taking a particular breed as an example, it is possible to infer a model to assess the genetic and demographical structure of other international endangered donkey populations. Then, we can plot selection strategies to implement once such populations have reached the sufficient number of individuals, and are supported by solid enough structures. Microsatellite-tested pedigree analyses were carried out to study the genetic diversity, structure and historical evolution of the Andalusian donkey breed since the 1980s. Despite mean inbreeding was low, highly inbred animals were present. The effective population size based on individual inbreeding rate was about half when based on individual coancestry rate. Nei's distances and equivalent subpopulations number indicated differentiated farms in a highly structured population. Although genetic diversity loss since the founder generations could be considered small, intraherd breeding policies and the excessive contribution of few ancestors to the gene pool could lead to narrower pedigree bottlenecks. Long average generation intervals could be considered when reducing inbreeding. Wright's fixation statistics indicated slight inbreeding between farms. Pedigree shallowness suggested applying new breeding strategies to reliably estimate descriptive parameters and control the negative effects of inbreeding, which could indeed, mean the key to preserve such valuable animal resources avoiding the extinction they potentially head towards. Diversity studies render especially important in donkeys as they reveal the genetic background in the populations and the starting point for making decisions on whether to apply conservation or breeding plans in this functionally misallocated species. Once genetic diversity parameters are balanced, finding new niches for donkeys becomes potentially the most relevant aim to approach in the midterm future for the species. Selection strategies in donkeys are approached from three different perspectives; donkey-assisted therapy and therapeutic riding, fertility and disease resistance, not only as a way to widen the functional spectrum of opportunities of donkeys but also to lengthen their useful lives, and improve their life quality and welfare. Studying the specific genetic background behind functional traits enables quantifying the degree in which such features pass from jacks and jennies onto the new foal generations. As a genetic term, environment means all influences other than inherited factors. Controlling the environmental factors conditioning the expression of certain functional features help to build animal models shedding light in the genetic fraction involved in such functional traits. The functional performance of 300 microsatellite-assisted parentage tested donkeys was studied using REML and Gibbs sampling Bayesian methods for the obtention of genetic parameters and breeding values using BLUP methodology. The first functional niche for which donkeys may be well-suited is linked to their special psychological nature and physical characteristics as facilitators of learning processes and for the development of key life skills and confidence building for a wide spectrum of vulnerable people. Therapeutic riding and asinotherapy take advantage of the physical and psychological interaction between donkeys and patients given the potential application of donkey's characteristics and abilities for the treatment of specific human disorders. The selection of donkeys when the breeding criteria is their suitability for equine-assisted therapies was implemented following two different approaches; the selection for coping styles and cognitive processes and the selection for gaits and kinetics. Aiming at developing suitable models seeking the consolidation of equine assisted-therapy breeding criteria, we studied 29 factors that may potentially influence several cognitive processes in donkeys. These factors not only affect donkeys' short-term behaviour but may also determine their long-term cognitive skills from birth. Thus, animal behaviour becomes a useful tool to obtain past, present or predict information from the situation of a certain animal in a particular area. Operant conditioning and Qualitative Behavioural Assessment (QBA) synergism can provide valuable information about animals' extinction/learning and emotional status. All noncognitive animal inherent features significantly affected four variables ($P<0.001$), although some were not linearly correlated. On the other hand, the effect power of meteorological factors ranged from 7.9% for the birth season on learning ($P<0.05$) to 38.8% for birth moon phase on mood ($P<0.001$). Psychometric testing enables quantifying animal cognitive capabilities and their genetic background. Among these cognitive capabilities, the study of problem-solving coping styles achieves a special relevance as it brings together the need genetically select donkeys displaying a neutral reaction during training, given its implication with handler/rider safety and trainability. Heritabilities for coping style traits were moderate, 0.18 to 0.21. Phenotypic correlations between intensity and mood/emotion or response type were –0.21 and –0.25, respectively. Genetic correlations between the same variables were –0.46 and –0.53, respectively. Phenotypic and genetic correlations between mood/emotion and response type were 0.92 and 0.95, respectively. Principal components and Bayesian analyses were used to compute the variation in cognitive capabilities explained by 13 cognitive processes and their genetic parameters, respectively. Heritabilities ranged 0.06 to 0.38 suggesting the same patterns previously reported for humans and other animal species. By contrast, when considering the selection for therapeutic riding, gaits' heritability estimates ranged from 0.53 to 0.67 for walk and trot, respectively. Genetic correlations ranged from 0.28 to 0.42, for walk/trot and amble/trot, respectively. Our results suggest that gait genetic lines could be developed. Among other breeding criteria, disease resistance and reproduction offer two functional niches to consider given their relationship with donkey life quality and welfare.

Breeding programs selecting for disease resistance could address food safety and quality issues in products such as donkey milk, and may be perceived to be more humane. Cutaneous habronematidosis (CH) is a highly prevalent parasitic seasonally recurrent skin disease causes distress and relapsing wounds to the animals. CH hypersensibility heritability was 0.0346. Genetic parameters and breeding values for functional traits enable planning strategies for endangered donkey breed preservation and breeding what may turn into a measure to improve animal welfare indirectly.Multiple births in equids are dangerous situations that compromise the life of the dam and offspring. However, embryo collection techniques take advantage of individuals whose multiple ovulations allow flushing more fertilized embryos from the oviduct. Heritabilities ranged from 0.18 to 0.24. Genetic and phenotypic correlations ranged from 0.496 to 0.846 and 0.206 to 0.607, respectively.

Resumen

Las herramientas que permiten entender la evolución de las poblaciones de burros en el tiempo, las tendencias futuras que estas poblaciones describen, y los factores condicionantes de dichas tales tendencias, se vuelven incalculablemente críticas cuando se pretende preservar y luego recuperar dichas poblaciones de su estado de peligro. Basándose en la falta de información característica sobre el trasfondo genealógico de las poblaciones de burros y tomando una raza particular como ejemplo, es posible inferir un modelo para evaluar la estructura genética y demográfica de otras poblaciones de burros en peligro. Entonces, podemos trazar estrategias de selección para implementar una vez que dichas poblaciones han alcanzado el número suficiente de individuos, y están apoyadas por estructuras suficientemente sólidas. Se realizaron análisis de pedigrí probados por microsatélites para estudiar la diversidad genética, la estructura y la evolución histórica de Raza Asnal Andaluza desde la década de 1980. A pesar de que la consanguinidad media era baja, animales altamente consanguíneos estaban presentes. El tamaño efectivo de la población basado en la tasa de consanguinidad individual fue aproximadamente la mitad que cuando se basaba en la tasa de coascendencia individual. Las distancias de Nei y el número de subpoblaciones equivalentes indicaron explotaciones diferenciadas en una población altamente estructurada. Aunque la pérdida de diversidad genética desde las generaciones fundadoras podría considerarse pequeña, las políticas de cría intra rebaño y la contribución excesiva de pocos antepasados al patrimonio génico podría conducir a cuellos de botella en el pedigrí más estrechos. Los largos intervalos de generación medios podrían considerarse para reducir la consanguinidad. Los estadísticos de fijación de Wright indicaron una consanguinidad leve entre explotaciones. La superficialidad del pedigrí sugería aplicar nuevas estrategias de cría para estimar parámetros descriptivos fiablemente y controlar los efectos negativos de la consanguinidad, lo que podría significar la clave para preservar estos valiosos recursos animales evitando la extinción hacia la que potencialmente se dirigen. Los estudios de diversidad adquieren una especial importancia en los burros, ya que revelan el trasfondo genético de las poblaciones y el punto de partida para tomar decisiones sobre la aplicación de planes de conservación o de selección en esta especie funcionalmente desubicada. Una vez que se equilibran los parámetros de diversidad genética, encontrar nuevos nichos para los burros se convierte en el objetivo más

relevante a afrontar para el futuro a medio plazo para la especie. Las estrategias de selección en los burros se afrontan desde tres perspectivas diferentes; terapia asistida por burro y la equitación terapéutica, la fertilidad y la resistencia a enfermedades, no sólo como una manera de ampliar el espectro funcional de las oportunidades de los burros, sino también para alargar su vida útil, y mejorar su calidad de vida y bienestar. Estudiar el trasfondo genético específico detrás de los caracteres funcionales permite cuantificar el grado en que tales características pasan de garañones y burras a las nuevas generaciones de ruchos. Como término genético, el ambiente implica a todas las influencias que no sean factores heredados. Controlar los factores ambientales que condicionan la expresión de ciertas características funcionales ayuda a construir modelos animales arrojando luz en la fracción genética involucrada en tales rasgos funcionales. El rendimiento funcional de 300 burros con filiación confirmada con microsatélites fue estudiado utilizando métodos REML y bayesianos por muestreo de Gibbs para la obtención de parámetros genéticos y valores de cría utilizando la metodología BLUP. El primer nicho funcional para el que los burros pueden ser adecuados, está ligado a su especial naturaleza psicológica y características físicas como facilitadores de los procesos de aprendizaje y para el desarrollo de habilidades clave de la vida y la construcción de confianza para un amplio espectro de personas vulnerables. La equitación terapéutica y la asinoterapia aprovechan la interacción física y psicológica entre los burros y los pacientes, dada la posible aplicación de las características y capacidades del burro para el tratamiento de trastornos humanos específicos. La selección de burros cuando los criterios de cría son su idoneidad para las terapias asistidas por équidos se implementó siguiendo dos enfoques diferentes; la selección de estrategias de afrontamiento y procesos cognitivos, y la selección de la marcha y el movimiento. Con el objetivo de desarrollar modelos adecuados que buscan la consolidación de criterios de selección para terapia asistida equina, estudiamos 29 factores que potencialmente pueden influir en varios procesos cognitivos en burros. Estos factores no sólo afectan el comportamiento a corto plazo de los burros, sino que también pueden determinar sus habilidades cognitivas a largo plazo desde el nacimiento. Por lo tanto, el comportamiento animal se convierte en una herramienta útil para obtener información pasada, presente o futura de la situación de un determinado animal en un área en particular. La sinergia entre el condicionamiento operante y la evaluación cualitativa del comportamiento (QBA) pueden proporcionar información valiosa sobre la extinción/aprendizaje y estado emocional de los animales. Todas las características no cognitivas inherentes a los animales afectaban significativamente a las cuatro variables testadas ($P<0.001$), aunque algunas no estaban correlacionadas linealmente. Por otro lado, la potencia de efecto de los factores meteorológicos osciló entre el 7.9% para la estación de nacimiento sobre el aprendizaje ($P <0.05$) y el 38.8% para la fase lunar en el momento del nacimiento sobre el estado de ánimo ($P<0.001$). Las pruebas psicométricas permiten cuantificar las capacidades cognitivas de los animales y su origen genético. Entre estas capacidades cognitivas, el estudio de las estrategias de afrontamiento para resolución de problemas logra una relevancia especial, ya que aúna la necesidad de seleccionar genéticamente burros que muestran una reacción neutral durante el entrenamiento, dada su implicación con la seguridad del entrenador/jinete y la capacidad de entrenamiento. Las heredabilidades para las

estrategias de afrontamiento eran moderadas, de 0.18 a 0.21. Las correlaciones fenotípicas entre intensidad de respuesta y estado de ánimo/emoción o el tipo de respuesta fueron −0.21 y −0.25, respectivamente. Las correlaciones genéticas entre las mismas variables fueron –0.46 y –0.53, respectivamente. Las correlaciones fenotípicas y genéticas entre el estado de ánimo/emoción y el tipo de respuesta fueron 0.92 y 0.95, respectivamente. Los componentes principales y análisis bayesianos se utilizaron para calcular la variación en las capacidades cognitivas explicadas por 13 procesos cognitivos y sus parámetros genéticos, respectivamente. Las heredabilidades variaron entre 0.06 a 0.38 sugiriendo los mismos patrones reportados previamente para los seres humanos y otras especies animales. Por el contrario, al considerar la selección para la equitación terapéutica, las estimaciones de la heredabilidad de los aires variaron de 0.53 a 0.67 para el paso y el trote, respectivamente. Las correlaciones genéticas variaron entre 0.28 a 0.42, entre el paso/trote y la ambladura/trote, respectivamente. Nuestros resultados sugieren que podrían desarrollarse líneas genéticas de acuerdo a los aires. Entre otros criterios de cría, la resistencia a enfermedades y la reproducción ofrecen dos nichos funcionales a considerar dada su relación con la calidad de vida del burro y el bienestar. Los programas de cría que seleccionan la resistencia a las enfermedades podrían abordar problemas de inocuidad y calidad de los alimentos en productos como la leche de burro, y pueden percibirse como más humanitarios. La habronematidosis cutánea (CH) es una enfermedad parasitaria recurrente de la piel de alta prevalencia, que causa angustia y heridas recidivantes a los animales. La heredabilidad de la hipersensibilidad de CH fue de 0.0346. Los parámetros genéticos y los valores de cría de rasgos funcionales permiten estrategias de planificación para la preservación y la selección de las razas de burro en peligro que pueden convertirse en una medida para mejorar el bienestar animal indirectamente. Los nacimientos múltiples en équidos son situaciones peligrosas que comprometen la vida de la madre y la descendencia. Sin embargo, las técnicas de recolección de embriones aprovechan individuos cuyas ovulaciones múltiples permiten la obtención de un número mayor de embriones más fecundados del oviducto. Las heredabilidades oscilaron entre 0.18 y 0.24. Las correlaciones genéticas y fenotípicas variaron entre 0.496 a 0.846 y de 0.206 a 0.607, respectivamente.

Introduction

Not only did worldwide studies on the domestic donkey raise concerns about the current endangerment risk of donkey breeds individually, but also about the whole species [1]. Tools allowing to understand the evolution of the different donkey populations in time, the future trends that they describe, and the factors conditioning such trends, become invaluable critical points when aiming at preserving and later recovering those donkey populations from their endangerment status.

Basing on the characteristic lack of background information that donkey breeds present and the commonly applied husbandry systems, it is possible to infer a model to assess the genetic and demographical structure of a certain donkey population taking a particular breed as an example.

The Andalusian donkey breed is believed to be closely related to or even the direct descendent of the 'White' Egyptian donkey breed, also known as Hassawi riding donkeys [2] and other rather undefined north African breeds as direct ancestors, the first precursors of the Andalusian donkey breed would have been introduced into the peninsula in the sixth century b.C., as already domestic animals with the transhumant movements by the Chamites, later components of the Iberian people, a fact that makes this breed stand out among the rest, as one of the direct remnants of the connection with the first African donkey ancestors.

The only existing remains from the early presence of this species in the Iberian Peninsula, appear at the Celtiberian levels in sites in the Basque Country and Navarra, supporting the theory that Celtiberians would have introduced donkeys from Africa through the north. The first human migrations left Africa through the Bab-el-Mandeb Arabian strait and reached Europe around 45 000 years ago, ascendingly following the Afro-Mediterranean coast, to finally settle along the Southeastern Iberian territories, according to the most probable genetic and anthropological hypotheses [3].

The Andalusian donkey breed would reach its maximum concentration in the early nineteenth century, surpassing the frontiers of the basin of the Guadalquivir River, something that has not excluded it from being recognized as endangered by the Spanish Official Catalogue of Livestock Breeds. The different census obstacles the breed had to face start dating from the end of the Cordobesian Umayyad Caliphate (756–1031 a.C.) and culminate with the industrialization process of the region since halfway through the nineteenth Century.

This would bring about the last drastic reduction of the census of the breed and its current endangered status, as a consequence of the disappearance of the causes originating its creation, breeding and selection –crop transport through an area with an inefficient transport network and mule production–. Although it started being scientifically noticed in the 1910s, the first reference to the studbook of the Andalusian donkey breed would not appear until 1932. However, its genetic structure has remained unknown.

The studbook information, genetic diversity status, population structure, and the assessment of breeding practices carried out, have become indispensable tools for the development of conservation programmes, as donkeys appraisal and valuation is still done considering their ancestry, what confers a more strictly economic basis to the control of endogamy and mating management [4]. Its traditional context and breeding methods may have led to an inbreeding increase, an effective population size reduction, and a consequent genetic diversity loss. This common context to almost all donkey breeds makes it likely for genetic analyses, especially when selecting for functional skills, to face compromises in terms of sample size, population structure, and functional data collection.

Superstition conjoined consequences together with the psychological misunderstanding of the donkey species relegated this animal to become one of the most misunderstood species of all times, as reported by the multiple derogatory literature references found in several languages and cultures worldwide [5-8]. This historical context parallelly evolved with the species and not only contributed to donkey's path towards the risky endangerment situation it faces today, but also relegated the role of these valuable animals to an afunctional secondary place within society [9], what in the end constitutes one of the most preventive tools for the donkeys not to be considered by the scientific community [10].

Being domesticated prior to the horse, the suitability of the donkey species for mankind has been documented through History. Considering its overall docile nature, donkeys have been proved to be especially suitable for women and children, who use them for traction and draught power when compared to oxen or larger equines. In areas where donkeys are no longer used, owners and breeders are left to find alternative uses otherwise endangered breeds vanish. This sets an optimal framework for new donkey application niches to arise, as for example, their use in leisure and equine assisted therapy [11], which are supported by scientifically reported beneficial effects on human health [12]. Donkeys used in such settings must be tested and selected for their abilities to develop cognitive processes, especially those relating to their overall behaviour and coping style levels, as this may translate in reducing the money and time invested in their education.

Their similarities with horses led to the misconception of the species [13]. Donkeys evolved in a particularly harsh context that modelled their psychology and conduct since domestication. Their tendency to freeze when facing unfamiliar situations characterizes a cautious and intelligent species that needs longer than others to interpret the stimuli around. In addition, their stoic nature, rarely displaying evidence of pain, makes them silently and emotionally suffer from hard treatment more than other species, hence making behavioural assessment become critical for their welfare [14].

These behavioural particularities are strengthened by a higher ability to use their power and endurance in their benefit, and therefore, to exert a stronger opposite response [15] what has often been confused with stubbornness, cooperation reluctance and silliness. Zucca*, et al.* [16] would suggest that such especial way to spatially interact with the environmental conditions around, their extinction/learning skills [17-19] and thus, the success of specific techniques to implement when educating donkeys may rely on cerebral laterality-based differences when compared to other affine species such as horses.

All these behavioural features suggest that a rather educational approach should be considered when teaching donkeys to develop certain tasks, contrasting the regular training methods applied in horses. Body language becomes then a useful tool when interpreting animal mood or emotions [20]. Even more in long-term neglected donkeys or when aiming at counteracting undesirable features, providing us with an efficient communication tool for the fulfilment of any human-related activity.

Donkeys' unmerited conception of a useless behaviourally problematic species curiously came into the scene at the same age in which the species was probably enjoying one of the most productive times for their functionality. During the Egyptian pharaonic times [21,22], donkeys were not just herded for milk or meat production, but also were usually ridden among the most notable personalities [23,24], what provided them with a distinguished role in society. The smooth riding characteristics of donkeys were already reported in text fragments by Al-Maqrīzī dating back to the 13th Century.

Donkeys can perform all the gaits that other affine species such as the horse develop. However, these gaits should be considered analogous variations as donkeys are conditioned by their anatomical and physiological characteristics [21]. These facts together with the close bonds that they form with humans, the application of each gait modality in the treatment of specific human conditions and their kinetic versatility are key advantages when setting the base for their sustainable functional future.

Assisted therapy has stepped into the functional scene of donkeys as not only have they been reported to facilitate the effective recovery of spontaneous communication in people with affective and emotional disorders due to their empathic nature [25], what may rely on the way they use their cognitive abilities to interact with humans [26], but also to improve reduced gross and fine motor skills [27-34]. Increasing the scarce information relative to interindividual variability in cognition in donkeys through research [35] may open a new path towards finding equine specific genes involved in assisted therapy desirable behavioural traits, such as coping styles or other cognitive processes, and gaits for donkey assisted therapy (physical, occupational, and speech-language therapy treatment strategy that utilizes equine movement as part of an integrated intervention program to achieve functional outcomes) and therapeutic riding (riding lesson specially adapted for people with special needs).

In psychology, among other cognitive processes, coping refers to the conscious efforts of an individual to solve personal and interpersonal problems in order to master, minimize or tolerate stress [36]. Coping mechanisms are commonly termed coping strategies, and they normally comprise adaptive strategies or strategies which reduce stress [37]. Benus*, et al.* [38] rodent experiments concluded that the response to external stimuli could mainly be classified into two equally valuable strategy alternatives to face daily environmental demands, passive and active animals. Koolhaas*, et al.* [39], suggested updating these 'styles' to proactive and reactive, as the former confusing terms did not consider fundamental differences. One of such fundamental differences is the degree in which behaviour is influenced by environmental stimuli. To sum up, the performance of routine rather intrinsically driven rigid types of behaviour found in proactive animals, contrasts the generally more flexible and reactive attitude to environmental stimuli of reactive animals. Thus, when we speak about coping, we generally refer to reactive coping or the coping response after the presentation of the stressor. This differs from proactive coping, in which a coping response aims to neutralize a future stressor. Rather subconscious or non-conscious strategies such as defence mechanisms are generally excluded from the field of coping [40].

The effectiveness of the coping effort depends on the type of stressful stimulus, the individual, and the circumstances. Coping responses are partly controlled by personality and mood, but also partly by the stressful nature of the environment around [41].

Among the four strategies that Weiten and Lloyd [36] identified as coping styles in humans, problem-focused coping styles address those adaptive behavioural responses aimed at reducing, adapting or eliminating stressors. Although equids' reactiveness could clearly fit within these coping styles, a remarkable dimorphism has been described among species. Some equids describe a rather reactive strategy or tendency to freeze (such as donkeys) when they are involved in a challenging situation while others proactively flee, i.e. zebras or horses [42].

Domesticated donkeys' wild ancestors often lived solitarily or in very small groups of two animals in which running away was not always such a successful survival method compared to that of the horse that lives in larger hierarchical groups and forms stronger bonds with its congeners [43,44]. Conversely, wild or even feral donkeys' close bonds remain more solitary, normally being established between the jenny and its foal. When facing a potentially threating stimulus, donkeys may display to the predator (or observer) apparently normal behavioural patterns. However, these "normal" behavioural patterns could also be associated with misunderstood negative affective states [45]. Apart from clear psychological differences, which may have an ancestral social basis, Koolhaas and Bohus [46] suggest that each of these strategies may be catalyzed by different endocrine responses. These endocrine responses may be the basis and therefore, influence the mechanisms adopted by animals to maintain control over potentially threatening situations.

Most of human-equid accidents result from unexpected animal reactions [47]. Daily human-animal interaction helps deepening the mutual interspecific bonds that are established (that is, improves the familiarity of the animals towards their handlers). These concepts have been suggested to be the basis for a better performance when obtaining neutrally responding individuals in very evolutionarily distant species [48,49]. Training processes can be conducted following different approaches. Thus, although a greater difficulty training certain donkeys may reduce their working life and increase the time and costs needed to obtain fully functional animals, this should not make us exclude such animals from their use in riding or therapy [50], but to tailor a different approach to educate or select them.

Methodology to select for coping styles or reactivity levels may be useful for breeders and owners. Identifying the coping styles displayed by donkeys or their reactivity level when facing diverse kinds of stimuli from the beginning enables appropriate training protocols to be implemented from day one to work with the animal's innate response and tailor training programmes to meet the animal's needs. Such implications and knowledge may improve their final destination to develop the tasks that they may be better fit for.

Meta-analytic studies of the fixed or random effects to be considered in genetic models become particularly necessary in functional genetics [51]. These effects may present small effect sizes on particular traits; however, they may still be statistically significant. In unison, these effects can explain quite a large proportion of the phenotypic variation for the traits studied in a population, hence, conditioning the estimates for genetic parameters of such traits. We should carefully consider which effects represent mere experimental design effects and which of them are biologically relevant for our trait and should therefore be included in our genetic models.

Qualitative Behavioural Assessment (QBA) enables the translation of animal body language signs into human personality and emotional familiar terms to develop models that users can consider when interacting with animals under a welfare framework [52]. Not only these QBA models lay the basis for studies assessing the suitability of different techniques to educate donkeys, but help quantify their body language signals, attempting to develop a nonverbal owner-donkey bidirectional communication [53]. Cognitive skills and their correlations with body signals may allow us to quantify the behavioural responses of animals [54,55]. Furthermore, the application of QBA animal models in human studies could lead to a better understanding of the treatment of human behavioural problems to improve our quality of life [56,57].

The knowledge on the factors conditioning cognitive processes (coping styles and intelligence related ones among others) is especially relevant to assess the genetic variability behind them, as it may help develop accurate selection programmes, aiming at preserving such variability, one of the keys for survival in endangered breeds.

Contrary to what authors such as Hausberger*, et al.* [58] have recommended, functional traits have never comprised the selection criteria included in the breeding programmes of donkeys, as only morphological and phaneroptical (mainly coat) features had been considered.

There are many internal and external factors that may affect equid behaviour and therefore, the cognitive processes that equids develop. Researchers have measured how factors such as environment [59], handling conditions [60], age, sex, breed, sire [58], season, diurnal cycles [61] and year [62] may modulate donkey behaviour from a phenotypical perspective. Although such factors have been reported to be significant for the development of different ethological patterns, no study has focused on assessing reliable quantitative methods for their integration in linear genetic models in donkeys.

The hypothetical conditioning effects of weather, moon and climate oscillations on animal behaviour and cognition have been widely but unscientifically reported. Popular knowledge has even provided untested testimony of the possibility to predict short-term future meteorological conditions basing on how animals react to the environment around them. This framework has promoted the appearance of the first empirical studies on the clinical and productive implications of such environmental factors in different animal species.

Research has focused on the study of the climatological alteration of physiological processes such as reproduction, and animal biorhythms in populations of different species [63]. By contrast, cognitive or behavioural alterations affecting animal populations may remain unnoticed due to being attributed to other more probable causes.

The study of the effects of factors such as season and weather on animal behaviour and mood has typically focused on understanding the changes in the ethological patterns conditioning animal routine and daily activities. These changes may globally appear as a consequence of the evolution of certain areas, which may no longer fulfil the unique set of requirements of the animal populations inhabiting them [64].

Among the functional traits to consider in the breeding programmes of donkey breeds, there is a need to assess those traits that may be related to welfare as they may imply a direct repercussion on the development activity that the animals perform and their productive and economic profitability [65]. Disease resistance or reproductive linked ones, may be interesting traits to consider given their direct implication with the enhancement of the zootechnical handling conditions of donkey populations.

Cutaneous habronematidosis (CH) is an Equidae specific skin disease that occurs when stomach worm larvae from the spirurid species comprising the superfamily Habronematidae (*Habronema* or *Draschia*, for instance) are deposited on injured or irritated skin tissue or mucous membranes [66]. Although donkey cutaneous habronematidosis (summer sores) would not be scientifically described until a few decades ago [67], current research suggests this dermatological condition causes more severe lesions in donkeys than it does in other equids such as horses and their hybrids [68]. Traditional nomenclature ("Summer or Jack sores") not only highlights a higher disease incidence and severity reported in donkeys [68], but also the progressively increasing incidence of this disease when weather conditions become warmer in late spring or early summer (late April through June, generally after March rainy periods), partially regressing or even disappearing in winter [69].

A higher predisposition to develop cutaneous habronematidosis has been suggested for grey or diluted coat equines [70,71], such as the Andalusian donkey. However, neither breed, sex nor age different predilections seems to exist in horses [72], and no statistically proven information has been reported for donkeys up to the date. Moist body orifices and areas (eyes, lip commissures, ears, ventral abdomen, prepuce, penis and urethral process) are more commonly affected as they are more likely to attract the attention of parasite carriers such as flies. Areas on the limbs, especially from the fetlock to the coronary band, are frequently prone to mild cuts, scrapes, and trauma and thus can also be susceptible to summer sores. In addition, biting flies prefer to alight on shaded parts of animals lower on their bodies [67,73,74]. The results can range from annoying and unsightly to fatal. Young foals, thin-skinned and poor body condition animals are especially hypersensible to the action of carrier flies [66]. In the particular case of donkeys, these parts are so thin that are easily harmed by the larvae, which cause discomfort and distress as they progress in their life cycle, what becomes a critical point for the welfare of the species.

Although equids are the final host of the parasites responsible for this condition, the cutaneous myiasis caused by the larvae of these gastrointestinal parasites occurs because of an abnormal step in the normal life cycle of the parasites. These misplaced larvae cannot grow into their adult forms in such locations, but still induce a severe local inflammatory reaction characterized by intense swelling, ulceration, redness, and itching. Donkeys produce self-inflicted injuries during the subsequent rubbing and scratching to alleviate the itching produced by the simultaneous action of carrier or vector parasites, such as flies, and the action of the larvae, what apart from irritating the animals, damages the skin and makes it easier for the larvae to access the stomach through the mouth [73].

The selection of other species against their enhanced hypersensibility to gastrointestinal parasites has been suggested as an alternative to develop the sustainable control of parasite infections [75,76]. Apparently, some equids tend to be more predisposed to suffer from cutaneous habronematidosis than others, exhibiting clinical signs on consecutive years, whereas other individuals on the same premises never develop this condition [73]. Despite CH is a highly prevalent condition, with 94.5% of the Andalusian donkeys affected at least once in their lives, there is a simultaneous inexistence of studies testing for the conditioning factors that may be involved or the genetic background existing behind cutaneous habronematidosis hypersensibility in donkeys. The present model not only computes the strength of the effects of highly predisposing factors on the appearance of this skin condition, which may enable enhancing the implementation of prophylactic measures, but also isolates the additive genetic component laying underneath CH hypersensibility. This way, we approach the hypothetical possibility of the implementation of a selective breeding plan for the individuals, which may indirectly reduce the incidence of cutaneous habronematidosis.

Breeding for less CH sensitive donkeys together with the implementation of proper husbandry techniques may translate into the avoidance of detrimental repercussions for donkey welfare derived from the development of this disease.

Similarly, welfare problems related to the way equids are bred have been given no discrete consideration within the academic literature [77]. Studies of the genetic background of multiple pregnancies are anecdotal as fertility, in general, has a very low heritability as common to other reproductive traits. These studies are even more limited when we focus on studying equids such as horses [78,79] or donkeys, for which no study has been reported.

The occurrence of multiple births has been addressed one of the main causes of fetal and neonatal loss in equids [80]. The majority of twin pregnancies in horses (73%) terminates in abortion or stillbirth of both twins from eight months to term. The likelihood of one or both twin foals to be born alive or to survive after birth complications reduces to around 11%. The foals are usually born stunted or emaciated, what does not allow them to survive further from 2 weeks of age [80].

In the case of the donkey species, Quaresma*, et al.* [81] addressed the overall neonatal mortality for the first month of life to be of near 9%. 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.

By contrast, the donkey is a species for which the most of its breed populations have been classed as endangered [1] and that has been reported to be highly reproductively compromised as it happens to many other endangered populations [21] what may be attributed to the deleterious effects of inbreeding in such populations [9]. The long gestation cycle (a norm of 12 months to give birth in the 13th month [42]), a fertility that steadily decreases over generations [81] and the highly inbred status of donkey breed populations [9,82] only contribute to worsening the endangerment risk situation that donkey breeds face worldwide. Furthermore, highly standardised reproduction techniques in horses and other equids [83] such as artificial insemination with frozen semen and embryo transfer still represent a challenge in donkeys [84-86].

Under this context, embryo vitrification and freezing 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 [87], overcome the best currently reported results for pregnancy rate (28%) obtained for uterine horn insemination using frozen-thawed semen [88]. The efficiency 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.

Objetivos

En la presente Tesis Doctoral se propone sentar las bases científicas del programa de mejoramiento genético de la Raza Asnal Andaluza como objetivo genérico para asegurar la conservación y la puesta en valor de la raza, a la vez que se ofrece un modelo de aplicación en otras poblaciones asnales. Para conseguir este objetivo genérico, se plantearon diversos objetivos específicos, desarrollados en cada uno de las nueve publicaciones incluidas en el Compendio:

- Primer objetivo. (Primera publicación: "Navas, F.J.; Jordana, J.; León, J.M.; Barba, C.; Delgado, J.V. A model to infer the demographic structure evolution of endangered donkey populations. *animal* 2017, *11*, 2129-2138.):

  Desarrollar un estudio demográfico en la población genealógica de la raza Asnal Andaluza, como base para el diseño de su programa de conservación.

Segundo objetivo. (Segunda publicación: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; McLean, A.K.; Delgado Bermejo, J.V. Non-parametric analysis of the noncognitive determinants of response type and response intensity, mood and learning in donkeys. Submitted to *Journal of Veterinary Behavior: Clinical Applications and Research.*"):

  Evaluar los efectos de 15 factores no cognitivos sobre las variables conductuales del tipo de respuesta, la intensidad de la respuesta, el estado de ánimo/emoción y la capacidad de aprendizaje o extinción. Estudiar las correlaciones entre el lenguaje corporal y doce categorías de estado de ánimo. Evaluar qué tratamientos de refuerzo fueron más adecuados para promover los procesos de extinción/aprendizaje de los burros.

- Tercer objetivo. (Tercera publicación: "Navas González, F.J.; Jordana Vidal, J.; Pizarro Inostroza, G.; Arando Arbulu, A.; Delgado Bermejo, J.V. Can Donkey Behavior and

Cognition Be Used to Trace Back, Explain, or Forecast Moon Cycle and Weather Events? *Animals* **2018**, *8*, 215."):

Estudiar a qué nivel factores ambientales, meteorológicos y las oscilaciones climáticas pueden afectar el tipo de respuesta y la intensidad, el estado de ánimo y las habilidades de aprendizaje de los burros. Diseñar ecuaciones de regresión para explicar, rastrear y predecir las posibles repercusiones conductuales que ciertas situaciones ambientales pueden tener, y cómo estas consecuencias pueden alterar los patrones de comportamiento que los burros muestran a lo largo de sus vidas.

- Cuarto objetivo. (Cuarta publicación: "Navas, F.J.; Jordana, J.; León, J.M.; Arando, A.; Pizarro, G.; McLean, A.K.; Delgado, J.V. Measuring and modeling for the assessment of the genetic background behind cognitive processes in donkeys. *Research in Veterinary Science* **2017**, *113*, 105-114."):

Evaluar los efectos que factores inherentes al animal (sexo y edad) y factores ambientales externos (año de evaluación, estación, estímulo y sistema de cría) tienen sobre los procesos cognitivos en los burros, y, en segundo lugar, describir la posible aplicación de modelos genéticos cuantificables para la inclusión de tales procesos cognitivos en los programas de cría y conservación a través de una metodología rutinaria de pruebas *in situ*.

- Quinto objetivo. (Quinto publicación: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; Arando Arbulu, A.; McLean, A.K.; Delgado Bermejo, J.V. Genetic parameter and breeding value estimation of donkeys' problem-focused coping styles. *Behavioural Processes* **2018**, *153*, 66-76."):

Describir un modelo para calcular los efectos que influyen en el tipo de respuesta, estado de ánimo y la intensidad de respuesta para aislar el fondo genético detrás de las estrategias de afrontamiento en burros. Estimar los parámetros genéticos de dichas estrategias o los patrones de reactividad expresados por los burros cuando enfrentan estímulos visuales y auditivos. Desarrollar un índice que aborde la posibilidad de seleccionar genéticamente animales hiporeactivos, neutralmente reactivos e hiperreactivos.

- Sexto objetivo. (Sexta publicación: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; McLean, A.K.; Delgado Bermejo, J.V. Dumb or smart asses? Donkey's cognitive capabilities *(Equus asinus)* share the heritability and variation patterns of human's cognitive capabilities *(Homo sapiens)*. Submitted to *Journal of Veterinary Behavior: Clinical Applications and Research.*"):

Desarrollar una puntuación análoga al cociente intelectual humano en animales y estudiar la variación poblacional y los patrones de herencia descritos en los burros, determinando la estimación de los componentes de la (co)varianza y los parámetros genéticos, para después predecir los valores de cría y sus precisiones para dos conjuntos de trece procesos cognitivos generales y específicamente relacionados con la inteligencia, respectivamente, en burros de raza Andaluza utilizando el software MTGSAM.

- Séptimo objetivo. (Séptima publicación: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M..; McLean, A.K.; Pizarro Inostroza, G.; Delgado Bermejo, J.V. Genetic parameter estimation and implementation of the genetic evaluation for gaits in a breeding program for assisted-therapy in donkeys. *Veterinary research communications* 2018, *42*, 101-110."):

Estimar los parámetros genéticos y los valores de reproducción y sus precisiones para las modalidades de marcha, caminata y trote en los burros. Para calcular los índices de selección utilizando diferentes combinaciones posibles de estas modalidades de marcha para encontrar los métodos de selección de mejor ajuste cuando el objetivo de cría era la locomoción, sentar las bases para el desarrollo de diferentes líneas de cinética terapéutica, teniendo en cuenta las modalidades de marcha para las que cada burro evaluado puede ser más adecuado.

- Octavo objetivo. (Octava publicación: "Navas González, F.J.; Jordana Vidal, J.; Camacho Vallejo, M.E.; León Jurado, J.M.; de la Haba Giraldo, M.R.; Barba Capote, C.; Delgado Bermejo, J.V. Risk factor meta-analysis and Bayesian estimation of genetic parameters and breeding values for hypersensibility to cutaneous habronematidosis in donkeys. *Veterinary Parasitology* **2018**, *252*, 9-16."):

Aislar y estudiar la potencia de los posibles factores ambientales que influyen en la hipersensibilidad a la habronematidosis cutánea en burros infectados de forma natural. Cuantificar el fondo genético detrás de los fenotipos limitadamente variables para el carácter de la hipersensibilidad a la Habronematidosis Cutánea (HC) y su herencia como un carácter binario. Estimar los parámetros genéticos para la hipersensibilidad de HC y los valores de cría previstos de los individuos en la población histórica de burros andaluces.

- Noveno objetivo. (Novena publicación: "Navas González, F.J.; Jordana Vidal, J.; McLean, A.K.; León Jurado, J.M.; Barba, C.J.; Arando, A.; Delgado Bermejo, J.V. Modeling for the Inheritance of Endangered Equid Multiple Births and Fertility: Determining Risk Factors and Genetic Parameters in Donkeys *(Equus asinus)*. Submitted to *Research in Veterinary Science.*"):

Investigar la ocurrencia de gestaciones múltiples en la población histórica de burros andaluces y la influencia que factores no genéticos como la explotación, el sistema de manejo, la ubicación, el año de nacimiento, la estación de nacimiento, el mes de nacimiento o la edad pueden haber tenido sobre la prevalencia de estas gestaciones múltiples. Estimar los parámetros genéticos de fertilidad y gestaciones múltiples a través del análisis del número de histórico de ruchos nacidos por animal, el número máximo de ruchos por nacimiento y el número de nacimientos múltiple por animal. Predecir valores de cría para todos los rasgos como una forma de evaluar la posible implementación de una estrategia de selección bidireccional.

Aims

This PhD thesis aims at laying the scientific basis of the breeding program of the Andalusian donkey breed as a generic objective to ensure the conservation and value of the breed, while offering a model of application in other donkey populations. The following specific objectives were approached and achieved, as developed on each of the nine publications included in the Compendium:

- First objective. (First publication: "Navas, F.J.; Jordana, J.; León, J.M.; Barba, C.; Delgado, J.V. A model to infer the demographic structure evolution of endangered donkey populations. *animal* 2017, *11*, 2129-2138.):

  To develop a demographic study in the genealogical population of the Andalusian asinine race, as a basis for the design of its conservation program.

Second objective. (Second publication: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; McLean, A.K.; Delgado Bermejo, J.V. Non-parametric analysis of the noncognitive determinants of response type and response intensity, mood and learning in donkeys. Submitted to *Journal of Veterinary Behavior: Clinical Applications and Research.*"):

  To assess the effects of 15 non-cognitive factors on the behavioural variables of response type, response intensity, mood/emotion, and learning capacity or extinction. To study the correlations between body language and twelve categories of mood. To assess which reinforcement treatments were best suited to promote the extinction/learning processes of donkeys.

- Third objective. (Third publication: "Navas González, F.**J.**; Jordana Vidal, J.; Pizarro Inostroza, G.; Arando Arbulu, A.; Delgado Bermejo, J.V. Can Donkey Behavior and Cognition Be Used to Trace Back, Explain, or Forecast Moon Cycle and Weather Events? *Animals* **2018**, *8*, 215."):

To study at which level environmental factors such as season, year, moon cycle, meteorological factors, and climate oscillations may affect the response type and intensity, mood and learning abilities of donkeys. To design regression equations to explain, trace back, and predict the possible behavioural repercussions that certain environmental situations may have, and how these consequences may alter the behavioural patterns that donkeys display through their lives.

- Fourth objective. (Fourth publication: "Navas, F.J.; Jordana, J.; León, J.M.; Arando, A.; Pizarro, G.; McLean, A.K.; Delgado, J.V. Measuring and modeling for the assessment of the genetic background behind cognitive processes in donkeys. *Research in Veterinary Science* **2017**, *113*, 105-114."):

To assess the effects that inherent factors (sex and age) and external environmental factors (assessment year, season, stimuli and husbandry system) have on cognitive processes in donkeys, and second, to describe the potential implementation of quantifiable genetic models for the inclusion of such cognitive processes in breeding and conservation programmes through a routine *in-situ* test methodology.

- Fifth objective. (Fifth publication: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; Arando Arbulu, A.; McLean, A.K.; Delgado Bermejo, J.V. Genetic parameter and breeding value estimation of donkeys' problem-focused coping styles. *Behavioural Processes* **2018**, *153*, 66-76."):

To describe a model to compute the effects influencing response type, mood and response intensity to isolate the genetic background behind coping strategies in donkeys. To estimate the genetic parameters the coping styles or reactivity patterns expressed by donkeys when facing visual and auditory stimuli. To develop an index addressing the possibility to genetically select for hyporeactive, neutrally responsive and hyperreactive animals.

- Sixth objective. (Sixth publication: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; McLean, A.K.; Delgado Bermejo, J.V. Dumb or smart asses? Donkey's cognitive capabilities *(Equus asinus)* share the heritability and variation patterns of human's cognitive capabilities *(Homo sapiens)*. Submitted to *Journal of Veterinary Behavior: Clinical Applications and Research.*"):

To develop an animal human-analogous IQ score and to study the populational variation and the inheritance patterns described in donkeys, determining the estimation of (co)variance components and genetic parameters, and to predict breeding values and their accuracies for two sets of thirteen general and specifically related to intelligence cognitive processes, respectively in Andalusian breed donkeys.

- Seventh objective. (Seventh publication: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M..; McLean, A.K.; Pizarro Inostroza, G.; Delgado Bermejo, J.V. Genetic parameter estimation and implementation of the genetic evaluation for gaits in a breeding program for assisted-therapy in donkeys. *Veterinary research communications* 2018, *42*, 101-110."):

  To estimate genetic parameters and breeding values and their accuracies for amble, walk and trot gait modalities in donkeys. To compute selection indexes using different possible combinations of these gait modalities to find the best fitting selection methods when the breeding goal was locomotion, to lay the basis for the development of different therapeutic kinetic lines, considering the gait modalities for which every donkey assessed may be better suited.

- Eighth objective. (Eighth publication: "Navas González, F.J.; Jordana Vidal, J.; Camacho Vallejo, M.E.; León Jurado, J.M.; de la Haba Giraldo, M.R.; Barba Capote, C.; Delgado Bermejo, J.V. Risk factor meta-analysis and Bayesian estimation of genetic parameters and breeding values for hypersensibility to cutaneous habronematidosis in donkeys. *Veterinary Parasitology* **2018**, *252*, 9-16."):

  To isolate and study of the strength of potential predisposing environmental factors influencing the hypersensibility to cutaneous habronematidosis in naturally infected donkeys. To quantify the genetic background behind the limitedly variable phenotypes for the CH hypersensibility trait and its inheritance as a binary trait. To estimate the genetic parameters for CH hypersensibility and the predicted breeding values of the individuals in the historical population of Andalusian donkeys.

- Ninth objective. (Ninth publication: "Navas González, F.J.; Jordana Vidal, J.; McLean, A.K.; León Jurado, J.M.; Barba, C.J.; Arando, A.; Delgado Bermejo, J.V. Modeling for the Inheritance of Endangered Equid Multiple Births and Fertility: Determining Risk Factors and Genetic Parameters in Donkeys *(Equus asinus)*. Submitted to *Research in Veterinary Science*"):

  To investigate the occurrence of multiple pregnancies in the historical population of Andalusian donkeys and the influence that non-genetic factors such as farm, husbandry system, location, year of birth, birth season, birth month or age may have had on the prevalence of these multiple gestations. To estimate genetic parameters of fertility and multiple births through the analysis of historical foal number born per animal, maximum foal number per birth and multiple birth number per animal. To predict breeding values for all the traits as a way to assess the potential implementation of a bidirectional breeding strategy.

Chapter 1

Navas, F.J.; Jordana, J.; León, J.M.; Barba, C.; Delgado, J.V.

A model to infer the demographic structure evolution of endangered donkey populations.

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animal

A model to infer the demographic structure evolution of endangered donkey populations

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Stemming from The Worldwide Donkey Breeds Project, an initiative aiming at connecting international researchers and entities working with the donkey species, molecularly tested pedigree analyses were carried out to study the genetic diversity, structure and historical evolution of the Andalusian donkey breed since the 1980s to infer a model to study the situation of international endangered donkey breeds under the remarkably frequent unknown genetical background status behind them. Demographic and genetic variability parameters were evaluated using ENDOG (v4.8). Pedigree completeness and generation length were quantified for the four gametic pathways. Despite mean inbreeding was low, highly inbred animals were present in the pedigree. Average coancestry, relatedness, and non-random mating degree trends were computed. The effective population size based on individual inbreeding rate was about half when based on individual coancestry rate. Nei's distances and equivalent subpopulations number indicated differentiated farms in a highly structured population. Although genetic diversity loss since the founder generations could be considered small, intraherd breeding policies and the excessive contribution of few ancestors to the gene pool could lead to narrower pedigree bottlenecks. Long average generation intervals could be considered when reducing inbreeding. Wright's fixation statistics indicated slight inbreeding between farms. Pedigree shallowness suggested applying new breeding strategies to reliably estimate descriptive parameters and control the negative effects of inbreeding, which could indeed, mean the key to preserve such valuable animal resources avoiding the extinction they potentially head towards, making the present model become an international referent when assessing endangered donkey populations.

Keywords: ass, endangered breed, genetic diversity, inbreeding, population genetics

Implications

Genetic diversity loss in domestic populations becomes especially important in apparently unsustainable species like donkeys, meaning the simultaneous loss of important functional traits. The Andalusian donkey breed fits the common framework of small endangered genetically unknown populations at the beginning of their conservation programmes, and thus can describe a translatable model systematizing the measures to take when the information available is reduced. Our findings enable quantifying the real risk of extinction such populations face, avoiding under-estimating population intrarelationships and reporting an unreal population structure, hindering proper handling measures, reducing the effectiveness of the techniques implemented and worsening their situation.

Introduction

Not only did worldwide studies on the domestic donkey raise concerns about the endangerment risk of donkey breeds individually, but also about the whole species (Kugler *et al*., 2008). Tools allowing us to understand the evolution of the different donkey populations in time, the future trends that they describe, and the factors conditioning such trends, become invaluable critical points when aiming at preserving and recovering those donkey populations from their complex status. Based on the characteristic lack of background information and the commonly applied husbandry systems, it is possible to infer a model to assess the genetic and

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demographical structure of certain donkey populations using a particular breed as an example. With the Egyptian ass and other rather undefined north African breeds as direct ancestors, the first precursors of the Andalusian donkey breed would have been introduced into the peninsula in the 6th century B.C., as already domestic animals with the transhumant movements by the Chamites, later components of the Iberian people, a fact that makes this breed stand out among the rest, as one of the direct remnants of the connection with the first African donkey ancestors. The only existing remains from the early presence of this species in the Iberian Peninsula, appear at the Celtiberian levels in sites in the Basque Country and Navarra, supporting the theory that Celtiberians would have introduced donkeys from Africa through the north. The first human migrations left Africa through the Bab-el-Mandeb Arabian strait and reached Europe around 45 000 years ago, ascendingly following the Afro-Mediterranean coast, to finally settle throughout the Southeastern Iberian territories, according to the most probable genetic and anthropological hypotheses (Melé *et al*., 2012). The Andalusian donkey breed would reach its maximum concentration in the early 19th century, surpassing the frontiers of the basin of the Guadalquivir River, something that has not excluded it from being recognized as endangered by the Spanish Official Catalogue of Livestock Breeds. The different census obstacles the breed had to face start dating from the end of the Cordobesian Umayyad Caliphate (756–1031 A.C.) and culminate with the industrialization process of the region since halfway through the 19th century. This would bring about the last drastic reduction of its census and current endangered status, as a consequence of the disappearance of the causes originating its creation, breeding and selection – crop transport through an inefficient transport network area and mule production. Although it started being scientifically noticed in the 1910s, the first reference to the studbook of the Andalusian donkey breed would not appear until 1939. However, its genetic structure has remained unknown. The studbook information, genetic diversity status, population structure and the assessment of breeding practices have become indispensable tools for the development of conservation programmes, as donkeys appraisal and valuation is still done considering their ancestry, what confers a more strictly economic basis to the control of endogamy and mating management (Santana and Bignardi, 2015). Its traditional context and breeding methods may have led to an inbreeding increase, an effective population size reduction and a consequent genetic diversity loss. Therefore, the aim of this study is the development of a model to perform the analysis of the pedigree completeness downwards, checking the repercussions of ancestors and founders, evaluating the structure of the population, its genetic variability and connections between its genetic and demographic parameters, measuring the existing gene flux and quantifying the risk of genetic diversity loss; evaluating its endangerment degree to suggest effective conservation strategies for donkeys and other endangered animal small populations (Oldenbroek, 1999).

Material and methods

Data registries and software tool

The complete pedigree file provided by the Union of Andalusian Donkey Breeders (UGRA) included 1015 animals constituting the historical population – 272 jackstocks and 743 jennies – born between January 1980 and July 2015. All registries were genotyped and parentage tests for the offspring included in the pedigree were performed with 28 molecular markers recommended by the International Society of Animal Genetics. Demographic and genetic parameters of the existing variability in the pedigree were evaluated and traced back to ancestors. Analyses were carried out using ENDOG (v4.8) software (Gutiérrez *et al*., 2005) on the complete pedigree file (historical population described above), on the current population, or alive animals in the historical population (914, 246 males and 668 females, born from January 1980 to July 2015) and a contrast population set (453 alive animals, 199 jackstocks and 254 jennies), including those donkeys in the current population from which both parents were known.

New-born annual increase, pedigree completeness index, breeding animals, generation interval and mean age of parents at offspring's birth

The study of the number of births was carried out to assess the maximum and mean progeny number per jack or jenny. Pedigree completeness index (PCI), which summarizes the proportion of known ancestors of each ascending generation, was evaluated through: (1) the maximum number of generations traced; (2) the number of complete traced generations; (3) the number of complete equivalent generations, calculated as (1/2n) where n is the number of generations setting the individual apart from each known ancestor (Maignel *et al*., 1996), equal to $\sum_{i=1}^{n_j} \frac{1}{2^{g_{ij}}}$, where n_j is the total number of ancestors of the animal, j and g_{ij} the number of generations between j and its ancestor i (Boichard *et al*., 1997); and (4) the quality of the information of the pedigree through the proportion of known parents, grandparents, great-grandparents and great-great-grandparents registered in the studbook. Generation intervals (James, 1977) and the average age of parents at the birth of their offspring were calculated for each of the four gametic pathways: sire to son, sire to daughter, dam to son and dam to daughter, from birthdate records for every animal together with those of its parents'. The jenny/jack ratio was computed through the percentage of females and males with progeny selected for breeding and the number of reproductive animals selected.

Inbreeding, coancestry and degree of non-random mating

First, individual inbreeding (F) was computed according to Meuwissen and Luo (1992). Second, the average relatedness (AR) of each individual was computed as proposed by Gutiérrez *et al*. (2005). Leroy *et al*. (2013) stated inbreeding F and coancestry C coefficients are identity estimators by descent (IBD), a probability that differs whether the alleles considered belong to a single individual or two individuals, respectively. Third, the individual rate of inbreeding $(\overline{\Delta F})$ for the generation, computed as suggested by Gutiérrez *et al.* (2009) was calculated using $\Delta F_i = 1 - \sqrt[t_i - 1]{1 - F_i}$, where t_i is the number of complete equivalent generations and F_i the inbreeding coefficient of the individual *i*. Mean inbreeding per generation was used to form a regression equation testing linear and quadratic functions, which predicted further inbreeding up to 15 generations onwards. Fourth, the individual rate of coancestry $(\overline{\Delta C})$ for the generation was computed as suggested by Cervantes *et al.* (2011) through $\Delta C_{ij} = 1 - \sqrt[\frac{t_i + t_j}{2}]{1 - C_{ij}}$, where t_i and t_j are the number of equivalent complete generations and C_{ij} the coancestry coefficient for the individuals *i* and *j*. Finally, non-random mating was calculated as described by Caballero and Toro (2000) relating to the inbreeding coefficients by $(1 - F) = (1 - C)\ (1 - \alpha)$ (Wright, 1969) indicating the existing deviation degree from Hardy–Weinberg proportions.

Probabilities of gene origin and ancestral contributions

First, the effective number of founders (f_e) was computed as $f_e = \frac{1}{\sum_{k=1}^{f} q_k^2}$, where q_k is the probability of gene origin of the *k*th founder and *f* the real number of founders (Lacy, 1989). Second, the effective number of ancestors (f_a) was determined by $f_a = \frac{1}{\sum_{k=1}^{f} p_k^2}$, where p_k is the marginal contribution of an ancestor *k* (Boichard *et al.*, 1997). Third, the effective number of founder genomes (f_g) was obtained by calculating the inverse of twice the average coancestry (Caballero and Toro, 2000). Fourth, the expected marginal contribution of each major ancestor *j* (the largest genetic contributing founders or not), which is not already explained by a previously chosen ancestor was computed as (Boichard *et al.*, 1997) and the contributions to inbreeding of nodal common ancestors, that is forming inbreeding loops, were computed according to Colleau and Sargolzaei (2008). Fifth, the mean effective population size $(\overline{N_e})$ (Wright, 1969) was calculated as $\overline{N_e} = \frac{1}{(2\overline{\Delta IBD})}$. Sixth, the number of equivalent subpopulations (Cervantes *et al.*, 2008) was calculated through $S = \frac{\overline{N_e C_i}}{\overline{N_e F_i}}$, in which $\overline{N_e C_i} = \frac{1}{(2\overline{\Delta C})}$ is the mean effective population size considering the coancestry coefficient and $\overline{N_e F_i} = \frac{1}{(2\overline{\Delta F})}$, considering the inbreeding coefficient. Seventh, genetic diversity (GD) was computed as $GD = 1 - \frac{1}{2 f_g}$. Eighth, GD lost in the population since the founder generation was estimated by $1 - GD$. Ninth, GD loss derived from the unequal contribution of founders was estimated by $1 - GD^*$, where $GD^* = 1 - \frac{1}{2 f_e}$ (Caballero and Toro, 2000). The difference between *GD* and *GD** indicates the *GD* loss owed to genetic drift accumulated since the foundation of the population (Lacy, 1989). Finally, the effective number of non-founders (N_{ef}) was computed through $N_{ef} = \frac{1}{\frac{1}{f_g} - \frac{1}{f_e}}$ (Caballero and Toro, 2000).

Herd relationships and breeding strategy

The existing 145 subpopulations were computed considering 272 owners/farms. Minimum Nei's genetic distance (Nei, 1987) between subpopulations *i* and *j* was computed as $D_{ij} = [(C_{ii} + C_{jj})/2] - C_{ij}$, in which C_{ij} is the average pairwise coancestry between individuals of the subpopulations *i* and *j*, including all $N_i \times N_j$ pairs. C_{ii} and C_{jj} are the average pairwise coancestries within subpopulations *i* and *j*, to assess interherd relationships. The maximum limit of relationship coefficient between mated animals is assessed to maintain ΔF in a generation equal or below 1% ($N_e = 50$), level below which the fitness of a population steadily decreases (Meuwissen and Woolliams, 1994). Relatedness is the probability that two individuals share an allele because of common ancestry. This probability is expressed as the coefficient of relatedness (*R*), and ranges from 0 (unrelated) to 1 (clones or identical twins), excluding alleles simply shared because of belonging to the same species or population. In total, five mating groups in which the relationship coefficient between mated animals was kept below 1.00%, 2.00%, 3.00%, 4.00% and 6.00% (the greatest feasible limit considering all possible matings among all 914 living animals) were assessed. The inbreeding coefficient (*F*) for the offspring of each mating was calculated as one-half of the parental relationship coefficient. The inbreeding rate (Gutiérrez *et al.*, 2009) was estimated by averaging the individual inbreeding increase through $\Delta F_i = 1 - \sqrt[t_i - 1]{1 - F_i}$, where t_i is the number of complete equivalent generations and F_i the inbreeding coefficient of the individual *i*. In total, 60 random matings were selected within each group, basing on the number of births in 2015 (56 births) and on the assumption of one foal/jenny using SPSS Inc. (2008). Then, 30 replicates were analysed to calculate the average effective population size (N_e) (Gutiérrez *et al.*, 2009).

Results

New-born annual increase, pedigree completeness index, breeding animals, generation interval and mean age of parents at offspring birth

The average number of foals born per year was 28.19, reaching the highest number (71) in 2003. The average equivalent complete generations number during the last decade was 1.38 and increased almost linearly over the years, until it reached a value of 2.59 in 2015. The PCIs for one, two, three and four generations, the statistics for the average maximum number of traced generations, average number of complete generations and average number of equivalent generations are shown in Table 1. Maximum progeny per jacks (41) and jennies (7) was equal in the three population sets. However, jenny/jack ratio (2.73/1 historically and 2.71/1 currently) decreased in the contrast population (0.78/1). The mean number of progeny for males and females was slightly and progressively lower in each population; 1.78 in the historical, 1.76 in the current and 1.24 in the contrast population for males and 0.63, 0.60 and 0.54 for females in the same population sets, respectively. The percentage of females with progeny selected for breeding was 10.76% and 25% for males in the historical population; 11.82% for females and 26.42% for males in the current population; and 30.74% for females and 35.42% for males in the contrast population. Historically breeding jacks were 2.98 years older

Table 1 *Summary statistics of the pedigree analysis, average inbreeding, average coancestry and degree of non-random mating for the historical population (n = 1015), the current population (n = 914) and the contrast population (first generation, both ancestors known animals) (n = 453) of the Andalusian donkey breed*

Item	Historical	Current	Contrast
n	1015	914	453
Maximum number of generations traced (mean ± SD)	1.16 ± 1.47	1.09 ± 1.43	2.45 ± 1.28
Maximum number of complete generations (mean ± SD)	0.55 ± 0.68	0.52 ± 0.67	1.24 ± 0.46
Number of complete equivalent generations (mean ± SD)	0.79 ± 0.93	0.75 ± 0.90	1.70 ± 0.62
One generation pedigree completeness index (known parents %)	47.14	52.08	100
Two generations pedigree completeness index (known grandparents %)	23.30	21.03	25.83
Three generations pedigree completeness index (known great-grandparents %)	8.60	9.54	3.90
Four generations pedigree completeness index (known great-great-grandparents %)	1.98	2.19	0.44
Males (%)	26.89	26.91	43.93
Average age of males in reproduction (year)	15.22	14.95	14.71
Females (%)	73.11	73.09	56.07
Average age of females in reproduction (year)	12.24	12.34	12.11
Average inbreeding (F) (%)	0.67	0.70	1.51
Average individual increase in inbreeding (ΔF) (%)	0.55	0.57	1.23
Maximum inbreeding coefficient (%)	28.12	28.12	28.12
Inbred animals (%)	5.42	5.80	11.92
Average coancestry (C) (%)	0.43	0.44	0.78
Average relatedness (AR) (%)	0.85	0.81	1.53

than breeding jennies on average, but this difference shortened in the current (2.61) and contrast population sets (2.6). The average generation interval for the historical population was 7.40 years and 7.34 years for the current population.

Inbreeding, coancestry and degree of non-random mating
Although average inbreeding was low (0.67% in the historical population, 0.70% in the current population and 1.51% in the contrast population), highly inbred animals were present (maximum inbreeding coefficient of 28.12%). The percentage of inbred animals was 5.42%, 5.80% and 11.92%; the average coancestry was 0.43%, 0.44% and 0.78%, and the degree of non-random mating reached a progressively increasing value of 0.0025, 0.0027 and 0.0074 for the three populations sets, respectively (Figure 1). The average inbreeding coefficient reached a 1.73% maximum in 2015, whereas 1.59% maximum average coancestry was reached in 2014. The average degree of non-random mating reached a maximum of 0.50% in 2006. Matings between highly inbred animals, 0.20% matings between full sibs, 1.18% matings between half sibs and 1.18% parent-offspring matings have occurred. Regression equations testing linear and quadratic functions are shown in Figure 2.

Probabilities of gene origin and ancestral contributions
The results for the analysis of the gene origin probabilities, ancestral contributions and genetic diversity loss are shown in Table 2. Considering the marginal genetic contribution, a single ancestor (identification number: 1) explained from 9.18% to 14.44% of the genetic pool of the contrast population and from 7.56% to 9.92% of the genetic pool of the current population, and was also responsible for 43.63% to 60% of total inbreeding. The top 10 ancestors contributing to the inbreeding accounted for 17.45% to 68.35% of the total inbreeding of the animals born in recent years. The effective population size based on the individual inbreeding rate (N_eF_i) (±SD) was 17.81 ± 8.45, whereas based on the individual coancestry rate (N_eC_{ij}) (±SD) was 41.88 ± 2.56. The number of equivalent subpopulations (±SD) was 2.35 ± 1.13.

Herd relationships and breeding strategy
The mean number of donkeys per farm was 4.75 ranging from 1 to 56. A total of 10 585 Nei's genetic distances were considered. Nei's average genetic distance between them was 8.29%. Mean coancestry within subpopulations was 8.73% and mean inbreeding 0.66%, whereas the mean coancestry in the metapopulation was 0.44% and the self-coancestry was 50.33%. Studying Wright's F parameters, the inbreeding coefficient relative to the total population (F_{IT}) was 0.0022 and the inbreeding coefficient relative to the subpopulation was 0.0883 (F_{IS}). The correlation between random gametes drawn from the subpopulation relative to the total population (F_{ST}) was 0.0832. The assessment of the herd structure revealed none of the herds could be considered the population nucleus. The number of farms, which did not use own fathers was almost three times higher than the one of those that did it, and none of the herds was totally isolated. In total, 43 pairs of farms held the greatest Nei's genetic distance among them (56.25%), whereas the shortest distance among farms was 0.98%. Mean Nei's minimum distance/average homozygosity was 8.28%. A cladogram is shown in Figure 3, where all the relationships among farms are represented. Descriptive statistics and effective population size for each relationship coefficient level are shown in Table 3.

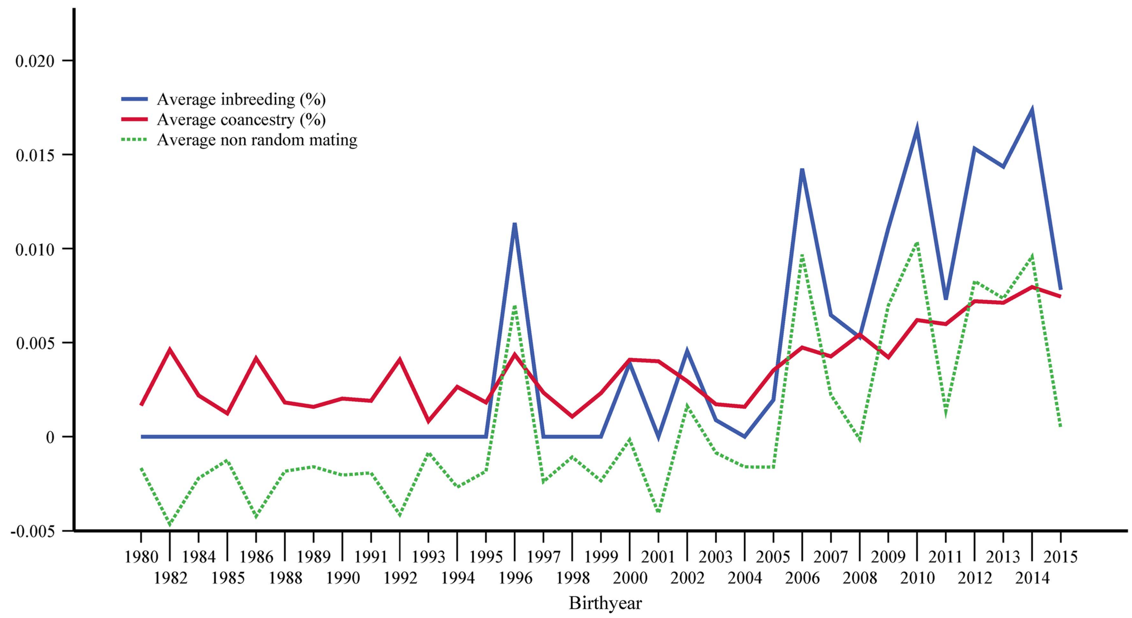

Figure 1 Evolution of the average coefficient of inbreeding, average coancestry and degree of non-random mating of the Andalusian donkey breed population according to the maximum number of complete generations from 1980 to 2015.

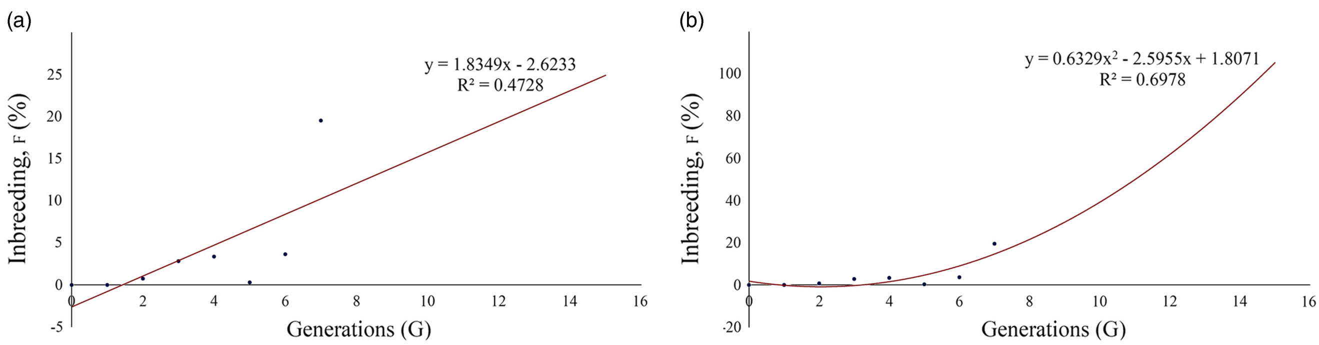

Figure 2 Linear (a) and quadratic (b) regression equations for mean inbreeding from the 1st to 5th generation, and predicted inbreeding from the 6th to 15th generation in the Andalusian donkey breed.

Table 2 *Measures of genetic variation and analysis of the probabilities of gene origin, genetic diversity loss for the current (*n *= 914) and contrast (*n *= 453) Andalusian donkey breed population sets under study*

Item	Current	Contrast
Total number of founders (*f*) (*n*)	524	226
Total number of ancestors (*n*)	503	219
Effective number of ancestors (f_a)	142	45
Effective number of non-founders (N_{ef})	344.58	130.59
Founder genome equivalents (f_g)	106.06	39.01
Total number of founder equivalents (f_e)	153.23	55.62
f_a/f_e ratio	0.93	0.81
f_g/f_e ratio	0.69	0.70
Genetic diversity (GD) (%)	99.53	98.72
GD loss due to bottlenecks and genetic drift since founders (GL) (%)	0.47	1.28
GD loss due to unequal founder contributions (%)	0.33	0.90
GD loss due to genetic drift (%)	0.14	0.38
Ancestors explaining 25 % of the gene pool (*n*)	17	4
Ancestors explaining 50 % of the gene pool (*n*)	96	19
Ancestors explaining 75% of the gene pool (*n*)	266	67

Table 3 *Descriptive statistics for (N_eF_i) effective size of the population of the Andalusian donkey breed through the individual inbreeding rate (ΔF_i), considering all possible matings at five different lower than 6% coefficient relatedness levels (R)*

$R \leqslant$	Matings	Replicates	Minimum	Maximum	Mean	SEM	SD
0.01	641	30	29.60	1134.88	209.63	47.00	257.46
0.02	783	30	0	1668.66	245.18	59.04	323.40
0.03	860	30	49.00	1275.55	223.96	51.10	279.88
0.04	906	30	29.60	1134.88	209.63	47.00	257.46
0.06	914	30	29.60	1134.88	209.63	47.00	257.46

Discussion

New-born annual increase, pedigree completeness index, breeding animals, generation interval and mean age of parents at offspring's birth

The knowledge about the pedigree of the breed has substantially increased in recent years, as a result of the increase in the number of foals whose genealogical information is known, but it is not deep rooted enough yet. Researchers like Gutiérrez *et al.* (2005) reported a mean number of equivalent generations of 2.5 for the Catalonian donkey; Rizzi *et al.* (2011) reported a slightly higher value (4.17) for Martina Franca donkeys and Quaresma *et al.* (2014) found that almost 80% of Miranda donkeys had unknown parents. The relatively poor pedigree completeness levels of many donkey breeds, result in the underestimation of inbreeding level trends, as individuals with unknown parents and their offspring are assigned a 0 inbreeding coefficient even if they are somehow related (Lutaaya *et al.*, 1999; Cassell *et al.*, 2003). Assessing the breeds individually, there was a great difference according to whether population control measures had been already implemented or not. In Amiata donkeys, the greatest number of traced generations was four and the average maximum, complete and equivalent generations were 1.4, 0.53 and 0.78, respectively (Cecchi *et al.*, 2006). Similarly, for the Catalonian donkey and its subpopulations, the number of complete generations ranged from 0.81 to 1.83, whereas the equivalent generations ranged from 1.2 to 2.78 (Gutiérrez *et al.*, 2005). This contrasts the Martina Franca donkey, for which the greatest number of traced generations was 11 and the average maximum, complete and equivalent generations were 4.67 ± 2.91, 1.97 ± 1.25 and 3.01 ± 1.83, respectively. Historically, in the first Andalusian donkey generation, the percentage of known ancestors was lower (47.14%) than in the current population (52.08%), evidencing a successful conservation programme. The Andalusian pedigree completeness level in the following generations was lower (1.98%) than in Catalonian (Folch and Jordana, 1998) or Martina Franca donkeys (Rizzi *et al.*, 2011), with around 20% of known ancestors in the fifth generation or the Pêga donkey, in which the proportion of know ancestors in the third generation was 43% (Santana and Bignardi, 2015).

The generation intervals for the four different pathways were similar and relatively long. A slightly greater mean generation interval was observed between dams and sons (7.93 years) when compared with the mean generation interval between dams and daughters (6.83 years). However, it is worth noting that the greatest value, shown by the dam to son pathway may have probably occurred because of a higher number of jennies whose age at delivery was above the mean, when compared with the same value for jackstocks. The mean age of parents when their offspring was born was slightly greater between dams and sons (8.23 years) than between dams and daughters (7.84 years). These values pointed out the promotion of the breeding use of some donkeys depending on the characteristics sought in particular, as a consequence of the differences in the taste that owners usually show towards the external features that certain donkeys present, which made the use of the animals for breeding also divergent. In Catalonian (Folch and Jordana, 1998) and Amiata donkeys (Cecchi *et al.*, 2006), the average generation intervals found (6.74 and 6.65 years, respectively) were shorter than in the Martina Franca (8.86 years) (Rizzi *et al.*, 2011), Miranda (8.18 years) (Quaresma *et al.*, 2014) or the Pêga donkey (10.70 years) (Santana and Bignardi, 2015). Long generation intervals could be mainly ascribed to the slow turnover rate because the most favoured and popular sires and dams continued contributing with their progeny on subsequent generations for years. Prolonging the generation interval may be useful to increase the number of sires and dams selected for breeding, thereby progressively increasing the effective population size, which is inversely proportional to the rate of inbreeding (Meuwissen, 1999) and therefore, contributing to preserving the genetic diversity of the population. The percentage of females with progeny selected for breeding was less than half the same percentage for males for the historical and the current population sets, but this difference almost disappeared when we considered the contrast population set. Historically breeding jacks were, on average, 2.98 years older than breeding jennies. This difference shortened in the current and contrast population sets, in which it was 2.61 and 2.6 (Table 1), indicating 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, hence the higher age of breeding jackstocks and the greater percentage of males with progeny selected for breeding. The jenny/jack ratio inverted in the contrast population, because of a higher number of males with both parents known than females, a sign of the historical lack of attention paid to the genealogy

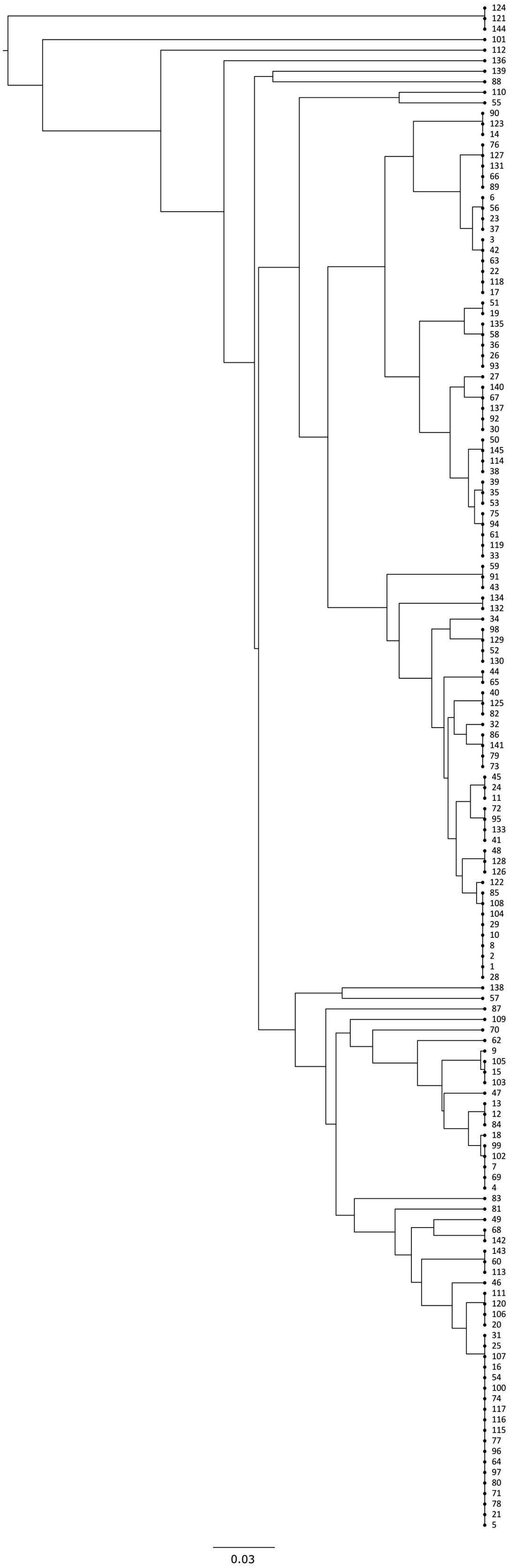

Figure 3 Cladogram constructed from Nei's distances among farms/owners of Andalusian donkey breed.

of females. The small age difference between breeding males and females suggested both sexes had the same reproductive life. The jenny/jack ratio was similar for Martina Franca donkeys (1.48/1) (Rizzi *et al*., 2011), higher for Pêga donkeys (4.97/1) (Santana and Bignardi, 2015) and Miranda donkeys (3.63/1) (Quaresma *et al*., 2014), and slightly higher for Amiata donkeys (2.90/1) (Cecchi *et al*., 2006).

Inbreeding, coancestry and degree of non-random mating

Inbreeding (F) and (coancestry coefficients) AR were slightly lower in the historical population set than in the current population set, showing an increasing trend over the years. The value of 1.51% for F and of 1.53% for AR in the contrast population showed that the more information about a certain individual was known the greater F was, reaching values over 1%, evidencing a high number of related matings and responding to the appearance of highly inbred animals in the pedigree (Table 2). In clinical genetics, a consanguineous mating is generally defined as a union between two individuals related as second cousins or closer ($F \leqslant 0.0156$). Beyond second cousins matings ($F < 0.0156$) often arise in small isolated populations. Although remote levels of consanguinity seem not to have a major adverse impact on health, they can result in a notable increase in homozygosity, often sought when defining a breed according to a certain standard; either it is morphological, functional or zootechnical. AR increased through the years, indicating breeders mated more related individuals, especially from 2006 ahead, when the census dramatically decreased and AR was more than twice the value of F. Very small values of mean F and AR (0.0029 and 0.0094, respectively) were reported for Amiata and (0.0008 and 0.0033, respectively) Miranda donkeys, which presented a shallow and incomplete pedigree, whereas in the Pêga donkey (0.0285 and 0.0126, respectively), the PCI made such values more reliable. By contrast, greater F and AR coefficients in Catalonian donkeys ($F = 0.0336$ and $AR = 0.0376$) and in the Berga subpopulation ($F = 0.072$ and $AR = 0.066$) were historically reported. The values found in Martina Franca donkeys must be attributed to the lack of genetic management and matings between only a few related animals and were similar to those reported for the Berga subpopulation (6.87% and 9.80%, respectively), in which contrarily they seemed to result from the attempts to obtain a highly selected morphologically homogenous herd (Folch and Jordana, 1998; Cecchi *et al*., 2006; Rizzi *et al*., 2011; Quaresma *et al*., 2014; Santana and Bignardi, 2015). Andalusian donkeys presented a worse endangerment situation as high inbreeding levels and relatedness coefficients were present in a shallow pedigree in the contrast population, showing an uncontrolled increasing tendency, which could get worse in time if measures were not implemented. Inbreeding was lower than coancestry for the population studied almost through all the years (Figure 1), suggesting that matings were not performed intentionally between close relatives and/or mainly within subpopulations, but still the information on the pedigree was inconsistent. Noteworthily, farms were scattered across a vast territory, which made it difficult to set a proper breeding relation with other genetic resources. These estimates were consistent when compared with the degree of non-random mating obtained. More positive values meant that positive assortative mating patterns were generally being adopted to seek particular phenotypic characteristics, although traditionally, no attention had been paid to this as shown by the trends described by this parameter until 2005, when further selection measures started being implemented to fit the existing population to a standard regarding the height and its emblematic grey coat. The rates of inbreeding per generation and the related *Ne* reflected the estimates of AR and *F*. The ΔF found in the contrast population exceeded the recommended maximum ΔF level (1%) and *Ne* (50) to maintain genetic variation and fitness in a population. By contrast, the values obtained historically were $\Delta F = 0.55\%$ and $N_e = 17.81 \pm 8.45$, which meant that the greater the number of new animals with reliable genealogical information added to the pedigree, and subsequently the higher the level of completeness were, the greater the presence of inbred animals revealed to be, therefore, evidencing the underestimation of the ΔF values previously obtained. In view of the observed inbreeding and coancestry differences the effective populations sizes based on these two parameters were also different, being N_eC_i almost twice higher than N_eF_i. Comparing the effective population size based on inbreeding and coancestry, the number of equivalent subpopulations was slightly lower than 3, indicating that the Andalusian donkey population was highly structured. According to Fernández *et al.* (2011), maintaining subdivided populations has the advantage of reducing the risk of extinction because of accidental or health factors as these events would only cause the extinction of a single group. In addition, the maximum long-term genetic diversity of a population is achieved by subdivision into as many separate groups as possible. However, population subdivision can exert a negative effect given each subpopulation will have a smaller effective size and therefore a higher level of inbreeding. The effective population size based on the individual inbreeding rate for Andalusian donkeys (17.81 ± 8.45) was similarly small to the value found for the same in the Pêga donkey (35), but yet it was smaller than for Martina Franca donkeys (Rizzi *et al.*, 2011). Although population structure greatly affects the individual inbreeding increase, it little affects the coancestry increase, therefore N_eC_i is more accurate than N_eF_i to calculate the effective population size (Leroy *et al.*, 2013).

Probabilities of gene origin and ancestral contributions

The ratio between the f_e and the f (f_e/f) obtained in the historical and contrast population sets was 0.31, suggesting the loss of genetic information from two out of three founders. Given the magnitude of f_e and f_e/f, it may be assumed that the frequent use of only a few individuals for breeding led to a loss of genetic variability. This was confirmed by the small number of founders contributing to 50% of genetic variability, 96 for the current population set and 20 for the contrast population set. Founder genome equivalents (*fg*) and the ratio between f_a and f_e, pointed out the unequal contribution of founders as the main cause for the current loss of genetic diversity. In the Pêga (Santana and Bignardi, 2015), Catalonian (Gutiérrez *et al.*, 2005), Miranda (Quaresma *et al.*, 2014) and Amiata (Cecchi *et al.*, 2006) donkeys, f_e value was similar to the value found in the Andalusian donkey (55.62), but higher than that of Martina Franca donkeys (Rizzi *et al.*, 2011), although the decreased level of pedigree completeness could have caused an overestimation of these parameters. The difference between f_e and f_a suggested a decrease in the genetic variation because of the several bottlenecks the breed has historically suffered (especially in the 1990s) and was confirmed by the increase in the number of births after the 1990s. The current loss of genetic variation is confirmed by the values of *fg* (106.06), and the greater decrease in f_e (39.01) found in the current population. The values recorded in the contrast population set (70%) and in the current population set (69%), were almost the same indicating the intensive breeding use of certain individuals. The reduced *fg* in the current population bases on the greater average inbreeding and the smaller number of individuals. Similar bottlenecks were reported in European donkey breeds with a similar degree of genetic diversity loss from the late 1940s to early 1990s, as a result of rural mechanization. The f_e/f_a ratio (0.81 : 0.93) for Andalusian donkeys compares with the values of 0.89 for Miranda (Quaresma *et al.*, 2014) or 0.82 for Martina Franca donkeys (Rizzi *et al.*, 2011), and contrasts the values of 0.38 for Catalonian (Gutiérrez *et al.*, 2005) or 0.37 for Amiata donkeys (Cecchi *et al.*, 2006), possibly addressing for narrower bottlenecks, basing on the misestimation caused because of their low pedigree completeness level, as reported by the difference between f_a/f_e ratio in the current and contrast Andalusian donkey populations. The 0.92 to 0.94 values for Pêga donkeys (Santana and Bignardi, 2015) infer such bottlenecks may not have been so sharp. These results do not only account for founder genotypes misrepresentation and disappearance from local populations, but also highlight a global critical situation affecting the whole species. Bottlenecks neither necessarily lead populations towards extinction, nor free them from becoming extinct even if their effectives are recovered. Bottleneck-based diversity loss is a double-edged sword as deleterious mutations could either be erased and harmless or otherwise, fixed in the population declining them into extinction, as they may produce an increased disease or climate change sensitivity which may remain unnoticed, until it may be too late (Frankham *et al.*, 2010).

Herd relationships and breeding strategy

The minimum Nei's genetic distance, effective population size and F_{ST} statistics evidenced herd based differenced subpopulations, traditionally stemming from the historical interherd breeding trends followed according to which, the origin of the donkeys founding the rest of the herds reduced to three main herds from which the main population founders were distributed, and the latter intraherd breeding policy and tendency to use a few selected animals for breeding, what could resemble the patterns described by other naturally temporarily isolated endangered wild species, such as the Bison (Halbert *et al*., 2012). Breeders should mate animals with relationship coefficients lower than 6%, helping minimize the inbreeding rate and increasing the effective population size, to counteract the risk of extinction.

Conclusions

Genetic diversity loss since the founder generations can be considered small in Andalusian donkeys and similar to breeds with a common unknown genetic background, however, monitoring is always a compulsorily reasonable decision. The typical excessive contribution of few ancestors to the gene pool of small critically endangered populations may lead to narrower bottlenecks in the near future whose hidden effects can only be controlled by tracking the populations. The generation intervals found may be considered an advantage to reduce the inbreeding increase maintaining the existing genetic diversity of donkey breeds. Our major concern falls on the productive sustainability as *in situ* conservation is clearly affected by a rising international demand, increasing feeding costs and a decrease in governmental subsidies as the main contributors to the loss of discarded or exported individuals, whose genealogical information is no longer considered. Tracking back 36 years of genetic history, the shallow level of pedigree completeness does not permit the reliable estimation of genetic variability parameters. However, the trends described by smaller population sets from which a greater level of genetical information is known, help us quantify the virtual inbreeding decrease, and the underrated distortion of genetic diversity loss describing parameters (f_e, f_a and f_g), enabling the counteraction of potential deleterious effects. Part of founders' genetic variability has been lost in the course of the years, and especially, the increased percentage of males and females exhibiting high AR values warn that the threat of extinction still looms over the breed. The considerably to slightly low effective population size may balance the inbreeding depression, approaching the estimated minimum viable effective size for the preservation of endangered species (Meuwissen and Woolliams, 1994). Although inbreeding rate in the current and historical populations was acceptable (under 1%), its value in the contrast population set alarmingly differs from the recommended value (+0.23%) and indicates that the more genealogical information is known, the more endangered the breed reveals it actually is. In conclusion, careful genetic management is necessary to minimize inbreeding practices and enhance genetic variation. Thus, measures such as the use of artificial insemination or embryo vitrification need to be implemented to contain the inbreeding rate and increase the effective population size, assessing the percentage of relationships that reproductive pairs share in each case and selecting individuals for mating when these relationships are kept below a 6% coancestry level.

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Chapter 2

Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; McLean, A.K.; Delgado Bermejo, J.V.

Non-parametric analysis of the noncognitive determinants of response type and response intensity, mood and learning in donkeys

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Abstract

Operant conditioning, Quantitative and Qualitative Behavioural Assessment (QBA) synergism can provide valuable information about animals' extinction/learning and emotional status. 300 donkeys were exposed to six reinforcement treatments to lead them to complete an operant conditioning test. Simultaneously, we studied the effects 15 animal-inherent, environmental location, and test related factors had on response type and intensity, mood and learning ability variables. We aimed at testing three hypotheses using non-parametric categorical analyses. First, we studied which of the 15 noncognitive factors could explain the variability of the 4 behavioural variables and their explanatory power. Second, we assessed the frontal and lateral ear position donkeys displayed when each reinforcement treatment was implemented to study the correlations between ear position and twelve mood QBA categories. Third, we assessed which reinforcement treatment was more suitable to promote donkeys' learning and welfare, studying their success rate at completing the operant conditioning test, and welfare related behavioural signs, respectively. Almost all noncognitive factors significantly affected four variables ($P<0.001$), although some were not linearly correlated. Our results address body language as an efficient tool to report translatable information on donkey's mood. They suggest neutral or luring/positive reinforcement techniques are the most learning-promoting and welfare-friendly methods to educate donkeys.

Keywords	Behaviour; Cognition; Reinforcement; Body Language; Welfare Signs.

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Highlights

- Our problem-solving test is highly sensitive to cognitive performance variations.
- Inherent, environmental and test factors significantly affect donkey cognition.
- Positive reinforcement methods promote welfare friendly learning in donkeys.
- Ear 3D position is a highly significant indicator for behavioural mood in donkeys.

Non-parametric analysis of the noncognitive determinants of response type and response intensity, mood and learning in donkeys

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Abstract

Operant conditioning, Quantitative and Qualitative Behavioural Assessment (QBA) synergism can provide valuable information about animals' extinction/learning and emotional status. 300 donkeys were exposed to six reinforcement treatments to lead them to complete an operant conditioning test. Simultaneously, we studied the effects 15 animal-inherent, environmental location, and test related factors had on response type and intensity, mood and learning ability variables. We aimed at testing three hypotheses using non-parametric categorical analyses. First, we studied which of the 15 noncognitive factors could explain the variability of the 4 behavioural variables and their explanatory power. Second, we assessed the frontal and lateral ear position donkeys displayed when each reinforcement treatment was implemented to study the correlations between ear position and twelve mood QBA categories. Third, we assessed which reinforcement treatment was more suitable to promote donkeys' learning and welfare, studying their success rate at completing the operant conditioning test, and welfare related behavioural signs, respectively. Almost all noncognitive factors significantly affected four variables ($P<0.001$), although some were not linearly correlated. Our results address body language as an efficient tool to report translatable information on donkey's mood. They suggest neutral or luring/positive reinforcement techniques are the most learning-promoting and welfare-friendly methods to educate donkeys.

Keywords

Behaviour; Cognition; Reinforcement; Body Language; Welfare Signs.

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Introduction

The imbalance between superstition and worshipping experimented by donkeys throughout the antiquity (Bough, 2012) not only provided them with their bad etymological connotations (Korostenskienė and Tarnauskaitė, 2015) but also with the general misconception of a difficult temperament. This attribution has parallelly evolved with donkey's social role and has traditionally resulted in the application of unnecessary negative reinforcement techniques and hard mistreatment towards them (Lochi et al., 2014).

Their similarities with horses led to the misconception of the species (Gallion, 2011). Donkeys evolved in a particularly harsh context that modelled their psychology and conduct since domestication. Their tendency to freeze when facing unfamiliar situations characterizes a cautious and intelligent species that needs longer than others to interpret the stimuli around. In addition, their stoic nature, rarely displaying evidence of pain, makes them silently and emotionally suffer from hard treatment more than other species, hence making behavioural assessment become critical for their welfare (Duncan and Hadrill, 2008).

These behavioural particularities are strengthened by a higher ability to use their power and endurance in their benefit, and therefore, to exert a stronger opposite response (Navas et al., 2013) what has often been confused with stubbornness, cooperation reluctance and silliness. Zucca et al. (2011) would suggest that such especial way to spatially interact with the environmental conditions around, their extinction/learning skills (Miltenberger, 2011; VanElzakker et al., 2014; Mackintosh 2018) and thus, the success of specific techniques to implement when educating donkeys may rely on cerebral laterality-based differences when compared to other affine species such as horses.

All these behavioural features suggest that a rather educational approach should be considered when teaching donkeys to develop certain tasks, contrasting the regular training methods applied in horses. Body language becomes then a useful tool when interpreting animal mood or emotions (Ainslie and Ledbetter, 1980). Even more in long-term neglected donkeys or when aiming at counteracting undesirable features, providing us with an efficient communication tool for the fulfilment of any human-related activity. Qualitative Behavioural Assessment (QBA) enables the translation of animal body language signs into human personality and emotional familiar terms to develop models that users can consider when interacting with animals under a welfare framework (Wemelsfelder, 2007). Not only these QBA models lay the basis for studies assessing the suitability of different techniques to educate donkeys, but help quantify their body language signals, attempting to develop a nonverbal owner-donkey bidirectional communication (Minero et al., 2016). Cognitive skills and their correlations with body signals may allow us to quantify the behavioural responses of animals (Paul et al., 2005; Mendl et al., 2009). Furthermore, the application of QBA animal models in human studies could lead to a better understanding of the treatment of human behavioural problems to improve our quality of life (Hausberger et al., 2011; Fureix et al., 2012).

This study aimed at solving three hypotheses using non-parametric categorical statistical analyses. First, while the donkeys were performing the operant conditioning test, we collaterally assessed the effects that 15 noncognitive factors may have on the behavioural variables of response type, response intensity, mood/emotion and extinction/learning ability. This way, we studied which of these noncognitive factors could account for the variation among individuals for such behavioural variables and at what level these variables were influenced. Then, through categorical regression, we issued equations to predict how the combination of the fifteen noncognitive factors could condition the four behavioural variables studied. Second, we registered the ear position that donkeys displayed when the reinforcement treatments were implemented through a QBA model, aiming at studying the correlations between body language and twelve mood categories, as a way to improve donkey-human bidirectional communication and their interaction during field experiences. Third, we assessed which reinforcement treatments were more suitable to promote donkeys' extinction/learning processes, studying the success rate of the donkeys at completing the operant conditioning test to which they were exposed. We studied extinction learning processes (VanElzakker et al., 2014), rather than habituation learning processes (Miltenberger, 2011; Mackintosh 2018) as for the second forms of learning, the donkeys may decrease or cease their responses to each stimulus after repeated or prolonged presentations, not because of the reinforcement event. Simultaneously, we assessed which of the reinforcement treatments made the donkeys display welfare related behavioural signs to study which reinforcement treatments could be considered emotionally-friendly techniques.

Materials and methods

Animal sample

Our study considered direct observations from 78 Andalusian studbook registered entire jacks and 222 jennies (N=300), born from 1990 to 2012. As age range was not normally distributed ($P<0.05$ for both Kolmogorov-Smirnov and Shapiro-Wilk's tests for normality) we used minimum, Q1, median, Q3 and maximum to describe the age range in our sample. Minimum age in the range was 0.27 months, Q1 age was 29.76 months, median age was 77.04 months, Q3 age was 129.07 months and maximum age was 270.40 months. The donkeys in the sample were the progeny of 48 jacks and 113 jennies.

Operant conditioning behavioural test

The operant conditioning behavioural test was carried out in an open area to which the donkeys were previously accustomed (it was part of the area over which the donkeys developed their daily activities). Each animal was exposed to six reinforcement treatments consecutively, one at each of the 6 stages within the operant conditioning test. At each stage, handler A and handler B used each of the 6 different reinforcement treatments to lead the donkeys to cross over an oilcloth laying on the floor. These treatments could comprise unknown elements (the animal had not been familiarized to them) or known elements (to which the animal had already been familiarized). These elements could be visual (elements fell within the visual areas of the donkeys) and/or acoustic (elements generated sounds, i.e., "motivator" or claps, although they may or may not fall within visual areas), and were presented to the donkeys from different positions (from the front or from a rear position always at 2 metres away from the animals). A cameraman (Handler C) simultaneously videotaped the experiences (1080 p, 50 Hz, shutter speed: 1/250 seconds) to assess the donkey's performance after the field experiences and to test for intra-observer discrepancies. Cameraman (Handler C) controlled timing. A detailed description of the operant conditioning test is shown in Figure 1.

Frankl "Mercalli" scale and Qualitative Behavioural Assessment

Qualitative Behaviour Assessment (QBA) evaluates the expressive quality of animal behaviour and emotions. It integrates and summarises the different aspects of an animal's dynamic style of interaction with the environment and the elements in it and can be used in addition to other welfare indicators or classical ethological measures (ethograms) (Wemelsfelder et al., 2000). The use of QBA enables the identification of the main dimensions of mood states (Mendl et al. (2010)) and facilitates bridging the gap that traditionally exists between subjective judgments and scientific measurement approaches (Wemelsfelder, 2007). In this context, some authors (Venham et al., 1980; Riba et al., 2017) have suggested the use of quantitative interval rating scales as the connector between categorical subjective descriptors and objectively measurable elements. Each donkey's mood/emotion when the operant conditioning test was carried out was registered by the same trained judge following the protocols developed by Navas et al. (2017a) which based on Minero et al. (2016) and then ranked in a Frankl "Mercalli" ordinal scale according to the interest presented towards the stimuli presented during the tests. To develop the ordinal scale used here we followed the principles of a Mercalli Intensity Scale used to measure the intensity of an earthquake by observing its effect on people, the environment and the earth's surface. Hence, we measured the intensity of the effect of stimuli by observing donkey's behaviour. Navas et al. (2013) generated the descriptor lists for the use in subsequent studies as the present one. QBA description was extracted from Minero et al. (2016), except for distracted, which was added as a category to describe the mood of those animals in which no attention was paid towards stimuli presented, and curious/cautious/mistrustful which were added by borrowing the concept of the middle point in the Likert scale (Likert, 1932) as in (Riba et al., 2017). The inclusion of those extra categories could be justified as, although they could be ascribed to same QBA descriptor (Calm/at ease), there was a gradually increasing interest towards the stimuli presented. The qualitative behavioural assessment procedure followed in this study and inter-observer and intraobserver reliability techniques applied to ensure the soundness and reliability of the scales used is described in Navas González et al. (2018) following the premises in (Wemelsfelder et al., 2000). A summary of the mood/emotion descriptors used is shown in Table 1.

Information registration

Information on the response type and response intensity, mood/emotional collateral responses and extinction/learning ability from the donkeys was registered during the development of a six-stage operant conditioning test (Figure 1). All the information concerning the 4 behavioural variables and 15 noncognitive factor was registered by the same trained judge for all the stages and animals. No intra-observer discrepancies were appreciated as all the information obtained on field matched that obtained after reviewing the video recordings. The donkeys were each given a maximum of 450 seconds to successfully complete the operant conditioning test (75 seconds per stage and treatment implemented). No additional time was provided for the donkeys to complete the test. The information registered corresponded to the first immediate reaction described by each animal when each of the stages was started. In 75 seconds, an animal can shift attention many times. However, to simplify the observations, our study tested for the first reaction of the animals, further actions implemented through the development of the test were discarded.

The records for each animal consisted of information on 19 categorical variables divided into two sets. The first set of 4 dependent behavioural categorical variables assessed the cognitive performance of donkeys through their response type, response intensity, mood/emotion, and extinction/learning ability. The variables in this first set could possibly be conditioned by a second set of independent variables comprising 15 noncognitive factors. A summary of the variables and categories included in the first variable set is described in Table 2, while a summary of the factors and categories included in the second categorical factor set is shown in Table 3.

Noncognitive categorical factors

Noncognitive categorical factors could be divided into three groups. Environmental location included farm/owner, husbandry system, province, ground type. Test related ones included treatment order, type of elements included, familiarity towards the elements used in each treatment and type of reinforcement treatment implemented. We also registered animal inherent information regarding the sex, age category (in months), sire, dam, inbreeding level (ΔF). The last group of factors comprised body language lateral and frontal ear position. The categories for independent noncognitive factors in the second set are shown in Table 3.

The information was registered during the yearly behaviour assessment sessions carried out on four random days per year, from June to November for three consecutive years from 2013 to 2015 at twenty-two different farms all over Andalusia (southern Spain).

The 22 farms involved, reared their animals under four husbandry systems (extensive, semi extensive, semi intensive and intensive) and were located in 5 Andalusian provinces (Southern Spain). 6% of the donkeys were tested during the breed's Official Morphological Contest held by the Union of Andalusian Donkey Breeders (UGRA). The differences between the categories present in the husbandry system categorical factor are shown in Table 4. Age categories were defined considering the distribution found in the population and the studbook regulations of UGRA. UGRA provided the pedigree file used to compute the inbreeding coefficient of the donkeys in the sample. The levels for inbreeding were set considering the distribution found in the population according to Navas et al. (2017b).

The ear bi-dimensional (frontal and lateral) body language ethogram is summarised in Table 5 and Figures 1 and 2. Table 5 shows the description of the levels in the body language factor, i.e., lateral and frontal ear position. A single global score was given to each position as although both ears can move independently, when they do not direct towards the same direction it may be attributed to the animal paying attention to different elements at the same time, therefore being unrelated to mood expression.

Behavioural categorical variables

The reaction developed by the donkeys when they faced the six consecutive treatments provided information on four behavioural categorical variables (Table 2). To name the mood/emotion variable, we considered the definitions by Cabanac (2002) and Mendl et al. (2010). A description of the different categories in the response type and mood/emotion variables is shown in Table 1. Intensity of response and extinction/learning ability variables were classified comprising five categories each described as shown in Table 2. Animals were sorted according to the intensity at which their responses were displayed from low intensity responses to high intensity responses whatever the mood/emotion displayed by them was (Tables 2 and 3). As animals were only scored once, apparently opposite behaviours were not scored correlatively in the same animal. That is to say, the response of an animal displaying a high intensity calm mood/emotion (very calm animal) was not registered as a low intensity nervous mood/emotion (slightly nervous mood/emotion) simultaneously. The reason for this is the fact that an animal cannot be nervous and calm at the same time whatever it is the intensity level at which such animal expresses its mood/emotion status (see Table 1).

Statistical analysis

Categorical variables represent a qualitative method of scoring data. As all the variables and factors considered in our study were categorical we used nonparametric tests to statistically assess the information recorded. A Chi-square test for independence was used to analyse whether the variables in the first set (Table 2) were randomly and significantly influenced by the factors in the second set (Table 3). Chi square is neutral to the parametric or non-parametric nature of the distribution and is relatively robust to situations with a limited number of data (N>50). The most appropriate statistic to use as a measure of Chi-square association is Cramér’s V. Cramér's V was computed to measure for the strentgth of linear correlation and significance between each variable from the first set with each variable from the second set using the Crosstabs procedure from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016) according to the indications of (Nolan and Heinzen, 2017).

Categorical regression (CATREG) on the data was used to describe how the variables in our study depended on the factors considered. The resulting regression equation could be used to predict behaviour or cognitive abilities for any combination of the 15 independent factors. 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).

Categorical Principal Components Analysis (CATPCA) was used to quantify categorical factors while reducing the dimensionality of the data and Categorical regression to establish the most important descriptive and discriminative noncognitive factors on the variables considered using the Optimal Scaling procedure from the Dimension reduction task from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016).

Justification for Statistical tests

The most appropriate statistic to use as a measure of Chi-square association is Cramér’s V. According to Cohen (1988), when using Cramér’s V small effect associations range from 0.0 to 0.10, medium effect associations from 0.3 to 0.5 and large effect associations from 0.5 to anything above. The same author would recommend that the interpretation of effect size should consider a statistically significant measure ($P<0.05$) with a small effect size or greater to indicate a meaningful difference, especially for behavioural or psychological studies.

Categorical variables can be included as independent variables in a regression analysis or as dependent variables in logistic regression or probit regression but must be converted to quantitative data for us to be able to analyse the data. Ordinary Linear Regression models could only be used when the dependent variable is quantitative and predictive variables are either quantitative or dummy. But in most of the cases, predicting variables from survey data are categorical. In this case, dummy binary variables have to be designed to apply traditional linear regression but the results would be hard for interpretation and impossible for further recalibration. In such situations, Categorical Regression Analysis could be preferred as an alternative modelling method. Categorical Regression Analysis (CATREG) is a non-parametric multiple regression analysis could be implemented when variables are all categorical or both categorical and numeric. CATREG's logic bases on the nonlinear transformation of dependent and independent variables. CATREG is also the name of the program in SPSS that uses the Categorical Regression Analysis algorithm (van der Kooij and Meulman, 1999). 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 system (Gifi, 1990). Determining the quantitative values for the variable categories, alternating least squares (als) iterative prediction method is used. The value determination after optimal scaling can be saved as a new variable set. With the results from CATREG, it is still required to verify the statistical significance of the predictors. Consequently, CATREG is equivalent to an ordinary linear regression when the qualitative predictors are substituted by the transformed (quantified) values (China et al., 2010). In this study, stepwise method used to prevent possible Multicollinearity problem in the linear multiple regression model formed by transformed variables.

As the independent noncognitive categorical factors registered in our study were categorical and the data was sorted into categories following different criteria, we used standardized coefficients to interpret and compare their effects on our behavioural dependent categorical variables. Standardized coefficients simply represent regression results with standard scores. By default, most statistical software, like SPSS, automatically converts both criterion (DV) and predictors (IVs) to Z scores and calculates the regression equation to produce standardized coefficients. When most statisticians refer to standardized coefficients, they refer to the equation in which one converts both DV and IVs to Z scores. In a simple model with two factors involved the coefficients for Z scores for each variable (Z'y) may be interested as follows:

β_1 mean a standard deviation increase in Z_{X1} is predicted to result in a β_1 standard deviation increase in Z'y holding constant Z_{X2}.

β_2 mean a standard deviation increase in Z_{X2} is predicted to result in a β_2 standard deviation increase in Z'y holding constant Z_{X1}.

Summarizing, the standardized partial coefficient represents the amount of change in Zy for a standard deviation change in Zx. So, if X1, one factor involved, was increased by one standard deviation, then one would anticipate a β_1 standard deviation increase in the variable being tested, holding constant the effect of X2 and vice versa.

With Z_{X1} and Z_{X2}, being the Z scores for each factor, and $\beta_{1\ \text{and}}\ \beta_2$ the standard coefficients for each of the, respectively.

As the above example shows, conversion of raw scores to Z scores simply changes the unit of measure for interpretation, the change from raw score units to standard deviation units.

As a rule, we assume standardized results reported used full standardization (both DV and IVs were converted to standard scores), and that the Z formula was used for standardization. The general standardized regression equation may follow the following model $Z'y = \beta_1 Z_{X1} + \beta_2 Z_{X2} + \ldots$, where Z'y is the predicted value of Y in Z scores; β_1 represent the standardized partial regression coefficient for X1; β_2 represent the standardized partial regression coefficient for X2; and Z_{X1} and Z_{X2} are the Z score values for the variables X1 and X2, respectively.

The intercept will always equal 0.00 when standardization is based upon Z scores and both DV and IVs are standardized.

Once the regression equation is standardized, then the partial effect of a given X upon Y, or Zx upon Zy, becomes somewhat easier to interpret because interpretation is in sd units for all predictors.

CATPCA is appropriate for variable selection and dimension reduction in categorical variables as it analyses the interrelationships among a large number of variables and explain these variables in terms of their common underlying dimensions (Hair et al., 1998). The objective is to find a few linear combinations of the variables (factors) that can be used to summarize the data without losing too much information in the process. CATPCA is a nonparametric method that quantifies categorical variables through a process called optimal scaling (Meulman et al., 2004). Optimal scaling uses category quantifications in such a way that as much as possible of the variance in the quantified variables is accounted for. The most important characteristic of CATPCA is that it can handle and discover nonlinear relationships between variables. Because CATPCA directly analyses the data matrix and not the derived correlation matrix, there need not be the usual concern to have at least five times as many observations as the variables. In fact, CATPCA is suited for analysis in which there are more variables than objects (Meulman et al., 2004). In behavioural sciences many of the variables used are qualitative, nominal or ordinal, thus indicating the use of CATPCA, which has been demonstrated to be more robust than PCA when assessing categorical variables (Vilela et al., 2017).

Results

Noncognitive factor analysis

The results from Chi-Square and Cramér's V, testing for the existence of linear correlation are shown in Table 6. Cramér's V effectively measured the strength of colinearility that the noncognitive factors considered have on the behavioural variables studied, given the high significance ($P<0.001$) that they report for most of the factor-variable combinations (Table 6). CATREG was performed to the 15 qualitative independent variables (factors) with the four behavioural categorical variables (response type, mood/emotion, intensity of response and extinction/learning) as dependent variables. Then stepwise linear regression to the data with the resulted quantifications was applied and the summary results with the significant variables are presented in Tables 8 and 9. The standardized coefficients (β) are listed in Table 8. The results from Chi-Square and Cramér's V, testing for the strength of linear correlation compared to the results found for CATREG, testing for factor-variable dependence except for inbreeding level (Supplementary Table 3). CATREG reported all of the independent variables except for sex, ground type and familiarity towards the elements to be significant for response type. Sex, ground type, and treatment type were nonsignificant for mood/emotion. Inbreeding level, ground type, treatment familiarity and reinforcement type were nonsignificant for the response intensity variable. Inbreeding level, ground type, treatment type, familiarity towards the elements, reinforcement type and lateral view of the ears were nonsignificant for extinction learning ability. Generally, dam had around 10% higher repercussion than sire on the variables tested (Supplementary Table 3). In donkeys, according to our results, such mother care may account for from a 2 to 6.1% of the variation in behaviour (Table 6), as reported by Cramér's V and CATREG standardized coefficients.

Our results (Table 4 and Supplementary Table 1) reported widely variable outputs when considering the offspring from a same sire or dam.

Inbreeding reported similar values for Cramér's V (0.120 to 0.240) and CATPCA loadings (0.113 to 0.367 and) for the behavioural variables studied (Supplementary Table 3). The results of CATREG for inbreeding moderately differed from the results of Cramér's V and CATPCA loadings (0.289 to 0.319, with inbreeding being nonsignificant for response intensity). CATREG describes how a variable depends on another, while Cramér's V is a measure of the correlation between two nominal variables where the relationship between the variables is linear in nature, what could account for such differences, as their nature may not be linear.

Cramér's V for sex ranged from 0.123 to 0.180 for response intensity and mood/emotion, respectively, while CATREG Standardized Coefficients (β) ranged from 0.051 to 0.186 for response intensity and extinction/learning, respectively. Absolute values for CATPCA loadings ranged from 0.077 to 0.536.

Cramér's V for age ranged from 0.187 to 0.211 for mood/emotion and response type, respectively. CATREG standardized coefficients ranged from 0.149 to 0.220 for response intensity and response type. Absolute values for the loadings in the CATPCA ranged from 0.066 to 0.702.

Farm/Owner Cramér's V ranged from 0.276 to 0.344 for mood/emotion and response intensity and extinction/learning, respectively, while the same parameter for the husbandry system factor ranged from 0.226 to 0.264 for response intensity and extinction/learning and mood/emotion, respectively. CATREG standardized coefficients (β) for farm/owner factor ranged from 0.472 to 0.601 for response type and mood/emotion, respectively. For husbandry system, the same values were 0.598 to 0.566 for response type and response intensity and extinction/learning. The absolute values for farm/owner CATPCA loadings ranged from 0.196 to 0.835 for and the same parameters for husbandry system range from 0.235 to 0.686.

Cramér's V for the province factor ranged from 0.175 to 0.249 for response type and response intensity and extinction/learning, respectively. CATREG standardized coefficient (β) ranged from 0.143 to 0.598 for extinction/learning and response intensity, respectively. CATPCA loadings absolute values ranged from 0.128 to 0.808.

Cramér's V for ground type ranged from 0.103 to 0.203 for response type and mood emotion, respectively. CATREG standardized coefficients ranged from 0.003 to 0.033 for response intensity and response type, respectively. CATPCA loadings for ground type absolute values ranged from 0.224 to 0.567.

Cramér's V for treatment type ranged from 0.077 to 0.364 for response intensity and mood/emotion respectively, while the same parameter for familiarity ranged from 0.043 to 0.232 for response intensity and extinction/learning, respectively. CATREG standardized coefficients for treatment type ranged from 0.023 to 0.293 for mood/emotion and extinction/learning, respectively, while the same parameters for familiarity ranged from 0.087 to 0.182 for response intensity and extinction/learning, respectively. CATPCA loadings absolute values for treatment type ranged from 0.117 to 0.756 and for familiarity ranged from 0.121 to 0.841. For treatment order, Cramér's V ranged from 0.074 to 0.197 for response intensity and mood/emotion. CATREG standardized coefficients ranged from 0.144 to 0.175 for response type and extinction/learning, respectively. CATPCA loadings absolute values ranged from 0.135 to 0.901.

Cramér's V ranged from 0.110 to 0.225, for the variable referring to the lateral view of the ears. For the same body language variable, the results for CATREG standardized coefficients of lateral vie of the ears ranged from 0.015 to 0.173 for extinction/learning and mood/emotion related variables. CATPCA loadings absolute values ranged from 0.237 to 0.773.

Frontal ear position accounted for a Cramér's V value that ranged from 0.121 to 0.230 for response intensity and a CATREG standardized coefficient ranging from 0.063 to 0.205 for extinction/learning and response intensity, respectively. CATPCA loadings absolute value ranged from 0.233 to 0.71.

Reinforcement type Cramér's V ranged from 0.073 to 0.173 for response intensity and mood/emotion variables. However, reinforcement CATREG standardized coefficients ranged from 0.006 to 0.049 for extinction/learning and mood/emotion, respectively. CATPCA loadings absolute values for reinforcement ranged from 0.185 to 0.756.

The factors affecting the four behavioural variables in order of importance according to the CATREG standardized coefficients (β) are shown in Supplementary Table 2. Since the stepwise method was used, there is no multicollinearity problem. The standardized solution for the regression equations can be found in Table 9.

A Categorical Principal Components Analysis (CATPCA) was applied on the total data set of 15 noncognitive factors with the aim of establishing and interpreting the factors determining the four behavioural variables tested (response type, mood/emotion, intensity of response and extinction/learning) to evaluate for redundancies among them. Two, three and four-dimensional model results are shown in Table 10.

Only 12 of the noncognitive factors studied contributed to the two–dimensional model in a meaningful way, 14 of them meaningfully contributed to the three-dimensional model and 10 of them meaningfully contributed to the four-dimensional model (factor loadings>0.5, Table 11), then the different components (PC1, PC2, PC3 and PC4) were best described by the factors highlighted in bold in Table 11.

The two-dimensional model has an internal consistency coefficient (Cronbach's Alpha) of 0.849 and yields an eigenvalue of 4.812 for the first component, indicating that 32.078% of the variance is accounted by this component (Table 10). For the second component, the internal consistency coefficient is 0.784 with an eigenvalue of 3.729, indicating that its proportion of variance is 24.860%. On the whole, the internal consistency coefficient (Cronbach's Alpha) for the bi-dimensional model was 0.946 and the eigenvalue yielded of 8.541, explaining a total of 56.938% of variability.

The internal consistency coefficients (Cronbach's Alpha), eigenvalues and percentage of variability explained by each of the components of the three and four-dimensional models are shown in Table 10. On the whole, the internal consistency coefficient (Cronbach's Alpha) for the three and four-dimensional models were 0.964 and 0.978, respectively. The eigenvalue yielded for the three and four-dimensional models were of 10.010 and 11.408, respectively, and they explained a total of 66.732% and 76.502% of variability, respectively.

Behavioural variables

Response type

According to Cramér's V, all the factors presented a statistically highly significant ($P<0.001$) effect on the response type except for reinforcement which was still significant ($P<0.05$) so that a linear correlation existed. The strength of such linear correlation and statistical significance of the factors on the response type are shown in Table 6. Total and relative frequencies for hyporeactive, neutral and hyperreactive levels can be found in the Supplementary Table 1.

CATREG results report all effects except for sex, ground type and familiarity towards the element presented had a significant effect on response type (Table 8). All of the coefficients for the factors were positive in the model. This shows that the response type does not depend on the sex of the animals, the ground type on which the test is developed and the familiarity of the donkeys towards the elements being faced.

Mood/emotion

The lowest total frequency was found for cautious donkeys while the highest frequency was reported for calm donkeys. Cramér's V reported all the factors considered had a highly significant effect ($P<0.001$) on mood/emotion highlighting a significant linear correlation. Total and relative frequencies for distracted, depressive, indifferent, calm, awaiting, curious, cautious, mistrustful, surprised, nervous, fearful and rejection levels can be found in the Supplementary Table 1. The statistical significance and strength of the factors on mood (ranging from 16.6% for reinforcement techniques and 52.0% for dam) are shown in Table 6.

CATREG reported the noncognitive factors of sex, ground type and treatment type had a nonsignificant effect on the mood/emotion variable so that the behavioural variables tested do not depend on them, even though there is a linear correlation. All of the coefficients for the factors were positive in the model.

Response intensity

According to Cramér's V, all the factors considered had a highly significant effect (P<0.001) on response intensity. Total and relative frequencies for low, mid-low, mid, mid-high and high intensity can be found in the Supplementary Table 1. Factor statistical significance and strength on the degree or intensity of response are shown in Table 6. CATREG reported Ground type, inbreeding level, reinforcement type and familiarity towards the elements presented to the donkeys had a nonsignificant effect on the response intensity. All of the coefficients for the factors were positive in the model.

Extinction/learning ability

On one hand, all the effects considered except for the familiarity towards the stimulus and reinforcement type applied had a highly significant effect (*P*<0.001) on extinction/learning rate according to Cramér's V. The statistical significance and strength of the effects on extinction/learning rate are shown in Table 6. Total and relative frequencies for the refusal to cross, surface dodging, erratically crossing laterally deviating, doubtful crossing and complete crossing without problems tendencies can be found in the Supplementary Table 1. On the other hand, CATREG reported ground type, reinforcement type, body language lateral and frontal view of the ears, familiarity towards the elements being presented to the donkeys and sex had a nonsignificant effect on extinction/learning. All of the coefficients for the factors were positive in the model.

Model and operant conditioning test behavioural variability explanatory quality

CATREG R squared coefficient obtained ranged from 0.614 to 0.704 for the response type and extinction/learning variables, respectively. In the same way, when CATPCA was implemented, four and three-dimensional models accounted for 76.052% and 66.732% of the total variance of behavioural variables, respectively. These results could compare to those obtained by CATREG. These findings address the fact that two of the components of the study could be summarized into one, with a low loss (9.32%) in the variability explanatory power, what could stem in the fact the response type variable was obtained classifying the levels in the mood/emotion variable, so that response type variable somehow derived from the mood/emotion variable. This percentage of loss is around the same value shown by CATPCA for the explanatory power of the 4th component (10.507%).

Discussion

Our statistical outputs (Tables 7 to 12) suggest that the operant conditioning tests and model designed and used for our study efficiently and successfully enable quantifying the variation in the adaptive and cognitive behavioural response of donkeys (Tables 8 and 11).

While studying our first hypothesis, Chi-Square and Cramér's V highlighted there was a significant linear correlation between factors and variables (Table 6), although the behavioural variables tested were not dependent on some of them as shown in the result section.

The slight to moderate increase in the dam effect strength respecting to the sire's may suggest a greater implication of jennies in the raising up process of donkey foals. Foyer et al. (2016) quantified such maternal behaviour in dogs and reported that different maternal care affected the behaviour and temperament of the puppies later in their adulthood; scoring a higher social and physical engagement and aggression than those brought up by less attentive mothers, what may account for such slight to moderate differences. Dam and Sire were the most highly determinant factors for all the models designed for all the statistical tests considered in this study.

Studies of sire effect on behaviour (Grandin and Deesing, 2013) clearly stated the differences in behaviour of the offspring from different sires in calves and lambs. A sire effect in the response to novel stimuli has been reported in horses as well (Minero et al., 2006). Deesing and Grandin (2007) reported Holstein heifers from certain sires to have higher activity levels, to be more nervous and excitable and to display a greater extinction/learning ability. A dam effect has been widely discussed but rarely studied (Lin et al., 2016) as it has always been taken for granted considering the behavioural transmission existing during mother care.

Inbreeding has largely been reported to influence cognitive abilities in general; still, the influence of inbreeding behaviour has not deeply been studied. Our results suggest that the effect of inbreeding may follow simultaneous recessive and dominant inheritance relationships which may differ depending on the behavioural features considered. Alarmingly inbred animals were more likely display low intensity responses and low extinction/learning rate responses as denoted by the progressive increase in refusal reactions. McMillan et al. (2011) found significantly higher rates of emotional behavioural patterns in dogs for fear (both social and non-social), house-soiling, and compulsive staring; and significantly lower rates of intra or interspecific aggression, trainability, chasing small animals, excitability, and energy, what compares and supports our results. As our results suggest, the multilevel inbreeding derived affection may result in less intelligent animals and therefore much harder to train.

Sex and even sexual status behavioural differences have been reported for jennies, jackstocks and geldings (Duncan and Hadrill, 2008), describing the general patterns found in our study. The effect of gender on memorisation and other cognitive problem solving related components have already been assessed in species such as horses (Wolff and Hausberger, 1996). A higher depressive prevalence was found for women and rat females in literature (Zanier-Gomes et al., 2015), what still may support our results as only jennies displayed depressive signs.

Age is often a highly confounding effect as experience, training and education background may distort the results, making it difficult to quantify the effects of age on basic temperament. Still, some generalizations are made in literature (McDonnell, 1999). For example, a higher curiosity, playfulness and reactivity has been found in young horses when compared to mature horses. In our study, the extinction/learning rate describes two frequency peaks around 1 to 3 years old and from 10 years old on as shown by the relative frequencies reported for such effects (Supplementary Table 1). Scientific studies indicate that younger horses learn quicker than mature horses. They are likely to adapt more readily to changes in physical and social environment too. On the other hand, very old animals are likely to be more sensible, quiet, and even more docile than young or middle-aged horses, what does not differ much from the ones described by the donkeys in our study.

French (1993) would conclude that a donkey's behaviour may be altered by the social system in which it lives but also by its previous experience, not only attributing a general effect to farms and their microenvironmental conditions but to the husbandry and management procedures to which donkeys may have been exposed (Urban-Chmiel, 2016). Our results deepen in the knowledge exposed by such authors, as they suggest those husbandry systems, like semi intensive or semi extensive systems, in which the animals are compelled to face diverse environments or those to which the animal may not be accustomed like contest situations, are likely to make donkeys be less prone to hyperreacting and rather observe and assess the situation around, than those living under extensive or intensive conditions.

The effect of different regions on behavioural traits has already been studied by Hansson (1996) in North and South Scandinavian bank voles. Multivariate analyses revealed two main components of activity and sociability, both with regional variation. Activity components (also including "freezing" behaviour, which could be associated to the kinds of behaviours described by donkeys under stressful or potentially dangerous situations) were chiefly related to age while sociability showed mainly regional variations. Following the same trend, our results report clear intra Western (Sevilla and Cádiz) regional similarities in frequency distribution and inter regional differences when compared to Eastern regions (Granada and Málaga). Cultural heritage differences condition the husbandry practices applied even in very delimited areas (Bostedt and Lundgren, 2010). Our results suggest that these differences may also condition the evolution of the behavioural patterns described by relatively close local fractions of the same population. These fractions may adapt to the particular functions to which they are required while being strongly influenced by the conditions that can occur in widely environmentally diverse extensive areas. Córdoba described average frequencies for all the variables and levels scored, so may be the key behavioural pattern to consider when comparing the individuals of the breed, in fact, the previous naming of the breed was traditionally addressed as Cordobesian or Lucentinan donkey, what may account for such relevance.

Equid hooves play an essential role in modulating their behaviour as it has been reported by Urban-Chmiel (2016). In particular, their especially quite sensitive bottom part makes horses and donkeys recognise soil types and find suitable paths to walk along, to which they react with different adaptive or mood specific responses, as supported by our results. For instance, those ground types resembling those in which donkeys naturally evolved tend to ease and neutralise animal responses.

When the stimulus was unknown and presented for the first time, the frequencies for neutral and calm responses considerably increased and the frequency for hyperreactive (fearful) responses considerably decreased. This may be because of the generally described trend in donkeys to freeze not flee (unlike horses) and analyse a potentially dangerous situation, which may derive in a more neutral response development when exposed to unknown stimuli (Gallion, 2011). However, when only a visual stimulus was presented, rejective attitudes were more frequently displayed than when a rear acoustic stimulus was presented. Behavioural responses have been reported to depend on the kind of stimulus presented and the visual and auditory capabilities of the individuals (Christensen et al., 2005). From a biological perspective, visual and acoustic stimulus are especially relevant in precocial species such as equids, as these senses may be primary immediate predator detection elements to enable scape when facing potentially harmful situations, which in horses are implemented through behavioural changes. The responses to the visual and the auditory stimulus probably reflect the fact that in this test, the donkeys were able to localise the stimulus, what induced avoidance behaviours (frozen, fear or rejective moods), as reported for horses.

To assess our second hypothesis, we registered the ear position that donkeys displayed when the reinforcement treatments were presented for the first time with a QBA model, aiming at studying the correlations between body language and twelve mood categories, as a way to improve donkey-human bidirectional communication and their interaction during field experiences.

For the assessment of the second hypothesis, we studied the relationship between body language related factors and the behavioural variables in our study with Chi-Square and Cramér's V, CATPCA (Categorical Principal Component Analysis) and CATREG (Categorical regression). Mood/emotion, response type and response intensity showed a moderate-high dependence on ear position (0.173 and 0.195, for lateral and frontal view of the ears, respectively) and a moderate linear correlation (22.5% and 23.0%, for lateral and frontal view of the ears, respectively). When assessing body language in the donkey species, ear position achieves a remarkable importance as it is one of their most mobile and expressive body parts (Navas et al., 2016) and which therefore can report very interesting information that can be used to assess donkey welfare (Geiger and Hovorka, 2015). Although several studies have successfully considered ear position as a donkey welfare assessment tool, our study is the first attempt to describe the 3D ear spatial configuration from a lateral and frontal view and its translation to response type, mood or intensity and correlations with extinction/learning.

The results of this research match the description of ear language signals involved in a part of the extensive behavioural repertoire described in previous studies. The decision about starting a thorough assessment of ear movement over the rest of the parts of the body reporting behavioural information was taken after Regan et al. (2014) reported the strong evidence for the robustness of these signs over time appointing the suitability for their use in donkey ethogram mood translation. Other studies (Geiger and Hovorka, 2015) comparatively correlated tense backwards or sideward ears with unresponsiveness, avoidance, and disinterest (what we considered hyporeactive responses), while those exhibiting a happy curiosity or interest demeanour presented sideward or forward relaxed or neck relaxed ears. Similarly, Minero et al. (2016) reported distressed, agitated, responsive, playful seem to be aligned with laid back ears, an apparently contradictory finding, but which supports our results. The ethogram and ear position description in Figure 2 and Table 5 can help us to assess which is the mood of a certain animal relying on the body signs it displays.

Last but not least, for our third hypothesis, we assessed which reinforcement treatments were more suitable to promote the donkeys' extinction/learning processes, studying the success rate of the donkeys at completing the operant conditioning test to which they were exposed. Simultaneously, we assessed which of the reinforcement treatments made the donkeys display welfare related behavioural signs.

Luring/Positive reinforcement techniques tended to trigger and promote mistrustful responses what may rely on the time the animals use to assess the situation which is presented and as the techniques applied may not be very pressing. The frequency for awaiting donkeys slightly increased when using negative reinforcement techniques. The impossibility of the animals to see a stimulus located in one of their rear blind spots (Navas et al., 2016), may result in an increase in the frequencies of fearful or rejective responses, while the possibility of the donkey to see the stimulus promoted neutral and calm responses as denoted by the higher frequency obtained.

The frequency for donkeys presenting refusal to cross attitudes and low intensity responses progressively increased with the consecution of the different test phases from 1 to 6. This trend inverted for complete successful crossing and high intensity responses whose frequency progressively increased. Consequently, although negative reinforcement techniques could state to be more successful to compel donkeys to complete a certain task, their effect on mood and the presentation of fearful attitudes may highlight welfare issues in the consecution of such techniques. Positive reinforcement techniques reported the highest frequency of neutral responses while the frequency of hyperreactive responses increased when applying negative reinforcement techniques. This supports the results obtained by Innes and McBride (2008) who reported significant differences suggesting that animals trained through positive reinforcement were more motivated to participate in the training sessions and exhibited more exploratory or 'trial and error' type behaviours in novel situations.

Conclusions

Location, management and farm characteristics and test related factors can condition the responses, their intensity, the mood and extinction/learning ability of donkeys. However, there is still a remarkable inherent component modulated by age, sex or parental background. The multistage operant conditioning test applied enables efficiently and significantly quantifying several factors related to donkey cognition and behaviour. The ethogram that we describe faces the popular knowledge on how different body language signals report a certain donkey's feelings and stablishing a formal description of the collateral signs that donkeys regularly display when describing certain mood or temperament patterns could be the key to the early diagnoses and treatment of potentially life-threatening conditions. Furthermore, The application of luring/positive reinforcement techniques overcomes the cognitive results obtained by negative reinforcement as they do not only allow the animals to accomplish certain tasks but also let them do it in a closer to natural way, generating less welfare related problems, which given the endangerment risk to which the donkey species is exposed may be vital for the preservation of new functional niches promoting the reintroduction of these valuable animals back to their relevant role in human practices.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

Welfare declaration

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. 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. 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.

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Table 1. Description for the mood and response type behavioral categorical variables and "Mercalli" scales.

Scale	Mood/Attitude	QBA definitions[d]	Scale	Response type	Attitude towards the element presented
1	Distracted Agitated	Restless, an animal can stand still and be agitated, fidgety, worried or upset, excited, disturbed, troubled[e].	1	Hyporeactive	Unable to concentrate, draws attention away from the primary taskPays attention[a] and moves towards other elements around, without paying attention to the elements[c] presented in the test.
2	Dejected Depressed[b] Withdrawn	Secluded or remote, shy, not searching for contact with others.	1	Hyporeactive	Overall, body posture shows lowered head and neck, roundness to spine and tucked tail. It does not pay attention to any elements around.
3	Indifferent Nonresponsive Apathetic	Having or showing little or no emotion; indifferent.	1	Hyporeactive	Normal posture. Pays no attention to the element presented, but it is not distracted by other elements around.
4	Calm At ease	In a relaxed attitude or frame of mind.	2	Neutral	Does not get startled. Stands still. Pays attention to other elements around at the same time that it pays attention to the element presented.
5	Awaiting Responsive	Receptive, aware of the environment.	2	Neutral	Does not get startled. Stands still. Only focuses on the element presented.
6	Curious	Eager to learn, inquisitive, wishing to investigate but stands still.	2	Neutral	Does not get startled. Stands still. Only focuses on the element presented. Moves its head towards the element presented.
7	Cautious	Eager to learn, inquisitive, wishing to investigate but approaches less than 1 m.	2	Neutral	Does not get startled. Pays attention and moves slightly towards the element (less than 1 m).
8	Mistrustful	Eager to learn, inquisitive, wishing to investigate, approaches completely.	2	Neutral	Does not get startled. Pays attention to and moves towards the element until approaching it completely.
9	Surprised Agitated	Restless, an animal can stand still and be agitated, fidgety, worried or upset, excited, disturbed, troubled.	3	Hyperreactive	Only focused on the element being presented. Gets startled but moves towards the element.
10	Nervous Anxious	Worried/tense, troubled, apprehensive, distressed.	3	Hyperreactive	Only focused on the element being presented. Gets startled and tries to move away from the element presented at first. Able to move towards the element presented if led by the operator.
11	Fearful	Having fear, afraid, even not linked with something going on in the environment, flight response, look anxious, back up/away, not move further.	3	Hyperreactive	Gets startled. Only focused on the element being presented. Tries to move away from the element presented. Unable to move towards the element presented if led by the operator.
12	Rejection Distressed	Much troubled, upset, afflicted, panicking.	3	Hyperreactive	Only focused on the element being presented. Gets startled and moves away from the element presented noticeably. Pulls away from the leading rope when the operator tries to move towards the element presented.

[a] By paying attention we mean that the donkey held direct visual contact with and/or directed its ear/s towards the element being presented. [b] All the animals displaying a dejected/depressed status had been born after the last third of their gestation had taken place during the cold wave occurring in Spain in 2005. Studies in rats have reported that the pregnancies of mothers who had been exposed to extreme cold conditions presented a resulting offspring at increased risk to experience future developmental, learning and emotional disorders. [c] Elements presented in the test are described in Table 2. Accessed from Navas et al. (2017a). [d] QBA description was extracted from Minero et al. (2016), except for distracted, which was added as a category to describe the mood of those animals in which no attention was paid towards stimuli presented, and curious/cautious/mistrustful which were added by borrowing the concept of the middle point in the Likert scale. The inclusion of those extra categories could be justified as, although they could be ascribed to same QBA descriptor (Calm/at ease), there was a gradually increasing interest towards the stimuli presented.[e]Merriam-Webster, 2019 (https://www.merriam-webster.com/dictionary/distracted#synonyms).

Table 2. Category description for response type, intensity of response, mood/emotion, and extinction/learning variables directly controlled during the operant conditioning test.

Behavioral categorical variables	Categories
Type of response	Hyporeactive, neutral, hyperreactive (Table 1).
Intensity of response	Low, mid-low, mid, mid-high, high.
Mood/emotion	Distracted, dejected/depressed, indifferent/unresponsive, calm, awaiting, curious, cautious, mistrustful, surprised, nervous, fearful, rejective (Table 1).
Extinction/learning	Stops and refuses to cross, dodges the surface, erratically crosses laterally deviating if compelled to do it, crosses but shows doubt signs, crosses completely without problems.

Table 3. Categories, description and levels for environmental location, test properties, inherent characteristics and body language noncognitive factors.

Cluster	Factor	Level
Environmental location	Farm/Owner	22 farms/owners
	Husbandry systems	Extensive, semi extensive, semi intensive, Official Morphological contest, intensive
	Province	Córdoba, Sevilla, Granada, Málaga, Cádiz
	Ground type	Concrete, soil
Test	Stimulus order	1, 2, 3, 4, 5, 6
	Type of stimulus	Frontal (visual), Frontal (visual) and rear (acoustic)
	Familiarity	Known, Unknown stimulus
	Reinforcement	Negative, Neutral, Positive/Luring
Animal inherent characteristics	Sex	Jack or jenny
	Age (in months)	<3 months, 3 months to 1 year, 1-3 years, 3-5 years 5 to 10 years, 10 more or older
	Sire	48 jackstocks
	Dam	113 jennies
	Inbreeding (ΔF)	Good/acceptable ($\Delta F<0.06$), admissible ($\Delta F=0.07-0.13$), alarming ($\Delta F>0.13$)
Body language	Lateral ear position	1-5 (From front to rear) see Figures 1 and 2, and Table 5
	Frontal ear position	1-5 (From front to rear) see Figures 1 and 2, and Table 5

Table 4. Description of the categories included in the husbandry system categorical factor.

Husbandry system	Live in reduced space facilities	Live in wider extension territories	Minimum punctual handling (sanitary inspection and stud book inclusion)	Daily human contact and regular handling	Donkey is familiar with the owners' requests	Unknown conditions for the animal
Intensive	X			X	X	
Semi-intensive		X		X	X	
Semi-extensive		X			X	
Contest					X	X
Extensive		X	X			

Table 5. Lateral and frontal ear position category description.

Frontal view	Lateral view
Ears forwards (90º)	Ears sideward and forwards (90º)
Ears describe a 45º imaginary line forwards (Figure 3)	Ears describe a 45º imaginary line fore and sideward (Figure 3)
Ears erected (90º)	Ears sideward (90º)
Ears describe a 45º imaginary line backwards (Figure 3)	Ears describe a 45º imaginary line back and sideward (Figure 3)
Ears backwards (90º)	Ears sideward and backwards (90º)

Table 6. Statistical significance and strength of different factors on the behavioural and cognitive variables tested in donkeys.

Cluster	Variable	N	Response type			Mood/emotion			Response intensity			Extinction/learning		
			χ^2	p-value	Cramer's V	χ^2	p-value	Cramer's V	χ^2	p-value	Cramer's V	χ^2	p-value	Cramer's V
Environmental Location	Farm/Owner	1800	402.658	<0.001**	0.334	1511.221	<0.001**	0.276	853.981	<0.001**	0.344	853.981	<0.001**	0.344
	Husbandry systems	1800	215.879	<0.001**	0.245	501.908	<0.001**	0.264	367.974	<0.001**	0.226	367.974	<0.001**	0.226
	Province	1800	110.715	<0.001**	0.175	423.146	<0.001**	0.242	445.251	<0.001**	0.249	445.251	<0.001**	0.249
	Ground type	1800	19.172	<0.001**	0.103	73.994	<0.001**	0.203	35.010	<0.001**	0.139	35.010	<0.001**	0.139
Test	Treatment order	1800	118.456	<0.001**	0.181	347.658	<0.001**	0.197	39.677	<0.001**	0.074	142.564	<0.001**	0.141
	Treatment Type	1800	90.714	<0.001**	0.224	238.731	<0.001**	0.364	10.589	<0.05*	0.077	50.993	<0.001**	0.168
	Familiarity	1800	60.768	<0.001**	0.184	174.508	<0.001**	0.311	3.274	0.513	0.043	97.287	<0.001**	0.232
	Reinforcement type	1800	38.677	<0.001**	0.104	107.513	<0.001**	0.173	19.002	<0.001**	0.073	75.021	<0.001**	0.144
Animal	Sex	1800	32.408	<0.001**	0.134	58.630	<0.001**	0.180	27.266	<0.001**	0.123	27.266	<0.001**	0.123
	Age (in moths)	1800	160.452	<0.001**	0.211	314.841	<0.001**	0.187	256.926	<0.001**	0.189	256.926	<0.001**	0.189
	Sire	1056	528.978	<0.001**	0.500	1903.740	<0.001**	0.425	711.272	<0.001**	0.410	711.272	<0.001**	0.410
	Dam	1050	662.010	<0.001**	0.561	2840.950	<0.001**	0.520	1303.051	<0.001**	0.557	1303.051	<0.001**	0.557
	Inbreeding	1800	52.125	<0.001**	0.120	207.838	<0.001**	0.240	55.078	<0.001**	0.124	55.078	<0.001**	0.124
Body language	Lateral ear position	1800	80.372	<0.001**	0.149	353.729	<0.001**	0.225	86.916	<0.001**	0.110	185.225	<0.001**	0.160
	Frontal ear position	1800	57.565	<0.001**	0.126	379.452	<0.001**	0.230	105.135	<0.001**	0.121	219.158	<0.001**	0.174

P < 0.001**: highly significant; *P* < 0.05*: significant.

Table 7. Model summary of stepwise linear regression with transformed variables.

Variable	R	R Square	Adjusted R Square	Sig.
Response type	0.783	0.614	0.561	0.000
Mood/emotion	0.828	0.685	0.642	0.000
Intensity of response	0.808	0.653	0.606	0.000
Extinction/learning	0.839	0.704	0.664	0.000

Table 8. Standardized Coefficients and significance of CATREG model.

Variable	Response type		Mood/emotion		Response intensity		Extinction/learning	
Parameter / Factor	Standardized Coefficients (β)	Significance	Standardized Coefficients (β)	Significance	Standardized Coefficients (β)	Significance	Standardized Coefficients (β)	Significance
Sire	0.692	0	0.586	0	0.768	0	0.769	0
Dam	0.917	0	0.919	0	0.667	0	0.669	0
Inbreeding level	0.307	0	0.289	0	0.047	0.212	0.051	0.135
Sex	0.059	0.135	0.052	0.124	0.186	0	0.182	0
Farm/Owner	0.545	0	0.601	0	0.472	0	0.473	0
Province	0.294	0	0.330	0	0.598	0	0.598	0
Husbandry System	0.566	0	0.594	0	0.320	0	0.319	0
Ground Type	0.033	0.321	0.018	0.553	0.003	0.931	0.004	0.916
Treatment Order	0.144	0	0.152	0	0.175	0	0.293	0
Treatment Type	0.148	0	0.023	0.344	0.137	0	0.015	0.490
Familiarity	0.109	0.052	0.13	0.023	0.087	0.095	0.058	0.182
Reinforcement type	0.046	0.013	0.049	0.009	0.031	0.140	0.006	0.858
Frontal view of Ears	0.112	0.023	0.195	0.003	0.205	0	0.143	0.008
Lateral view of Ears	0.116	0.017	0.173	0.007	0.124	0.024	0.063	0.276
Age (in months)	0.220	0	0.189	0	0.149	0	0.150	0

Table 9. Regression equations for the behavioural variables assessed.

	Regression equation	Legend
General model	$Z'y_{tmil} = \beta_{Sire}Z_{Sire} + \beta_{Dam}Z_{Dam} + \beta_{Inbreeding}Z_{Inbreeding} + \beta_{Sex}Z_{Sex} + \beta_{Farm}Z_{Farm} + \beta_{Province}Z_{Province} + \beta_{System}Z_{System} + \beta_{Ground}Z_{Ground} + \beta_{Order}Z_{Order} + \beta_{Type}Z_{Type} + \beta_{Familiarity}Z_{Familiarity} + \beta_{Reinforcement}Z_{Reinforcement} + \beta_{FrontalEars}Z_{FrontalEars} + \beta_{LateralEars}Z_{LateralEars} + \beta_{Age}Z_{Age}$	$Z'y_{tmil}$= Z score for each behavioural categorical variable (Response type, response intensity, mood/emotion and extinction/learning). β=standardized coefficient for each of the noncognitive categorical factors appearing in the subindex. Z=Z score for each of the noncognitive categorical factors appearing in the subindex.
Behavioural Variables	Regression equation	Legend
Response type	$Z'y_t = 0.692(Z_{Sire}) + 0.917(Z_{Dam}) + 0.307(Z_{Inbreeding}) + 0.545(Z_{Farm}) + 0.294(Z_{Province}) + 0.566(Z_{System}) + 0.144(Z_{Order}) + 0.148(Z_{Type}) + 0.046(Z_{Reinforcement}) + 0.112(Z_{FrontalEars}) + 0.116(Z_{LateralEars}) + 0.220(Z_{Age})$	$Z'y_t$= Z score for response type variable. $\beta_{Sire}Z_{Sire}=0.692(Z_{Sire})$ $\beta_{Dam}Z_{Dam}=0.917(Z_{Dam})$ $\beta_{Inbreeding}Z_{Inbreeding}=0.307(Z_{Inbreeding})$ $\beta_{Farm}Z_{Farm}=0.545(Z_{Farm})$ $\beta_{Province}Z_{Province}=0.294(Z_{Province})$ $\beta_{System}Z_{System}=0.566(Z_{System})$ $\beta_{Order}Z_{Order}=0.144(Z_{Order})$ $\beta_{Type}Z_{Type} =0.148(Z_{Type})$ $\beta_{Reinforcement}Z_{Reinforcement}=0.046(Z_{Reinforcement})$ $\beta_{FrontalEars}Z_{FrontalEars}=0.112(Z_{FrontalEars})$ $\beta_{LateralEars}Z_{LateralEars}=0.116(Z_{LateralEars})$ $\beta_{Age}Z_{Age}=0.220(Z_{Age})$
Mood/Emotion	$Z'y_m = 0.586(Z_{Sire}) + 0.919(Z_{Dam}) + 0.289(Z_{Inbreeding}) + 0.601(Z_{Farm}) + 0.330(Z_{Province}) + 0.594(Z_{System}) + 0.152(Z_{Order}) + 0.130(Z_{Familiarity}) + 0.049(Z_{Reinforcement}) + 0.195(Z_{FrontalEars}) + 0.173(Z_{LateralEars}) + 0.189(Z_{Age})$	$Z'y_m$= Z score for the mood/emotion variable. $\beta_{Sire}Z_{Sire}=0.586(Z_{Sire})$ $\beta_{Dam}Z_{Dam}=0.919(Z_{Dam})$ $\beta_{Inbreeding}Z_{Inbreeding}=0.289(Z_{Inbreeding})$ $\beta_{Farm}Z_{Farm}=0.601(Z_{Farm})$ $\beta_{Province}Z_{Province}=0.330(Z_{Province})$ $\beta_{System}Z_{System}=0.594(Z_{System})$ $\beta_{Order}Z_{Order}=0.152(Z_{Order})$ $\beta_{Familiarity}Z_{Familiarity}=0.130(Z_{Familiarity})$ $\beta_{Reinforcement}Z_{Reinforcement}=0.049(Z_{Reinforcement})$ $\beta_{FrontalEars}Z_{FrontalEars}=0.195(Z_{FrontalEars})$ $\beta_{LateralEars}Z_{LateralEars}=0.173(Z_{LateralEars})$ $\beta_{Age}Z_{Age}=0.189(Z_{Age})$
Response intensity	$Z'y_i = 0.768(Z_{Sire}) + 0.667(Z_{Dam}) + 0.186(Z_{Sex}) + 0.472(Z_{Farm}) + 0.598(Z_{Province}) + 0.320(Z_{System}) + 0.175(Z_{Order}) + 0.137(Z_{Type}) + 0.205(Z_{FrontalEars}) + + 0.149(Z_{Age})$	$Z'y_i$= Z score for the response intensity variable. $\beta_{Sire}Z_{Sire}=0.768(Z_{Sire})$ $\beta_{Dam}Z_{Dam}=0.667(Z_{Dam})$ $\beta_{Sex}Z_{Sex}=0.186(Z_{Sex})$ $\beta_{Farm}Z_{Farm}=0.472(Z_{Farm})$ $\beta_{Province}Z_{Province}=0.598(Z_{Province})$ $\beta_{System}Z_{System}=0.320(Z_{System})$ $\beta_{Order}Z_{Order}=0.175(Z_{Order})$ $\beta_{Type}Z_{Type} =0.137(Z_{Type})$ $\beta_{Familiarity}Z_{Familiarity}=0.087(Z_{Familiarity})$ $\beta_{Reinforcement}Z_{Reinforcement}=0.031(Z_{Reinforcement})$ $\beta_{FrontalEars}Z_{FrontalEars}=0.205(Z_{FrontalEars})$ $\beta_{LateralEars}Z_{LateralEars}=0.124(Z_{LateralEars})$ $\beta_{Age}Z_{Age}=0.149(Z_{Age})$
Extinction/learning ability	$Z'y_l = 0.769(Z_{Sire}) + 0.669(Z_{Dam}) + 0.051(Z_{Inbreeding}) + 0.182(Z_{Sex}) + 0.473(Z_{Farm}) + 0.598(Z_{Province}) + 0.319(Z_{System}) + 0.293(Z_{Order}) + 0.143(Z_{FrontalEars}) + 0.150(Z_{Age})$	Z'yl= Z score for the extinction/learning ability variable. $\beta_{Sire}Z_{Sire}=0.769(Z_{Sire})$ $\beta_{Dam}Z_{Dam}=0.669(Z_{Dam})$ $\beta_{Sex}Z_{Sex}=0.182(Z_{Sex})$ $\beta_{Farm}Z_{Farm}=0.473(Z_{Farm})$ $\beta_{Province}Z_{Province}=0.598(Z_{Province})$ $\beta_{System}Z_{System}=0.319(Z_{System})$ $\beta_{Order}Z_{Order}=0.293(Z_{Order})$ $\beta_{FrontalEars}Z_{FrontalEars}=0.143(Z_{FrontalEars})$ $\beta_{AgeZAge}=0.150(Z_{Age})$

Table 10. CATPCA model summary.

Dimension	Cronbach's Alpha	Total (Eigenvalue)	% of Variance	Dimension	Cronbach's Alpha	Total (Eigenvalue)	% of Variance	Dimension	Cronbach's Alpha	Total (Eigenvalue)	% of Variance
1	0.798	3.924	26.157	1	0.839	4.64	30.931	1	0.849	4.812	32.078
2	0.757	3.406	22.705	2	0.784	3.73	24.867	2	0.784	3.729	24.860
3	0.644	2.502	16.682	3	0.424	1.64	10.935				
4	0.392	1.576	10.507								
Total	0.978[a]	11.408	76.052	Total	0.964[a]	10.01	66.732	Total	0.946[a]	8.541	56.938

[a]Total Cronbach's Alpha is based on the total Eigenvalue.

Table 11. CATPCA Component Loadings.

	Dimension			Dimension				Dimension			
Noncognitive factors	1	2	Noncognitive factors	1	2	3	Noncognitive factors	1	2	3	4
Farm/ Owner	**-0.958**	-0.12	Farm/ Owner	**0.962**	-0.083	0.06	Farm/ Owner	**0.835**	-0.332	-0.308	-0.196
Dam	**0.928**	0.12	Dam	**0.927**	-0.103	-0.169	Province	**0.808**	-0.32	-0.231	-0.128
Sire	**0.883**	0.126	Province	**0.903**	-0.075	0.029	Husbandry system	**0.686**	-0.271	-0.363	-0.235
Province	**-0.871**	-0.109	Sire	**0.882**	-0.11	-0.221	Ground type	**0.567**	-0.224	-0.483	-0.32
Husbandry system	**-0.834**	-0.119	Husbandry system	**0.813**	-0.072	0.123	Treatment Order	0.36	0.901	0.135	-0.202
Ground type	**-0.716**	-0.098	Ground type	**0.661**	-0.042	0.354	Familiarity	0.341	0.841	0.121	-0.16
Age (in months)	-0.336	-0.037	Treatment Order	0.083	**0.95**	-0.228	Reinforcement type	-0.299	-0.756	-0.117	0.185
Inbreeding	0.142	0.024	Familiarity	0.078	**0.889**	-0.203	Treatment Type	0.299	0.756	0.117	-0.185
Treatment Order	-0.111	**0.951**	Treatment Type	0.072	**0.813**	-0.169	Age (in months)	-0.066	0.104	**-0.702**	-0.126
Familiarity	-0.104	**0.888**	Reinforcement type	-0.066	**-0.777**	0.23	Sire	**0.558**	-0.322	**0.696**	0.028
Treatment Type	-0.098	**0.818**	Frontal view of ears	0.112	**0.622**	**0.524**	Dam	**0.632**	-0.345	**0.647**	0.022
Reinforcement type	0.087	**-0.771**	Lateral view of ears	0.208	**0.577**	0.499	Sex	-0.212	0.171	**-0.536**	0.077
Frontal view of ears	-0.195	**0.607**	Age (in months)	0.024	0.062	**0.590**	Inbreeding	0.252	-0.141	0.367	0.113
Lateral view of ears	-0.273	**0.565**	Sex	-0.184	0.074	**0.552**	Lateral view of ears	**0.519**	0.243	-0.237	**0.773**
Sex	0.029	0.046	Inbreeding	0.222	-0.029	-0.257	Frontal view of ears	**0.514**	0.26	-0.233	**0.771**
Numbers in bold highlight meaningfully contributing factors to each model (>│0.5│)											

Figure 1. Operant conditioning behavioral test to assess for the thirteen cognitive processes in the study.

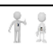
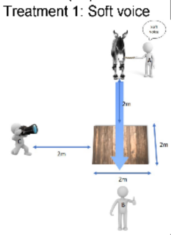
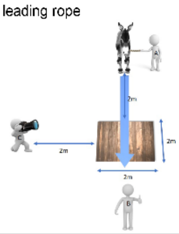
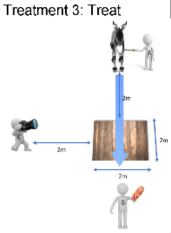
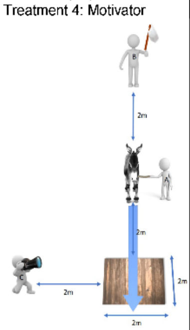
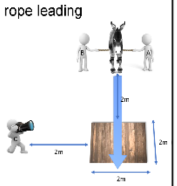
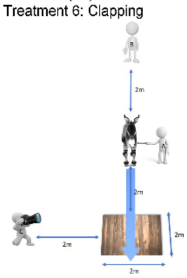
Time per stage/treatment presentation	75 seconds per stage/treatment presentation. The application of the reinforcement treatments that Handler A, Handler B or both implemented to lead the donkey across the oilcloth lasted for the whole 75 seconds. These treatments were applied to check the response of the animals to the different types of reinforcement. No additional time was supplied for the donkeys to complete the stages, so that, once the 75 seconds, provided to the donkeys to interact with the elements presented, had expired, the following stage started and the next treatment was implemented.		
Test duration	450 seconds.		
Test stages	1 to 6. Each stage corresponded to the implementation of each of the six reinforcement treatments.		
Previous considerations	▪ The oilcloth was the element (obstacle) that the donkeys were led to cross over. No donkey had been in contact with the oilcloth previous to the test. Handlers A and B, used 6 reinforcement treatments to lead the donkeys cross over such obstacle. ▪ The donkeys were accustomed to the area in which the test took place as it was an open area on which the donkeys used to carry out their daily activities. ▪ The donkeys that were taking the test were not present while the oilcloth was being laid on the floor for the first time. The donkeys were assessed one at a time, so no additional donkey was present while the test was taking place. ▪ The test started when Handler B raised the oilcloth and relayed it again on the floor in front of the donkey being tested. This action only took place 1 minute before stage 1 (before the 1st treatment was implemented) and was not repeated further in the test. Cameraman started controlling time after the oilcloth had been relayed, when Handler A gave the first step forward towards the oilcloth. ▪ Frontal and visual elements fell within the visual scope of the donkeys, while we considered rear elements those that fell into a blind area. Acoustic elements could be frontal or rear and emitted sounds. ▪ Reinforcement treatments comprised different elements. Known elements were those which had already been presented to the donkeys at any point in their lives (relying on owner's information), while unknown elements were those to which, according to the owner, the donkeys were not acquainted. ▪ All the reinforcement treatments were implemented sequentially and consecutively from stage 1 to 6, one after another, without any stop between each of them, whether the donkey had completed each stage (crossed the obstacle) completely or not (avoided it). That is to say, the fact that an animal crossed/avoided the oilcloth completely in one of the treatments from 1 to 6, did not prevent the rest of treatments from being implemented.		
Legend		Donkey being tested	
		2x2 m oilcloth with a wooden print	
		Rope leader/Handler A	
		Handler B/Lurer (in Stage 4)/2nd Rope leader (in Stage 5)/Clapper (in Stage 6)	
		Cameraman (C)/Time controller	
		Treat (bread, carrots, feed or sugar lumps). Carried by Handler B.	
		Motivator. Plastic bag attached to a wooden stick. Carried by Handler B.	
STAGE 1 (S1) Treatment 1: Soft voice 	▪ Oilcloth presented to the donkey for the first time *(Frontal unknown element)*. ▪ The donkey is given 75 seconds to complete Stage 1, that is to cross over the oilcloth. ▪ Using a lead rope and soft voice, Handler A tried to comfort the donkey to make it cross the oilcloth on the floor, but without pulling from the rope if the donkey refused to move *(Neutral reinforcement)*.	STAGE 2 (S2) Treatment 2: Pressure to leading rope 	▪ Donkey had already had contact with the oilcloth in Stage 1 *(Frontal known element)*. ▪ Using a lead rope with applied pressure to make the donkey cross over the oilcloth. Handler A released the pressure when the donkey moved to cross the oilcloth *(Negative reinforcement)*.
STAGE 3 (S3) Treatment 3: Treat 	▪ Donkey had already had contact with the oilcloth in Stage 1 and 2 and was familiar to the treat given *(Frontal known elements)*. ▪ Handler B offered a familiar treat to lead the donkey to cross over the oilcloth (the treat offered depended on the owner's tastes and therefore the animals were familiar to it. Handler B used the treat that the owner of each donkey normally offered them to tease them. All animals did not accept any other treat that had not been offered to them by their owners previous to the test, as the field experiences reported) *(Positive reinforcement/Luring)*	STAGE 4 (S4) Treatment 4: Motivator 	▪ Donkey had already had contact with the oilcloth in Stage 1, 2 and 3 *(Frontal known element and rear unknown element)*. ▪ Handler A applied pressure to the lead rope at the same time Handler B made a noise from behind the donkey with a so-called "donkey motivator" (plastic bag tied on the end of a stick. The donkey was led by slightly pulling the rope until it crossed the oilcloth completely *(Negative reinforcement)*.
STAGE 5 (S5) Treatment 5: Double rope leading 	▪ Donkey had already had contact with the oilcloth in Stage 1, 2, 3 and 4 *(Frontal known element)*. ▪ Using two lead ropes attached on either side of the halter, Handlers A and B encouraged the donkey across, releasing the pressure when the donkey moved and then reapplied when it stopped until it crossed the oilcloth completely *(Negative reinforcement)*.	STAGE 6 (S6) Treatment 6: Clapping 	▪ Donkey had already had contact with the oilcloth in Stage 1, 2, 3, 4 and 5 *(Frontal and rear known elements)*. ▪ Handler B clapped his hands from behind the donkey to make it move forward. Handler A applied pressure on the lead rope and while the donkey was led across by the auditory sound of the claps, pressure and sound were released or stopped when the donkey moved and reapplied when it stopped until the donkey had completed the task *(Negative reinforcement)*.
Accessed from Navas, *et al.* 2018.			

Figure 2. Ear position illustrative ethogram. In (A), ears are laid backwards, attached to the surface of the neck and the sclerotic is shown as a threat signal, whereas (B) depicts an attentive attitude with ears backwards but slightly sideward to listen its educator orders. (C) represents a state of tranquillity, fatigue or boredom. (D) shows a depressed possibly ill animal, as it can be appreciated by the ear position and the slight inclination of the neck. While in (E), ears are diverted backwards and sideward as a warning and attention sign. (F) is the typical expression that an environmentally interested or curious donkey would display, while (G) depicts a typical expression of alert or fear (Navas et al., 2012).

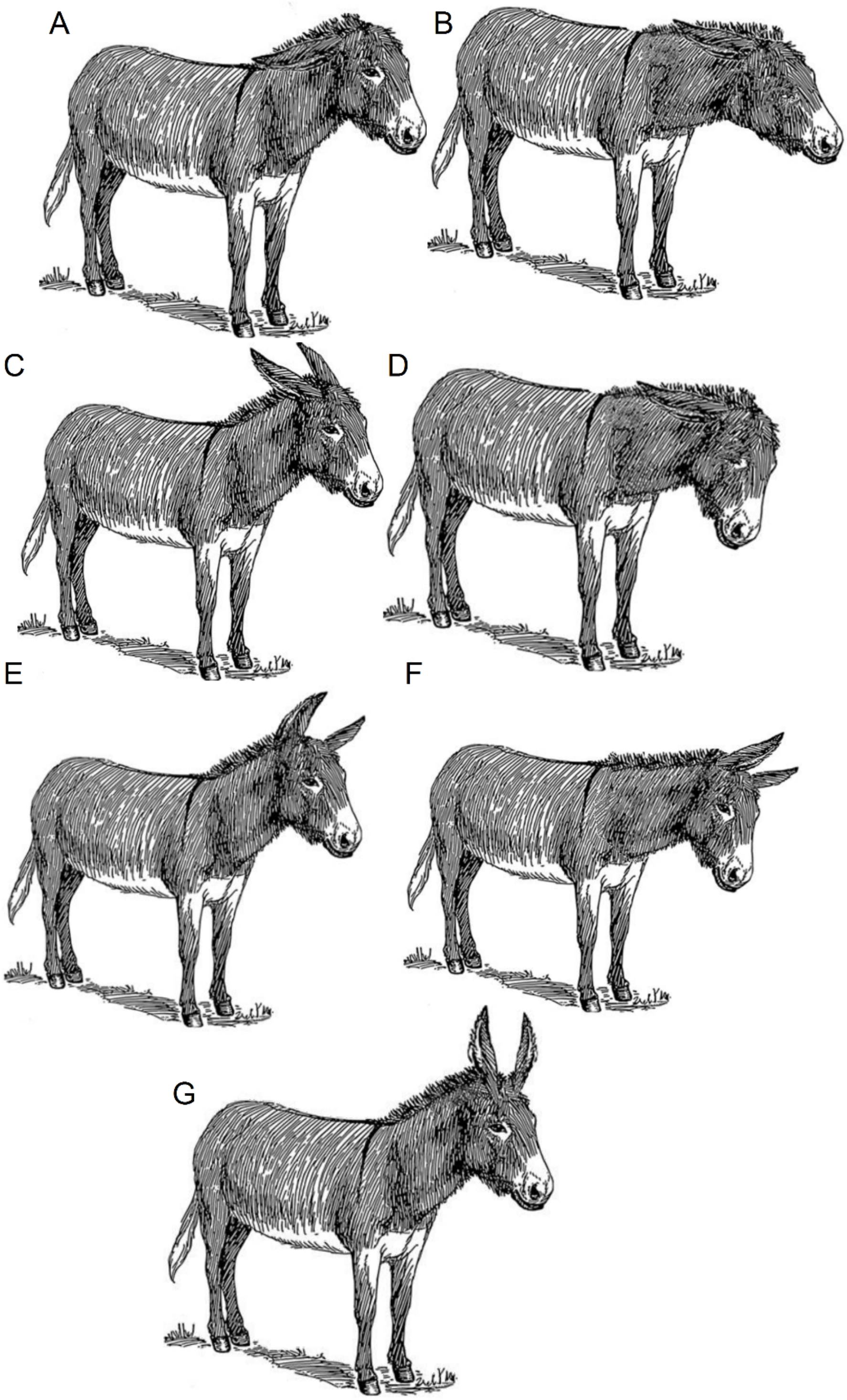
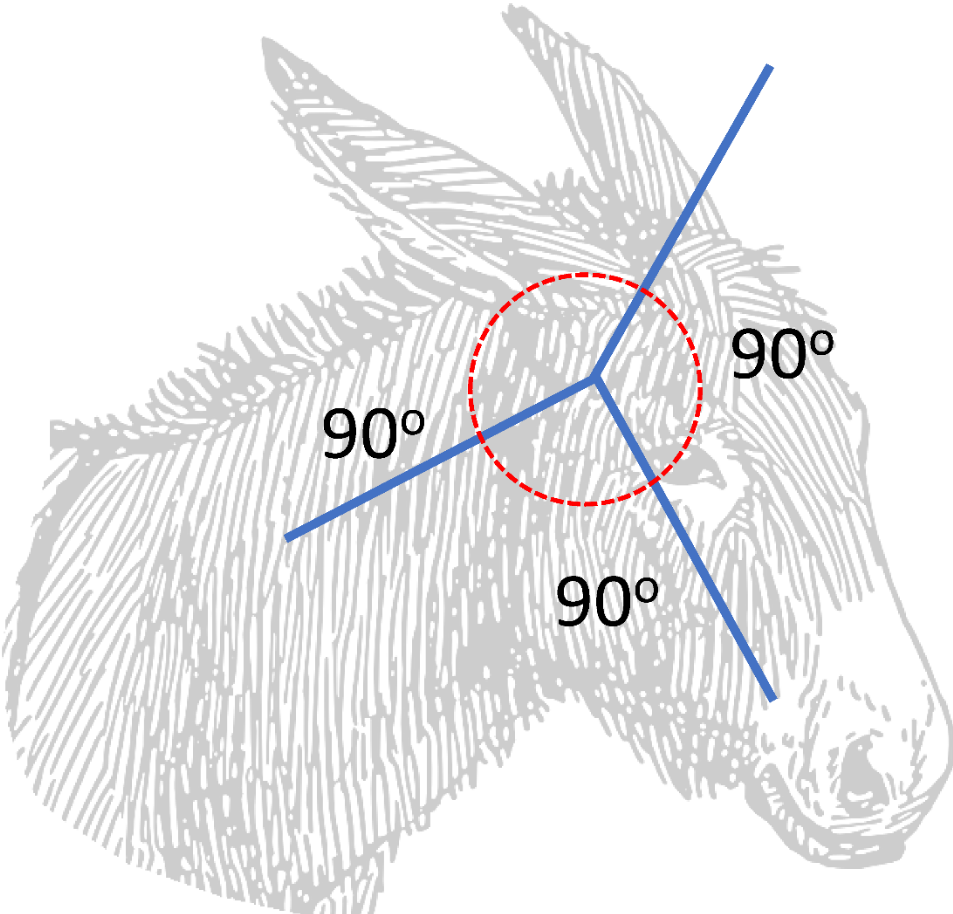


Figure 3. Lateral and frontal straight angles described by the ears to be used as a reference.



Chapter 3

Navas González, F.J.; Jordana Vidal, J.; Pizarro Inostroza, G.; Arando Arbulu, A.; Delgado Bermejo, J.V.

Can Donkey Behavior and Cognition Be Used to Trace Back, Explain, or Forecast Moon Cycle and Weather Events?

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Can Donkey Behavior and Cognition Be Used to Trace Back, Explain, or Forecast Moon Cycle and Weather Events?

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Simple Summary: Donkeys have been traditionally attributed the ability to inform humans about the environment. Carefully observing the behavior and cognitive reactions of donkeys in their habitat may enable to quantify such reactions to develop informative mathematical models. These models can be used to explain present environmental situations, trace back past events or even predict future conditions. Our results suggest, environmental stressing situations may affect donkeys in a way that they register the cognitive adaptations or sequels derived from such situations. Furthermore, such environmental events may not only affect the present cognitive status of the animals, but they may drive this cognitive record affecting the behavioral patterns donkeys display through their lives. Our model is able to explain 75.9% of the variability in response type and intensity, mood, or learning capabilities. Conclusively, donkeys can be used as an environment informative sensitive tool and may therefore, predict and register slight human-unappreciable climatic variations to which they may behaviorally adapt beforehand.

Abstract: Donkeys have been reported to be highly sensitive to environmental changes. Their 8900–8400-year-old evolution process made them interact with diverse environmental situations that were very distant from their harsh origins. These changing situations not only affect donkeys' short-term behavior but may also determine their long-term cognitive skills from birth. Thus, animal behavior becomes a useful tool to obtain past, present or predict information from the environmental situation of a particular area. We performed an operant conditioning test on 300 donkeys to assess their response type, mood, response intensity, and learning capabilities, while we simultaneously registered 14 categorical environmental factors. We quantified the effect power of such environmental factors on donkey behavior and cognition. We used principal component analysis (CATPCA) to reduce the number of factors affecting each behavioral variable and built categorical regression (CATREG) equations to model for the effects of potential factor combinations. Effect power ranged from 7.9% for the birth season on learning ($p < 0.05$) to 38.8% for birth moon phase on mood ($p < 0.001$). CATPCA suggests the percentage of variance explained by a four-dimension-model (comprising the dimensions of response type, mood, response intensity and learning capabilities), is 75.9%. CATREG suggests environmental predictors explain 28.8% of the variability of response type, 37.0% of mood, and 37.5% of response intensity, and learning capabilities.

Keywords: cognition; cold wave; learning abilities; lunar phases; meteorological conditions

1. Introduction

The hypothetical conditioning effects of weather, moon and climate oscillations on animal behavior and cognition have been widely but unscientifically reported. Popular knowledge has even provided untested testimony of the possibility to predict short-term future meteorological conditions basing on how animals react to the environment around them. This framework has promoted the appearance of the first empirical studies on the clinical and productive implications of such environmental factors in different animal species.

Great scale migration of animal populations, adaptation, or even census reduction or extinction have become proved symptoms of how life cycles may be affected by this progressively changing environmental situation. However, the alteration of the particular environmental characteristics of specific areas has also been suggested to lead the lower scale evolutionary process of local animal life cycles [1].

Research has focused on the study of the climatological alteration of physiological processes such as reproduction, and animal biorhythms in populations of different species [2]. By contrast, cognitive or behavioral alterations affecting animal populations may remain unnoticed due to being attributed to other more probable causes.

The study of the effects of factors such as season and weather on animal behavior and mood has typically focused on understanding the changes in the ethological patterns conditioning animal routine and daily activities. These changes may globally appear as a consequence of the evolution of certain areas, which may no longer fulfil the unique set of requirements of the animal populations inhabiting them [3].

Parallel to these more or less quantifiable effects, there is also a simultaneous repercussion on animal cognitive or behavioral health [4]. These effects may not only alter the components of disorder incidence but may also condition animal physiology, as they increase the levels of sensitivity or even distort the cognitive status of specific populations producing long-lasting consequences.

When we consider these behavioral and cognitive registers under a local specific context, we can trace back their origin up to potential weather or meteorological condition related situation or event [5].

Scientists have paid attention to the study of the environmental changes that may distort seasonal and circadian rhythms in different species. However, the effects of factors such as the moon cycle on animal behavior have only been approached assessing the alterations occurring on daily animal patterns or physiological rhythms [6]. Not to mention the inexistence of research assessing other traditionally folklore-reported environmental effects on cognition, such as the hypersensibility to anticipate particular events. The role on neuroanatomy, ethology, and endocrinology and the activity and effects of neurohormones releasing cycles may be triggered and regulated by the electromagnetic radiation and the gravitational pull of the moon and light cycles during the different moon phases, which may reflect in psychological processes such as mood or cognitive abilities.

The first aim of this research is to study at which level environmental factors such as season, year, moon cycle, meteorological factors, and climate oscillations may affect the response type and intensity, mood and learning abilities of donkeys. Second, we used categorical principal component analyses (CATPCA) to study the possibility to reduce our set of environmental variables to a smaller set that still contains most of the information in the previous one, hence reducing the likelihood of Type I error that can derive when testing for the effects of a large number of explanatory and predictor variables. Third, using this reduced information, we designed regression equations using categorical regression (CATREG) to explain, trace back, and predict the possible behavioral repercussions that certain environmental situations may have, and how these consequences may alter the behavioral patterns that donkeys display through their lives, in order to provide clues on how behavior can become a useful tool for daily care.

2. Materials and Methods

2.1. Animal Sample

Our study sample comprised 78 Andalusian uncastrated jacks and 222 unneutered jennies ($n = 300$), born from 1990 to 2012 and officially registered in the national studbook of the Andalusian donkey breed. As the age range was not normally distributed ($p < 0.05$, Kolmogorov–Smirnov test for normality) we used minimum, Q1, median, Q3 and maximum to describe the age range in our sample. Minimum age in the range was 0.27 months, Q1 age was 29.76 months, median age was 77.04 months, Q3 age was 129.07 months, and the maximum age was 270.40 months.

2.2. Information Registration

We registered the information on the response type and response intensity, mood/emotional collateral responses and learning ability from the donkeys in our sample during the development of a six-stage operant conditioning test (Table 1). Reinforcement treatments, stimuli descriptions, their classification, and their constituting elements are provided in Tables 1 and 2. The same trained judge registered all the information concerning the four behavioral variables and 15 noncognitive factor for all the stages and animals. The donkeys were each given a maximum of 450 s to complete the operant conditioning test (75 s per stage and treatment implemented). No additional time was provided for the donkeys to complete the test. The information registered corresponded to the first immediate reaction described by each animal when each of the stages was started. In 75 s, an animal can shift attention many times. However, to simplify the observations, our study tested for the first reaction of the animals, further reactions shown through the development of the test were discarded.

The records for each animal consisted of information on 18 categorical variables divided into two sets. The first set of 4 dependent behavioral categorical variables assessed the cognitive performance of donkeys through their response type, response intensity, mood/emotion, and learning ability. The variables in this first set could be conditioned by a second set of independent variables comprising 14 environmental factors. A summary of the variables and categories included in the first variable set is described in Table S1, while Table S2 shows a summary of the factors and categories included in the second categorical factor set. Table S3 shows the descriptive statistics, and numerical parametrization of all the variables analyzed. Table S1 presents Category description and definition for response type, the intensity of response, mood/emotion, and learning variables directly controlled during the operant conditioning test.

2.3. Categorical Behavioral Variables

The reaction developed by the donkeys when they faced the six consecutive treatments provided information on four categorical behavioral variables (Table S1). To name the mood/emotion variable, we considered the definitions by Cabanac [7] and Mendl et al. [8]. Table S4 shows a description of the scales used to score the response type and mood/emotion variables. The intensity of response and learning ability variables were subdivided into five categories each described as shown in Table S1. The appraiser scored the animals relying on the intensity of their responses from low intensity responses to high intensity responses whatever the mood/emotion displayed by them was (Tables S2 and S3). As animals were only scored once, opposite behaviors were not scored correlatively in the same animal. That is to say, the response of an animal displaying a high intensity calm mood/emotion (very calm animal) was not registered as a low intensity nervous mood/emotion (slightly nervous mood/emotion) simultaneously. The reason for this is the fact that an animal cannot be nervous and calm at the same time whatever it is the intensity level at which such animal expresses its mood/emotion status (see Table S4).

2.4. Qualitative Behavioral Assessment

The same trained judge registered each donkey's mood/emotion following the protocols developed by Navas et al. [9] which based on Minero et al. [10]. Navas et al. [9] generated the descriptor lists for the use in subsequent studies as the present one. Table S4 shows a summary of the mood/emotion descriptors used concerning Table 2.

2.5. Noncognitive Categorical Factors

Environmental categorical factors could be divided into two groups. Meteorological and environmental conditions included year of evaluation, the season of evaluation, weather conditions, temperature, moon phase at evaluation, relative humidity, windspeed, sunlight hours, barometric pressure, rainfall on the day of evaluation, and rainfall on the following day. Animal birth characteristics included season of birth, year of birth and moon phase at birth. Table S2 shows the categories for independent noncognitive factors in the second set.

The information was registered during the yearly behavior assessment sessions carried out on four random days per year, from June to November for three consecutive years from 2013 to 2015 at twenty-two different farms all over Andalusia (Southern Spain).

The 22 farms involved, reared their animals under four husbandry systems (extensive, semi extensive, semi intensive and intensive) and were located in 5 Andalusian provinces (Southern Spain). The 6% of the donkeys were tested during the breed's Official Morphological Contest held by the Union of Andalusian Donkey Breeders (UGRA).

2.6. Meteorological and Moon Cycle Records

Day records for temperature, relative humidity, windspeed, sunlight hours, barometric pressure, rainfall per day and rainfall prediction (on the following day) were obtained from the State Meteorological Agency (AEMET) (http://www.aemet.es/). Moon phase at evaluation and moon phase at birth records were obtained from the Astronomical Applications Department of the US Naval Observatory (http://aa.usno.navy.mil).

2.7. Operant Conditioning Behavioral Test

The operant conditioning behavioral test was carried out in an open area to which the donkeys were previously accustomed (it was part of the area over which the donkeys developed their daily activities). During the operant conditioning test, the donkeys were made cross over a 200 × 200 cm oilcloth with a wooden print on it using increasingly aversive reinforcement methods (from stimuli 1 to 6). We exposed each animal to six reinforcement treatments consecutively, one at each of the six stages within the operant conditioning test. At each stage, handler A and handler B used each of the six different reinforcement treatments to lead the donkeys to cross over an oilcloth laying on the floor. These treatments/stimuli could comprise unknown elements (the animal had not been familiarized to them) or known elements (to which the animal had already been familiarized). These elements could be visual (elements fell within the visual areas of the donkeys) and/or acoustic (elements generated sounds, i.e., "motivator" or claps, although they may or may not fall within visual areas) and were presented to the donkeys from different positions (from the front or from a rear position always at 2 m away from the animals). A cameraman (Handler C) simultaneously videotaped the experiences (1080 p, 50 Hz, shutter speed: 1/250 s) to assess the donkey's performance after the field experiences and to test for intra-observer discrepancies. Cameraman (Handler C) controlled timing. A detailed description of the operant conditioning test, the reinforcement treatments, stimuli descriptions and classification and their constituting elements are described in Navas et al. [9] and Navas González et al. [11], and summarized in Tables 1 and 2.

Table 1. Description of the operant conditioning test used in the study.

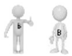
Test Factors	Descriptions
Time per stage/treatment presentation	75 s per stage/treatment presentation. The application of the reinforcement treatments that Handler A, Handler B or both implemented to lead the donkey across the oilcloth lasted for the whole 75 s. These treatments were applied to check the response of the animals to the different types of reinforcement. No additional time was supplied for the donkeys to complete the stages, so that, once the 75 s, provided to the donkeys to interact with the elements presented, had expired, the following stage started and the next treatment was implemented.
Test duration	450 s.
Test stages	1 to 6. Each stage corresponded to the implementation of each of the six reinforcement treatments.
Previous considerations	• The oilcloth was the element (obstacle) that the donkeys were led to cross over. No donkey had been in contact with the oilcloth previous to the test. Handlers A and B, used 6 reinforcement treatments to lead the donkeys cross over such obstacle. • The donkeys were accustomed to the area in which the test took place as it was an open area on which the donkeys used to carry out their daily activities. • The donkeys that were taking the test were not present while the oilcloth was being laid on the floor for the first time. The donkeys were assessed one at a time, so no additional donkey was present while the test was taking place. • The test started when Handler B raised the oilcloth and relayed it again on the floor in front of the donkey being tested. This action only took place 1 minute before stage 1 (before the 1st treatment was implemented) and was not repeated further in the test. Cameraman started controlling time after the oilcloth had been relayed, when Handler A gave the first step forward towards the oilcloth. • Frontal and visual elements fell within the visual scope of the donkeys, while we considered rear elements those that fell into a blind area. Acoustic elements could be frontal or rear and emitted sounds. • Reinforcement treatments comprised different elements. Known elements were those which had already been presented to the donkeys at any point in their lives (relying on owner's information), while unknown elements were those to which, according to the owner, the donkeys were not acquainted. • All the reinforcement treatments were implemented sequentially and consecutively from stage 1 to 6, one after another, without any stop between each of them, whether the donkey had completed each stage (crossed the obstacle) completely or not (avoided it). That is to say, the fact that an animal crossed/avoided the oilcloth completely in one of the treatments from 1 to 6, did not prevent the rest of treatments from being implemented.
Legend	 Donkey being tested.
	 2 × 2 m oilcloth with a wooden print.
	 Rope leader/Handler A.
	 Handler B/Lurer (in Stage 4)/2nd Rope leader (in Stage 5)/Clapper (in Stage 6).
	 Cameraman (C)/Time controller.
	 Treat (bread, carrots, feed or sugar lumps). Carried by Handler B.
	 Motivator. Plastic bag attached to a wooden stick. Carried by Handler B.

Table 1. *Cont.*

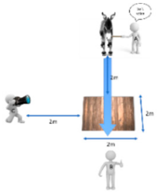
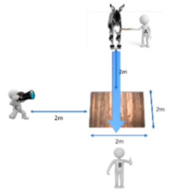
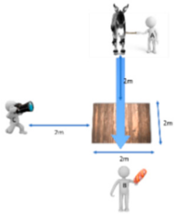
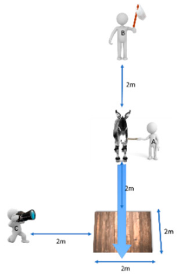
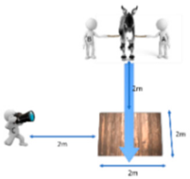
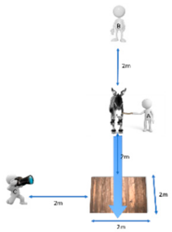
Test Stage	Descriptions	Test Stage	Descriptions
STAGE 1 (S1) Treatment 1: Soft voice 	• Oilcloth presented to the donkey for the first time (Frontal unknown element). • The donkey is given 75 s to complete Stage 1, that is to cross over the oilcloth. • Using a lead rope and soft voice, Handler A tried to comfort the donkey to make it cross the oilcloth on the floor, but without pulling from the rope if the donkey refused to move (Neutral reinforcement).	STAGE 2 (S2) Treatment 2: Pressure to leading rope 	• Donkey had already had contact with the oilcloth in Stage 1 (Frontal known element). • Using a lead rope with applied pressure to make the donkey cross over the oilcloth. Handler A released the pressure when the donkey moved to cross the oilcloth (Negative reinforcement).
STAGE 3 (S3) Treatment 3: Treat 	• Donkey had already had contact with the oilcloth in Stage 1 and 2 and was familiar to the treat given (Frontal known elements). • Handler B offered a familiar treat to lead the donkey to cross over the oilcloth (the treat offered depended on the owner's tastes and therefore the animals were familiar to it. Handler B used the treat that the owner of each donkey normally offered them to tease them. All animals did not accept any other treat that had not been offered to them by their owners previous to the test, as the field experiences reported) (Positive reinforcement/Luring).	STAGE 4 (S4) Treatment 4: Motivator 	• Donkey had already had contact with the oilcloth in Stage 1, 2 and 3 (Frontal known element and rear unknown element). • Handler A applied pressure to the lead rope at the same time Handler B made a noise from behind the donkey with a so-called "donkey motivator" (plastic bag tied on the end of a stick. The donkey was led by slightly pulling the rope until it crossed the oilcloth completely (Negative reinforcement).
STAGE 5 (S5) Treatment 5: Double rope leading 	• Donkey had already had contact with the oilcloth in Stage 1, 2, 3 and 4 (Frontal known element). • Using two lead ropes attached on either side of the halter, Handlers A and B encouraged the donkey across, releasing the pressure when the donkey moved and then reapplied when it stopped until it crossed the oilcloth completely (Negative reinforcement).	STAGE 6 (S6) Treatment 6: Clapping 	• Donkey had already had contact with the oilcloth in Stage 1, 2, 3, 4 and 5 (Frontal and rear known elements). • Handler B clapped his hands from behind the donkey to make it move forward. Handler A applied pressure on the lead rope and while the donkey was led across by the auditory sound of the claps, pressure and sound were released or stopped when the donkey moved and reapplied when it stopped until the donkey had completed the task (Negative reinforcement).

Table 2. Description of the treatments and stimuli presented, their reinforcement classification and terminology considered.

Treatment/Stimulus	Stimulus Description	Stimulus Type	Reinforcement
Treatment 1 (S1): Soft voice	Handler (B) uses a lead rope and soft voice, trying to comfort the donkey to make the donkey cross the oilcloth on the floor, but without pulling the rope if the donkey refuses to move.	Unknown frontal visual stimulus.	Neutral [a]
Treatment 2 (S2): Pressure to leading rope	Handler (B) uses a lead rope with applied pressure to make the donkey cross over the oilcloth. Handler (B) releases the pressure when the donkey moves as it crosses the oilcloth.	Known frontal visual stimulus.	Negative [b]
Treatment 3 (S3): Treat	A familiar treat is used to lure the donkey (dry bread, carrots or feed, depending on the owner's tastes and to which the donkeys on each farm were accustomed). We use the treat that the owner regularly uses as a treat for all of the donkeys in the same farm (the attraction or attention of the animals to the treats depends on whether they are used to the treats presented or not as empirical observations had revealed at a preliminary stage when developing the operant conditioning test). When the donkeys are not familiar to the treats presented, they do not respond to the stimulus by handler (C). The treat is given to the donkey once the task is completed.	Known frontal visual stimulus.	Positive/Luring [c]
Treatment 4 (S4): Motivator	Handler (B) applies pressure to the lead rope, and handler C makes noise from behind the donkey with a so-called "donkey motivator" (plastic bag tied on the end of a stick) [12]. Handler (B) leads the donkey by slightly pulling the rope until the donkey crosses the oilcloth completely.	A known frontal visual stimulus and an unknown rear auditory stimulus.	Negative
Treatment 5 (S5): Double rope leading	Two handlers (B and C) using two lead ropes attached on either side of the halter to encourage the donkey across. The handlers (B and C) release the pressure when the donkey moves and then reapply the pressure when it stops until the donkey crosses the oilcloth completely.	Known frontal visual stimulus.	Negative
Treatment 6 (S6): Clapping	Handler (B) applies pressure on the lead rope, and handler (C) encourages the donkeys across by an acoustic sound. Handler C claps their hands from behind the donkey to make it move forward [13]. Pressure and sound are released or stopped when the donkey moves and reapplied when it stops until the donkey had completed the task.	A known frontal visual stimulus and an unknown rear acoustic stimulus.	Negative

A full description of the protocols, scales, and methods used in this study is described in Navas et al. [9] and Navas González et al. [11]. The terminology used to classify stimuli throughout this paper rests on classical concepts, as applied by Sankey et al. [14]. According to these authors stimuli can be perceived as negative, neutral or positive. [a] Neutral reinforcement training implies the donkey perceives the tasks to be neither positive nor aversive and therefore the stimulus does not act to reinforce or punish the donkey's behavior. Therefore, the animal fails to respond to the stimuli and continues quietly and calmly with the task uninterrupted [15]. [b] *Negative reinforcement* implies delivering an unpleasant stimulus and terminating it when an individual performs a presented task in the desired manner or expresses the desired behavior [16]. [c] *Positive/luring reinforcement* implies the presentation of a pleasant stimulus (lure) when an individual fulfils a task in the desired manner or expresses the desire and the behavior [16].

2.8. Test and Scoring System Reliability

Statistical tests did not report intra-observer discrepancies as all the scores obtained on the field matched those obtained after reviewing the tapes again. Aiming at eliminating the effect of appraiser to reduce the likelihood of subjective evaluations, 50 individuals (16.67% of the total sample) were tested using the operant conditioning test described scoring for the categorical variables of response type, mood and response intensity at a preliminary stage of the study. Cohen's κ determined whether the repeatability of the model was enough to delete the effect of the appraiser from the model, providing a measure of the accuracy of scoring of the appraisers. Then 95% confidence intervals (95% kappa IC) were computed according to 95% kappa IC = $\kappa \pm 1.96$ SEκ, where; SEκ = $[(po(1-po)/n(1-pe)^2]^{0.5}$ with the Crosstabs procedure of SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016, Armonk, NY, USA). This preliminary analysis aimed at testing for interobserver reliability, i.e., the reliability of the scoring system, which proved to be highly reliable as there was highly statistically significant perfect agreement between the three appraisers' judgements when scoring for response type and response intensity for the six stimuli/treatments presented. Each stimulus corresponded to one of the six stages in the test (Table 1). When testing for mood/emotion, there was highly statistically significant almost perfect agreement among the three observers at the preliminary test for repeatability for all the traits and stimuli, except when testing for mood at the presentation of stimulus/treatment 3. In this case, the strength of agreement between appraisers 1 and 2 and 2 and 3 was substantial and at the presentation of stimuli/treatments 1 and 6, for appraisers 2 and 3 between whom inter-observer agreement was substantial. The slight distortion occurring may be attributed to the change in the kind of reinforcement applied to make the donkeys cross over the oilcloth on the floor occurring in stimuli/treatments 1, 3, and 6. At the presentation of stimulus/treatment 1, the animal passed from being at rest to start the operant conditioning test. At the presentation of stimulus/treatment 3, the animals went from being exposed to negative reinforcement (stimulus/treatment 2) to being exposed to positive/neutral reinforcement (stimulus/treatment 3). Finally, at the presentation of stimulus/treatment 6, the stimulus changed from being presented at the visible area of the donkey to be located at a rear position (blind area). Table S5 shows the results for interobserver reliability tests at this preliminary study.

2.9. Statistical Analysis

Categorical variables represent a qualitative method of scoring data. As all the variables and factors considered in our study were categorical, we used nonparametric tests to assess the information recorded statistically. A Chi-square test for independence was used to analyze whether the factors in the second set (Table S2) randomly and significantly influenced the variables in the first set (Table S1). Chi square is neutral to the parametric or non-parametric nature of the distribution and is relatively robust to situations with a limited number of data ($n > 50$). The most appropriate statistic to use as a measure of Chi-square association is Cramér's V. Cramér's V is used to measure the strength of linear correlation, that is to test for the multicollinearity and significance between each variable from the first set with each variable from the second set using the Crosstabs procedure from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016, Armonk, NY, USA) according to the indications of Nolan [17]. Table S6 shows total and relative frequencies for the associations of the four dependent categorical variables with the environmental variables.

Categorical principal components analysis (CATPCA) was used to quantify categorical factors while reducing the dimensionality of the data and Categorical regression to establish the most important descriptive and discriminative noncognitive factors on the variables considered using the Optimal Scaling procedure from the Dimension reduction task from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016, Armonk, NY, USA). Reducing the dimensionality of relatively large sets of variables prevents type I errors from occurring, as we may strip our model to the core independent variables affecting the dependent variables studied by our model. A lower number of variables means we may need stronger evidence against the null hypothesis H_0 (via a lower p-value) before we will reject the null. Therefore, if the null hypothesis is true, we will be less likely to reject it by chance. This reduced information was used later at the categorical regression (CATREG) analysis.

We used CATREG to describe regression models to study how the variables assessed depended on the factors considered. The resulting regression equations could be used to trace back, explain, or predict behavior or cognitive abilities for any combination of the 14 independent factors. 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, Armonk, NY, USA).

2.10. Justification for Statistical Tests

The most appropriate statistic to use as a measure of Chi-square association is Cramér's V. Cramer's V is a measure of association for nominal variables. Effectively it is the Pearson chi-square statistic rescaled to have values between 0 and 1 as follows:

$$V = \sqrt{\frac{\chi^2}{n_{obs(\min(n_{cols},\ n_{rows}))} - 1}} \tag{1}$$

where χ^2 is the Pearson chi-square, n_{obs} represents the number of observations included in the table, and where n_{cols} and n_{rows} are the number of columns and rows in the table, respectively. For a 2 by 2 table, of course, this is just the square root of chi-square divided by the number of observations, which is also known as the phi coefficient. Cramer's V squared is the average of the squares of the canonical correlation coefficient between two categorical variables. Such canonical-correlation analysis will find the strength that linear combinations of the X_i and Y_j have on each other. When using Cramér's V small effect associations range from 0.0 to 0.10, medium effect associations from 0.3 to 0.5 and large effect associations from 0.5 to anything above. The same author would recommend that the interpretation of effect size should consider a statistically significant measure ($p < 0.05$) with a small effect size or higher to indicate a meaningful difference, especially for behavioral or psychological studies.

CATPCA is appropriate to reveal the inherent overlapping nature of behavioral variables, hence becomes suitable for variable selection and dimension reduction in categorical variables. This statistical test analyses the interrelationships among a large number of variables and explains these variables regarding their common underlying dimensions. The objective is to find a few linear combinations of the variables (factors) that can be used to summarize the data without losing too much information in the process. CATPCA is a nonparametric method that quantifies categorical variables through a process called optimal scaling. Optimal scaling uses category quantifications in such a way that they account for as much as possible of the variance in the quantified variables. The most relevant characteristic of CATPCA is that it can handle and discover nonlinear relationships between variables. Because CATPCA directly analyses the data matrix and not the derived correlation matrix, so that, we can avoid the usual concern to have at least five times as many observations as the variables. CATPCA suits analysis in which there are more variables than objects. In behavioral sciences many of the variables used are qualitative, nominal or ordinal, thus indicating the use of CATPCA, which has been demonstrated to be more robust than PCA when assessing categorical variables.

CATPCA eigenvalues are indicators of how many dimensions are needed. As a general rule, when all variables are either single nominal, ordinal, or numerical, the eigen value for a dimension should be larger than 1. For multiple nominal variables, there is no easy rule of thumb to determine the appropriate number of dimensions. If we replace the number of variables by the total number of categories minus the number of variables, the above rule still holds. However, this rule alone would probably allow more dimensions than are needed. When choosing the number of dimensions, the most useful guideline is to keep the number small enough so that meaningful interpretations are possible. The model summary table also shows Cronbach's alpha (a measure of reliability), which is maximized by the procedure. In this study, the stepwise method was used to prevent the possible multicollinearity problem that could arise in the linear multiple regression model formed by transformed variables. The resulting reduced set of variables can be used to perform a categorical regression analysis to build significant behavioral descriptive equations that enable quantifying the result of the effects of specific combinations of environmental factors on behavioral variables, such as response type or intensity, mood or learning abilities.

When assessing non-parametrical data, categorical variables can be included as independent variables in a regression analysis but must be converted to quantitative data for us to be able to analyze them. Ordinary linear regression models could only be used when the dependent variable is quantitative and predictive variables are either quantitative or dummy. The analysis of such ordinary linear regression models involves minimizing the sum of squared differences between a response (dependent) variable and a weighted combination of predictors (independent). Variables are typically quantitative, with (nominal) categorical data recoded to binary or contrast variables. As a result, categorical variables serve to separate groups of cases, and the technique estimates separate sets of parameters for each group. The estimated coefficients reflect how changes in the predictors affect the response. Prediction of the response is possible for any combination of predictor values. CATREG extends the standard approach by simultaneously scaling nominal, ordinal, and numerical variables. The procedure quantifies (transforms) categorical variables so that the quantifications reflect characteristics of the original categories. The procedure treats quantified categorical variables in the same way as numerical variables. Using nonlinear transformations allow variables to be analyzed at a variety of levels to find the best-fitting model. R-squared evaluates the scatter of the data points around the fitted regression line. It is also called the coefficient of determination, or the coefficient of multiple determination for multiple regression. For the same data set, higher R-squared values represent smaller differences between the observed data and the fitted values. R-squared is the percentage of the dependent variable variation that a linear model explains. As the independent noncognitive categorical factors registered in our study were categorical and the data was sorted into categories following different criteria, we used standardized coefficients to interpret and compare their effects on our behavioral dependent categorical variables. When we apply a stepwise linear regression model to the transformed variables, the standardized and unstandardized coefficients are equal. Hence, we can interpret the unstandardized coefficients. Standardized coefficients represent regression results with standard scores. By default, most statistical software, like SPSS, automatically converts both criterion (DV) and predictors (IVs) to Z scores and calculates the regression equation to produce standardized coefficients. When most statisticians refer to standardized coefficients, they refer to the equation in which one converts both DV and IVs to Z scores. In a simple model with two factors involved the coefficients for Z scores for each variable (Z'y) may be interested as follows:

β_1 mean a standard deviation increase in Z_{X1} is predicted to result in a β_1 standard deviation increase in Z'y holding constant Z_{X2}.

β_2 mean a standard deviation increase in Z_{X2} is predicted to result in a β_2 standard deviation increase in Z'y holding constant Z_{X1}.

Therefore, the standardized partial coefficient represents the amount of change in Zy for a standard deviation change in Z_X. So, if X1, one factor involved, were increased by one standard deviation, then one would anticipate a β_1 standard deviation increase in the variable tested holding constant the effect of X2 and vice versa.

With Z_{X1} and Z_{X2}, being the Z scores for each factor, and β_1 and β_2 the standard coefficients for each of the, respectively.

As the above example shows, conversion of raw scores to Z scores changes the unit of measure for interpretation, the change from raw score units to standard deviation units.

As a rule, we assume standardized results reported used full standardization (both DV and IVs were converted to standard scores), and that the Z formula was used for standardization. The general standardized regression equation may follow the following model $Z'y = \beta_1 Z_{X1} + \beta_2 Z_{X2} + \ldots$, where Z'y is the predicted value of Y in Z scores; β_1 represents the standardized partial regression coefficient for X1; β_2 represents the standardized partial regression coefficient for X2; and Z_{X1} and Z_{X2} are the Z score values for the variables X1 and X2, respectively.

The intercept will always equal 0.00 when standardization is based upon Z scores, and both DV and IVs are standardized.

Once the regression equation is standardized, then the partial effect of a given X upon Y, or Z_X upon Zy, becomes somewhat easier to interpret because interpretation is in sd units for all predictors.

3. Results

3.1. Noncognitive Factor Analysis

Table 3 shows the results from Chi-Square and Cramér's V, testing for the existence of linear correlations. Cramér's V effectively measured the strength of collinearity that the noncognitive factors considered have on the behavioral variables studied, given the high significance ($p < 0.001$) that they report for all the factor-variable combinations except for season at birth and response type (Table 3). CATREG was performed to the 14 qualitative independent variables (environmental factors) with the four behavioral categorical variables (response type, mood/emotion, the intensity of response and learning ability) as dependent variables. Then stepwise linear regression to the data with the resulted quantifications was applied, and Tables 4 and 5 present the summary results with the significant variables. Table 5 lists the standardized coefficients (β). CATREG reported all of the independent variables except for season at evaluation to be significant for response type (Table S7). Season at evaluation and the rainfall on that day were nonsignificant for mood/emotion. Weather conditions, temperature, and barometric pressure were nonsignificant for response intensity and learning ability.

According to Cramér's V, there was a moderate linear correlation between sunlight hours and the four behavioral variables tested (0.194 to 0.274), which was as well supported by the percentage of variance explained by this factor according to CATREG standardized coefficients. However, CATPCA addressed the correlations with three of the dimensions were inverse (from strong −0.954 to moderately weak −0.110) as reported by the values of the negative component loading (Tables 3, 5 and S8). By contrast, there was a moderate positive component, thus direct correlation with dimension 2.

For the year of birth, the Cramér's V values ranged from 0.192 to 0.310 what reported a moderately high linear correlation. Moderately high CATREG standardized coefficients reported a moderate dependence for the four variables on this factor. Component loading for dimension 1 was negligible. However, there was a moderately strong negative loading for dimension 2 (inverse correlation) and strong positive loadings for dimensions 3 and 4 (strong direct correlation) (Tables 3, 5 and S8).

There was a moderate linear correlation between windspeed and the four behavioral variables tested (Cramér's V ranging from 0.182 to 0.248), which was as well supported by the percentage of variance explained by this factor according to CATREG standardized coefficients. CATPCA addressed these correlations with two of the four dimensions (dimensions 1 and 3) were strongly inverse as reported by the high negative component loadings, while the other two were moderately positive thus direct (dimensions 2 and 4) (Tables 3, 5 and S8).

For the season of evaluation, the Cramér's V values ranged from 0.196 to 0.252 what reported a moderate linear correlation. Moderate to high CATREG standardized coefficients reported a moderate to strong dependence on the four variables on this factor. Component loading for dimension 1 was high, describing a strong direct correlation. However, there was a moderately strong negative loading for dimension 3 (inverse correlation). CATPCA component loadings for dimensions 2 and 4 were positive moderately low (moderately low direct correlation) (Tables 3, 5 and S8). Season of evaluation Cramér's values ranged from 0.049 to 0.122 (response type and mood/emotion, respectively). The CATREG standardized coefficients ranged from 0.053 to 0.075, what resembled the low to moderately low values found for Carmér's V. CATPCA component loadings were positive and moderately low to moderate for dimensions 1, 3, and 4, and negative and moderate for dimension 2.

Table 3. Statistical significance and strength of the effects on the different variables tested in donkeys in this study.

Variable	N	Response Type			Mood/Emotion			Response Intensity			Learning Ability		
		χ^2	*p*-Value	Cramer's V	χ^2	*p*-Value	Cramer's V	χ^2	*p*-Value	Cramer's V	χ^2	*p*-Value	Cramer's V
Environmental/Meteorological													
Year of evaluation	1800	76.99	<0.001 ***	0.146	256.34	<0.001 ***	0.267	138.40	<0.001 ***	0.196	138.40	<0.001 ***	0.196
Season of evaluation	1800	70.54	<0.001 ***	0.198	114.27	<0.001 ***	0.252	49.60	<0.001 ***	0.166	49.60	<0.001 ***	0.166
Weather conditions	1800	16.71	<0.001 ***	0.096	87.12	<0.001 ***	0.220	77.51	<0.001 ***	0.208	77.51	<0.001 ***	0.208
Temperature	1800	81.46	<0.001 ***	0.150	152.10	<0.001 ***	0.206	136.99	<0.001 ***	0.195	136.99	<0.001 ***	0.195
Moon phase at evaluation	1800	50.52	<0.001 ***	0.118	159.28	<0.001 ***	0.121	66.72	<0.001 ***	0.096	66.72	<0.001 ***	0.096
Relative humidity	1800	49.39	<0.001 ***	0.117	275.41	<0.001 ***	0.226	56.35	<0.001 ***	0.102	56.35	<0.001 ***	0.102
Windspeed	1800	146.78	<0.001 ***	0.202	332.77	<0.001 ***	0.248	178.81	<0.001 ***	0.182	178.81	<0.001 ***	0.182
Sunlight hours	1800	135.56	<0.001 ***	0.194	271.25	<0.001 ***	0.274	266.23	<0.001 ***	0.272	266.23	<0.001 ***	0.272
Barometric pressure	1800	109.42	<0.001 ***	0.174	362.36	<0.001 ***	0.317	189.71	<0.001 ***	0.230	189.71	<0.001 ***	0.230
Rainfall per day	1800	112.73	<0.001 ***	0.177	325.54	<0.001 ***	0.301	221.94	<0.001 ***	0.248	221.94	<0.001 ***	0.248
Rainfall on the following day	1800	121.10	<0.001 ***	0.183	373.48	<0.001 ***	0.263	224.46	<0.001 ***	0.204	224.45	<0.001 ***	0.204
Animal Birth													
Season of birth	1800	6.88	0.194	0.049	80.90	<0.001 ***	0.122	34.12	<0.05 *	0.079	34.12	<0.05 *	0.079
Year of birth	1800	347.07	<0.001 ***	0.310	875.91	<0.001 ***	0.210	265.58	<0.001 ***	0.192	265.58	<0.001 ***	0.192
Moon phase at birth	1800	44.75	<0.001 ***	0.111	270.38	<0.001 ***	0.388	77.86	<0.001 ***	0.208	77.85	<0.001 ***	0.208

Levels of significance are indicated by * and *** for $p < 0.05$, statistically significant and $p < 0.001$, highly statistically significant, respectively.

Table 4. Model summary of stepwise linear regression with transformed variables.

Variable	R	R Square	Adjusted R Square	Significance
Response type	0.537	0.288	0.265	<0.001
Mood/emotion	0.608	0.370	0.350	<0.001
Intensity of response	0.612	0.375	0.355	<0.001
Learning ability	0.612	0.375	0.355	<0.001

Table 5. Standardized coefficients and significance of categorical regression (CATREG) model.

Variable	Response Type		Mood/Emotion		Response Intensity		Learning Ability	
	β	Sig.	β	Sig.	β	Sig.	β	Sig.
Year of birth	0.235	<0.001	0.212	<0.001	0.195	<0.001	0.195	<0.001
Season of birth	0.053	<0.001	0.075	<0.001	0.054	<0.001	0.054	<0.001
Relative humidity	0.136	<0.001	0.263	<0.001	0.106	<0.001	0.106	<0.001
Year of evaluation	0.196	<0.001	0.242	<0.001	0.065	0.031	0.065	0.042
Season of evaluation	0.129	0.058	0.116	0.113	0.621	<0.001	0.621	<0.001
Weather conditions	0.121	0.001	0.211	<0.001	0.029	0.257	0.029	0.267
Temperature	0.206	<0.001	0.230	<0.001	0.040	0.230	0.040	0.244
Moon phase at birth	0.098	<0.001	0.117	<0.001	0.093	<0.001	0.093	<0.001
Moon phase at evaluation	0.145	<0.001	0.111	<0.001	0.107	<0.001	0.107	<0.001
Windspeed	0.304	<0.001	0.395	<0.001	0.280	<0.001	0.280	<0.001
Sunlight hours	0.527	<0.001	0.596	<0.001	0.814	<0.001	0.814	<0.001
Barometric pressure	0.285	<0.001	0.365	<0.001	0.054	0.115	0.054	0.130
Rainfall on that day	0.166	0.044	0.103	0.105	0.231	0.013	0.231	0.011
Rainfall on the following day	0.387	<0.001	0.468	<0.001	0.670	<0.001	0.670	<0.001

β = Standardized coefficients; Sig. = Significance.

According to Cramér's V, there was a moderately high linear correlation between rainfall on the following day and the four behavioral variables tested (0.183 to 0.263), which was as well supported by the percentage of variance explained by this factor according to CATREG standardized coefficients. CATPCA component loading for dimension 1 was high, describing a strong direct correlation. However, there was a moderately strong negative loading for dimension 3 (inverse correlation). Component loadings for dimensions 2 and 4 were positive moderately low (moderately low direct correlation) (Tables 3, 5 and S8).

For rainfall on the same day, the range of the linear correlations of the four variables with the factor was slightly wider (Cramér's V from 0.177 to 0.301). This was supported by the percentage of variance explained by this factor according to CATREG standardized coefficients. CATPCA component loadings reported the same value patterns described above for rainfall on the following day (Tables 3, 5 and S8).

The range of the linear correlations of the four variables with barometric pressure ranged from 0.174 to 0.317), what was supported by the percentage of variance explained by this factor according to CATREG standardized coefficients with a dependence ranging from 0.054 to 0.365. CATPCA component loading reported positive and from moderate to strong values for the dimensions 1, 2 and 3, but the moderate negative value of the component loading for dimension 4 suggested a moderately strong negative inverse correlation (Tables 3, 5 and S8).

According to Cramér's V, there was a moderately high linear correlation between rainfall on the following day and the four behavioral variables tested (0.183 to 0.263), which was as well supported by the percentage of variance explained by this factor according to CATREG standardized coefficients. CATPCA component loading for dimension 1 was high, describing a strong direct correlation. However, there was a moderately strong negative loading for dimension 3 (inverse correlation). Component loadings for dimensions 2 and 4 were positive moderately low (moderately low direct correlation) (Tables 3, 5 and S8).

There was a moderate linear correlation between temperature and the four behavioral variables tested (Cramér's V ranging from 0.150 to 0.206), which was as well supported by the percentage of variance explained by this factor according to CATREG standardized coefficients. CATPCA addressed these correlations were positive and from low to high thus direct for the four dimensions (Tables 3, 5 and S8).

Year of evaluation reported Cramér's V values ranging from 0.146 to 0.267 and CATREG standardized coefficients ranging from 0.065 to 0.242 for the behavioral variables studied (Tables 3, 5 and S8). The results of CATPCA loadings were 0.017 to 0.700 for dimensions 4 and 2, respectively. These loadings suggested a low to strong direct correlation of this factor (Tables 5 and S8).

The range of Cramér's V for moon phase at evaluation for the four variables tested was narrower than the one for other factors (0.102 to 0.121). CATREG standardized coefficient range was narrow as well, ranging from 0.107 to 0.145. Values for the loadings in the CATPCA were negative and low to moderately high for dimensions 1 and 2 (weak to moderate inverse correlation), and positive and moderate to high for dimensions 3 and 4 (moderate to strong direct correlation), respectively. However, moon phase at birth reported a wider range for Cramér's V values than other factors (from 0.111 to 0.388). By contrast, CATREG standardized coefficient range was narrow, ranging from 0.093 to 0.117. Values for the loadings in the CATPCA were positive and from low to moderate (weak to moderate direct correlation) for all the dimensions except for dimension 3, for which the value was negative and moderate (moderate inverse correlation).

Relative humidity Cramér's V ranged from 0.117 to 0.226 for response type and mood/emotion, respectively. CATREG standardized coefficients (β) for relative humidity factor ranged from 0.106 to 0.263 for response intensity and learning, and mood/emotion, respectively. CATPCA loadings were negative and moderately high for dimensions 1 and 3 (moderately strong inverse correlation), and positive and moderate to high for dimensions 2 and 4, addressing a moderate to strong direct correlation.

For weather conditions, the range of the linear correlations of the four variables with the factor was from moderately low to moderate (Cramér's V from 0.096 to 0.220, for response type and mood/emotion, respectively). However, the percentage of variance explained by this factor according to CATREG standardized coefficients ranged from 0.029, for response intensity and learning ability, to 0.211 for mood/emotion. CATPCA component loadings were negative and moderately low for dimensions 1 and 4 (moderate inverse correlation), and positive and moderate to high for dimensions 2 and 3 (moderate to strong direct correlation) (Tables 3, 5 and S8).

A categorical principal components analysis (CATPCA) was applied on the total data set of 14 environmental factors with the aim of establishing and interpreting the factors determining the four behavioral variables tested (response type, mood/emotion, intensity of response, and learning) to evaluate for redundancies among them. Two, three, and four-dimensional model results are shown in Table 6. Table 7 shows the factors affecting the four behavioral variables in order of importance according to the CATREG standardized coefficients (β). Since we used the stepwise method, there was no multicollinearity problem. Only 8 of the environmental factors studied contributed to the two–dimensional model in a meaningful way 11 of them meaningfully contributed to the three-dimensional model and 12 of them meaningfully contributed to the four-dimensional model (factor loadings > 0.5, Table 6), then the different components (PC1, PC2, PC3, and PC4) were best described by the factors highlighted in bold in Table 7.

The outcomes of Cramér's V and CATPCA analyses were used to inform the CATREG regression analyses performed and thus configure the regression equations presented in Table 8, hence the reduction of factors on each predictive equation. This reduction affects both the likelihood of Type 1 errors and the likelihood that multiple significant findings are reported as independent observations, when in fact they represent the same underlying relationship, as it was discarded in Navas et al. [9]. Table 8 presents the standardized solution for the regression equations.

The two-dimensional model has an internal consistency coefficient (Cronbach's Alpha) of 0.880 and yields an eigen value of 5.471 for the first component, indicating that 39.075% of the variance is accounted by this component (Table 6). For the second component, the internal consistency coefficient is 0.602 with an eigen value of 2.269, indicating that its proportion of variance is 16.204%. On the whole, the internal consistency coefficient (Cronbach's Alpha) for the bi-dimensional model was 0.938, and the eigen value yielded of 7.739, explaining a total of 55.279% of the variability.

Table 6. CATPCA model summary.

Dimension	Cronbach's Alpha	Total (Eigenvalue)	% of Variance	Dimension	Cronbach's Alpha	Total (Eigenvalue)	% of Variance	Dimension	Cronbach's Alpha	Total (Eigenvalue)	% of Variance
1	0.849	4.733	33.804	1	0.876	5.351	38.225	1	0.880	5.471	39.075
2	0.618	2.347	16.767	2	0.594	2.228	15.914	2	0.602	2.269	16.204
3	0.530	1.968	14.058	3	0.451	1.721	12.296				
4	0.395	1.579	11.280								
Total	0.976 [a]	10.627	75.910	Total	0.961 [a]	9.301	66.435	Total	0.938 [a]	7.739	55.279

[a] Total Cronbach's Alpha is based on the total eigenvalue.

Table 7. Categorical principal component analyses (CATPCA) component loadings.

Environmental Factors	Dimension		Environmental Factors	Dimension			Environmental Factors	Dimension			
	1	2		1	2	3		1	2	3	4
Rainfall on the following day	**0.974**	0.127	Season	**0.974**	0.167	0.032	Rainfall on the following day	**0.964**	0.046	−0.209	0.115
Sunlight hours	**−0.974**	−0.148	Sunlight hours	**−0.973**	−0.180	−0.037	Rainfall per day	**0.964**	0.044	−0.211	0.116
Season	**0.973**	0.123	Rainfall on the following day	**0.972**	0.184	0.036	Sunlight hours	**−0.954**	−0.110	0.207	−0.149
Rainfall per day	**0.972**	0.132	Rainfall per day	**0.971**	0.188	0.039	Season	**0.954**	0.080	−0.225	0.139
Year of evaluation	**0.754**	0.142	Year of evaluation	**0.745**	0.052	0.370	Barometric pressure	**0.703**	0.155	**0.574**	−0.161
Barometric pressure	**0.666**	−0.372	Barometric pressure	**0.651**	−0.349	−0.220	Year of evaluation	0.183	**0.700**	**0.601**	0.017
Temperature	−0.448	−0.206	Temperature	−0.437	−0.350	0.377	Windspeed	−0.405	**0.694**	−0.33	0.476
Windspeed	−0.344	**0.871**	Windspeed	−0.336	**0.810**	0.342	Relative humidity	−0.489	**0.660**	−0.312	0.453
Relative humidity	−0.404	**0.846**	Relative humidity	−0.474	**0.738**	0.353	Temperature	0.274	**0.610**	0.329	0.035
Season of birth	0.095	−0.424	Season of birth	0.125	−0.444	0.180	Season of birth	0.149	−0.353	0.264	0.246
Year of birth	−0.331	−0.363	Moon phase at birth	0.068	0.375	−0.046	Weather conditions	−0.141	0.291	**0.634**	−0.198
Moon phase at birth	0.070	0.360	Year of birth	0.075	−0.436	**0.659**	Moon phase at evaluation	−0.075	−0.324	0.323	**0.659**
Moon phase at evaluation	−0.180	−0.220	Moon phase at evaluation	−0.008	−0.336	**0.589**	Year of birth	0.001	−0.329	0.392	**0.622**
Weather conditions	−0.173	−0.179	Weather conditions	−0.189	−0.095	**−0.576**	Moon phase at birth	0.002	−0.314	0.23	0.362

Numbers in bold highlight meaningfully contributing factors to each model (>|0.5|).

Table 8. Regression equations for the behavioral variables assessed.

Model	Regression Equation	Legend
General model	$Z'y_{tmil} = \beta_{RainfallPrediction}Z_{RainfallPrediction} + \beta_{Sunlighthours}Z_{Sunlighthours} + \beta_{Season}Z_{Season} + \beta_{Rainfall}Z_{Rainfall} + \beta_{Year}Z_{Year} + \beta_{BarometricPressure}Z_{BarometricPressure} + \beta_{Temperature}Z_{Temperature} + \beta_{Windspeed}Z_{Windspeed} + \beta_{Relativehumidity}Z_{Relativehumidity} + \beta_{BirthSeason}Z_{BirthSeason} + \beta_{BirthYear}Z_{BirthYear} + \beta_{BirthMoon}Z_{BirthMoon} + \beta_{Moonphase}Z_{Moonphase} + \beta_{Weather}Z_{Weather}$	$Z'y_{tmil}$ = Z score for each behavioral categorical variable (Response type, response intensity, mood/emotion and learning ability). β = standardized coefficient for each of the noncognitive categorical factors appearing in the subindex. Z = Z score for each of the noncognitive categorical factors appearing in the subindex.
Response type	$Z'y_t = 0.387(Z_{RainfallPrediction}) + 0.527(Z_{Sunlighthours}) + 0.166(Z_{Rainfall}) + 0.196(Z_{Year}) + 0.285(Z_{BarometricPressure}) + 0.206(Z_{Temperature}) + 0.304(Z_{Windspeed}) + 0.136(Z_{Relativehumidity}) + 0.053(Z_{BirthSeason}) + 0.235(Z_{BirthYear}) + 0.098(Z_{BirthMoon}) + 0.145(Z_{Moonphase}) + 0.121(Z_{Weather})$	$Z'y_t$ = Z score for response type variable. $\beta_{RainfallPrediction}Z_{RainfallPrediction} = 0.387(Z_{RainfallPrediction})$ $\beta_{Sunlighthours}Z_{Sunlighthours} = 0.527(Z_{Sunlighthours})$ $\beta_{Rainfall}Z_{Rainfall} = 0.166(Z_{Rainfall})$ $\beta_{Year}Z_{Year} = 0.196(Z_{Year})$ $\beta_{BarometricPressure}Z_{BarometricPressure} = 0.285(Z_{BarometricPressure})$ $\beta_{Temperature}Z_{Temperature} = 0.206(Z_{Temperature})$ $\beta_{Windspeed}Z_{Windspeed} = 0.304(Z_{Windspeed})$ $\beta_{Relativehumidity}Z_{Relativehumidity} = 0.136(Z_{Relativehumidity})$ $\beta_{BirthSeason}Z_{BirthSeason} = 0.053(Z_{BirthSeason})$ $\beta_{BirthYear}Z_{BirthYear} = 0.235(Z_{BirthYear})$ $\beta_{BirthMoon}Z_{BirthMoon} = 0.098(Z_{BirthMoon})$ $\beta_{Moonphase}Z_{Moonphase} = 0.145(Z_{Moonphase})$ $\beta_{Weather}Z_{Weather} = 0.121(Z_{Weather})$
Mood/Emotion	$Z'y_m = 0.468(Z_{RainfallPrediction}) + 0.596(Z_{Sunlighthours}) + 0.242(Z_{Year}) + 0.365(Z_{BarometricPressure}) + 0.230(Z_{Temperature}) + 0.395(Z_{Windspeed}) + 0.263(Z_{Relativehumidity}) + 0.075(Z_{BirthSeason}) + 0.212(Z_{BirthYear}) + 0.117(Z_{BirthMoon}) + 0.111(Z_{Moonphase}) + 0.211(Z_{Weather})$	$Z'y_m$ = Z score for the mood/emotion variable. $\beta_{RainfallPrediction}Z_{RainfallPrediction} = 0.468(Z_{RainfallPrediction})$ $\beta_{Sunlighthours}Z_{Sunlighthours} = 0.596(Z_{Sunlighthours})$ $\beta_{Year}Z_{Year} = 0.242(Z_{Year})$ $\beta_{BarometricPressure}Z_{BarometricPressure} = 0.365(Z_{BarometricPressure})$ $\beta_{Temperature}Z_{Temperature} = 0.230(Z_{Temperature})$ $\beta_{Windspeed}Z_{Windspeed} = 0.395(Z_{Windspeed})$ $\beta_{Relativehumidity}Z_{Relativehumidity} = 0.263(Z_{Relativehumidity})$ $\beta_{BirthSeason}Z_{BirthSeason} = 0.075(Z_{BirthSeason})$ $\beta_{BirthYear}Z_{BirthYear} = 0.212(Z_{BirthYear})$ $\beta_{BirthMoon}Z_{BirthMoon} = 0.117(Z_{BirthMoon})$ $\beta_{Moonphase}Z_{Moonphase} = 0.111(Z_{Moonphase})$ $\beta_{Weather}Z_{Weather} = 0.211(Z_{Weather})$

Table 8. *Cont.*

Model	Regression Equation	Legend
Response intensity	$Z'y_i = 0.670(Z_{RainfallPrediction}) + 0.814(Z_{Sunlighthours}) + 0.621(Z_{Season}) + 0.231(Z_{Rainfall}) + 0.065(Z_{Year}) + 0.280(Z_{Windspeed}) + 0.106(Z_{Relativehumidity}) + 0.054(Z_{BirthSeason}) + 0.195(Z_{BirthYear}) + 0.093(Z_{BirthMoon}) + 0.107(Z_{Moonphase})$	$Z'y_i$ = Z score for the response intensity variable. $\beta_{RainfallPrediction}Z_{RainfallPrediction} = 0.670(Z_{RainfallPrediction})$ $\beta_{Sunlighthours}Z_{Sunlighthours} = 0.814(Z_{Sunlighthours})$ $\beta_{Season}Z_{Season} = 0.621(Z_{Season})$ $\beta_{Rainfall}Z_{Rainfall} = 0.231(Z_{Rainfall})$ $\beta_{Year}Z_{Year} = 0.065(Z_{Year})$ $\beta_{Windspeed}Z_{Windspeed} = 0.280(Z_{Windspeed})$ $\beta_{Relativehumidity}Z_{Relativehumidity} = 0.106(Z_{Relativehumidity})$ $\beta_{BirthSeason}Z_{BirthSeason} = 0.054(Z_{BirthSeason})$ $\beta_{BirthYear}Z_{BirthYear} = 0.195(Z_{BirthYear})$ $\beta_{BirthMoon}Z_{BirthMoon} = 0.093(Z_{BirthMoon})$ $\beta_{Moonphase}Z_{Moonphase} = 0.107(Z_{Moonphase})$
Learning ability	$Z'y_i = 0.670(Z_{RainfallPrediction}) + 0.814(Z_{Sunlighthours}) + 0.621(Z_{Season}) + 0.231(Z_{Rainfall}) + 0.065(Z_{Year}) + 0.280(Z_{Windspeed}) + 0.106(Z_{Relativehumidity}) + 0.054(Z_{BirthSeason}) + 0.195(Z_{BirthYear}) + 0.093(Z_{BirthMoon}) + 0.107(Z_{Moonphase})$	$Z'y_i$ = Z score for the learning ability variable. $\beta_{RainfallPrediction}Z_{RainfallPrediction} = 0.670(Z_{RainfallPrediction})$ $\beta_{Sunlighthours}Z_{Sunlighthours} = 0.814(Z_{Sunlighthours})$ $\beta_{Season}Z_{Season} = 0.621(Z_{Season})$ $\beta_{Rainfall}Z_{Rainfall} = 0.231(Z_{Rainfall})$ $\beta_{Year}Z_{Year} = 0.065(Z_{Year})$ $\beta_{Windspeed}Z_{Windspeed} = 0.280(Z_{Windspeed})$ $\beta_{Relativehumidity}Z_{Relativehumidity} = 0.106(Z_{Relativehumidity})$ $\beta_{BirthSeason}Z_{BirthSeason} = 0.054(Z_{BirthSeason})$ $\beta_{BirthYear}Z_{BirthYear} = 0.195(Z_{BirthYear})$ $\beta_{BirthMoon}Z_{BirthMoon} = 0.093(Z_{BirthMoon})$ $\beta_{Moonphase}Z_{Moonphase} = 0.107(Z_{Moonphase})$

Table 6 shows the internal consistency coefficients (Cronbach's Alpha), eigenvalues and percentage of variability explained by each of the components of the three and four-dimensional models. On the whole, the internal consistency coefficient (Cronbach's Alpha) for the three and four-dimensional models were 0.961 and 0.976, respectively. The eigen value yielded for the three and four-dimensional models were of 9.301 and 10.627, respectively, and they explained a total of 66.435% and 75.910% of the variability, respectively.

3.2. Model and Operant Conditioning Test Behavioral Variability Explanatory Quality

CATREG R squared coefficient obtained ranged from 0.288 to 0.375 for the response type, and response intensity and learning ability variables, respectively (Table 4). In the same way, when CATPCA was implemented, four and three-dimensional models accounted for 75.910% and 66.435% of the total variance of behavioral variables, respectively. These results could compare to those obtained by CATREG. These findings address the fact that two of the components of the study could be summarized into one, with a low loss (9.475%) in the explanatory power of the variability. This low loss could stem from the fact that the response type variable was obtained classifying the levels in the mood/emotion variable, so that response type variable somehow derived from the mood/emotion variable. This percentage of loss is around the same value shown by CATPCA for the explanatory power of the 4th dimension (11.280%).

4. Discussion

Our statistical outputs suggest that the operant conditioning tests and model designed and used for our study efficiently and successfully enable quantifying the variation in the adaptive and cognitive behavioral response of donkeys (Tables 4 and 7).

Cramer's V has been stated to be the most suitable parameter for assessing factor strength and testing for significance after the results of cross-sectional studies relying on chi-square analyses. Although most meteorological or climatological variables could be assumed to be approximately normally distributed, some other such as rainfall, remarkably deviate from a Gaussian distribution [18]. Chi-square tests become then especially relevant, as they are neutral to the parametric or non-parametric nature of the distribution and relatively robust to situations in which there are only a limited number of data common to endangered populations, as it would be the case of donkey breeds.

As our results suggest, when we aim at comparing continuous environmental factors relying on linear scales with accurately described behavioral or cognitive categorical variables, it is useful to homogenize their nature, turning continuous variables into categorical ones. This homogenization may simplify establishing effective, easily-understandable relationships.

According to Cohen [19], when using Cramer's V, small effect associations may range from 0.0 to 0.10, medium effect associations from 0.3 to 0.5 and large effect associations from 0.5 to anything above. The same author would suggest this parameter to be especially suitable for behavioral or psychological studies, considering a statistically significant measure of $p < 0.05$ with a smaller or greater effect size to indicate a meaningful difference among the categories of a particular factor influencing the different categorical levels of the variables under study.

While studying our first hypothesis, Chi-Square and Cramér's V highlighted there was a significant linear correlation between environmental factors and variables (Table 3), although the behavioral variables tested were not dependent on some of them as shown in the result section.

Chi-Square and Cramér's V highlighted there was a highly statistically significant linear correlation ($p < 0.001$) between all environmental factors and variables, except for season at birth which was just significant ($p < 0.05$) for response intensity and learning ability and non-significant for response type (Table 3). However, the only factor behavioral variables tested were not dependent on some of them as shown in the result section.

Date of birth has been extensively reported to influence behavior and cognitive abilities in animal models which have later been applied to humans [20,21] with an underneath basis relying on circadian rhythms [22], frequently or exclusively focusing on the influence of birth months. However, the CATREG standardized coefficients and CATPCA component loadings reported found an almost three times lower variation and therefore a weaker factor strength for the birth season when compared to birth year. This low variation among seasons could rely on season shifting, one of the most widely discussed events of climate change [23]. The occurrence of shifting seasons is directly linked to warmer worldwide temperatures. According to Stine et al. [24], the amplitude component of the annual cycle (half the difference between summer and winter temperatures) has progressively decreased in most continental areas. This situation translates into the occurrence of warmer winters resulting in a lower seasonal weather variation through the year, as our results suggest. In the same way, the greater importance and higher relative frequency for birth year variations may support all of the long-term progressively increasing temperature records existing from one year to another since 1884 [25].

It may be worth noting that the late gestation of the animals displaying a depressive behavior pattern took place during the winter to early spring of 2005, when the cold wave accounting for the lowest temperature in the last 117 years, took place in Spain [26]. This situation may be worsened given the characteristics of the light grey coat of Andalusian donkeys which makes them more sensitive to cold weather. Furthermore, the animals born during that spring were all jennies. Studies in humans [27] and rats [28] have reported that the pregnancies of mothers who had been exposed to extreme weather conditions not only presented a resulting offspring with a lower weight at birth and at increased risk to experience developmental, learning, and emotional disorders, but also an altered sex ratio, lowering the occurrence of newborn male offspring in different species [29–31].

Moon phases have been reported to increase the number of deliveries in cows [32]. The same authors would report that apart from the higher birth rates of the dairy cows near and during the full moon, the predicted and real delivery dates significantly differed within the eight moon phases. Cows with predicted delivery dates before the first-quarter moon tended to deliver later than expected, whereas cows with delivery dates on a full moon to last-quarter phase tended to deliver on schedule. Although our study is the first to attempt the assessment of the effect of the moon phase at birth on mood or behavior, it is possible that this reported alteration on the times at delivery may be the basis for different degree alterations of cognitive development. These cognitive alterations may translate into future behavioral mood statuses, as suggested by the near 10% linearly correlated effect of moon phase at birth on learning abilities and 12% linearly correlated effect of moon phase at birth on mood found in our study through Cramér's V and CATREG. Figure 1 shows the relative frequency distribution for different mood/emotion patterns displayed by the donkeys relative to the phase of the moon at the moment of birth and at the time of evaluation.

Our results support the information found by Zakari et al. [33], according to which the behavioral repertoire of donkeys is modulated depending on the season. This seasonal evaluation effect has also been reported by equid welfare organizations such as The Brooke in working donkeys [34]. The study by Meyer et al. [35] in humans reported cognitive abilities to be distorted by a seasonal effect linked to serotonin levels in humans with better cognitive performance in summer, what extended to our experience could explain the increased frequency of animals refusing to cross the unknown surface. Donkeys' increased cognitive abilities have been mistaken with stubbornness. Therefore, refusal to cross new surfaces may be related with an increased ability to assess potentially harmful or dangerous situations.

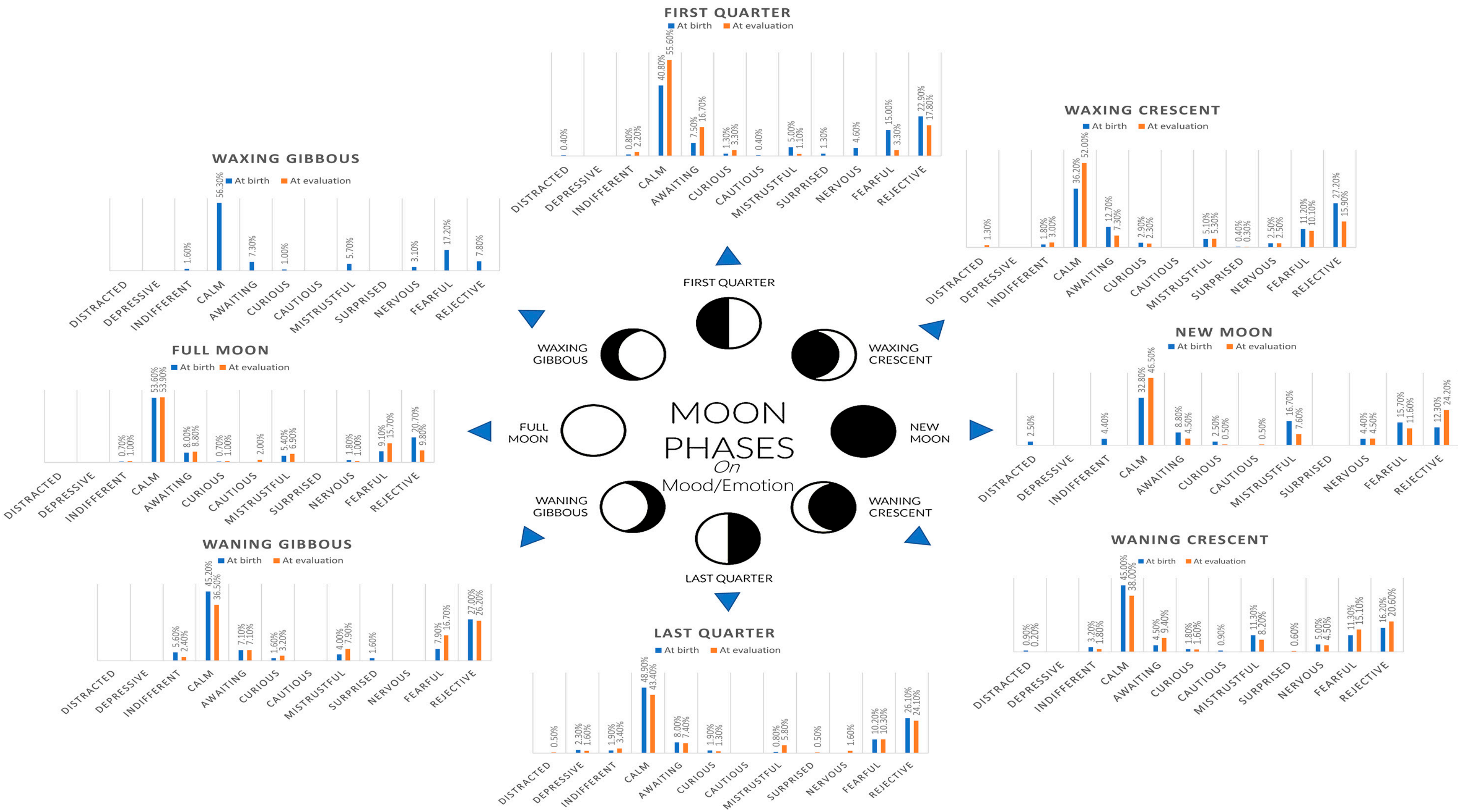


Figure 1. Relative frequency distribution for different mood/emotion patterns displayed by the donkeys relative to the phase of the moon at the moment of birth and at the time of evaluation.

Moon phase has been reported to alter both humans and animal at many different psychological and physiological levels [6]. A slight decrease in the strength of the effect of moon phase at the date of evaluation of more than half the strength for the effect of moon phase at birth was reported according to CATREG standardized coefficients. Cramér's V for moon phase at evaluation was around half the value for moon phase at birth, what suggested a stronger linear correlation between this factor and mood, response intensity, and learning ability variables. The power that the moon exerts on living beings may be mainly attributed to two factors or primary forces which differ along the consecution of the moon cycle; gravity and light changes, and their suggested effect on hormonal production and regulation. Folklore has reported a possibly calmer, hyporeactive status and low cognitive abilities in marine animals like the whale shark, which, as South Sea Islanders believe, are most easily caught a few days after a full moon. In the same way, the Miskito Indians of Eastern Nicaragua, believe that all animals respond to tides, that the woodpecker pecks when the tide is changing, and that hunting and fishing are best at the rising tide, but not at a new moon [36]. This has also been reported for hunting behavior in such large felines as lions, which were prone to hunt larger preys during new moon phases [37]. The time between two successive high or low tides is 12.4. A "lunar day" is 24.8 h. Tides are greatest at a new moon when the gravitational pull of the sun and moon are both acting in the same direction. Because the moon is moving relative to the Earth and the Sun, "lunar days" are not precisely 24 h [38], which at the same time alters normal light cycles. LeGates et al. [39] reported that when subjected to an abnormal light cycle, mice's cognitive and mood functions were directly affected through intrinsically photosensitive retinal ganglion cells, which may support the strength of the effects obtained for all variables in our study. The effect of the number of sunlight hours found in our study not only was the stronger one according to CATREG standardized coefficients but also the one holding the strongest inverse correlations for all the dimensions in the CATPCA. Exposure to unnatural lighting can induce significant changes in affection, increasing depressive-like and decreasing anxiety-like responses as it disrupts circadian rhythms of locomotor activity, body temperature, hormones, and the sleep-wake cycle in animals [40].

Behavioral responses and mood have been reported to be altered because of weather conditions and the effects of high and low extreme temperature and relative humidity, although still no previous study assessing the direct correlation with weather conditions or environment temperature has been carried out. The results by Denissen et al. [41] revealed the main effects of temperature, wind power, and sunlight on negative emotion patterns in humans and this could be extrapolated to donkeys as highlighted by the CATPCA loadings and CATREG standardized coefficients observed for the temperature, relative humidity and weather conditions on the four variables tested (Tables 3, 5, 8 and S8). The basis for this behavioral and possibly cognitive repercussion could be, as stated by [42], the fact that endothermic animals such as equids usually keep their body temperature within narrow limits with changing environmental conditions in an attempt to cool brain temperature. This advantage means a drawback as well, as it occurs at a high energetic cost, making endothermic animals face a two-fold challenge. This double challenge could be one of the reasons, as reported by Janczarek et al. [43], for adverse changes in the behavior of recreational horses that can occur if the horse is ridden when the air temperature is above 26 °C. These conditions may cause an alteration in mood, with donkeys showing more elusive and hyporeactive responses, and a reduction in the willingness to work in horses and other equids. In our study, this was supported by the increase on the refusal to cross and lack of cooperation when completing the problem-solving test, a decrease on the frequency for neutral responses and an increase in the frequencies for rejective and fearful attitudes when temperature ranged from 25 to 29 °C.

Relative humidity has been reported to be a thermally stressing factor from a welfare perspective and to affect donkey behavior and performance when it reaches extreme upper values as reported by Zakari et al. [5] and Gebresenbet et al. [44]. Heat loss mechanisms include evaporation, skin blood flow, and cardiovascular support for thermoregulation and exercise. Low temperatures have been reported to inhibit sweat gland in the donkey [45] and when simultaneously relative humidity is high this effects increase. Sweat does not readily evaporate from the body, and therefore it cannot reduce its temperature efficiently. When this rate is low, such evaporation rate is excessive therefore causing mucosa and skin dryness and increasing heart rate [44]. This situation alters performance in working donkeys and has been reported to reduce complex cognitive capacities in humans [46]. Parallelly, the low cooperative response frequency may be attributed to the fact that as temperature increases and relative humidity decreases, when kept around an optimal point for donkeys, they may be prone to display natural behaviors. Donkeys are energetic natural savers [47], and they will tend to slow moving and decrease their behavioral activity rather than display the compensative methods that they are likely to present under stressing meteorological situations [5].

Extreme high windspeed has been reported to be a welfare distorting factor for donkeys [5,48] to which individuals may adapt differently. Interestingly, as windspeed decreased, the responses of the donkeys became milder, and their attitudes turn less cooperative. White or light coat animals such as the Andalusian donkey have been reported to absorb more heat under higher to 3 m/s windspeeds, which may make them develop more stressful responses [49], hence, the high frequency for stress related moods and slightly lower intensity responses for calmer or cooperative moods. The low variation found, may account for the similar values obtained for almost all the variables. Similarly to our findings, studies in mice have reported a pronounced behavioral inhibition as well as a cognitive disruption because of an increase in the duration of light phases per day, which should be considered when testing animals for such traits [50].

Slight barometric pressure fluctuations have traditionally been reported to promote behavioral and feeding activity in fish. Fishers usually relate slight changes towards high pressure to clear sky occurrence during which fishing is medium to slow as fish may slowly be in deeper water or near cover. These trends progressively invert when there is falling pressure, the best attributed timing for fishing during degrading weather when fish are more active what may support our results [51], though still no previous scientific research has been carried out on the effects of slight variations on barometric pressure. Studies on rats have reported individuals to be more prone to develop depressive behavioral patterns when they are exposed to a sharp fall in barometric pressure (20 hPa below the natural atmospheric pressure) [52]. However, the animals in our study were not exposed to such extreme air pressure variations.

Rainfall has been reported to be especially crucial as a welfare distorting or stressful provoking factor in donkeys [48]. Curiously, donkeys have traditionally been attributed the ability to predict lousy weather (Graphical abstract) and rain occurrence [53,54] as it could be stated by this study, although this may be the first attempt to scientifically proof such ability.

5. Conclusions

Environmental conditions, seasonal, timing (year) and moon cycle phases are potential stress factors or behavioral modulators that affect the behavior and cognitive responses of donkeys, as well as may have potentially long lasting effects which can be traced back. Climate oscillation effects may affect donkeys altering their physiological biorhythms and produce severe behavioral and cognitive modifications. Deviations in behavioral patterns or on the abilities of the donkeys to perform complex tasks to which they may not be accustomed may become relevant indicators of welfare as well as they may address the most suitable techniques or methods to be applied in each case. Furthermore, behavior becomes a relevant tool when predicting future weather conditions as well as may report the potential distortion that they may cause, a prominent importance fact for veterinarians, practitioners and donkey owners, as it may allow them to anticipate such situations in order to counteract their effects.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/8/11/215/s1. Table S1. Category description and definition for response type, the intensity of response, mood/emotion, and learning variables directly controlled during the operant conditioning test; Table S2. Categorical variable description and levels for the effects of meteorological environment and birth characteristics collaterally controlled during the fulfilment of the test during the first phase of the study; Table S3. Descriptive statistics and numerical parametrization of all the variables analyzed; Table S4. Description for the mood and response type behavioral categorical variables and "Mercalli" scales; Table S5. Cohen's kappa and 95% confidence interval for inter-observer reliability testing; Table S6. Total and relative frequencies for the associations of the four dependent categorical variables (type and intensity/degree of response, mood/attitude and problem-solving success/learning rate) with eleven independent environmental factors (year, season and moon phase at evaluation, temperature, relative humidity, windspeed, sunlight hours, barometric pressure, rainfall per day, rainfall on the following day and weather conditions); and the three birth related environmental characteristics (season, year and moon phase at birth).; Table S7. CATREG Standardized Coefficients (β) sorted in order of importance on the variables tested; Table S8. Cramér's V (Chi squared), Standardized Coefficients (β) (CATREG) and loadings (CATPCA) output comparison.

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Conflicts of Interest: The authors declare no conflict of interest.

Ethics 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 guide 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 22 September of 2010.

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Chapter 4

Navas, F.J.; Jordana, J.; León, J.M.; Arando, A.; Pizarro, G.; McLean, A.K.; Delgado, J.V.

Measuring and modeling for the assessment of the genetic background behind cognitive processes in donkeys

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Measuring and modeling for the assessment of the genetic background behind cognitive processes in donkeys

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ABSTRACT

New productive niches can offer new commercial perspectives linked to donkeys' products and human therapeutic or leisure applications. However, no assessment for selection criteria has been carried out yet. First, we assessed the animal inherent features and environmental factors that may potentially influence several cognitive processes in donkeys. Then, we aimed at describing a practical methodology to quantify such cognitive processes, seeking their inclusion in breeding and conservation programmes, through a multifactorial linear model. Sixteen cognitive process-related traits were scored on a problem-solving test in a sample of 300 Andalusian donkeys for three consecutive years from 2013 to 2015. The linear model assessed the influence and interactions of four environmental factors, sex as an animal-inherent factor, age as a covariable, and the interactions between these factors. Analyses of variance were performed with GLM procedure of SPSS Statistics for Windows, Version 24.0 software to assess the relative importance of each factor. All traits were significantly ($P < 0.05$) affected by all factors in the model except for sex that was not significant for some of the cognitive processes, and stimulus which was not significant ($P > 0.05$) for all of them except for the coping style related ones. The interaction between all factors within the model was non-significant ($P > 0.05$) for almost all cognitive pro-cesses. The development of complex multifactorial models to study cognitive processes may counteract the inherent variability in behavior genetics and the estimation and prediction of related breeding parameters, key for the implementation of successful conservation programmes in apparently functionally misplaced endangered breeds.

1. Introduction

Being domesticated prior to the horse, the suitability of the donkey species for mankind has been documented through History. Considering its overall docile nature, donkeys have been proved to be especially suitable for women and children, who use them for traction and draught power when compared to oxen or larger equines. In areas where donkeys are no longer used, owners and breeders are left to find alternative uses otherwise endangered breeds vanish. This sets an optimal framework for new donkey application niches to arise, as for example, their use in leisure and equine assisted therapy (Rose et al., 2011), which are supported by scientifically reported beneficial effects on human health (Borioni et al., 2012). Donkeys used in such settings must be tested and selected for their abilities to develop cognitive processes, especially those relating to their overall behavior and coping style levels, as this may translate in reducing the money and time invested in their education.

The knowledge on the factors conditioning cognitive processes is especially relevant to assess the genetic variability behind them, as it may help develop accurate selection programmes, aiming at preserving such variability, one of the keys for survival in endangered breeds.

Contrary to what authors such as Hausberger et al. (2004) have recommended, functional traits have never comprised the selection criteria included in the breeding programmes of donkeys, as only morphological and phaneroptical (mainly coat) features had been considered.

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There are many internal and external factors that may affect equid behavior and therefore, the cognitive processes that equids develop. Researchers have measured how factors such as environment (French, 1993), handling conditions (Lansade et al., 2004), age, sex, breed, sire (Hausberger et al., 2004), season, diurnal cycles (Lamoot and Hoffmann, 2004) and year (Lamoot et al., 2005) may modulate donkey behavior from a phenotypical perspective. Although such factors have been reported to be significant for the development of different ethological patterns, no study has focused on assessing reliable quantitative methods for their integration in linear genetic models in donkeys. Hence, this study constitutes the first of its kind aiming at understanding the degree at which non-genetic factors influence cognitive processes under field conditions in donkeys.

The two main objectives of this study were, first, to assess the effects that inherent factors (sex and age) and external environmental factors (assessment year, season, stimuli and husbandry system) have on cognitive processes in donkeys, and second, to describe the potential implementation of quantifiable genetic models for the inclusion of such cognitive processes in breeding and conservation programmes through a routine *in-situ* test methodology.

2. Material and methods

2.1. Animals

Records from 300 Andalusian donkeys (n = 300, 78 jacks and 222 jennies), with ages ranging from 9 days to 23 years, were used in this study. All the donkeys were registered in the Andalusian donkey studbook and had been genotyped by the use of a filiation test for each mating with 24 microsatellite molecular markers recommended by the International Society of Animal Genetics (ISAG), especially suitable for donkeys (Table 1). The donkeys (n = 300) were the progeny of 93 jacks and 253 jennies.

2.2. Cluster definition context: etymological reasons and scale definitional issues

Intelligence or IQ-related cognitive processes have been suggested to be influenced by environmental factors, as opposed to other cognitive processes which may not necessarily be affected. This context suggests a potential hereditary or genetic background conditioning them and lays the basis for their quantification and qualification. The strategies used to measure cognitive processes and the etymological controversy raised when we intend to sort them into categories, to isolate intelligence or coping style related ones from the rest, often arrives at a point at which, although we cannot consider these processes to be synonyms, they may often overlap.

The practical study of complex traits, such as cognitive processes, always requires the thorough separate definition of the traits being considered, as concepts may outline traits better than terms themselves. In this study, we initially separated the cognitive processes assessed into three clusters to define and study them more accurately. The first of them or coping style cluster involved three traits describing the reactivity of the donkeys to visual and auditory stimuli presented from different positions. The two remaining clusters were divided considering the differences set by Sparrow and Davis (2000). According to these authors, a second cluster or cognition cluster comprised the traits that referred to the cognitive processes whereby individuals acquire knowledge from the environment. The third cluster or intelligence cluster considered intelligence in a very narrow sense, referring to those cognitive processes that are commonly evaluated by intelligence human IQ tests or by extension, g-factor animal related tests (Boring, 1929). Sparrow and Davis (2000) would address the agreement on the existence of multiple components that combine to produce complex cognitive processes (such as problem-solving), as the common point at which the different definitions and theories of cognition and intelligence converge. This dissertation sets the main behavioral context of our study, and is one of the main reasons for the design and use of the present problem-solving test (Table 2), as it enables the simultaneous quantification and classification of the ability of the donkeys under study to develop such complexly intertwined cognitive processes.

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 R: GTTCACTGAATGTCAAATTCTGCT	78–84
HTG10	F: CAATTCCCGCCCCACCCCCGGCA R: TTTTTATTCTGATCTGTCACATTT	83–103
HTG15	F: TCCTGATGGCAGAGCCAGGATTTG R: AATGTCACCATGCGGCACATGACT	116–134
LEX3	F:ACATCTAACCAGTGCTGAGACT R:AAGAACTAGAACCTACAACTAGG	194–220
VHL20	F: CAAGTCCTCTTACTTGAAGACTAG R: AACTCAGGGAGAATCTTCCTCAG	75–105
TKY287	F:ATCAGAGAACACCAAGAAGG R:TCTCTGCTATAGGTAAGGTC	215–245
TKY294	F:GATCTATGTGCTAGCAAACAC R:CTAGTGTTTCAGATAGCCTC	210–235
TKY297	F:GTCTTTTTGTGCCTCGGTG R:TCAGGGGACAGTGGCAGCAG	215–250
TKY301	F:AATGGTGGCTAATCAATGGG R:GTGTATGATGCCCCTCATCTC	140–170
TKY312	F:AACCTGGGTTTCTGTTGTTG R:GATCCTTCTTTTTATGGCTG	90–130
TKY321	F:TTGTTGGGTTTAGGTATGAAGG R:GTGTCAATGTGACTTCAAGAAC	175–210
TKY341	F:TATCCAGTCACCCATTTTAC R:TTGTGTCAGTACACTCTATG	135–160
TKY343	F:TAGTCCCTATTTCTCCTGAG R:AAACCCACAGATACTCTAGA	135–170
TKY344	F:GTGTCCATCAATGGATGAAG R:CTTAAGGCTAAATAATATCCC	75–115

F: Forward primer; R: Reverse primer.

Not only is the difficulty in isolating cognitive processes for their study, but also the fact that they may be measured differently, what determined the use of the test elected as well. IQ related or g factor (see Anderson, 2000) intelligence tests provide numerical values assigned on a scale. By contrast, although cognitive assessment does not necessarily use a numerical score, it enables categorical values to be translated into linear numerical scales, therefore connecting the quantification and qualification of the processes studied. The translations from the cognitive processes categorical scales to numerical scales for the three clusters described above are shown in Tables 3 and 4.

Table 2
Problem-solving test phase I and II description and treatment classification.

Treatment	Description	Stimulus type	Reinforcement
Phase I. Oilcloth test.			
Treatment 1 (S1)	Handler (B) uses a lead rope and soft voice, trying to comfort the donkey to make the donkey cross the oilcloth on the floor, but without pulling the rope if the donkey refuses to move.	Unknown frontal visual stimulus.	Positive
Treatment 2 (S2)	Handler (B) used a lead rope with applied pressure to make the donkey cross over the oilcloth. Handler (B) releases the pressure when the donkey moves as it crosses the oilcloth.	Known frontal visual stimulus.	Positive
Treatment 3 (S3)	The donkey was lured by a familiar treat (dry bread, carrots or feed, depending on the owner's tastes and to which the donkey was accustomed) by handler (C). The treat is given to the donkey when the task was completed.	Known frontal visual stimulus.	Positive
Treatment 4 (S4)	Handler (B) applied pressure to the lead rope and handler C made noise from behind the donkey with a so-called “donkey motivator” (plastic bag tied on the end of a stick) (McLean et al., 2012). Handler (B) led the donkey by slightly pulling the rope until the donkey crosses the oilcloth completely.	A known frontal visual stimulus and an unknown rear auditory stimulus.	Negative
Treatment 5 (S5)	Two handlers (B and C) using two lead ropes attached on either side of the halter to encourage the donkey across. The handlers (B and C) released the pressure when the donkey moves and then reapplied when it stops until it crosses the oilcloth completely.	Known frontal visual stimulus.	Negative
Treatment 6 (S6)	Handler (B) applies pressure on the lead rope and handler (C) encourages the donkeys across by an auditory sound. Handler C claps their hands from behind the donkey to make it move forward (Nansen and Blache, 2016). Pressure and sound are released or stopped when the donkey moves and reapplied when it stops until the donkey had completed the task.	A known frontal visual stimulus and an unknown rear acoustic stimulus.	Negative
Phase II. Response tests to object and sound recognition and association.			
Treatment 7 (S7)	Measured the donkeys' reaction towards the presence of the veterinarian when asked to complete the task.	Known visual and acoustic stimuli.	N/A
Treatments 8 and 9 (S8 and S9)	Measured the response of the donkeys to the sound of a horn. Handler (C) beeps a horn in front of the donkey once (Lanier et al., 2000). After that, handler (C) blares a horn in front of the donkey three times (Lanier et al., 2000).	Simultaneous unknown at first and later known frontal visual and acoustic stimuli.	N/A
Treatment 10 (S10)	A handler (C) played a car engine recording from a round red speaker in front of the donkey under study. All donkeys had previously been in contact with a car engine sound, but the stimulus came out of an unknown device.	Simultaneous unknown visual stimulus and a known acoustic stimulus.	N/A
Treatments 11 (S11) and 12 (S12)	Scored the reaction towards other donkeys in the same herd during all the tests and the reaction towards other species animals (cows, sheep, poultry, llamas, cats, and dogs) in the same farm to which the donkeys were accustomed.	Known visual and acoustic stimuli.	N/A

N/A: not applicable.

2.3. Problem-solving test and stimulus/treatment description

All behavioral responses were registered by only one trained judge during the annual behavior assessment sessions on four random days from June to November and from 2013 to 2015, as this is the period of time of the year during which the weather conditions are most consistent in the area where the study took place. Data were collected from 22 different farms in the Andalusian region of Spain.

The behavioral test used for this study consisted of two consecutive main phases that lasted for 15 min per animal on the whole, with no pause between the presentation of each of the consecutive treatments/stimuli. Time was evenly distributed throughout the consecution of the different treatments/stimuli. Each donkey was exposed to 12 external stimuli once. Phase I started when the animal was exposed to a 2 m^2

Table 3
Scale translation and description of the twelve mood or attitude reaction related adjectives considered and donkeys' response classification towards the twelve stimuli presented to them during the study.

Scale	Mood/Attitude	Description	Response	Scale	Degree/Intensity
1	Distracted	No reaction. Pays attention to other stimuli around.	Hyporeactive	1	Scored from 1 to 5
2	Depressive	No reaction. Pays reduced attention to it. Overall, body posture shows lowered head and neck, roundness to spine and tucked tail.	Hyporeactive	1	
3	Indifferent or nonresponsive	No reaction. Pays attention to it.	Hyporeactive	1	
4	Calm	Reaction, but stands still. Pays attention to other stimuli at the same time.	Neutral	2	
5	Awaiting	Reaction, but stands still. Only focuses on the stimulus presented.	Neutral	2	
6	Curious	Reaction. Pays attention and stands still moving its head towards the stimulus.	Neutral	2	
7	Cautious	Reaction. Pays attention and slightly moves towards the stimulus.	Neutral	2	
8	Mistrustful	Reaction. Pays attention and moves towards the stimulus slowly and doubtfully.	Neutral	2	
9	Surprised	Reaction. Only focused on the stimulus being presented. Gets startled but moves towards the stimulus calmly.	Hyperreactive	3	
10	Nervous	Reaction. Only focused on the stimulus being presented. Gets startled, and tries to move apart from it at first. Able to move towards it if led by the operator.	Hyperreactive	3	
11	Fearful	Reaction. Only focused on the stimulus being presented. Tries to move apart from it. Unable to move towards it if led by the operator.	Hyperreactive	3	
12	Rejection	Reaction. Only focused on the stimulus being presented. Gets startled, and moves apart from it noticeably. Pulls apart from the leading rope when the operator tries to move towards the stimulus.	Hyperreactive	3	

Table 4
Description of the thirteen traits comprising the intelligence and cognition clusters studied and definition of their scales in donkeys.

Trait	Definition	Scale	Description
Intelligence cluster			
Concentration	The animal collaborates during the assessment session and does not get distracted by the environment.	1	Distracted
		2	Poor
		3	Inconstant
		4	Intermediate
		5	Concentrated
Curiosity	The animal is interested in the novel stimuli being presented and moves towards them.	1	Never (0%)
		2	Rarely (5-10%)
		3	Sometimes (50%)
		4	Frequently (70%)
		5	Always (100%)
Memory	The animal remembers the stimuli being presented.	1	Scattered
		2	Poor short-term memory
		3	Average short-term memory
		4	Average long-term memory
		5	Good long-term memory
Stubbornness	The donkey rejects following the requests of the assessor.	1	Stubborn (Cautious)
		2	Indifferent
		3	Moaner
		4	Reluctant
		5	Obedient
Docility	The donkey easily follows the orders of the instructor.	1	Stubborn
		2	Indifferent
		3	Moaner
		4	Reluctant
		5	Obedient
Alertness	The animal shows a vigilant or alert status focusing on the stimulus around.	1	Untamed
		2	Unwilling
		3	Reticent
		4	Adaptable
		5	Docile
Cognition cluster			
Dependence	The donkey is comfortable when separated from the main herd	1	Dependent
		2	Restless
		3	Stable
		4	Adapted
		5	Calm
Trainability	Ability of the animal to be trained into the fulfillment of the tests	1	Never (0%)
		2	Rarely (5-10%)
		3	Sometimes (50%)
		4	Frequently (70%)
		5	Always (100%)
Cooperation	The donkey cooperates with its handlers during the daily tasks	1	Never (0%)
		2	Rarely (5-10%)
		3	Sometimes (50%)
		4	Frequently (70%)
		5	Always (100%)
Emotional stability	The animal is not predictable from one to another stimulus	1	Unpredictable
		2	Surprising
		3	Stable
		4	Balanced
		5	Predictable
Perseverance	The animal is patient when completing several sequential tests.	1	Inpatient
		2	Generally impatient but easily handled
		3	Patient but pushes the operator occasionally
		4	Patient without pushing the operator
		5	Awaits the operator's orders
Get in/out of stables	The animal shows no problem when leaving or entering its housing facilities.	1	Never (0%)
		2	Rarely (5-10%)
		3	Sometimes (50%)
		4	Frequently (70%)
		5	Always (100%)
Ease of handling	The animal shows sympathy towards humans.	1	Mistrustful towards humans in general
		2	Mistrustful towards unknown people
		3	Comfortable with familiar people, but mistrustful to unknown people
		4	Comfortable with the human presence
		5	Increased sympathy for human presence

oilcloth (vinyl fabric with a canvas-like cotton mesh backing featuring a wooden printed design) for the first time (novel object), and assessed the progressive response of the animals to stimuli one to six (Table 2), parallelly assessing the suitability of the use of negative, positive or lure reinforcement methods to effectively encourage donkeys to cross the oilcloth, to which they become progressively familiar, as the test continues (non-novel object).

The oilcloth was placed 2 m ahead in front of the donkey and relayed in the same position before testing every new animal. The response of the donkey was registered and quantified by the judge from the moment the oilcloth was relayed in front of the donkey by handler (A). Handler (B) was in charge of completing the task with the donkey by utilizing different treatments/stimuli (from one to six). Phase II assessed the response to treatments/stimuli seven to twelve (Table 2) and corresponds to the presentation of different acoustic or visual independent stimuli to the donkeys under study. The animals were videotaped (30 frames/s) at 2 m from the left side of the oilcloth, from the beginning of Phase I until the end of Phase II, for later further evaluation by the same person. The person videotaping the animals, was in charge of supervising each test followed the timing requirements mentioned above.

2.4. Cognitive process related traits definition and scales

Prior to the behavioral assessment, we conducted a telephone interview to survey the experience of the owners of the donkeys in the study to define the traits comprising the clusters to be considered in the model. First, we asked owners to identify the adjectives that they most commonly used to describe their donkeys' mood or attitude towards external stimuli. Among the answers that the respondents gave, we chose twelve adjectives as the most frequent ones to describe the response to external stimuli displayed to define the scales to assess the traits included in the coping style cluster (Table 3). We discarded the rest of adjectives because of the anecdotical occurrence of their use. This coping style cluster consisted of three scales. The first scale or mood/attitude scale translated the adjectives from the survey into a score ranging from 1 to 12, with increasing levels of arousal and evasive behavior. The second scale or response scale measured whether the donkeys were hyporeactive, neutral or hyperreactive, and ranged from 1 to 3, with one being hyporeactive, 2 meaning a neutral response and 3 describing a hyperreactive animal. We assigned a score number of 1 to highly hyporeactive or distracted donkeys, and a value of 12 to highly reactive or elusive donkeys moving apart from the stimuli. We used a third scale or degree/intensity scale to score the level at which each response in the mood/attitude scale was displayed from 1 to 5, with 1 meaning the lowest intensity response while a score of 5 describes the highest intensity response displayed. We simultaneously registered information on the relationship held with reinforcement techniques applied to educate donkeys on getting used to the novel stimuli presented (Table 2).

Secondly, we interviewed owners about their donkeys' inherent cognitive abilities, the tasks that they should routinely accomplish on their farms and the training/education methodology (or learning methods) owners regularly apply for their donkeys to learn such skills/tasks. Among the answers the respondents gave, they coincided on thirteen traits which were chosen as they were the ones that the owners most frequently allude to during the interviews (Table 4). We discarded the rest of traits because of the anecdotical occurrence of their use or because of being related to the use of different nouns to allude the same behavioral trait concept.

We organized the information deriving from the interview for the thirteen behavioral traits in two clusters. A 'cognition' cluster comprising seven traits that were directly related to unspecific cognitive processes considering the ability of donkeys to perceive information from their environmental situation, and an 'intelligence' cluster comprising the six remaining traits, describing the cognitive processes or mental capacities of the donkeys to retain information from the environment as knowledge to be applied towards adaptive responses within a specific context. We translated these categorical traits into different linear scales, in which the donkeys scoring one meant they presented the lowest extreme behavioral pattern and five the highest extreme one. The thirteen intelligence and cognition related traits considered, and a detailed definition of the scores present in the scale is described in Table 4.

2.5. Statistical analyses

The present study initially considered sixteen traits comprising three main clusters according to the cluster definition context described above. Coping style cluster comprised three of these traits and was assessed separately due to the higher number of observations (n = 3 600) and factors involved, while the other two clusters 'cognition' (7 traits) and 'intelligence' (6 traits) were assessed together (n = 300), as they did not include the stimulus effect, which was non-significant ($P > 0.05$) for all the thirteen traits included in both clusters. To statistically support the organization of clusters initially described in the cluster definition context, we computed Pearson's correlations between the cognitive processes tested to ensure that none of them demonstrated very strong multicollinearity (> 0.95) what may suggest excluding those traits possibly measuring for the same cognitive process. Then, we performed an agglomerative hierarchical cluster analysis (HCA) using the centroid joining method with squared Euclidean distances to classify cognitive processes into groups with shared similarities to confirm the soundness of the *a priori* cognitive clustering division, by means of the Classify procedure from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016). The dependent variables measured (Tables 5 and 6) were of a continuous level and were assumed to be approximately normally distributed. The independent variables (year of assessment, husbandry system, sex and stimulus/treatment) each consisted of two or more categorical, independent groups with independence of observations and no significant outliers were found. We also assumed homogeneity of variances for each combination of the groups of the two independent variables, therefore, we performed a one-way ANOVA and a posthoc Tukey Test using the Means procedure from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016) to compute the fraction of the variance explained by each factor separately. Because of the small size of the sample, we used ε^2 and ω^2 to compute the effect size in the model, as they use unbiased measures of the variance components and report the least mean root square errors, therefore becoming suitable for behavioral studies (Okada, 2013), according to $\varepsilon^2 = \frac{SS_b - df_b MS_w}{SS_t}$ and $\omega^2 = \frac{SS_b - df_b MS_w}{SS_t + MS_w}$, respectively.

We performed a two-way MANOVA using the GLM procedure from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016) to compute the existing interactions between factors, as they are discontinuous variables. We used non-linear regression from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016) for two different statistic models consisting of three fixed effects; i.e.: assessment year (AY), 3 levels; sex (Sex), 2 levels and system (Sys), 5 levels and a covariate, age in months, and their separate repercussion on each of the sixteen variables. In the case of the coping style cluster, an additional effect comprising the stimuli (Sti) consisting of 12 levels was included (Table 2). The model fitted for the coping style cluster was:

$$Y = \mu + AY + Sex + Sys + Sti + AY^*Sex + AY^*Sys + AY^*Sti + Sex^*Sys + Sex^*Sti + Sys^*Sti + AY^*Sex^*Sys + AY^*Sex^*Sti + AY^*Sys^*Sti + AY^*Sex^*Sys^*St + A + \varepsilon$$

While the model for the intelligence and cognition clusters was:

$$Y = \mu + AY + Sex + Sys + AY^*Sex + AY^*Sys + Sex^*Sys + AY^*Sex^*Sys + A + \varepsilon$$

where,

Y = behavioral traits (1–16)
μ = mean
AY = assessment year (1–3)
Sex = sex (1, 2)

Table 5
Descriptive statistics for variables, fixed effects and covariables of coping style, intelligence and cognition related traits in Andalusian donkeys (n = 300).

Clusters	Effects	n	Minimum	Maximum	Mean	SEM	SD	CV
Fixed effects	Year	3 600	1	3	1.97	0.011	0.653	0.33
	System	3 600	1	5	2.58	0.016	0.971	0.25
	Stimulus	3 600	1	12	6.50	0.058	3.453	0.38
	Sex	3 600	1	2	1.74	0.007	0.439	0.53
Covariate	Age (in Months)	3 600	0.267	270.400	84.078	1.023	61.405	0.73
Coping style cluster	Response	3 600	1	3	2.26	0.008	0.473	0.21
	Mood	3 600	1	12	6.28	0.054	3.223	0.51
	Degree	3 600	1	5	3.28	0.026	1.534	0.47
Intelligence cluster	Concentration	300	1	5	3.80	0.059	1.027	0.27
	Curiosity	300	1	5	4.10	0.054	0.933	0.23
	Memory	300	1	5	4.11	0.060	1.035	0.25
	Stubbornness	300	1	5	3.67	0.068	1.174	0.32
	Docility	300	1	5	3.99	0.054	0.943	0.24
	Alertness	300	1	5	4.74	0.033	0.573	0.12
Cognition cluster	Dependence	300	1	5	4.33	0.063	1.089	0.25
	Trainability	300	1	5	3.80	0.060	1.035	0.27
	Cooperation	300	1	5	4.13	0.062	1.081	0.26
	Emotional stability	300	1	5	3.78	0.057	0.983	0.26
	Perseverance	300	1	5	4.64	0.044	0.762	0.16
	Get In/Out of Stables	300	1	5	4.58	0.046	0.791	0.17
	Ease of Handling	300	1	5	4.03	0.065	1.119	0.28

Table 6

Summary of the results of the ANOVA, posthoc Tukey Test and the determinative coefficient of the effect of each factor on weight through ε^2 and ω^2 estimators on the sixteen cognitive process-related traits assessed in Andalusian donkeys.

Cluster	Trait	Factors	F (df)[D]	P value	Levels (Mean)[C]	ε^2	ω^2
Coping styles							
Response		Year	26.088 (2)	0.000	2013 (2.36)^{bc} 2014 (2.23)^{a} 2015 (2.23)^{a}	0.0138	0.0137
		Sex	31.139 (1)	0.000	♂ (2.33) ♀ (2.23)	0.0083	0.0083
		System[A]	39.667 (4)	0.000	I (2.46)^{bcd} SI (2.16)^{ace} SE (2.26)^{abe} C (2.23)^{ae} E (2.42)^{bcd}	0.0412	0.0412
		Stimuli[B]	34.417 (11)	0.000	S1 (2.30)^{dgjkl} S2 (2.30)^{dgkl} S3 (2.21)^{dfgjkl} S4 (2.46)^{abceghikl} S5 (2.32)^{dgkl} S6 (2.36)^{cgkl} S7 (2.07)^{abcdefhij} S8 (2.33)^{dgkl} S9 (2.31)^{dgkl} S10 (2.42)^{acgkl} S11 (2.01)^{abcdefhij} S12 (2.01)^{abcdefhij}	0.0927	0.0926
Mood		Year	29.639 (2)	0.000	2013 (7.03)^{bc} 2014 (6.1)^{a} 2015 (5.94)^{a}	0.0157	0.0157
		Sex	23.089 (1)	0.000	♂ (6.71) ♀ (6.12)	0.0061	0.0061
		System[A]	40.534 (4)	0.000	I (7.65)^{bcd} SI (5.64)^{ace} SE (6.24)^{abe} C (6.19)^{ae} E (7.44)^{bcd}	0.0421	0.0421
		Stimuli[B]	62.107 (11)	0.000	S1 (6.91) ^{dgjkl} S2 (6.87)^{dgjkl} S3 (6.24)^{dfgjkl} S4 (7.98)^{abceghikl} S5 (6.82)^{dgjkl} S6 (7.19)^{cdghkl} S7 (4.54)^{abcdefhij} S8 (6.26)^{dfgjkl} S9 (6.58)^{dgjkl} S10 (7.8)^{abceghikl} S11 (4.08)^{abcdefhij} S12 (4.05)^{abcdefhij}	0.1574	0.1573
Degree		Year	57.152 (2)	0.000	2013 (3.00)^{b} 2014 (3.52)^{ac} 2015 (2.94)^{b}	0.0303	0.0303
		Sex	13.899 (1)	0.000	♂ (3.12) ♀ (3.34)	0.0036	0.0036
		System[A]	55.021 (4)	0.000	I (2.95)^{bc} SI (3.76)^{ace} SE (3.05)^{bd} C (3.53)^{ace} E (2.71)^{bd}	0.0566	0.0566
		Stimuli[B]	45.763 (11)	0.000	S1 (2.56)^{ceghijkl} S2 (2.81)^{ghijkl} S3 (3.15)^{adghij} S4 (2.55)^{ceghijkl} S5 (3.1)^{adghij} S6 (2.78)^{ghijkl} S7 (3.82)^{abcdefjkl} S8 (3.66)^{abcdefj} S9 (3.96)^{abcdefkl} S10 (4.27)^{abcdefghkl} S11 (3.39)^{abdfgij} S12 (3.37)^{abdfgij}	0.1203	0.1203
Cognition							
Dependence		Year	8.817 (2)	0.000	2013 (3.29)^{c} 2014 (4.03)^{c} 2015 (3.62)^{ab}	0.0937	0.0934
		Sex	2.022 (1)	0.156	♂ (3.62) ♀ (3.84)	0.0070	0.0069
		System[A]	5.584 (4)	0.000	I (3.46) SI (4.05)^{ce} SE (3.65)^{b} C (4.18)^{e} E (3.53)^{bd}	0.0468	0.0467
Trainability		Year	3.850 (2)	0.022	2013 (3.34)^{b} 2014 (3.84)^{a} 2015 (3.57)	0.0257	0.0256
		Sex	1.665 (1)	0.198	♂ (3.58) ♀ (3.71)	0.0000	0.0000
		System[A]	3.987 (4)	0.004	I (3.00)^{b} SI (4)^{a} SE (3.65) C (3.59) E (3.47)	0.0567	0.0566
Cooperation		Year	8.067 (2)	0.000	2013 (3.74)^{b} 2014 (4.12)^{ac} 2015 (3.93)^{b}	0.0211	0.0210
		Sex	3.776 (1)	0.053	♂ (3.82) ♀ (4.05)	0.0085	0.0085
		System[A]	10.723 (4)	0.000	I (3.76)^{bc} SI (4.25)^{acde} SE (3.96)^{ab} C (3.82)^{b} E (3.35)^{b}	0.0499	0.0497
Emotional stability		Year	16.458 (2)	0.000	2013 (4.57)^{b} 2014 (4.73)^{ac} 2015 (4.95)^{b}	0.0400	0.0399
		Sex	3.099 (1)	0.079	♂ (4.9) ♀ (4.68)	0.0245	0.0244
		System[A]	4.672 (4)	0.001	I (4.86)^{b} SI (4.74)^{ac} SE (4.72)^{b} C (4.82) E (4.53)	0.0021	0.0021
Perseverance		Year	5.054 (2)	0.007	2013 (4.40)^{b} 2014 (4.74)^{a} 2015 (4.62)	0.0264	0.0263
		Sex	0.648 (1)	0.421	♂ (4.58) ♀ (4.66)	0.0000	0.0000
		System[A]	2.130 (4)	0.077	I (4.62) SI (4.8) SE (4.54) C (4.59) E (4.41)	0.0149	0.0149
Get In/Out of Stables		Year	13.800 (2)	0.000	2013 (4.26)^{b} 2014 (4.78)^{ac} 2015 (4.38)^{b}	0.0789	0.0786
		Sex	7.715 (1)	0.006	♂ (4.37) ♀ (4.66)	0.0220	0.0219
		System[A]	7.786 (4)	0.000	I (4.78)^{d} SI (4.85)^{cde} SE (4.41)^{b} C (4.12)^{ab} E (4.29)^{b}	0.0832	0.0830
Ease of Handling		Year	8.028 (2)	0.000	2013 (3.59)^{b} 2014 (4.22)^{a} 2015 (4.00)	0.0449	0.0448
		Sex	3.725 (1)	0.055	♂ (3.82) ♀ (4.10)	0.0090	0.0090
		System[A]	8.395 (4)	0.000	I (3.41)^{bc} SI (4.39)^{ae} SE (4.06)^{ae} C (3.76) E (3.29)^{bc}	0.0900	0.0898
Intelligence							
Concentration		Year	3.218 (2)	0.041	2013 (3.53)^{b} 2014 (3.90)^{a} 2015 (3.85)	0.0146	0.0146
		Sex	5.811 (1)	0.017	♂ (3.56) ♀ (3.89)	0.0158	0.0158
		System[A]	5.434 (4)	0.000	I (3.38)^{b} SI (4.13)^{ace} SE (3.72)^{b} C (3.82) E (3.35)^{b}	0.0560	0.0558
Curiosity		Year	3.997 (2)	0.019	2013^{bc} (4.43) 2014 (4.47)^{a} 2015 (3.82)^{a}	0.0497	0.0495
		Sex	0.610 (1)	0.435	♂ (4.18) ♀ (4.38)	0.0034	0.0034
		System[A]	2.809 (4)	0.026	I (4.08) SI (4.64)^{ce} SE (4.17)^{b} C (4.82)^{e} E (3.76)^{bd}	0.0578	0.0576
Memory		Year	15.276	0.000	2013 (3.50)^{bc} 2014 (3.91)^{a} 2015 (3.82)^{a}	0.0187	0.0186
		Sex	1.570 (1)	0.211	♂ (3.67) ♀ (3.84)	0.0022	0.0022
		System[A]	12.015 (4)	0.000	I (3.35)^{bcd} SI (4.05)^{ace} SE (3.77)^{ab} C (3.82)^{ae} E (3.41)^{bd}	0.0384	0.0383
Stubbornness		Year	4.943 (2)	0.008	2013 (3.82)^{b} 2014 (4.16)^{a} 2015 (4.23)	0.0197	0.0196
		Sex	0.710 (1)	0.400	♂ (4.03) ♀ (4.12)	0.0000	0.0000
		System[A]	5.497 (4)	0.000	I (3.68)^{bc} SI (4.17)^{a} SE (4.1)^{a} C (4.47) E (4.18)	0.0236	0.0235
Docility		Year	4.216 (2)	0.016	2013 (3.54)^{b} 2014 (4.33)^{a} 2015 (4.15)	0.0872	0.0869
		Sex	3.569 (1)	0.060	♂ (3.99) ♀ (4.16)	0.0019	0.0019
		System[A]	4.924 (4)	0.001	I (3.43)^{b} SI (4.53)^{ae} SE (4.04) C (4.35) E (3.41)^{b}	0.1284	0.1281
Alertness		Year	7.227 (2)	0.001	2013 (3.76)^{c} 2014 (4.33)^{c} 2015 (3.95)^{ab}	0.0451	0.0450
		Sex	8.504 (1)	0.004	♂ (3.92) ♀ (4.20)	0.0092	0.0092
		System[A]	1.158 (4)	0.329	I (3.46) SI (4.59) SE (4.06) C (3.82) E (3.65)	0.1151	0.1148

[A] Husbandry system classification: I (Intensive), SI (Semi intensive), SE (Semi extensive), C (Contest), E (Extensive).

[B] From S1 to S12, these are the stimuli to which the donkeys were exposed.

[C] Superindexes denote the levels of the traits among which there was a statistically significant difference $P < 0.05$. Levels: Year (^{a}2013, ^{b}2014, ^{c}2015); System (^{a}I, ^{b}SI, ^{c}SE, ^{d}C, ^{e}E); Stimuli (S1^{a}, S2^{b}, S3^{c}, S4^{d}, S5^{e}, S6^{f}, S7^{g}, S8^{h}, S9^{i}, S10^{j}, S11^{k}, S12^{l}).

[D] F(df): Snedecor's F (degrees of freedom).

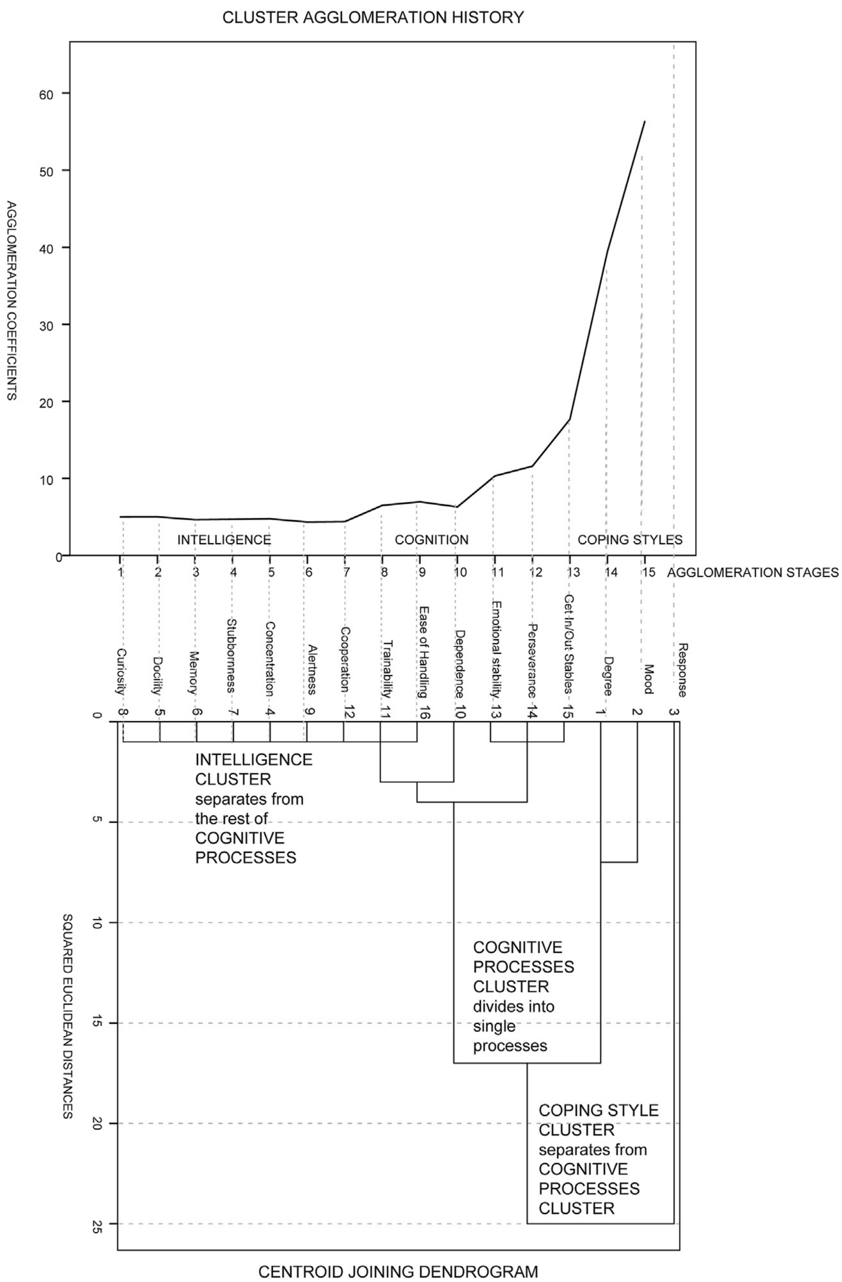

Fig. 1. Hierarchical cluster agglomeration history and centroid joining cluster dendrogram representation of the three clusters (coping styles, cognition and intelligence) comprising the sixteen cognitive processes related variables in the study.

Sys = system (1–5)
Sti = stimulus (1–12)

AY*Sex + AY*Sys + AY*Sti + Sex*Sys + Sex*Sti + Sys*Sti + AY*Sex*Sys + AY*Sex*Sti + AY*Sys*Sti + AY*Sex*Sys*Sti = interaction between several levels

A = age (months)
ε = residual error.

We used the age of the donkeys expressed in months as a linear and quadratic covariate to correct the phenotype observation of each behavioral variable. The reason for this inclusion is the fact that despite all donkeys were not born on the same day, they were scored together, so that assessed at different ages. We could expect the residual error of the model to be remarkably important given the increased likelihood of the existence of factors influencing the cognitive processes assessed that may not be controlled by the model, one of the main drawbacks when studying behavioral genetics.

3. Results

The three clusters initially set according to bibliography matched the results obtained for the preliminary HCA. HCA variable distribution and agglomeration coefficients and stages are shown in Fig. 1. The Pearson's correlations among all cognitive processes highlighted the individuality of the cognitive processes studied, ranged from − 0.084 to 0.812 and were highly statistically significant ($P < 0.001$) except for the alertness process, whose correlations were statistically significant ($P < 0.05$) for stubbornness, dependence, cooperation and emotional stability and were not significant for all the variables in the coping style cluster. A summary of the results of the descriptive statistics analysis is shown in Table 5. A summary of the main results of the one-way ANOVA, posthoc Tukey Test and effect size estimator, ε^2 and ω^2 is shown in Table 6. A summary of the determinative coefficients of the significant levels of factors, interactions, covariates and models obtained with MANOVA for all behavioral traits is shown in Tables 7 and 8.

Table 7
Signification (P values) and determinative coefficients (reduced or adjusted R^2) for each possible double and multiple factor interaction, covariates and models obtained with MANOVA for coping style cognitive process related traits in Andalusian donkeys.

Effects/traits	Response	Mood	Degree
Age (in months)	0.000	0.000	0.000
Year * sex	0.024	0.010	0.081
Year * system	0.000	0.000	0.000
Year * stimulus	0.023	0.000	0.996
Sex * system	0.000	0.000	0.000
Sex * stimulus	0.499	0.755	0.788
System * stimulus	0.000	0.000	0.000
Year * sex * system	0.000	0.000	0.010
Year * sex * stimulus	0.963	0.989	0.695
Year * system * stimulus	0.335	0.217	0.012
Sex * system * stimulus	0.079	0.066	0.852
Year * sex * system * stimulus	0.644	0.956	0.991
Reduced R^2	0.243	0.328	0.346

4. Discussion

The selection and registration of Andalusian donkeys occurs at the age of 3 years old, similarly to what happens in some horse breeds such as the Hanoverian, Dutch and Swedish Warmblood, Selle Français and Irish Sports Horse (Thorén Hellsten et al., 2006), when the individuals are assessed and included in or excluded from the studbook of the breed. This selective process has traditionally emphasized on the adherence of the individuals to morphological and phaneroptical standards exclusively.

The worldwide endangerment status of donkey breeds contrasts their potential new functional niches. This situation promotes research development to adapt the traditional standards to such new functional perspectives. Systematic data collection and genetic evaluation for functional traits may provide breeders with more objective tools when selecting their breeding stock to enhance selection response (Arnason and Van Vleck, 2000).

Organized breeding programmes have proved to be effective for other more profitable species like horses. At this point, the possibility of harmonizing selection programmes across different countries setting the same breeding objectives has been suggested as an interesting measure for the development of breeds.

However, the definition of donkey breeding objectives is not an easy task to accomplish as no selection has been carried out yet on this species. Therefore, there is no clue about which traits should be taken into account, nor which non-genetic factors should be controlled to face the new functional perspectives. In species such as the donkey, in which their functional roles are so closely related to humans, behavior becomes a key element to consider.

The quantitative study of behavior and especially cognitive processes, often deals with overlapping processes. To categorize such processes, we tested donkeys for their responses in a standardized test to prevent the behavioral traits assessed from containing elements of other distorting behavioral elements such as reactions to social separation.

Although we expected the statistical analysis to report some high Pearson's correlations because of the similar nature of the cognitive processes measured, we did not detect potential redundancies among processes (all Pearson's correlations ≤ 0.812). The results of the Centroid hierarchical cluster analysis successfully matched our previous cluster definition hypothesis as it organized the sixteen traits studied into the three clusters (Fig. 1). This analysis proceeds from each cognitive process constituting its own cluster, to all of the processes being iteratively and progressively combined into a single global cluster (Jarvis et al., 2003; Norušis, 2012). Then, we selected the iteration that best represented the three clusters that we had previously determined by examining the agglomeration stages and coefficients obtained (Fig. 1).

The study of behavior especially faces compromises when we try to define the terms involved in specific studies. These difficulties may be mainly ascribed to the existing inconsistency across situations because of the lack of accurate descriptions of the traits being studied or to the lack of a common training of the observers.

Age adds an additional difficulty as personality and cognition in humans (Soubelet and Salthouse, 2011) and equids (Wolff and Hausberger, 1994) seems to interconnectedly evolve in time, especially when considering which responses are presented and at which degree they are performed at different ages. The mean age of the donkeys at evaluation was 84.08 months, with a coefficient of variation (CV) of above 73% (Table 5). Because of the fact that behavioral processes are the result of a dynamic interaction between the genetic background and environmental factors such as previous experiences (Boissy et al., 2005), age may affect the result obtained. For instance, the study by Oki et al. (2007) generally considered young horses comparing to the heterogeneity of the age range in our present study, what may have affected our results. Age factor resulted highly significant ($P < 0.001$) except for the alertness trait included in the intelligence cluster (Table 5).

The fixed effects that comprise our model were chosen after performing a thorough bibliographic review on equine behavior and the factors significantly affecting it (Hausberger and Muller, 2002). Among the factors that influence equine behavior, sex and environment as described by Hausberger et al. (2004) or French (1993) and body condition (McCall, 1989) have generally proved to present a strong effect on equine behavioral traits. The rest of fixed effects controlled in our study consisted of the year (a 3-year period from 2013, 2014 and 2015), and the 12 stimuli presented and used to score the behavioral responses displayed (Table 2).

Table 8
Signification (P values) and determinative coefficients (reduced or adjusted R^2) for each possible double and multiple factor interaction, covariates and models obtained with MANOVA for intelligence and cognition traits in Andalusian donkeys.

Effects/traits	Age (in months)	Sex * year	Sex * system	Year * system	Sex * year * system	Reduced R^2
Concentration	0.000	0.453	0.430	0.375	0.335	0.161
Dependence	0.000	0.139	0.109	0.023	0.886	0.276
Trainability	0.000	0.074	0.516	0.645	0.716	0.202
Curiosity	0.000	0.130	0.073	0.889	0.465	0.143
Memory	0.000	0.718	0.099	0.034	0.550	0.372
Cooperation	0.000	0.413	0.080	0.316	0.592	0.311
Emotional stability	0.000	0.162	0.495	0.664	0.601	0.291
Stubbornness	0.000	0.260	0.427	0.448	0.368	0.198
Docility	0.000	0.001	0.352	0.785	0.113	0.233
Alertness	0.110	0.189	0.418	0.003	0.174	0.194
Perseverance	0.000	0.091	0.683	0.256	0.787	0.110
Get in/out of stables	0.000	0.280	0.702	0.000	0.391	0.286
Ease ot handling	0.000	0.050	0.533	0.394	0.665	0.297

The sample analyzed was unequally distributed in 22 farms all over Andalusia. Traditionally, one to three animals is kept on the farms in which breeding is not one of the primary productive objectives and locations gathering a higher number of individuals are anecdotical. This context made a farm/herd effect not to be considered, as the 40.91% of the 22 farms involved in this study only housed from 1 to 3 donkeys. With almost half of the farms accounting for only 12 animals from the sample, computing a herd effect would distort the results, hindering the estimation of the farm variation source. To overcome this difficulty, common farm characteristics were assessed to classify them into different husbandry systems, which helped to reduce such potential distortion.

Several specific studies in donkeys and other equids have reported the relevance of environment and handling on behavior patterns (French, 1993; Keeling et al., 2016; Lansade et al., 2004). All these factors are gathered in the husbandry system fixed effect that comprises 5 levels: Intensive, semi-intensive, semi-extensive, contest and extensive. The intensive level describes intensive farms in which the donkeys normally live in boxes or other reduced space facilities, but what is more important, in which the donkey contact with humans occurs on a daily basis, and which are frequently handled for more than just the minimum routinely hygienical and sanitary inspection tasks. The semi-intensive level describes farms in which the donkeys, apart from the previously described characteristics for the intensive level, are left roam in wider territory extensions but still keeping the daily contact basis with the people in their charge. This human contact time situation inverts in the semi-extensive level in which donkeys are kept in wider extensions and with whom the human contact is not kept daily, although the donkeys are still familiar and respond to the owner's requests. The contest level alludes to situations in which the animals are assessed under conditions that they are not accustomed to (Official Morphological Contest of the Breed), as the donkeys are transported to different facilities to theirs, and therefore they do not maintain the same human contact basis, nor they are surrounded by their home environment. Last, the extensive level gathers farms in which the contact with humans only occurs when sanitary inspection actions, vaccination campaigns or microchipping sessions are carried out or when the donkeys are being evaluated for their inclusion in the breed studbook, to then be left into a totally extensive nearly semi feral status.

The effect of the husbandry system was highly significant ($P < 0.001$) on all the behavioral traits assessed.

The effect of sires on the behavioral responses developed by their offspring has been highlighted by authors such as Hausberger et al. (2004) who reported a statistically significant effect. This is not surprising as the additive genetical component of behavioral traits imply both a sire and a dam effect on the traits assessed and therefore, both progenitors are half relevant when configuring the breeding value of a certain animal and not just the sire. The interaction of sex with behavioral traits has also been suggested in horses (Wolff and Hausberger, 1996) so that the model in our study included it as a fixed effect. Sexual dimorphism was evident in the breed for six of the sixteen traits. All the traits in the coping style cluster and concentration, alertness and the ability to get in or out stables (from the cognition and intelligence clusters) were significantly different between males and females ($P < 0.05$) as has been addressed in Table 6.

Only a few double and multiple interactions between the four factors controlled in the 'coping style' model are non-significant ($P > 0.05$) for the response, mood and degree traits, while the most of the double and triple interactions are significant ($P < 0.05$) for the three variables under study (Table 5). In the case of the 'intelligence' and 'cognition' model the interactions between the three factors involved were non-significant as well for all the thirteen traits studied. Double interactions between system and year were not significant ($P > 0.05$) except for dependence, memory, alertness and the ease of getting out or in stables, while the double interaction between system and sex was not significant ($P > 0.05$) for all traits (Table 5). ε^2 and ω^2 determinative coefficients for each trait assessed ranged between 15.74% and 0.19%.

Similar linear scales aiming at assessing behavioral traits and specifically coping style traits have similarly been studied in horses (Calviello et al., 2016), though the studies have not deepened or divided the components to study them separately and no genetical inference has been made yet.

The nature of the system that is currently used to evaluate the individuals being recognized as pure Andalusian breed donkeys could lead to an increase in environmental variability, considering the subjectivity inherently attached to the judgment of traits such as behavior (even though the judges are trained and experienced). The adoption of a linear scoring system in which the traits are evaluated in a continuous scale corresponding to the expression of cognitive or other behavioral process-related traits between two biological extremes may result in much better distribution properties enabling a better quantification of the traits measured (Rustin et al., 2009).

The Andalusian donkey breeding programme has resulted in the moderate genetic improvement of conformation, type, and phaneroptical traits, but some adjustment and refinement can be introduced to optimize selection responses. The formal definition of the breeding objectives is the key element of any genetic improvement programme (Van Vleck, 1993), and in the case of the Andalusian donkey, the need to include functional traits in the breeding goals, while maintaining selection for morphological and type characteristics, is essential.

The next step is to assess the information provided by these behavioral tests and to seek for genetic parameters when expanding the information and comparing it together with the genealogical data of the pedigree to implement a systematic genetic evaluation procedure, allowing the objective and early selection of breeding animals. An initial genetic assessment reported a mean heritability value of 0.20 ± 0.021 for the coping style cluster, 0.18 ± 0.13 for the cognition cluster and 0.21 ± 0.14 for the intelligence cluster, respectively, which will be studied and discussed in future studies. Simultaneously, the breeding programme can be further optimized by reducing generation intervals (through the registration of behavioral responses systematically at an earlier age and genetic evaluation of young animals).

This study sets the basis for behavioral traits to be considered as new selection criteria, hence, large studies carried out over several years and containing a higher number of animals is needed before any precise measures concerning the influence of the genetic and environmental effects can be determined. Nonetheless, selection for better-behaved donkeys would be potentially beneficial for donkeys' welfare and to reduce the number of accidents related to equestrian activities, as well as for the analysis of their suitability for assisted therapy programmes or any other human-related activity.

5. Conclusions

Statistical univariate and multivariate models can help isolate the effect of different variation factors on certain behavioral traits. The determinative coefficient for each of these factors becomes then an indicator of the fraction of the variation that such factors explain. The difficulty to find and control models to assess animal behavior especially increases when we intend to do it under field practical situations. The levels of significance found, show that the model used to assess the coping style cluster is more accurate and suitable than the one used to test for intelligence and cognition traits. This situation not only enables a more objective quantification methodology for coping styles related traits but also reports more reliable global results. The differences appearing because of the influence of the different fixed effects on the behavioral traits assessed may be attributed to the fact that the tests used may, in fact, evaluate the ability of certain owners to educate their donkeys rather than the inner cognitive capacity of the animals to develop a certain process. Although sexual dimorphism is evident on some of the cognitive processes, the variation may be ascribed to differences in the handling methods and routines applied to jacks and jennies. The husbandry system applied can help us group the animals to save the potential result distortion that may occur due to the unequal distribution of animals among the farms. The fraction of variance explained by external factors may be low when we considered them individually, but it can improve when their partial weights are summarized. The variance explained by these multifactorial models permits comparatively considering them to be efficient to quantify the sixteen cognitive processes in our study, as they provide very useful information for the design and ease of the complex models used in behavioral genetic analyses. Both double and triple interactions were mostly non-significant for intelligence and cognition clusters. This finding supports the fact that, in behavioral studies, the reliance on several factors individually, may help us quantify the factors or effects involved more accurately than their conjoint effects. Our results suggest the suitability of the proposed cognitive recording system to be applied in the routinely genetic selection of donkeys. These breeding criteria will be implemented in the future in order to make the donkey more commercially competitive and useful, not only aiming at saving animals but whole breeds from extinction.

Welfare declaration

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 guide for the care and use of laboratory and farm animals in research, receiving the approval from local and regional Welfare Committees.

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Chapter 5

Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; Arando Arbulu, A.; McLean, A.K.; Delgado Bermejo, J.V.

Genetic parameter and breeding value estimation of donkeys' problem-focused coping styles

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Genetic parameter and breeding value estimation of donkeys' problem-focused coping styles

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ABSTRACT

Donkeys are recognized therapy or leisure-riding animals. Anecdotal evidence has suggested that more reactive donkeys or those more easily engaging flight mechanisms tend to be easier to train compared to those displaying the natural donkey behaviour of fight. This context brings together the need to quantify such traits and to genetically select donkeys displaying a neutral reaction during training, because of its implication with handler/rider safety and trainability. We analysed the scores for coping style traits from 300 Andalusian donkeys from 2013 to 2015. Three scales were applied to describe donkeys' response to 12 stimuli. Genetic parameters were estimated using multivariate models with year, sex, husbandry system and stimulus as fixed effects and age as a linear and quadratic covariable. Heritabilities were moderate, 0.18 ± 0.020 to 0.21 ± 0.021. Phenotypic correlations between intensity and mood/emotion or response type were negative and moderate (−0.21 and −0.25, respectively). Genetic correlations between the same variables were negative and moderately high (−0.46 and −0.53, respectively). Phenotypic and genetic correlations between mood/emotion and response type were positive and high (0.92 and 0.95, respectively). Breeding values enable selection methods that could lead to endangered breed preservation and genetically selecting donkeys for the uses that they may be most suitable.

1. Introduction

In psychology, coping refers to the conscious efforts of an individual to solve personal and interpersonal problems in order to master, minimize or tolerate stress (Weiten and Lloyd 2008). Coping mechanisms are commonly termed coping strategies, and they normally comprise adaptive strategies or strategies which reduce stress (Lazarus and Folkman, 1984). Benus et al. (1991) rodent experiments concluded that the response to external stimuli could mainly be classified into two equally valuable strategy alternatives to face daily environmental demands, passive and active animals. Koolhaas et al. (1999), suggested updating these 'styles' to proactive and reactive, as the former confusing terms did not consider fundamental differences. One of such fundamental differences is the degree in which behaviour is influenced by environmental stimuli. To sum up, the performance of routine rather intrinsically driven rigid types of behaviour found in proactive animals, contrasts the generally more flexible and reactive attitude to environmental stimuli of reactive animals. Thus, when we speak about coping, we generally refer to reactive coping or the coping response after the presentation of the stressor. This differs from proactive coping, in which a coping response aims to neutralize a future stressor. Rather subconscious or non-conscious strategies such as defence mechanisms are generally excluded from the field of coping (Kramer, 2010).

The effectiveness of the coping effort depends on the type of stressful stimulus, the individual, and the circumstances. Coping responses are partly controlled by personality and mood, but also partly by the stressful nature of the environment around (Carver and Connor-Smith, 2010).

Among the four strategies that Weiten and Lloyd (2008) identified as coping styles in humans, problem-focused coping styles address those adaptive behavioral responses aimed at reducing, adapting or eliminating stressors. Although equids' reactiveness could clearly fit within

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these coping styles, a remarkable dimorphism has been described among species. Some equids describe a rather reactive strategy or tendency to freeze (such as donkeys) when they are involved in a challenging situation while others proactively flee, i.e. zebras or horses (Weaver, 2008).

Domesticated donkeys' wild ancestors often lived solitarily or in very small groups of two animals in which running away was not always such a successful survival method compared to that of the horse that lives in larger hierarchical groups and forms stronger bonds with its congeners (Proops et al., 2012; The Donkey Sanctuary, 2014). Conversely, wild or even feral donkeys' close bonds remain more solitary, normally being established between the jenny and its foal. When facing a potentially threating stimulus, donkeys may display to the predator (or observer) apparently normal behavioural patterns. However, these "normal" behavioural patterns could also be associated with misunderstood negative affective states (Moehlman, 1998). Apart from clear psychological differences, which may have an ancestral social basis, Koolhaas and Bohus (1989) suggest that each of these strategies may be catalyzed by different endocrine responses. These endocrine responses may be the basis and therefore, influence the mechanisms adopted by animals to maintain control over potentially threatening situations.

Most of human-equid accidents result from unexpected animal reactions (Keeling et al., 1999). Daily human-animal interaction helps deepening the mutual interspecific bonds that are established (that is, improves the familiarity of the animals towards their handlers). These concepts have been suggested to be the basis for a better performance when obtaining neutrally responding individuals in very evolutionarily distant species (Simianer and Köhn 2010; Cibulski et al., 2014). Training processes can be conducted following different approaches. Thus, although a greater difficulty training certain donkeys may reduce their working life and increase the time and costs needed to obtain fully functional animals, this should not make us exclude such animals from their use in riding or therapy (Batt et al., 2008), but to tailor a different approach to educate them.

Methodology to select for coping styles or reactivity levels may be useful for breeders and owners. Identifying the coping styles displayed by donkeys or their reactivity level when facing diverse kinds of stimuli from the beginning enables appropriate training protocols to be implemented from day one to work with the animal's innate response and tailor training programmes to meet the animal's needs. Such implications and knowledge may improve their final destination to develop the tasks that they may be better fit for.

Meta-analytic studies of the fixed or random effects to be considered in genetic models become particularly necessary in behavioural genetics (Navas et al., 2017a). These effects may present small effect sizes on particular traits; however, they may still be statistically significant. In unison, these effects can explain quite a large proportion of the phenotypic variation for the traits studied in a population, hence, conditioning the estimates for genetic parameters of such traits.

The higher the determinative coefficient (R^2) in a general linear model is, the lower the residual variance unexplained by the effects that we have controlled in our model will be. Among other determinative coefficients, ε^2 and ω^2 use unbiased measures of the variance components and report the least mean root square errors, therefore becoming suitable for behavioral studies with a large number of effects involved (Okada, 2013).

In genetic analyses, the variation for a certain trait in a population, depends on the number of animals that represent each of the possible combinations among the effects affecting a certain trait included in the model testing for such trait. When our sample is so small that it lacks a high enough fraction of animals representing each of these possible combinations, the model turns invalid to measure for the trait that it was aimed at measuring. That is, this model may misrepresent the real biological variation found for that trait in particular in the population under study, considering all the possible combinations of effects involved.

We should carefully consider which effects represent mere experimental design effects and which of them are biologically relevant for our trait and should therefore be included in our genetic models.

Limited research has studied the genetic background of coping styles or reactivity in horses and none has focused on studying such traits in donkeys. Oki et al. (2007) estimated the heritability of behavioural responses at veterinary inspections for three consecutive years and found highly repeatable (0.97–0.98) heritabilities (0.23–0.28). The lower limit for horses' heritability of reactivity in literature is 0.17, reported by Rothmann et al. (2014). However, the accuracy of the heritability reported by these authors was low, probably because to the low number of horses in the study. Therefore, the aim of this study is first, to describe a model to compute the effects influencing response type, mood and response intensity to isolate the genetic background behind coping strategies in donkeys. Second, to estimate the genetic parameters for the three above-mentioned variables aiming at outlining the possibly existing overlapping among the behavioural variables tested. Third, to assess the genetic and phenotypic correlations of the coping styles or reactivity patterns expressed by donkeys when facing visual and auditory stimuli. Fourth, the development of an index addressing the possibility to genetically select for hyporeactive, neutrally responsive and hyperreactive animals suggesting the possible inclusion of these traits as breeding programme selection criteria.

2. Material and methods

2.1. Animal sample and study background

Direct records included the information from 300 Andalusian breed donkeys, 78 jacks and 222 jennies. As age range was not normally distributed ($P < 0.05$ for both Kolmogorov-Smirnov and Shapiro-Wilk's tests for normality) we used minimum, Q_1, median, Q_3 and maximum to describe the age range in our sample. Minimum age in the range was 0.27 months, Q_1 age was 29.76 months, median age was 77.04 months, Q_3 age was 129.07 months and maximum age was 270.40 months. Such wide age range was considered, as the stimuli battery used to test for coping styles/reactivity was suitable for all animals included in the study and given the fact that we assess an endangered breed from which the information belonging to each individual is indispensable. The donkeys in the sample were the progeny of 93 jackstocks and 253 jennies. All the donkeys were registered in the breeds' Spanish studbook. Their pedigree is routinely genetically tested through microsatellite-assisted genotyping and parentage tests for the resulting offspring of each mating.

2.2. Behavioural tests, scales and phenotyping

The records were measured during the yearly behaviour assessment sessions carried out over four randomly chosen days from June to November per year from 2013 to 2015 at twenty-two different farms all over Andalusia (southern Spain). Such sessions were developed to fulfil the requirements of the Order of 22nd September 2011, establishing the regulatory bases for the concession of grants to officially recognized entities for the management of the studbooks of livestock breeds for the conservation of livestock resources in the framework of the programme of Rural development of Andalusia 2007–2013. Each record comprised 3 scores for each animal which described the coping strategies that the animals developed when they were made face twelve consecutive stimuli which combined different elements (people, animals or objects) (Fig. 2). These elements could be unknown (the animal had not been familiarized with them) or known (the animal had already been familiarized), visual (could be perceived with the eyes, i.e. all of the stimuli) or visual and acoustic (apart from being perceived with the eyes, they generated sounds, i.e., a horn and a red speaker to play a car engine playback). These elements were presented to the donkeys from

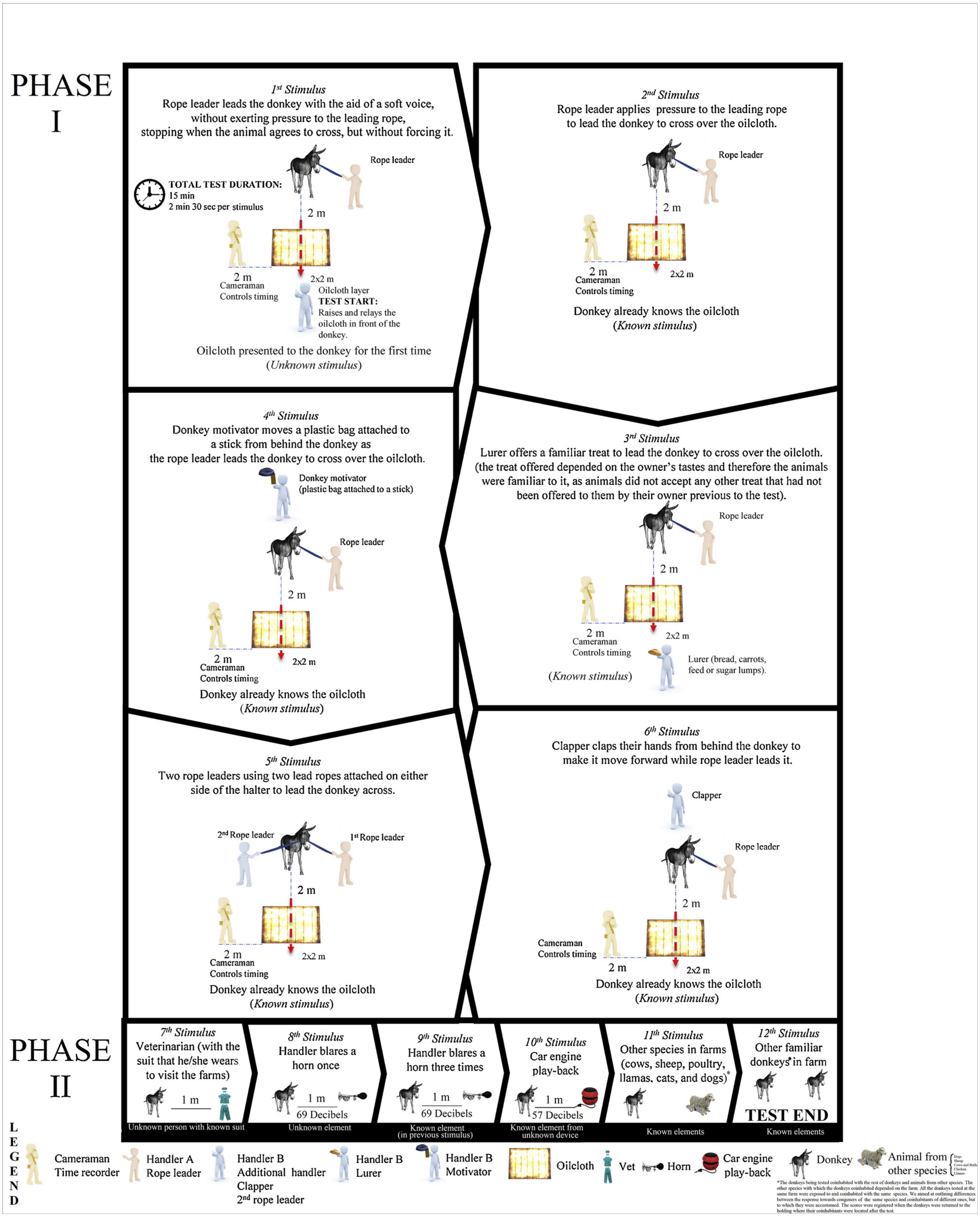

Fig. 1. Behavioural test and stimuli presentation description.

different positions (from the front or from a rear position always at 2 m away from the animals) (see Fig. 1 for the description of each possible element combination comprising each of the twelve stimuli). By coping strategy or style, we understand the increasingly aversive responses described by the animal implemented by the donkeys as a way to approach or avoid the potential threat represented by each of the twelve stimuli. The three scores given to each animal were simultaneously registered and they described the coping strategies displayed by the animals in three different traits (1 score per trait): mood/emotion when facing the stimuli response type towards the stimuli presented and intensity of such response. By mood/emotion we refer to the emotional/psychological state of the donkey. This emotional/psychological state

Table 1
Scale level description for the mood or attitude trait.

Scale	Mood/Attitude	Response type	Attitude towards the stimulus presented
1	Distracted	Hyporeactive	Pays attention[a] and moves towards other stimuli around, without paying attention to the stimulus presented in the test.
2	Dejected/Depressed	Hyporeactive	Overall, body posture shows lowered head and neck, roundness to spine and tucked tail. It does not pay attention to any stimuli around.
3	Indifferent/Nonresponsive	Hyporeactive	Normal posture. Pays no attention to the stimulus presented, but it is not distracted by other stimuli around.
4	Calm	Neutral	Does not get startled. Stands still. Pays attention to other stimuli around at the same time that it pays attention to the stimulus presented.
5	Awaiting	Neutral	Does not get startled. Stands still. Only focuses on the stimulus presented.
6	Curious	Neutral	Does not get startled. Stands still. Only focuses on the stimulus presented. Moves its head towards the stimulus presented.
7	Cautious	Neutral	Does not get startled. Pays attention and moves slightly towards the stimulus (less than 1 m).
8	Mistrustful	Neutral	Does not get startled. Pays attention to and moves towards the stimulus until approaching it completely.
9	Surprised	Hyperreactive	Only focused on the stimulus being presented. Gets startled but moves towards the stimulus.
10	Nervous	Hyperreactive	Only focused on the stimulus being presented. Gets startled, and tries to move away from the stimulus presented at first. Able to move towards the stimulus presented if led by the operator.
11	Fearful	Hyperreactive	Gets startled. Only focused on the stimulus being presented. Tries to move away from the stimulus presented. Unable to move towards the stimulus presented if led by the operator.
12	Rejection	Hyperreactive	Only focused on the stimulus being presented. Gets startled, and moves away from the stimulus presented noticeably. Pulls away from the leading rope when the operator tries to move towards the stimulus presented.

Accessed from (Navas et al., 2017a).
[a] By paying attention we mean that the donkey held direct visual contact with and/or directed its ear/s towards the stimulus being presented.

can last for a short or a longer period of time and is usually a result of an external stimulus as those presented at our test (for the mood/emotion scale present in Table 1 we considered the definitions by Cabanac (2002) and Mendl et al. (2010)). We developed the scale to measure for the response type following the study by Budzyńska (2014), classifying the animals according to the coping strategy that they implemented. This is whether the donkeys did not pay attention to the stimulus presented or they adopted a reactive or proactive strategy towards it. The response intensity scale measured the degree at which the emotional/psychological states in the first scale mentioned were displayed following the studies by Berger et al. (2013), and Geuens and De Pelsmacker et al., 2002.

The behavioural test was carried out in an open area to which the donkeys had been previously accustomed (it was part of the area over which the donkeys engaged in their daily activities) and comprised two phases or groups of stimuli following two different approaches, an operant conditioning test and a single-stimulus presentation test (Phase I and II), described in Fig. 1.

During phase I (operant conditioning test, first group of stimuli from stimulus 1 to 6), the donkeys were made cross over a 200 × 200 cm oilcloth with a wooden print on it using increasingly aversive methods (stimuli 1 to 6). Phase I starts when handler B raises and relays the oilcloth on the floor 2 m away in front of the animals. The donkeys were led by handler A using a leading rope (stimuli 1 to 4, and 6). An additional handler (handler B) and leading rope were used in stimulus 5 to lead the animals over the oilcloth. In stimuli 1 and 2, only handler A interacts with the donkey to lead it cross over the oilcloth. From stimulus 3 to 6, handler B presented the above-mentioned increasingly aversive methods, 2 m away from the animal (methods described in Fig. 1 and in Navas et al., 2017a) to lead the donkey cross over the oilcloth. No pressure was exerted to the rope during the presentation of stimulus 1. From stimulus 2 to 6, pressure was applied to the rope (two ropes in stimulus 5) until the animal accepted to move or the time left for it to do it was consumed (Fig. 1). An additional test person (cameraman) stood at two metres from the side of the oilcloth to videotape the experiences (to review the scores *posteriori*, cheeking for inter and intra-observer accuracy comparing field scores to videotape scores) and control that phase I lasted for a total of 450 s (75 s per stimuli presented). No pause was left between phase I and II.

During phase II (single-stimulus presentation test, second group of stimuli from stimulus 7 to 12), the donkeys were made to face 6 additional external stimuli (Fig. 1) presented to the donkey by handler B, from a metre away while the animal was held by handler A with the same previously used leading rope. The same additional test person (cameraman) stood at two metres by the side of the animal to videotape the experiences and control that phase II lasted for a total of 450 s (75 s per stimuli).

All stimuli were presented to all donkeys. Whether an animal crossed/refused the oilcloth completely at the presentation of any of the stimulus from 1 to 6, did not prevented the rest of stimuli from being presented. During Phase I, the donkey being tested was led back to cross the oilcloth again once it had crossed over it for the first time (stimulus 1) for each remaining stimulus from 2 to 6. Stimuli 7 to 12 were all consecutively presented indistinctly from the response displayed by the animal to any of them. All the stimuli were standardized and presented equally to the donkeys, except for stimulus 12 (animals from different species). In stimulus 12, the species of the animals presented depended on the species of the animals coinhabiting the farm with the donkeys being tested.

Zapata et al. (2016) reported aggression towards unfamiliar humans and aggression towards unfamiliar dogs to be associated with highly relevant genes at two different genome regions and possibly linked at a genetic level. While developing our model at a previous stage, we hypothesized such genetic connection may exist as well in the case of reactivity when the individuals are familiar whatever their species is. Our empirical data showed there were slight differences when we compared the reaction towards familiar donkeys and other familiar animals but there were not differences when comparing familiar animals from different species (in the cases in which such comparison was possible, i.e. multiple species on farm). This may stem in the previous socialization process occurring before the test took place.

No additional time was supplied for the donkeys to fulfil the experiences. Once the 75 s provided to the donkeys to react to the each of the stimulus being presented passed, the next stimulus was presented, following an increasing order from 1 to 12. There was no pause between stimuli. The test started when the animal was made cross over the oilcloth for stimulus 1 and finished when the reaction towards other animals was assessed in stimulus 12 (according to Fig. 1) and lasted for a total of 900 s.

In 75 s, an animal can shift attention many times. However, as our study intended to test for the coping styles/reactivity of the animals, further reactions implemented through the development of the test

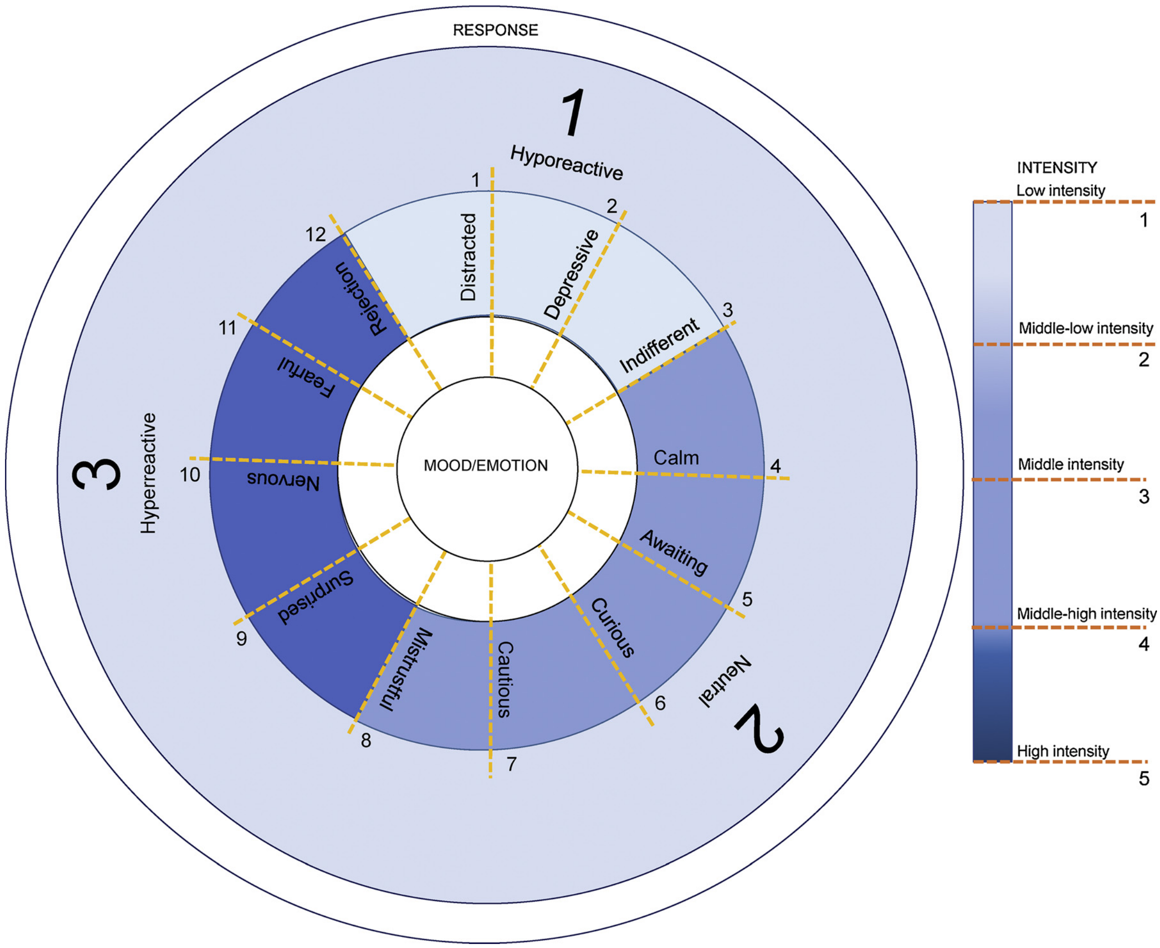

Fig. 2. Scale translation and description for the three coping style/reactivity related traits assessed in Andalusian donkeys. Response type (1–3), mood/emotion (1–12) and degree/intensity of response (1–5).

after the presentation of the different stimuli were discarded and used for other studies. For the same reasons, this study did not focus on whether the animals crossed the oilcloth completely or not, but their response/coping strategies implemented when each stimulus was presented. Each donkey was given 3 scores, one for each of the three traits to describe its coping strategy at the presentation of each of the 12 stimuli; one for response type, one for mood/emotion and one for response intensity. Each of the traits considered relied on a different scale (Fig. 2). The scores registered corresponded to the first immediate coping strategy described by each animal towards each stimulus from 1 to 12. These scores exclusively described the response of the animal when it was facing the stimulus being presented during the test without considering the attention paid to other elements in the testing environment. The animals had been previously accustomed as they were present in the area in which the donkeys engaged in their daily activities. This familiarization process aimed at preventing the presence of possible new elements from distorting the response of the animal to the stimuli presented in the test.

The first scale scored the animal mood/emotion when crossing or facing the stimuli being presented. A full description of the levels included in the mood/emotion scale is provided in Table 1. The second scale relied on the first one and scored the type of response that the animals displayed towards the stimulus presented from 1 to 3, with 1 measuring a hyporeactive animal (from 1 to 3 mood/emotion scores in the first scale), 2 meaning a neutrally responding animal (from 4 to 8 mood/emotion scores in the first scale) and 3, and hyperreactive donkey (from 9 to 12 mood/emotion scores in the first scale). To implement such classification we followed the premises stated in by Budzyńska (2014). Hyporeactive donkeys were those who did not pay attention to the stimulus being presented so that did not implement any coping strategy. Those donkeys displaying neutral responses fitted the reactive or passive copers classification stated by Budzyńska (2014). The passive (reactive) coping style involves behavioural inhibition (e.g., lower locomotion, immobility, withdrawal, freezing behaviour). On the other hand, hyperreactive donkeys were classified as active (proactive) copers according to the same authors. The active coping strategy is characterized by active behavioural reactivity ("fight–flight response") see Table 1.

Last, animals were given a score from 1 to 5 basing on the intensity at which their responses were displayed, with 1 meaning a low intensity response and 5 a high intensity response whatever the mood/emotion displayed was (Table 1 and Fig. 2). Our response intensity scale considers the findings of the research carried out by Berger et al. (2013) and adapts the affect intensity scale in Geuens and De Pelsmacker et al., 2002. We conjoined the time for the donkeys to be startled by the stimulus -latency-, and the scale by Geuens and De Pelsmacker et al., 2002 into our scale to measure the intensity of response. According to the later, the levels in determined for the response intensity scale were as follows; 1: the donkey does not startle more than 60 s after the stimulus was presented. Low intensity or negative startle responses; 2: the donkey startles from 40 to 60 s after the stimulus was presented. Middle-low intensity or mild negative startle responses; 3: the donkey startles from 20 to 40 s after the stimulus was presented. Middle intensity or serenity responses; 4: the donkey startles from 10 to 20 s after the stimulus was presented. Middle-high intensity or mild positive startle responses, and 5: the donkey startles in less than 10 s after the stimulus was presented. High intensity, positive intensity or strong startle responses.

As animals were only scored once, opposite behaviours were not scored correlatively in the same animal. For example, a very calm animal was not simultaneously registered as a slightly nervous animal, as this animal cannot be nervous and calm at the same time whatever it was the intensity level at which such animals maintained their mood/emotions. The translation and relationship between scales for the three traits is shown in Fig. 2. A full description and development of the tests and scales used can be consulted in (Navas et al., 2017a). The scores for every individual were registered by the same trained judge for all the stimuli and animals. No intra-observer discrepancies were appreciated as all the scores obtained on field matched those obtained after reviewing the tapes again. Cohen's κ test was run at a preliminary stage of the study (Navas et al., 2013) to test for inter-observer reliability and determine if there was agreement between three appraisers' judgement on the scores of 50 individuals (16.67% of the total sample) for the categorical variables of response type, mood and response intensity. Cohen's κ determined whether the repeatability of the model was enough to delete the effect of appraiser from the model, providing a measure of the accuracy of scoring of the appraisers, following the guidelines from Altman (1999), adapted from Landis and Koch (1977). Then 95% confidence intervals (95% kappa IC) were computed according to 95% kappa IC = κ ± 1.96 SEκ, where; $SE\kappa = [(po(1\text{-}po)/n(1\text{-}pe)^2]^{0.5}$ with the Crosstabs procedure of SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016). This preliminary analysis aimed at testing for the reliability of the scoring system, which proved to be highly reliable as there was highly statistically significant perfect agreement between the three appraisers' judgements when scoring for response type and response intensity for the 12 stimuli presented. When testing for mood/emotion, there was highly statistically significant perfect agreement among the three observers at the preliminary test for repeatability for all the traits and stimuli, except when testing for mood at the presentation of stimulus 7, in which case, the strength of agreement between appraisers 1 and 2 and 1 and 3 was substantial, and between appraisers 2 and 3, between who it was moderate. Stimulus 7, was the turn out from Phase I to Phase II, what may have been a cause for the occurring slight distortion. The results for this preliminary study are presented in Supplementary Table 1.

2.2.1. Model design, variables and fixed effects

An analysis of the descriptive statistics for the variables response type, mood/emotion and degree/intensity and for the previously described fixed effects (year of assessment, sex, husbandry system and stimulus kind) and preliminary analyses of variance were carried out with the GLM procedure of SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016) to assess the relative importance of the fixed effects included in the linear model. The previous meta-analysis for the effects included in this model is described in Navas et al. (2017a).

2.2.2. Genetic parameter assessment

The first and one of the most relevant goals of our analyses was to obtain estimates of fixed effects (BLUE, Supplementary Table 2) and breeding values (BLUP) for coping style/reactivity related traits in Andalusian donkeys, by mixed model procedures using an Animal Model. Firstly, mixed models were used to obtain estimates of variance components by Restricted Maximum Likelihood, in univariate analyses using the MTDFREML package (Boldman et al., 1995).

Coping style/reactivity traits were scored for each stimulus only once in the lifetime of the individual. Although the stimuli could have been presented to each animal several times along the course of its life to collect multiple direct observations, we intended to assess the current status of the animals when mostly unknown stimuli were presented. Hence, multiple tries may have been detrimental because of the donkeys becoming educated to fulfil the tests, what may have influenced the responses obtained. Therefore, the statistical model used in the analysis of such traits was a single trait Animal Model with single records.

In matrix notation, the mixed model used in the analyses of coping style/reactivity related traits (Fig. 2): response type (3 levels: hyporeactive, neutral and hyperreactive), mood/emotion (12 levels: distracted, dejected/depressed, indifferent, calm, awaiting, curious, cautious, mistrustful, surprised, nervous, fearful and rejection) and degree/intensity (5 levels: 1–5) was

$$y = Xb + Za + e$$

where y is the vector of records for coping style/reactivity traits, b is the vector of fixed effects to be estimated and X the corresponding incidence matrix relating records to fixed effects, a is the vector of breeding values to be estimated and Z the corresponding incidence matrix, and e is the vector of residuals. In this case, the fixed effects included in vector b were year of assessment (3 levels: 2013, 2014, 2015), sex (2 levels: male and female), system (5 levels: Intensive, semi intensive, semi extensive, contest and extensive) and stimuli (12 levels, Navas et al., 2017a; and Fig. 1) plus the linear and quadratic effect of age at scoring as covariable. The levels comprising the fixed effects included in the model can be consulted in Navas et al., 2017a. The previously described combination of fixed effects chosen was used as, the bivariate correlation found between all variables were statistically significant ($P > 0.01$), with the exception of the year effect over the degree/intensity variable and the effect system on the variables response type and mood/emotion, which were not significant. Because of the lack of inter-observer and intra-observer discrepancies, we decided not to include the appraiser as a fixed effect in our model, as these findings support the fact that the appraisers were properly trained to homogeneously score the individuals being tested.

Secondly, the MTDFREML package (Boldman et al., 1995) was used to obtain estimates of genetic (σ_a^2), phenotypic (σ_p^2), environmental (σ_e^2) variance components and narrow sense heritability (h^2) estimates by Restricted Maximum Likelihood, iterating until a convergence criterion of 10^{-12} was obtained. The methods gathered by Behera (2007) were considered to estimate sire/paternal half-sib (σ_S^2), dam/maternal half-sib (σ_D^2), within progeny (σ_W^2) variance components; and paternal half-sib heritability (h_S^2), maternal half-sib heritability (h_D^2), pooled (sire-plus-dam) heritability (h_{S+D}^2).

The analyses involved the relationship matrix of animals with direct records related through at least one known ancestor, that is, the 1017 animals in the historical pedigree file of the breed. Considering the lack of previous experiences on donkeys, the only estimated variance components that had been reported for horses (Rothmann et al., 2014), were used as the starting point to compute our own specific variance components and estimates of fixed and random effects in univariate analyses.

After convergence was reached, breeding values were predicted for all animals in the relationship matrix and estimates of fixed effects were obtained. Afterwards, bivariate analyses were carried out among combinations of the different coping style/reactivity traits using MTDFREML and the same linear models used in univariate analyses, to obtain estimates of the corresponding phenotypic correlations (r_P) and their genetic (r_G) and environmental correlation components (r_E). The genetic correlation between two traits is the correlation between the genetic influences on a trait and the genetic influences on a different trait estimating the degree of pleiotropy or causal overlap between both traits. On the contrary, environmental correlations describe the relationships between the environments affecting two traits. The relationship between phenotypic correlations and their components is defined through $r_P = r_G + r_E$.

Finally, bivariate analyses were carried out to obtain estimates of the genetic correlation between each of the coping style/reactivity related traits assessed.

The standard errors of genetic correlations among coping style/reactivity related traits were obtained directly from the MTDFREML

Table 2
Variance components and heritability (h^2) for coping style/reactivity related traits in Andalusian donkeys, obtained from univariate analyses.

Trait	Response type	Mood/Attitude	Degree/Intensity
σ_a^2	0.034	1.698	0.398
σ_P^2	0.192	8.189	1.888
σ_e^2	0.157	6.491	1.490
$h^2 + SE$	0.18 ± 0.020	0.21 ± 0.021	0.21 ± 0.021
σ_S^2	0.016	0.759	0.189
σ_D^2	0.027	1.350	0.265
σ_W^2	0.743	31.633	6.516
$h_{S+D}^2 \pm SE$	0.11 ± 0.025	0.13 ± 0.025	0.13 ± 0.027
$h_S^2 \pm SE$	0.08 ± 0.025	0.09 ± 0.025	0.11 ± 0.028
$h_D^2 \pm SE$	0.14 ± 0.025	0.16 ± 0.026	0.15 ± 0.026

Estimated genetic (σ_a^2), phenotypic (σ_P^2), environmental (σ_e^2), sire/paternal half-sib (σ_S^2), narrow sense heritability (h^2), dam/maternal half-sib (σ_D^2), within progeny (σ_W^2)variances; full-sib pooled (sire plus dam) heritability(h_{S+D}^2), paternal half-sib heritability (h_S^2), maternal half-sib heritability (h_D^2); and (SE) Standard error.

analyses.

2.3. Index selection

The application of coping styles or reactivity as indicators in direct selection for functionality was investigated by standard index selection procedures (Van Vleck, 1993; Hazel, 1943). The possibility of improving functionality by directly selecting for coping style/reactivity related traits, and the accuracy of the three functional traits considered in our analysis was studied, assuming that only one record is available per animal. Then, basing on the estimated phenotypic relationship between coping style/reactivity related traits, and their estimated genetic relationship with each other, the possibility of selecting based on an index combining all the coping style/reactivity related traits was investigated. In matrix notation, the weights to be applied in the selection index combining individual partial scores were obtained as follows:

$$b = P^{-1}g$$

where b is the vector of weights to be applied to each of the coping style/reactivity related traits, P is the phenotypic (co) variance matrix of coping style/reactivity related traits, and g is the vector of genetic covariances of the coping style/reactivity related traits with each other. MatLab r2015a (The MathWorks, Inc. (2015)) was used to compute all selection indexes.

After solving for b, the variance of the selection index was obtained as follows:

$$\sigma_I^2 = b'Pb$$

and the accuracy of selection for the *i*th coping style/reactivity related trait was estimated as follows:

$$r_{API} = \sqrt{\frac{\sigma_I^2}{\sigma_{Ai}^2}} \quad or \quad r_{API} = \sqrt{h^2}$$

where r_{API} is the accuracy obtained from direct selection for the *i*th trait and σ_{Ai}^2 is the corresponding additive genetic variance, and h^2 is the heritability for that particular trait.

The relative weight given to each of the reactivity traits included in the selection index was assessed by constructing a reduced selection index where each of the reactivity traits is removed, and calculating the reduction or gain observed in weighted average accuracy relative to the optimum index (Cameron, 1997).

2.4. Welfare declaration and ethical approval

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 guide for the care and use of laboratory and farm animals in research. 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. Variables and fixed effects descriptive statistics

The pedigree file provided by the Union of Andalusian Donkey Breeders (UGRA) included 1017 animals (272 jacks and 745 jennies) born between January 1980 and July 2015 from which only 914 animals, 246 jacks and 668 jennies, born from January 1980 to July 2015 were alive during the development of the study. Our sample consisted of 300 donkeys from which we had direct observations from the field. Pedigree analyses allow to estimate genetic information from ancestors through their descendants with direct information. Thus, the cross compared genetic assessment of the direct information of the 300 donkeys with the pedigree genealogical knowledge supplied indirect observations (after computing predictive breeding values, PBV) from 724 ancestors, that is ¾ of the total historical pedigree. Then, the greater direct information from related animals we gathered, the greater the accuracy of prediction of such breeding values was as well. The 36 field observations per animal consisted of 12 observations per each of the three variables measured (mood/emotion, response type and degree/intensity) and donkey studied (N = 300)), making a total of 10,800 records (3600 per trait assessed).

For the variables studied, the highest estimate of additive genetic variance was obtained for mood/emotion, which also had the highest phenotypic variance (Table 2), while the lowest estimate of genetic variance was obtained for response type.

3.1.1. Genetic parameters and genetic correlations assessment

Variance components and heritability (h^2) estimates for all reactivity traits are shown in Table 2. For all estimates of h^2, the SE was between 0.020 and 0.028, indicating a good accuracy of the estimated parameters. Phenotypic (r_P) and genetic (r_G) correlations are shown in Table 3.

3.2. Selection index

The results for the selection index, variance of the selection index (σ_I^2), additive genetic variance (σ_{Ai}^2) and accuracy of selection (r_{APi}) for each reactivity trait (response type, mood/emotion and degree/intensity) are shown in Table 4.

Table 4 shows the results of the study of direct selection for

Table 3
Correlations for coping style/reactivity related traits in Andalusian donkeys, obtained in bivariate analyses.

Trait	Response type	Mood/Attitude	Degree/Intensity
Response type	–	0.92 ± 0.003	−0.21 ± 0.017
Mood/Attitude	0.95 ± 0.010	–	−0.25 ± 0.016
Degree/Intensity	−0.46 ± 0.073	−0.53 ± 0.066	–

Phenotypic correlations (r_P) (above diagonal) and genetic correlations (r_G) (below diagonal).

Table 4
Summary of the parameters of the selection index when the direct selection goals are reactivity or coping styles in Andalusian donkeys.

Item	Direct Selection Goal: Coping Styles/Reactivity		
	Response type	Mood/ Attitude	Degree/ Intensity
Vector of selection index weights (b)	0.080	0.246	0.193
Variance of the selection index (σ_I^2)	0.007	0.376	0.089
Vector of standardized index weights[a]	0.434	0.189	0.306
Additive genetic variance (σ_{Ai}^2)	0.034	1.698	0.398
Additive genetic standard deviation (σ_{Ai})	0.185	1.303	0.631
Accuracy of selection (r_{APi})	0.445	0.471	0.473
Relative loss[b]/gain[c] in selection accuracy[b] (%)	8.37[c]	10.76[b]	0.43[c]

[a] Index weight standardized per additive genetic standard deviation.
[b] Relative loss/gain in accuracy if each trait is individually removed from the selection index.

functional traits considering only one record, revealed the accuracy of selection obtained would be similar for mood/emotion and degree/intensity traits, i.e. 0.4706, 0.4725, respectively, and slightly lower for response type (0.4456). To assess the relative importance of each partial item, Table 4 also includes the index weights per unit of genetic standard deviation of the reactivity items, as well as the relative loss in accuracy of index selection if each trait is individually removed from the index. When index weights are computed per genetic standard deviation results were positive and ranged from medium-strong (0.4336) for the response type trait, while it was medium-low for the other two traits assessed (Table 4).

Weighted average accuracy for the three traits was 0.4674. The potential loss in accuracy resulting from excluding a given trait from the selection index indicates that mood/emotion is a trait to retain when selection is for reactivity, 10.76%. The potential gain in accuracy resulting from excluding the Response type or Degree traits from the selection index was 8.37% and 0.43%, respectively (Table 5).

3.3. Breeding values and accuracy

The results for the estimates of predicted breeding values (PBV) ranged between −0.37 and 0.48 for the response type trait, −2.26 and 3.42 for the mood/emotion trait and −1.50 and 1.37 for the degree/intensity trait, while the existing range of accuracy (RAP) for all reactivity traits ranged from 0 to 0.85 (Table 6) in the Andalusian donkey.

4. Discussion

The information on the genetic background behind behavioural traits in donkeys is nonexistent and is still in the first research stages for other species (Wolff et al., 1997; Marsbøll and Christensen, 2015; Le Scolan et al., 1997). Our estimates supply objective information to breeders and are the base to develop a systematic genetic evaluation platform including reactivity traits as breeding criteria (Dubois et al., 2008).

The compromises concerning data size and structure common to donkey populations prevent us from excluding animals whose valuable information could contribute to increase the accuracy of the estimates computed whatever their age it is, on the condition that the scoring of such animals is feasible and age is controlled and included as a covariate in the model. We are studying a genetically-tested sample that represents around 30% of the historically registered population of an endangered breed, and a 300 animal sampling may probably be near the minimum required to obtain reliable heritability estimates, therefore any observation that can be reliably tested is worth considering, even if it involves considering animals from a wide range of age.

The decision on which fixed effects would comprise the model was preliminarily developed by Navas et al. (2017a) after performing a review of the studies on donkey behaviour (Hausberger and Muller, 2002), considering the balance between statistically significant and biologically relevant factors. For example, Hausberger et al. (2004) reported the sire to statistically influence novel object fearful reactions phenotypically, as the offspring of the same sire tended to develop the same responses. However, considering the genetic variability to be expected, both sire and dam should theoretically equally contribute to the breeding value (BV) of an individual. By contrast, our results suggest a greater genetic influence of the dam (Table 2) for coping styles.

Andalusian donkeys' studbook registration only considers morphology and coat. Thus, our system could increase population's variability, especially, considering the judgment subjectivity inherently attached to behaviour (notwithstanding judges are trained and experienced). The linear scoring system which, rather than scoring traits on their desirability, evaluates them in a continuous scale between two biological extremes, results in much better distribution properties and a better selection accuracy (Calviello et al., 2016; Rustin et al., 2009; Samoré et al., 1997).

Positive genetic and phenotypic correlations for response type and mood were even higher than those values obtained for horses (approximately around 0.9) (Oki et al., 2007). This may reflect differences in the scoring system applied and the fact that response type could be considered a synthetization of the mood/emotion variable. The negative genetic and phenotypic correlations of response type and mood/emotion, when compared to response intensity should be noticed. Donkeys which presented a certain extreme response type or mood when facing external stimuli were not bound to develop an extremely high/low intense response. This highlighted the genetic independence of response intensity from the rest of traits, a sign of a possible indirect

Table 5
Weighted average accuracy values from reduced selection indexes where each coping style/reactivity related trait is removed.

Item	Response type	Mood/Attitude	Degree/Intensity	Weighted average accuracy (r_{APi})
Selection Index (b)	0.080	0.246	0.193	0.467
r_{APi}	0.446	0.471	0.473	
Item	Response	Mood/Attitude	Degree/Intensity	Weighted average accuracy (r_{APi})
Selection Index (b)	Excluded	0.191	0.195	0.507
r_{APi}		0.501	0.512	
Item	Response	Mood/Attitude	Degree/Intensity	Weighted average accuracy (r_{APi})
Selection Index (b)	0.167	Excluded	0.201	0.452
r_{APi}	0.435		0.466	
Item	Response	Mood/Attitude	Degree/Intensity	Weighted average accuracy (r_{APi})
Selection Index (b)	0.072	0.271	Excluded	0.454
r_{APi}	0.435	0.459		

Table 6
Predicted Breeding Values (PBV) and accuracy descriptive statistics for coping style/reactivity traits in the Andalusian donkey.

Trait	Item	Sex							
		Jacks (N = 272)				Jennies (N = 745)			
		Minimum	Maximum	Mean	SEM	Minimum	Maximum	Mean	SEM
Response type	PVB	−0.337	0.358	0.006	0.006	−0.373	0.479	0.004	0.003
	SEP	0.070	0.190	0.146	0.002	0.090	0.200	0.152	0.001
	RTI	0.000	0.920	0.495	0.018	0.000	0.890	0.355	0.014
	RAP	0.000	0.846	0.337	0.017	0.000	0.792	0.264	0.012
Mood/Attitude	PVB	−2.259	2.500	−0.023	0.044	−1.882	3.420	0.010	0.021
	SEP	0.500	1.320	1.018	0.016	0.580	1.390	1.072	0.010
	RTI	0.000	0.920	0.504	0.019	0.000	0.900	0.362	0.014
	RAP	0.000	0.846	0.348	0.018	0.000	0.810	0.274	0.012
Degree/Intensity	PVB	−1.173	1.134	−0.063	0.019	−1.496	1.368	−0.033	0.011
	SEP	0.240	0.640	0.493	0.008	0.280	0.670	0.519	0.005
	RTI	0.000	0.920	0.505	0.019	0.000	0.900	0.363	0.014
	RAP	0.000	0.846	0.350	0.018	0.000	0.810	0.276	0.012

Error of prediction (SEP), precision (RTi), accuracy (RAP).

selection for the traditionally described mild-mannered lively temperament of Andalusian donkeys.

This general antagonism between response type or mood/emotion and the response degree/intensity described is of particular relevance in donkeys, for which both traits could represent important breeding objectives. Given the favourable genetic relationships existing between traits, coping style traits can play an important role in a selection programme aimed at improving the suitability of donkeys for mule production, animal-assisted therapy or leisure riding. This fact may make interesting to consider the possibility of developing and maintaining bloodlines within the Andalusian donkey setting different selection aims separately.

The potential loss in accuracy resulting from excluding mood/emotion and degree/intensity indicated they are traits to retain when selecting for coping styles. However, there was a potential gain in accuracy when response type was excluded. This could have been expected because of the lower heritability of this trait (respecting to the rest of reactivity traits), what translates in a lower variability provided by this trait to the selection index at the same time.

Using a standardized test can prevent coping styles from containing uncontrolled elements of, for example, social isolation reactions, one of the main problems to face in behavioural genetics. For some individuals, the test may have been primarily one of exposure to novel objects, for others, the test may have been primarily a test of social isolation and the anxiety caused by social isolation, what may have driven any observed response rather than a response of novelty per se. However, all donkeys were tested through the same methods and under the same premises. Given social isolation distorting effects had occurred, all animals may have been affected similarly. This situation prevents the scores obtained from being differently distorted. Furthermore, some of the main characteristics considered when classifying the husbandry system factor included in the model, were the kind and frequency of contact towards humans (Supplementary Table 3), what may help controlling such potentially distorting effects. Still, an index based on similar behaviour variables and their levels of arousal has been shown to be useful in ranking horses by their reactivity, particularly to assess fearfulness (Wolff et al., 1997).

The estimated heritability reported for coping style/reactivity is slightly higher than that in horses and similar to that reported for German shepherds or red junglefowl (Overall et al., 2014; Agnvall et al., 2012). Our estimated heritabilities of 0.18 for response type, 0.21 for mood/emotion and 0.21 for degree/intensity are generally in the upper range for reactivity traits reported in the literature, being slightly higher than the estimates reported for similar traits in other species (van der Steen et al., 1988; Visscher and Goddard, 1995), but still moderate when comparing them to other functional traits. Busjahn et al., (1999) and Kozak et al. (2005) found that genetic factors exerted a significant influence on coping style in human twins. These values for problem-solving coping styles (0.21–0.30) are the most similar ones found in literature.

By comparison, Hausberger et al. (2004) estimated the heritabilities of different temperament traits, including emotionality when being alone with a novel object, in the range of 0.29 (± 0.12) to 0.40 (± 0.24) which were much less accurate than those in the present study. In contrast, Oki et al. (2007) estimated the heritabilities of behavioural reactions to veterinarian inspections with higher accuracy, and the authors found slightly higher heritabilities (0.23–0.28). The high accuracy found by Oki et al. (2007) may be because the study was carried out over 3 years, as in our case, what contributes to the inclusion of more related animals. Furthermore, the high accuracy of the estimates may be related to the great number of stimuli testing the same variables. The similar heritabilities found by Hausberger et al. (2004) and Oki et al. (2007) could be caused by the fact that the tests in these studies have been carried out under controlled settings what means that they were less sensitive to environmental influence.

Accuracy was high and heritability standard error low. This means the model used to quantify the genetic background behind coping styles is accurate. This is likely because, despite the limited number of donkeys, our genetically-tested sample constituted around 30% of the total of registered endangered Andalusian donkeys alive and with direct observations. This sample may probably be near the minimum required to obtain reliable heritability estimates. In genetics, we can obtain indirect information from an animal from which neither there is information for the trait being measured, nor can it be measured at present (because of the animal being death or sold) by comparing the observations for that trait from descendants somehow related with such common ancestor. This percentage increased to 71.19% when we considered the animals with direct and indirect observations (¾ of the historical studbook) (Navas et al., 2017a,b).

Despite the moderate heritability estimates reported for coping style/reactivity in Andalusian donkeys, the predicted breeding values (PBV) for these traits show considerable variability, indicating that selection based on objective estimates of genetic merit could be effective. PBVs account for the potential genetic transmitting ability of an individual as a parent. They are estimated for individuals based on their own and their relatives' performance records after correcting for various environmental factors. When parents are selected based on highly reliable BV, a faster genetic progress is expected in the resultant population, what becomes critical in any breeding programme. The moderate coping styles/reactivity heritabilities and the high phenotypic variability compensate, resulting in a moderately wide PBV distribution.

The formal breeding objective definition is the key element of any breeding programme (Van Vleck, 1993). The selection for morphological and coat characteristics is of paramount importance in standardized donkey breed with such fixed breed standards. However, the inclusion of functional traits among donkey' breeding goals is essential.

Systematically enhancing the use of this information in selection decisions may enable the early selection of breeding animals. Parallelly, the breeding programme can be further optimized by reducing generation intervals (earlier registration and genetic evaluation of young animals), improving selection accuracy through multivariate animal models combining functional and morphological traits, and increasing selection intensity (reducing the number of breeding jacks) to levels compatible with an increased selection response, but considering the increased risk of extinction and detrimental problems caused by the increased inbreeding in donkey breeds with such a low effective population number (Haberland et al., 2012; Folch and Jordana, 1998; Quaresma et al., 2014; Cecchi et al., 2006; Rizzi et al., 2011; Santana and Bignardi, 2015; Gutiérrez et al., 2005; Aranguren-Méndez et al., 2001). The inclusion of genomic information in the Andalusian donkey's selection programme plays a major role and should be carefully investigated as it has been done in horses' behavioural genetic studies (Momozawa et al., 2005).

We do not aim at selecting/discarding animals, but to use them for what they may be better suited. Breeding for hyporeactive donkeys can be relevant for assisted-therapy. However, highly reactive animals may be desirable for work. Selection for reactivity is already performed in horses. Thoroughbreds and Arabians more easily implement flight and reactive strategies than Quarters or Warmbloods, however there are some bloodlines that produce calm and docile individuals. By contrast, some Warmblood and Quarter Horse bloodlines produce horses more easily engaging flight and reactive strategies (McDonnell, 1999).

Before coping styles can be set as new selection criteria, more research regarding development of simple and validated behaviour tests is required. Nonetheless, less reactive donkey selection would be potentially beneficial for their welfare and for reducing equestrian activity-related accidents, as well as for assessing their suitability for assisted-therapy and leisure programmes.

5. Conclusions

It can be concluded that moderate heritabilities for reactivity related traits such as response type, mood/emotion and degree/intensity were obtained after the evaluation of the behavioural tests and of the information of the field sheets associated. The accuracy of these estimates was high as well, even more when considering the limited number of donkeys in the study, what may highlight the efficiency of the behavioural test and model designed to assess such traits. Yet, it is essential to note that this study is the first to estimate a heritability of coping style/reactivity related traits measured in a practical situation related to a selection programme in donkey breeds. The findings indicate that selection for reduced reactivity and fearfulness in donkeys is achievable, although it requires more research including more animals, a difficult task to achieve if we work at a breed level, considering the existing extinction risk that they are exposed to.

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Statement of conflict of interests

The authors report that there are no conflicts of interest relevant to this publication.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.beproc.2018.05.008.

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Chapter 6

Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; McLean, A.K.; Delgado Bermejo, J.V.

Dumb or smart asses? Donkey's cognitive capabilities *(Equus asinus)* share the heritability and variation patterns of human's cognitive capabilities *(Homo sapiens)*

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Abstract

Scientific evidence for intelligence in donkeys could expose their historical unmerited cognitive derogatory status. Psychometric testing enables quantifying animal cognitive capabilities and their genetic background. Due to the impossibility to use the language dependent scales widely used to measure intelligence in humans, we used a nonverbal operant conditioning problem-solving test to compute a human-analogous IQ, scoring the information of thirteen cognitive processes from 300 genetically-tested donkeys. Principal components and Bayesian analyses were used to compute the variation in cognitive capabilities explained by the cognitive processes tested and their genetic parameters, respectively. According to our results, IQ may explain over 62% of the cognitive variance, and 0.06 to 0.38 heritabilities suggest that we could ascribe a significant proportion to interacting genes describing the same patterns previously reported for humans and other animal species. Our results address the existence of a human analogous heritable component and mechanisms underneath intelligence and cognition in probably one of the most traditionally misunderstood species from a cognitive perspective.

Keywords	Cognition; g; heritability; donkeys; intelligence quotient.

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Supplementary Table S1.docx [e-Component]

Supplementary Table S3.docx [e-Component]

Supplementary Table S4.docx [e-Component]

Supplementary Table S5.docx [e-Component]

- A remarkable variation in cognitive abilities among donkeys is found.
- The distribution and inheritance of cognition could be ascribed to a similar background in humans.
- The heritabilities for cognitive abilities reveal indirect selection may have been carried out.
- The ease at which animals interact with their housing is moderately related to stubbornness.
- Selecting for memory and curiousity, we select for stubborness, and emotional stability.

Dumb or smart asses? Donkey's cognitive capabilities *(Equus asinus)* share the heritability and variation patterns of human's cognitive capabilities *(Homo sapiens*)

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Abstract

Scientific evidence for intelligence in donkeys could expose their historical unmerited cognitive derogatory status. Psychometric testing enables quantifying animal cognitive capabilities and their genetic background. Due to the impossibility to use the language dependent scales widely used to measure intelligence in humans, we used a nonverbal operant conditioning problem-solving test to compute a human-analogous IQ, scoring the information of thirteen cognitive processes from 300 genetically-tested donkeys. Principal components and Bayesian analyses were used to compute the variation in cognitive capabilities explained by the cognitive processes tested and their genetic parameters, respectively. According to our results, IQ may explain over 62% of the cognitive variance, and 0.06 to 0.38 heritabilities suggest that we could ascribe a significant proportion to interacting genes describing the same patterns previously reported for humans and other animal species. Our results address the existence of a human analogous heritable component and mechanisms underneath intelligence and cognition in probably one of the most traditionally misunderstood species from a cognitive perspective.

Keywords

Cognition; *g*; heritability; donkeys; intelligence quotient.

Funding

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Introduction

Donkeys' unmerited conception of a problematic behaviour curiously came into the scene at the same age in which the species was probably enjoying one of the most productive times for their functionality. During the Egyptian pharaonic times (Rossel et al., 2008; Navas et al., 2016), donkeys were not just herded for milk or meat production, but also were usually ridden among the most notable personalities (Alkhateeb-Shehada, 2008; Bar-Oz et al., 2013), what provided them with a distinguished role in society. Superstition conjoined consequences together with the psychological misunderstanding of the species relegated this animal to become one of the most cognitively detracted species of all times, as reported by the multiple derogatory literature references found in several languages and cultures worldwide (Gregory, 2007; Bough, 2010; Estaji and Nakhavali, 2011; Way, 2014). This context has indirectly translated into this species being driven into one of the most worrying endangerment situations nowadays as a direct consequence of their lack of functionality (Navas et al., 2017b). Assisted therapy has stepped into the functional scene of donkeys as they have been reported to facilitate the effective recovery of spontaneous communication in people with affective and emotional disorders due to their empathic nature (Borioni et al., 2012), what may rely on the way they use their cognitive abilities to interact with humans (Sudekum Trotter and N. Baggerly, 2018). Increasing the scarce information relative to interindividual variability in cognition in donkeys through research (Osthaus et al., 2013) may open a new path towards finding equine specific genes involved in assisted therapy desirable behavioral traits. This new research possibility may enable psychometrically quantifying the degree in which such features are inherited providing us with methods to select for it. These objectives appear in a framework in which we have evolved from the simple identification of the genes related to behavioral responses like the dopamine D4 receptor (DRD4) gene polymorphism in the *Equus* genus (Momozawa et al., 2005b) from a decade ago to the current situation of large gene numbers involved in even the most basic behavioral features. The behavioral traits specifically related to intelligence and cognitive learning processes in animals have historically raised remarkable interest. Such interest was highlighted by the letters to the editor under the topic *Intelligence in animals* published by the Nature Journal from 1883 to 1904. This scientific context, basing on the unavoidable relation established to related human characters, derived into the definition of the general factor of intelligence or *g*.

As summarized in the first key point of the review by Deary et al. (2010), more than a century of empirical research provides conclusive evidence that a general factor of intelligence (also known as *g*, general cognitive ability, mental ability and IQ (intelligence quotient) exists, despite some claims to the contrary (Herrmann and Call, 2012). From their review, we could infer that *g* partially and remarkably accounts for 40 to above 50% of the differences in the performance between individuals on a given cognitive test (Reader et al., 2011; Locurto et al., 2013), and composite scores (IQ) based on different tests are frequently regarded as estimates of individuals' standing on *g*. Other authors such as Kamphaus and Frick (2005) and Frick, Barry, & Kamphaus (2010) suggest that the terms IQ, general intelligence, general cognitive ability, general mental ability, or intelligence are often used interchangeably to refer to this common core shared by cognitive tests.

In the normal population, *g* (one of the psychometric constructs that exist and which summarizes the correlations among different cognitive tasks in individuals) and IQ (what you score on a cognitive test from individuals) are roughly 90% correlated. Such a strong correlation enables using IQ score, with a high level of accuracy to predict *g*, and vice versa (OpenStax, 2014). Hence, IQ could be used as the standardized score of tests designed to measure *g*, a theoretical faculty of “general intelligence factor”. Matzel and Sauce (2017) would state that the rationale for most psychometric tests is roughly based on Spearman's (Spearman, 1904) observation that performance on a wide range of cognitive tasks is correlated and, as such, can be reduced to a single index of aggregate performance across a battery of diverse tests. That is, the more familiar term of intelligence quotient (IQ) used in humans as it summarizes the correlations observed between the scores of a particular individual on a wide range of cognitive abilities compared to the skills that such individuals must present considering their chronological age (Reader et al., 2011). The influence of language on intelligence has been reported to be one of the most determining factors setting human and animal cognition apart (Dennett, 1994). There appears to be no evidence to date that nonhuman species understand recursion (Corballis, 2007). Because animals lack recursion (and human language is recursive), animals lack language (Premack, 2007). Traditional tests put a premium on language skills, making it necessary to develop and assess intelligence through nonverbal tests, for instance, those used in children with language difficulties or disabilities (DeThorne and Schaefer, 2004). In contrast to human widely verbal or language-dependent scales, animals' cognitive ability assessment relies on different interactive and observational tools which focus on the ability of the animals to interact with their environment and everything on it, through innovation, habit reversal or inhibition, social learning, or the responses to known and unknown stimuli, for instance.

Only few examples of research involve cognitive processes from a genetic perspective, for instance, humans (Darst et al., 2015), mice (Galsworthy et al., 2005) or primates (Hopkins et al., 2014). Thus, research in the field still relies on phenotypical perspectives and rather suggest the genetic structure behind such processes than quantify it (Horowitz, 2014). In this context, human-nonhuman species extrapolations are rare (Anderson et al., 2017).

g has proved to be responsible for 47% to 60% of the individual genetic variance in cognitive ability measures in non-human species such as primates (Reader et al., 2011; Locurto et al., 2013). This percentage of explained variability is similar to the fraction of variance explained by IQ reported for humans (40-50%) (Kamphaus and Frick, 2005). Despite, some studies have reported the existence of large interspecific (Osthaus et al., 2013) and intraspecific (Baragli et al., 2011) variation for cognitive processes in donkeys, no wide-scale populational study has been carried out, and despite being suggested (Proops et al., 2012), the genetic background behind them remains unexplored yet.

Among other issues (Kaufman, 2018), two of the criticisms usually leveled at attempts to test for non-human *g* address the difficulty of developing standard tasks to be implemented across species and the presence of species specializations (Proops et al., 2009). Although these problems are lessened in studies in which comparisons are made among very similar species (Proops et al., 2009), literature rarely deals with the contrast between distant species. However, these difficulties could be overcome through the implementation of an extrapolation method.

The quantification of cognitive capabilities in humans can be performed considering tests of a very different nature, but which assess the same underlying cognitive processes (Eysenck, 2018). Despite tests measuring for the ability of individuals at specific cognitive processes may differ when it comes to what is measured and how, they commonly report a single psychometric construct per individual (Saklofske et al., 2017). Using the computation method to score cognitive capabilities widely applied in humans to compute IQ in animal populations can help to explore the existence of a contrastable interspecific underlying general intelligence factor or *g*. Therefore, the present research aims to develop an animal human-analogous IQ score and to study the populational variation and the inheritance patterns described in donkeys. The use of pedigree extensively genetically tested information can provide us with antidotal evidence of the popularly attributed dual misconception between intelligence and stupidity (according to Cambrigde Dictionary, asinine, derived from the term ass literally means, extremely stupid) in donkeys. Not only aiming at responding traditionally raised questions concerning the practical application of equine behavior and cognition genetics and the factors directly affecting them (Hausberger, 2002) but contrasting the populational distribution of donkey intelligence and human intelligence, at the same time.

Materials and methods

Study sample and study background

The whole pedigree file included 1017 Andalusian donkeys -272 jacks and 745 jennies- born between January 1980 and July 2015. As the age range was not normally distributed ($P<0.05$ Shapiro-Francia W' Test of normality) we used minimum, Q_1, median, Q_3 and maximum to describe the age range in our sample. The minimum age was 0.27 months, Q_1 age was 29.76 months, median age was 77.04 months, Q_3 age was 129.07 months, and the maximum age was 270.40 months. Such wide age range was considered, as the test battery used to assess cognitive processes was suitable for all animals included in the study and given the fact that we evaluate an endangered breed from which the information belonging to each individual is indispensable. The donkeys in the sample were the progeny of 93 jackstocks and 253 jennies. Parentage tests for each mating had been performed with twenty-four microsatellite molecular markers recommended by the International Society of Animal Genetics (ISAG) providing genetically tested pedigree extensive indirect information from 724 ancestors.

Behavioral record registration

Before carrying out the behavioral assessment, we conducted a telephone interview to survey the experience of the owners of the donkeys in the study to define the traits comprising the clusters to consider in the model. We interviewed owners about their donkeys' inherent cognitive abilities, the tasks that they should routinely accomplish on their farms and the training/education methodology (or learning methods) owners regularly apply for their donkeys to learn such skills/tasks. Among the answers the respondents gave, they coincided on thirteen traits chosen as they were the ones that the owners most frequently allude to during the interviews (Supplementary Table S1). We discarded the rest of the features because of the anecdotal occurrence of their use or because of being related to the use of different nouns to allude the same behavioral trait concept.

We organized the information deriving from the interview for the thirteen behavioral traits in two clusters. 'Cognition' cluster comprised seven traits that were directly related to unspecific cognitive processes considering the ability of donkeys to perceive information from their environmental situation. Second, 'Intelligence' cluster comprising the six remaining traits, describing the cognitive processes or mental capacities of the donkeys to retain information from the environment as knowledge to be applied towards adaptive responses within a specific context (Table 1). Table 1 not only defines what each cognitive process or trait assessed in donkeys is, but also what would be the human extrapolation as well. We translated these categorical traits into different linear scales, in which the donkeys scoring one meant they presented the lowest extreme behavioral pattern and five the highest extreme one. We show the thirteen intelligence and cognition related traits considered, and a detailed definition of the scores present in the scale in Supplementary Table S1.

We set the definition of the cognitive processes included in the study, defining the scales to measure them and establishing the possible non-genetic factors that may be exerting a modulating effect, relying on the protocols in Momozawa et al. (2005a) and establishing their analogies with human cognitive processes as it can be observed in Navas et al. (2017a), Figure 1, Table 1 and Supplementary Table S1. The thirteen cognitive processes were divided into seven direct on-field general cognitive process related traits and six specifically related intelligence cognitive process traits, attending to principal component analysis criteria, as described in Navas et al. (2017a). The standardization, development of the tests and scales was described in a previous stage of the study (Navas et al., 2017a; Navas González et al., 2018b) and is summarised in Figure 1. Statistical verification that tests being used are in fact measuring the constructs they are intended to measure, and whether they can do so with internal reliability was performed at two previous studies (Navas et al., 2017a; Navas González et al., 2018a) as it has been reported in the Test and scoring system reliability section of the present article.

We registered all records describing the cognitive ability of the donkeys during the development of a six-stage operant conditioning test (Figure 1). The same trained appraiser registered all the information concerning the four behavioral variables for all the stages and animals. The donkeys were each given a maximum of 450 seconds to complete the operant conditioning test (75 seconds per phase and treatment implemented). No additional time was provided for the donkeys to complete the test.

Operant conditioning behavioral test

The operant conditioning behavioral test was carried out in an open area to which the donkeys were previously accustomed (it was part of the area over which the donkeys developed their daily activities). We exposed each animal to six reinforcement treatments consecutively, one at each of the six stages within the operant conditioning test. At each phase, handler A and handler B used each of the six different reinforcement treatments to lead the donkeys to cross over an oilcloth laying on the floor. These treatments could comprise unknown elements (the animal was not familiar to them) or known factors (to which the animal was already familiar). These elements could be visual (elements fell within the visual areas of the donkeys) and/or acoustic (elements generated sounds, i.e., "motivator" or claps, although they may or may not fall within visual areas) and were presented to the donkeys from different positions (from the front or from a rear position always at 2 metres away from the animals). A cameraman (Handler C) simultaneously videotaped the experiences (1080 p, 50 Hz, shutter speed: 1/250 seconds) to assess the donkey's performance after the field experiences and to test for intra-observer discrepancies. Cameraman (Handler C) controlled timing. We show a detailed description of the operant conditioning test in Figure 1 and Navas González et al. (2018b).

Test and scoring system reliability

We did not appreciate intra-observer discrepancies as all the scores obtained on the field matched those obtained after reviewing the tapes again. We run a Cohen's κ test at a preliminary stage of the study to test for inter-observer reliability and determine if the three appraisers' judgment agreed on the scores of 50 individuals (16.67% of the total sample) for the score at the thirteen cognitive processes assessed. Cohen's κ determined whether the repeatability of the model was enough to delete the effect of appraiser from the model, providing a measure of the accuracy of scoring of the appraisers. Then 95% confidence intervals (95% kappa IC) were computed according to 95% kappa IC= $\kappa \pm 1.96$ $SE\kappa$, where; $SE\kappa = [(po(1-po)/n(1-pe)^2]^{0.5}$ with the Crosstabs procedure of SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016). This preliminary analysis aimed at testing for the reliability of the scoring system, which proved to be highly reliable as there was highly statistically significant perfect agreement between the three appraisers' judgments when scoring for the thirteen cognitive processes tested during the development of the operant conditioning test. There was highly statistically significant and from substantial to almost perfect agreement among the three observers at the preliminary test for repeatability for all the traits. We present the results for this preliminary study in Supplementary Table S2.

Donkey's intelligence quotient (IQ)

In human terms, mental age scores how an individual performs intellectually for a particular cognitive process, compared to the average performance that should be expected for that individual for that same cognitive process at its current chronological age (Gerrig and Zimbardo 2002).

Current human IQ tests set the median raw score of the norming sample as IQ 100, i.e., when chronological and mental ages are equal or when a particular individual can reach the score that it would be expected to reach considering its chronological age (Hunt, 2010). Then, each standard deviation unit (SD) from this value is scored up or down at increasing or decreasing intervals of 15 IQ points (Gottfredson 2009). We computed the mean score obtained by the donkeys in the population under study at the multi-phased operant conditioning test (Supplementary Videos 1 to 6) for each of the thirteen cognitive processes (scored 1 to 5) to develop an analogous animal scale. Then, using the variation reported for humans as a reference (Hunt 2010), we focused on the highest mean score in the scale (from 1 to 5) that was reached on average by any donkey of the lowest age level possible for each cognitive process (Figures 2 and 3). Then, we set such score as the average range (IQ 100), addressing the mental age at which a donkey, in particular, would be expected to reach that score for that specific cognitive process. This score set the starting point from which to move up or down in the scale from 1 to 5 (Table 2 and Supplementary Table S1) to set the IQ categories above the average (above average and very superior) (Figure 3). Quantitatively, we made these increases/decreases following 15 points intervals per standard deviation unit.

To extrapolate the results to humans, when this mental age matched the chronological age of a particular donkey, we considered its IQ to be within the average range and thus, analogous to human IQ 100. We classified the donkeys below this score at which mental age was equal to chronological age to be below the average IQ range (Figure 3). Overestimation of individuals very below or above the average is likely to occur due to the donkeys being able to succeed in reaching the highest average level (5) for the different processes at very early ages.

The mental age of each donkey, hence IQ, was computed as the average of the mental ages or IQs reported for all of the thirteen cognitive skills for each animal. We calculated IQ through the following mathematical equation; IQ=(Mental age/Chronological age)·100 (NCME, 2017).

Variance in Problem-solving multistage cognitive test

A principal component analysis (PCA) was carried out to compute the variation in IQ explained by the cognitive processes tested.

Human and donkey's IQ distribution comparison.

We compared humans and donkeys' IQ distributions through the calculation of polynomial regression equations (2^{nd} order) and R squared (R2) values as shown in Figure 2 and compared through an analogous scale in Figure 3. To score the difference between distributions, we calculated the percent of explained standard deviation or the percent by which the standard deviation of the errors is less than the standard deviation of the dependent variable, following the equation suggested by Nau, P. (2014):

Percent of explained standard deviation $=\left(1-\sqrt{1-R^2}\right)*100$, with R^2 being R squared.

Genetic analysis, Predicted breeding values and descriptive statistics ("PBV Bayesian accuracies")

Our study aimed at obtaining estimators for fixed effects and covariates, variance components, heritabilities and breeding values for cognitive process related traits in Andalusian donkeys, through single record mixed Animal Model procedures, as all the characters were scored only once in the lifetime of the individual through Bayesian multivariate analyses using the Multiple Trait Gibbs Sampling for Animal Models package (MTGSAM) (Van Tassell and Van Vleck, 1995). We obtained a single chain of 550000 cycles, discarding 50000 (burn-in), and using thinning intervals of 200 cycles to retain sampled values which reduced the lag correlation among thinned samples. The convergence criteria used implied the change in the Log-likelihood of the function in successive iterations and were less than 10^{-10}. Gibbs sampling procedures enable building and saving a random number or the total number of samples of variances obtained in the iterative process (2058 solutions in our case). Then, for each sample of variances saved, the genetic parameters could be calculated and assessed to obtain descriptive statistics such as mean, standard deviation, variance and standard errors, which could provide us with a perspective of the existing variability. Univariate analyses were carried out to compute the heritability of each trait to avoid the distortion that could be caused by the effects of epistasis among features (calculated then within residual variance). Then, bivariate analyses were used to calculate the correlations between each possible combination of the thirteen characters assessed to quantify such possible epistatic effects through genetic correlations. Then, we predicted breeding values (PBV) and systematic deviation for all animals in the relationship matrix. We calculated Bayesian PBVs directly with MTGSAM software (Supplementary Table S3). To assess the accuracy of PBVs, we calculated the posterior distribution of each parameter sampling uncorrelated realizations from the Gibbs chain with the PULLDAT application of the MTGSAM software. We thinned the chain of samples until the correlation of adjacent samplings were approximately 0 to assess the distribution, calculate mean, standard deviation, variance, and standard error of breeding values (Supplementary Table S4).

The multi-trait animal model used for the analyses is as follows:

$$y = Xb + Za + \varepsilon$$

where y is the vector of records for cognitive process related traits, b is the vector of fixed effects to be estimated and X the corresponding incidence matrix relating records to fixed effects, a is the vector of breeding values to be determined and Z the corresponding incidence matrix, and □ is the vector of residuals. In this case, the fixed effects considered in vector b were assessment year (3 levels: 2013, 2014, 2015), sex (2 levels: male and female) and husbandry system (5 levels: Intensive, semi-intensive, semi-extensive, contest and extensive) plus the linear and quadratic effect of age at scoring as a covariable. We chose the previously described combination of fixed effects as the bivariate correlations found between at least one of the fixed effects and e, ch of the thirteen-cognitive process related traits were statistically significant (P<0.05). A previous analysis was carried out to describe the effects and levels included in this model (Navas et al., 2017a).

The analyses included the relationship matrix of animals with direct records related through at least one known ancestor, considering the 1017 animals in the historical pedigree. Considering the lack of previous experiences for cognitive and intelligence traits in donkeys, we used the phenotypical variance of each character and the existing phenotypical correlations between each possible pair combination for the estimation of the starting point to seek for the convergence of additive genetic (multiplying them by 0.2).

Then we did the same for residual variances (multiplying them by 0.8) and genetic and phenotypic correlations to obtain specific variance components and estimates of fixed and random effects for each trait in multivariate analyses. The standard errors of genetic correlations were derived directly from the MTGSAM analyses. After the analyses reached convergence and we obtained genetic parameters, we predicted breeding values for all animals in the relationship matrix, and we obtained fixed effects estimates.

Results

Donkey's intelligence quotient (IQ)

Table 2 and Supplementary Table S5 show the mental age ranges, and descriptive statistics for each of the thirteen cognitive processes studied. Human (Minnesota, 2015) and donkey IQ distributions, polynomial regression equations (2nd order) and R squared (R2) values are shown in Figure 2 and compared through an analogous scale in Figure 3. The percent of explained standard deviation for donkey's IQ was of 27.62%, while for humans it was 33.23%.

Variance in Problem-solving multistage cognitive test

The PCA revealed two components whose eigenvalues were higher than 1 (Table 3), which together explained 72.14% of the cognitive variation between donkeys. However, the eigenvalue of the second component (PC2) was only slightly higher than 1. The first principal component (PC1) had strong positive loadings for all the cognitive processes studied suggesting that donkeys scoring high on this factor show signs that may be indicative of better cognitive performance. The first principal component (PC1) explained 62.78% of the cognitive variation. The second principal component (PC2) had weak negative loadings for all cognitive processes except for alertness and perseverance, and they only explained a 9.36% of the cognitive variation. We show a summary of the results for the PCA of the 300 donkeys assessed in Table 3.

Genetic parameters assessment

For the studied variables, the highest estimate of additive genetic variance was obtained for stubbornness, which also accounted for the highest phenotypic variance (Table 4), while the lowest additive genetic variance estimates were obtained for alertness and perseverance.

We show estimates for variance components for all cognitive and intelligence-related traits in Table 4. For all estimates of h^2, the SE was 0.01, indicating the high accuracy of the estimated parameters.

We show genetic and phenotypic correlations and heritability estimates for all the cognitive processes in Table 4. Phenotypic correlations (r_P) among all the seven general cognitive process related or 6 specific cognitive process intelligence associated traits were positive and from low to strong, with 0.12 (of alertness with dependence) being the lowest and 0.81 the strongest correlation (between memory and trainability) (Table 4). Genetic correlations (r_G) were generally positive and ranged from 0.11 to 0.97. However, all the correlations between alertness and the rest of traits, except for those with dependence, emotional stability, perseverance and the ability to get in/out stables were negative and from low to strong (-0.35 to -0.85), which were the lowest ones as well. Overall, the poorest correlation both phenotypically and genetically was obtained for alertness, while we got the strongest one for emotional stability (Table 5). The standard error for the phenotypical and genetic correlations was around 0.01 for all parameters (Table 5).

Predicted breeding values and descriptive statistics ("PBV Bayesian accuracies")

The results for the estimates of predicted breeding values (PBV) ranged between -1.60 to 0.50. We show a summary of the descriptive statistics of the breeding values obtained for each cognitive process sorted by sex in Supplementary Table S3. The dispersion measures ("PBV Bayesian accuracies") of the PBV for each of the thirteen cognitive processes estimated after Gibbs sampling procedures are shown in Supplementary Table S4.

Discussion

Modelling animal cognitive processes may enable understanding how human cognitive features interact or how they are inherited. However, leaving experimental conditions to assess species in their environment (Miklosi, 2015; Miklósi and Kubinyi, 2016) can be a challenging experience, especially when these species lack human-primate behavioral resemblance or mice in-depth knowledge of cognition genomics (Plomin, 1999).

Among the challenges found in the field, the study of donkey endangered populations makes us face compromises because of the low number of individuals and their population structure (Navas et al., 2017b). Such situation compels us to include donkeys from a wide age range as long as they are able to fulfil the tests that we want to carry out.

As age could be expected to affect the ability of the individuals to solve out multistage problem-solving cognitive test, the effect of age is assessed and included in the cognitive model as a covariate to correct for its possible distortion. The variation coefficient for age in our sample is 0.73, what bases on the population's age distribution depicted in Navas et al. (2018). This population distribution may compromise the evaluation of our sample in more narrowly-defined age ranges as they may not be representative of the whole population, due to the unequal distribution of animals among the groups.

Figures 2 and 3 suggest donkeys' IQ similarly describes the Gaussian distribution found in humans' IQ, although the curve is moderately deviated to the left. This is also shown by the polynomial regression equations (2nd order) and R squared (R^2) values that only differ 0.0781 (7.81%) in the percent of explained variance (determination coefficient or R^2). The percent of explained standard deviation for donkey's IQ was only 5.61% lower than that of human's IQ, suggesting confidence intervals may overlap.

Standard deviations are measured in the same units as the variables, hence directly determine the widths of confidence intervals. Nau, R. (2014) suggests, 5% decrease in R^2 would increase the error standard deviation by about 10% in relative terms. That begins to rise to the level of a perceptible widening in confidence intervals, what means both IQs may distribute similarly with human IQ confidence interval being slightly narrower.

Results indicate that the highest sample percentage (97%) that gathers at 15-125 IQ in donkeys is gathered around a narrower IQ range in humans (70-130 IQ). However, when we extrapolated the results (Figure 3), we found more dissimilar sample percentages, that is sharper differences between donkey individuals. Donkeys exceeding IQ 130 appeared because of the nature of the cognitive processes scored. Some of them, such as getting in/out stables were likely to be already significantly developed by very young animals what slightly distorted the results for animals in the very lowest or highest IQ range.

In human psychometric testing, individuals' test scores are positively correlated across tasks assessing several cognitive domains, with a general factor typically accounting for 40 to 50% of total variance (Plomin, 2001; Deary et al., 2007). We found from low to strong significant positive correlations between almost all cognitive processes, loading positively on the first component of PCA (PC1) and extracted with an eigenvalue >1. PC1 captured almost 63% of the variance in cognitive performance in donkeys, what has also been reported for primates for which *g* has proved to be responsible for 47% to 60% of the individual genetic variance in cognitive ability measures (Reader et al., 2011; Locurto et al., 2013) and about 55-60% of the individual variance in tests of cognitive ability in mice (Locurto, & Scanlon, 1998).

Plomin (2001) suggested 'cognitively complex' tasks present higher *g* loadings. Thus, low *g* loadings are consistent with the suggestion that certain cognitive processes may not be a good measure of animal cognitive ability (Boogert et al., 2011), as prior experience may have influenced their learning performance. Additionally, the positive cognitive process intercorrelations could be further evidence that animals' previous knowledge may not affect these abilities (Boogert et al., 2011). Our studies are consistent with those by Woodley Of Menie et al. (2015) on the fact that those cognitive abilities being more *g*-loaded would be more heritable and present larger additive genetic and phenotypic variance values (Tables 2 and 3).

Our estimated heritabilities ranged from 0.06 for dependence to 0.38 for the ability of the donkeys to enter or leave their stables what suggests cognitive processes are complexly and moderately inheritable in donkeys. These heritability values are generally moderate and similar to those for cognitive processes related traits in literature, and slightly higher than similar traits' estimates reported in other species, even more, when we consider the low standard error (higher accuracy) obtained, considering the limited sample size and matches the results found in the literature. Darst et al. (2015) obtained similar heritability values from 0.10 to 0.64 (Standard error of the mean= 0.12 to 0.15, respectively) for cognitive traits in human siblings with a parental history of Alzheimer's disease.

The only existing animal examples are those in mice by Galsworthy et al. (2005), who would report a heritability upper limit value ranging from 0.34 to 0.42 after Plomin (2001) discussed how a mouse *g* model could provide a human translatable analytic tool for exploring functionally gene-linked cognitive processes and how they overlap. After it, the principal component analysis (PCA) of thirteen cognitive traits carried out in the study by

Hopkins et al. (2014) reported heritability values for g in chimpanzees from 0.012 to 0.538. This value remarkably improved after retesting almost the same sample of animals for two consecutive years, scoring a value of 0.624±0.242, suggesting repeated measures may considerably improve the results obtained. This value was noticeably higher for h^2, maybe because of the controlled laboratory conditions applied, but presented a much higher standard error than our results did. Early attempts aiming at clarifying behavioral hereditary and additive components-environmental factor interaction (sex, age, breed and handling conditions) suggest that, even with little environmental variation, individual genetic variation occurs (French, 1993; Wolff and Hausberger, 1996; Hausberger et al., 2004).

The low standard error in heritability and correlation estimates suggest that the model used to study cognitive processes' genetic background is efficient. Literature low to moderate behavioral heritability values and high standard prediction errors evidence the inability of scientists to infer accurate and suitable models from studying the fraction of the total variation that can be accounted for by genetics. Analyzing and improving heritabilities may derive in the ability to enhance traits through selection.

The negative genetic correlation between alertness and the most of the traits reflects that donkeys describing extreme alert signs when facing external stimuli were prone not to be curious for the stimuli being presented and not likely to approach them. Simultaneously, these donkeys were difficult to handle or educate, uncooperative, less likely to concentrate and memorize the task introduced and tended to display freezing coping styles strategies as highlighted by the negative correlations with stubbornness and docility. These values suggested the independent location of the alertness trait at a different locus than the rest, what had also been outlined by the results of the Principal Components analysis (Table 3).

The occurrence of a negative genetic correlation between a pair of traits that holds a positive phenotypical correlation, for example, alertness with other cognitive processes (Table 5), has traditionally been attributed to countervailing environmental effects to which the animal adapts (Sgro and Hoffmann, 2004). The concept of behavioral plasticity (Mery and Burns, 2010), accounts for such ability of organisms to change their behavior as a result of the exposure to certain stimuli. In this way, the effects of training, learning or education can condition the expression of specific cognitive processes and translate into phenotypical changes that differ from the genetic basis underlying.

From a genetic perspective, the genetic correlation between two traits is the correlation between the genetic influences on a trait and the genetic influences on a different trait estimating the degree of pleiotropy or causal overlap between both traits while, phenotypic correlation is a measure of the strength (consistency, reliability) of the relationship between performance in one trait and performance in another trait. On the contrary, environmental correlations describe the relationships between the environments affecting two traits. The relationship between phenotypic correlations and their components is defined through rP=rG+rE.

A high phenotypic correlation linked to such high underlying genetic correlation enables a successful selection of the individuals in favor of their concentration skills when visually selecting for those animals that display better memory skills, are more stubborn, are more easily trainable, are more willing to cooperate and are easier to handle. By contrast, if we aimed at selecting for more curious donkeys, we may only choose those displaying better memorizing skills, stubborn and more easily trainable individuals.

When selecting for donkeys for their memorizing skills we indirectly select for individuals who concentrate easily, that are more curious, are more stubborn, more docile, more easily trainable, more cooperative, more emotionally stable and easier to handle.

Phenotypically selecting for stubborn animals, we may genetically select for animals that concentrate better, are more curious, have better memorizing skills, are more docile, cooperative, and easier to train and handle. Animals more easily engaging an alertness status will be less curious as well, both from a genetic and phenotypic perspective, thus we should promote indirect selection strategies to select for one of both.

The low to moderate genetic correlations for dependence towards the owner with the rest of processes suggest it is not a good criterion to follow to visually select donkeys for any other cognitive ability. However, more trainable and cooperative animals will genetically be more prone to concentrate better, be more curious, have better memorizing skills, be more stubborn and docile. Moreover, the more stubborn the donkeys are the more emotionally stable they are as well. Perseverance does not hold any quantitatively important correlation, so as to be able to use it as a criterion for selection of other cognitive processes.

The ease at which animals enter their stables or leave them is moderately related to how stubborn the animal is, what may rely on the nature of donkeys which rather than flying freeze and try to avoid potentially stressing factors coming back to a place where they feel safe. Easily trainable animals are correlated to more cooperative ones, those more easily concentrating and more docile ones.

The correlations we have found suggest remarkable synergism between the most of the cognitive processes, as reported in chimpanzees (Hopkins et al., 2014). Visscher et al. (2008) reported a 0.5 to 0.8 human IQ heritability range attributing the IQ related traits' moderate-high standard error to the narrow range of sibling identity by descent.

From this finding, we can infer the fact that although the genes controlling for some behavioural traits may be topographically close or these traits may be features of the same cognitive process (enabling a simultaneous selection for both), some behavioural traits may be controlled by genes located at different loci or should be attributed to very distant cognitive processes (compelling to carry out an inverse selection strategy). Therefore, adding more data to the sample may reveal more reliable and independent personality components with higher heritabilities and may help to outline the relationships established between traits. Some traits may be under strong genetic control, but the particular population studied may have no genetic variation as a result of selection, also resulting in low heritability values. The values for additive variance enable the selection of individuals according to their cognitive abilities. Donkeys that may present a better cognitive development may potentially make the most of the elements present in their environments as well as may make educational or training plans easier and more effective, both regarding the money expended and the time devoted for a trainer/educator to get the donkey achieving the progress intended, hence, are more profitable.

Although we may be able to collaterally assess cognitive processes developed during the fulfillment of standardized tests, we may often be exposed to several drawbacks. For instance, the likelihood of measuring a superficial behavior portion, other behavioral elements or the possibility of testing the owner's ability to educate donkeys instead of specific traits may translate into the moderate heritability values and standard errors found.

The use of well-defined and objective criteria assessed through proper standardized tests by few well-trained judges reports typically much higher heritabilities. High correlations may suggest such skills may have been split into too numerous pieces or overlapping among cognitive traits involving more than one cognitive process and the cognitive process themselves individually. Therefore, reanalyzing data may reveal more reliable and independent personality components with higher heritabilities. Some traits may be under strong genetic control, but the particular population studied may have no genetic variation as a result of selection, also resulting in low heritability values. Still, our results provide some of the first evidence that an analogous factor to human *g* may underpin cognitive performance in donkeys and accounts for a similar distribution in the human population.

Conclusions

Our results suggest donkeys could be considered somehow intelligent animals when comparatively scoring them relying on an analogous human scale. However, we do not intend to assert that some donkeys may account for a higher IQ than humans compared through the same scale, what would be nonsense. The cognitive processes and methods to score them widely differ from one species to another. Furthermore, the more complex the cognitive development of the species being tested is, the more complex these methods should be (Gómez, 2005). However, still, a remarkable variation among donkeys is found, i.e., there are donkeys which are more intelligent than others, and the present methodology enables quantifying such differences. The remarkably similar phenotypical distribution and inheritance patterns described in asses (compared to birds (Shaw et al., 2015), or other mammals (Hopkins et al., 2014), including humans (Mortensen et al., 2005; Hunt, 2010)) may suggest intelligence could be ascribed to a similar scientific background or even be supported by a similar genetic structure to the one widely studied in humans. Such finding lays the basis for future research to deepen in the field of animal cognition. Our results suggest that donkey cognition heritable mechanisms may be attributed to human's similar genetic background. This study opens the door to selection and breeding for better cognitively performing animal generations. Our methodology comprises a novel approach to the animal intelligence controversy, using a standard human applied method to score individual intelligence quotient.

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Conflict of interest statement

Francisco Javier Navas González declares that he has no conflict of interest, Jordi Jordana Vidal declares that he has no conflict of interest, José Manuel León Jurado declares that he has no conflict of interest, Amy K. McLean declares that she has no conflict of interest, and Juan Vicente Delgado Bermejo declares that he has no conflict of interest.

Welfare declaration

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. 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. 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.

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Table 1. Definition of the thirteen cognitive traits comprising the intelligence and cognition clusters studied in donkeys and their human analogy.

Intelligence Cluster		
Cognitive Process/Trait	Definition	Human Analogy
Concentration	The animal collaborates during the assessment session and does not get distracted by the environment.	Attention (Moran, 2011)*
Curiosity	The animal is interested in the novel stimuli being presented and moves towards them.	Curiosity (Kidd and Hayden, 2015)*
Memory	The animal remembers the stimuli being presented.	Memory (Goshen and Yirmiya, 2007)*
Stubbornness	The donkey rejects following the requests of the assessor.	Cognitive rigidity (Buzzichelli et al., 2018) /Decision Making (Secchi and Bardone, 2009)**
Docility	The donkey easily follows the orders of the instructor.	Docility/Decision Making (Secchi and Bardone, 2009)**
Alertness	The animal shows a vigilant or alert status focusing on the stimulus around.	Alertness (Oken et al., 2006)*
Cognition cluster		
Dependence	The donkey is comfortable when separated from the main herd	Separation anxiety (Littenberg et al., 1971)**
Trainability	Ability of the animal to be trained into the fulfillment of the tests	Cognitive training (Sternberg, 1981)**
Cooperation	The donkey cooperates with its handlers during the daily tasks	Cognitive cooperation (Wilson et al., 2004)*
Emotional stability	The animal is not predictable from one to another stimulus	Anticipation (Roca et al., 2011; Murphy et al., 2015)/Predictability (Namikawa et al., 2013)**
Perseverance	The animal is patient when completing several sequential tests.	Patience (Yingxu & Guenther, 2007). Related to decision making. Patience is studied as a decision-making problem, involving the choice of either a small reward in the short-term, against a more valuable reward in the long-term (Coutlee and Huettel, 2012)**
Get In/Out of Stables	The animal shows no problem when leaving or entering its housing facilities.	Fear (Hofmann, 2008)/Cognitive appraisal (Folkman et al., 1986)/ Coping (Lazarus and Folkman, 1984)**
Ease of Handling	The animal shows sympathy towards humans.	Cognitive empathy (Smith, 2006)/Attitudes towards animals (Taylor and Signal, 2005; Sharp et al., 2006)**

Definitions and clustering criteria accessed from Navas et al. (2017) and Sparrow and Davis (2000).

*Addressed as cognitive processes in literature themselves.

**Addressed to involve several underlying cognitive processes in literature.

Table 2. Mental age ranges (in months) in Andalusian donkeys for the thirteen cognitive processes studied.

Cluster	Items/Scores	1	2	3	4	5
Intelligence cognitive process related traits	Concentration	Below average	Below average	Average	3	17
	Curiosity	Below average	Below average	Below average	Average	21
	Memory	Below average	Below average	Average	3	17
	Stubbornness	Below average	Below average	Below average	Average	27
	Docility	Below average	Below average	Below average	Average	27
	Alertness	Below average	Below average	Below average	Average	3
General cognitive processes related traits	Dependence	Below average	Below average	Average	3	21
	Trainability	Below average	Below average	Average	3	38
	Cooperation	Below average	Below average	Below average	Average	17
	Emotional stability	Below average	Below average	Below average	Average	27
	Perseverance	Below average	Below average	Below average	Average	3
	Get In/Out of Stables	Below average	Below average	Below average	Below average	Average
	Ease at Handling	Below average	Below average	Average	3	17

The average level was set at the mean score reached for each cognitive process at the age range of ≤1 month.

Table 3. Results of the principal components analysis for the 300 Andalusian donkeys.

Cluster	**Cognitive Process**	**PC1**	**PC2**
Cognition	Trainability	**0.898**	-0.114
Intelligence	Stubbornness	**0.894**	-0.190
Cognition	Ease at Handling	**0.889**	-0.045
Intelligence	Memory	**0.888**	-0.117
Cognition	Cooperation	**0.883**	-0.111
Cognition	Emotional stability	**0.861**	-0.109
Intelligence	Docility	**0.860**	-0.047
Intelligence	Concentration	**0.851**	-0.073
Intelligence	Curiosity	**0.753**	-0.085
Cognition	Dependence	**0.727**	0.075
Cognition	Perseverance	**0.711**	0.400
Cognition	Get In/Out of Stables	0.590	0.426
Intelligence	Alertness	0.210	**0.875**
Eigenvalue		8.162	1.216
% Variance explained		62.781	9.357

The loadings and percentage of variance explained for each principal component (PC) with an eigenvalue >1 are shown. Loadings >0.6 are in bold.

Table 4. Estimated genetic (σ_a^2), phenotypic (σ_p^2) and residual (σ_e^2) variances for intelligence and cognitive traits in Andalusian donkeys, obtained from univariate analyses.

Cluster	**Trait**	$\boldsymbol{\sigma_a^2}$	$\boldsymbol{\sigma_p^2}$	$\boldsymbol{\sigma_e^2}$
Intelligence cognitive process related traits	Concentration	0.2574	0.9022	0.6448
	Curiosity	0.1218	0.7636	0.6418
	Memory	0.0487	0.7012	0.6525
	Stubbornness	0.1537	1.1456	0.9919
	Docility	0.0856	0.7103	0.6247
	Alertness	0.0617	0.3041	0.2424
General cognitive processes related traits	Dependence	0.1806	0.8523	0.6717
	Trainability	0.1845	0.8753	0.6908
	Cooperation	0.0815	0.8057	0.7242
	Emotional stability	0.1304	0.6973	0.5669
	Perseverance	0.0534	0.5298	0.4764
	Get In/Out of Stables	0.1882	0.4949	0.3067
	Ease at Handling	0.0874	0.8925	0.8049

Table 5. Estimated heritabilities (diagonal), phenotypic (r_P) (above diagonal) and genetic correlations (r_G) (below diagonal) for intelligence and cognitive traits in Andalusian donkeys.

	Trait	Intelligence cluster						Cognition cluster						
		Concentration	**Curiosity**	**Memory**	**Stubbornness**	**Docility**	**Alertness**	**Dependence**	**Trainability**	**Cooperation**	**Emotional stability**	**Perseverance**	**Get In/Out of Stables**	**Ease at Handling**
Intelligence cluster	Concentration	0.28±0.01	0.51±0.01	0.68±0.01	0.70±0.01	0.56±0.01	0.37±0.01	0.40±0.01	0.70±0.01	0.61±0.01	0.54±0.01	0.51±0.01	0.32±0.01	0.65±0.01
	Curiosity	0.87±0.01	0.16±0.01	0.67±0.01	0.60±0.01	0.52±0.01	0.35±0.01	0.42±0.01	0.63±0.01	0.52±0.01	0.48±0.01	0.46±0.01	0.56±0.01	0.51±0.01
	Memory	0.88±0.01	0.60±0.01	0.06±0.01	0.74±0.01	0.66±0.01	0.17±0.01	0.45±0.01	0.81±0.01	0.72±0.01	0.62±0.01	0.51±0.01	0.50±0.01	0.70±0.01
	Stubbornness	0.69±0.01	0.69±0.01	0.64±0.01	0.13±0.01	0.73±0.01	0.17±0.01	0.34±0.01	0.73±0.01	0.71±0.01	0.72±0.01	0.48±0.01	0.39±0.01	0.73±0.01
	Docility	0.87±0.01	0.50±0.01	0.54±0.01	0.72±0.01	0.22±0.01	0.31±0.01	0.32±0.01	0.73±0.01	0.65±0.01	0.62±0.01	0.42±0.01	0.48±0.01	0.69±0.01
	Alertness	-0.59±0.01	-0.85±0.01	-0.70±0.01	-0.71±0.01	-0.54±0.01	0.20±0.01	0.12±0.01	0.24±0.01	0.17±0.01	0.62±0.01	0.39±0.01	0.45±0.01	0.29±0.01
Cognition cluster	Dependence	0.92±0.01	0.80±0.01	0.87±0.01	0.97±0.01	0.89±0.01	0.63±0.01	0.21±0.01	0.47±0.01	0.47±0.01	0.37±0.01	0.43±0.01	0.35±0.01	0.43±0.01
	Trainability	0.84±0.01	0.82±0.01	0.63±0.01	0.83±0.01	0.77±0.01	-0.35±0.01	0.93±0.01	0.20±0.01	0.65±0.01	0.65±0.01	0.46±0.01	0.38±0.01	0.70±0.01
	Cooperation	0.89±0.01	0.67±0.01	0.64±0.01	0.71±0.01	0.59±0.01	-0.46±0.01	0.94±0.01	0.86±0.01	0.10±0.01	0.64±0.01	0.45±0.01	0.39±0.01	0.72±0.01
	Emotional stability	0.92±0.01	0.87±0.01	0.65±0.01	0.76±0.01	0.61±0.01	0.61±0.01	0.97±0.01	0.88±0.01	0.67±0.01	0.18±0.01	0.46±0.01	0.48±0.01	0.63±0.01
	Perseverance	0.62±0.01	-0.18±0.01	0.54±0.01	0.50±0.01	0.66±0.01	0.50±0.01	0.86±0.01	0.80±0.01	0.66±0.01	0.61±0.01	0.10±0.01	0.40±0.01	0.54±0.01
	Get In/Out of Stables	0.49±0.01	-0.50±0.01	-0.42±0.01	0.66±0.01	-0.29±0.01	0.07±0.01	0.94±0.01	0.11±0.01	0.86±0.01	-0.20±0.01	0.71±0.01	0.38±0.01	0.29±0.01
	Ease at Handling	0.85±0.01	0.49±0.01	0.58±0.01	0.59±0.01	0.82±0.01	-0.64±0.01	0.94±0.01	0.78±0.01	0.77±0.01	0.63±0.01	0.80±0.01	-0.64±0.01	0.10±0.01

Figure 1. Operant conditioning behavioral test to assess for the thirteen cognitive processes in the study.

Time per stage/treatment presentation: 75 seconds per stage/treatment presentation. The application of the reinforcement treatments that Handler A, Handler B or both implemented to lead the donkey across the oilcloth lasted for the whole 75 seconds. These treatments were applied to check the response of the animals to the different types of reinforcement. No additional time was supplied for the donkeys to complete the stages, so that, once the 75 seconds, provided to the donkeys to interact with the elements presented, had expired, the following stage started and the next treatment was implemented.

Test duration: 450 seconds.

Test stages: 1 to 6. Each stage corresponded to the implementation of each of the six reinforcement treatments.

Previous considerations:

- The oilcloth was the element (obstacle) that the donkeys were led to cross over. No donkey had been in contact with the oilcloth previous to the test. Handlers A and B, used 6 reinforcement treatments to lead the donkeys cross over such obstacle.
- The donkeys were accustomed to the area in which the test took place as it was an open area on which the donkeys used to carry out their daily activities.
- The donkeys that were taking the test were not present while the oilcloth was being laid on the floor for the first time. The donkeys were assessed one at a time, so no additional donkey was present while the test was taking place.
- The test started when Handler B raised the oilcloth and relayed it again on the floor in front of the donkey being tested. This action only took place 1 minute before stage 1 (before the 1st treatment was implemented) and was not repeated further in the test. Cameraman started controlling time after the oilcloth had been relayed, when Handler A gave the first step forward towards the oilcloth.
- Frontal and visual elements fell within the visual scope of the donkeys, while we considered rear elements those that fell into a blind area (Weaver, 2008). Acoustic elements could be frontal or rear and emitted sounds.
- Reinforcement treatments comprised different elements. Known elements were those which had already been presented to the donkeys at any point in their lives (relying on owner's information), while unknown elements were those to which, according to the owner, the donkeys were not acquainted.
- All the reinforcement treatments were implemented sequentially and consecutively from stage 1 to 6, one after another, without any stop between each of them, whether the donkey had completed each stage (crossed the obstacle) completely or not (avoided it). That is to say, the fact that an animal crossed/avoided the oilcloth completely in one of the treatments from 1 to 6, did not prevent the rest of treatments from being implemented.

Legend

Donkey being tested

2x2 m oilcloth with a wooden print

Rope leader/Handler A

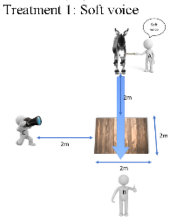
Handler B/Lurer (in Stage 4)/2nd Rope leader (in Stage 5)/Clapper (in Stage 6)

Cameraman (C)/Time controller

Treat (bread, carrots, feed or sugar lumps). Carried by Handler B.

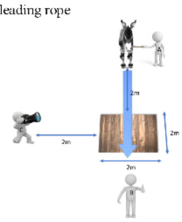
Motivator. Plastic bag attached to a wooden stick. Carried by Handler B.

STAGE 1 (S1)
Treatment 1: Soft voice



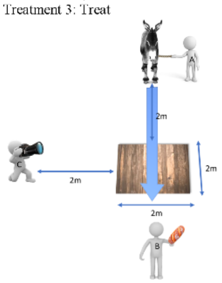
- Oilcloth presented to the donkey for the first time *(Frontal unknown element).*
- The donkey is given 75 seconds to complete Stage 1, that is to cross over the oilcloth.
- Using a lead rope and soft voice, Handler A tried to comfort the donkey to make it cross the oilcloth on the floor, but without pulling from the rope if the donkey refused to move *(Neutral reinforcement).*

STAGE 2 (S2)
Treatment 2: Pressure to leading rope



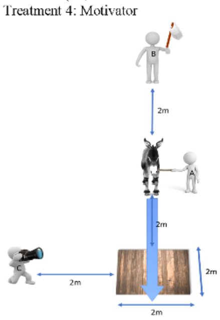
- Donkey had already had contact with the oilcloth in Stage 1 *(Frontal known element).*
- Using a lead rope with applied pressure to make the donkey cross over the oilcloth. Handler A released the pressure when the donkey moved to cross the oilcloth *(Negative reinforcement).*

STAGE 3 (S3)
Treatment 3: Treat



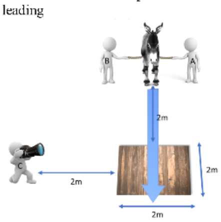
- Donkey had already had contact with the oilcloth in Stage 1 and 2 and was familiar to the treat given *(Frontal known elements).*
- Handler B offered a familiar treat to lead the donkey to cross over the oilcloth (the treat offered depended on the owner's tastes and therefore the animals were familiar to it. Handler B used the treat that the owner of each donkey normally offered them to tease them. All animals did not accept any other treat that had not been offered to them by their owners previous to the test, as the field experiences reported) *(Positive reinforcement/Luring)*

STAGE 4 (S4)
Treatment 4: Motivator



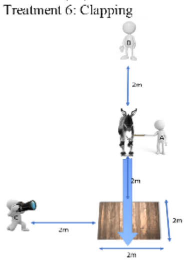
- Donkey had already had contact with the oilcloth in Stage 1, 2 and 3 *(Frontal known element and rear unknown element).*
- Handler A applied pressure to the lead rope at the same time Handler B made a noise from behind the donkey with a so-called "donkey motivator" (plastic bag tied on the end of a stick. The donkey was led by slightly pulling the rope until it crossed the oilcloth completely *(Negative reinforcement).*

STAGE 5 (S5)
Treatment 5: Double rope leading



- Donkey had already had contact with the oilcloth in Stage 1, 2, 3 and 4 *(Frontal known element).*
- Using two lead ropes attached on either side of the halter, Handlers A and B encouraged the donkey across, releasing the pressure when the donkey moved and then reapplied when it stopped until it crossed the oilcloth completely *(Negative reinforcement).*

STAGE 6 (S6)
Treatment 6: Clapping



- Donkey had already had contact with the oilcloth in Stage 1, 2, 3, 4 and 5 *(Frontal and rear known elements).*
- Handler B clapped his hands from behind the donkey to make it move forward (Nansen and Blache, 2016). Handler A applied pressure on the lead rope and while the donkey was led across by the auditory sound of the claps, pressure and sound were released or stopped when the donkey moved and reapplied when it stopped until the donkey had completed the task *(Negative reinforcement).*

Figure 2. Donkey sample and human population IQ distribution graphic, R squared, and Polynomial Regression equation (2nd order).

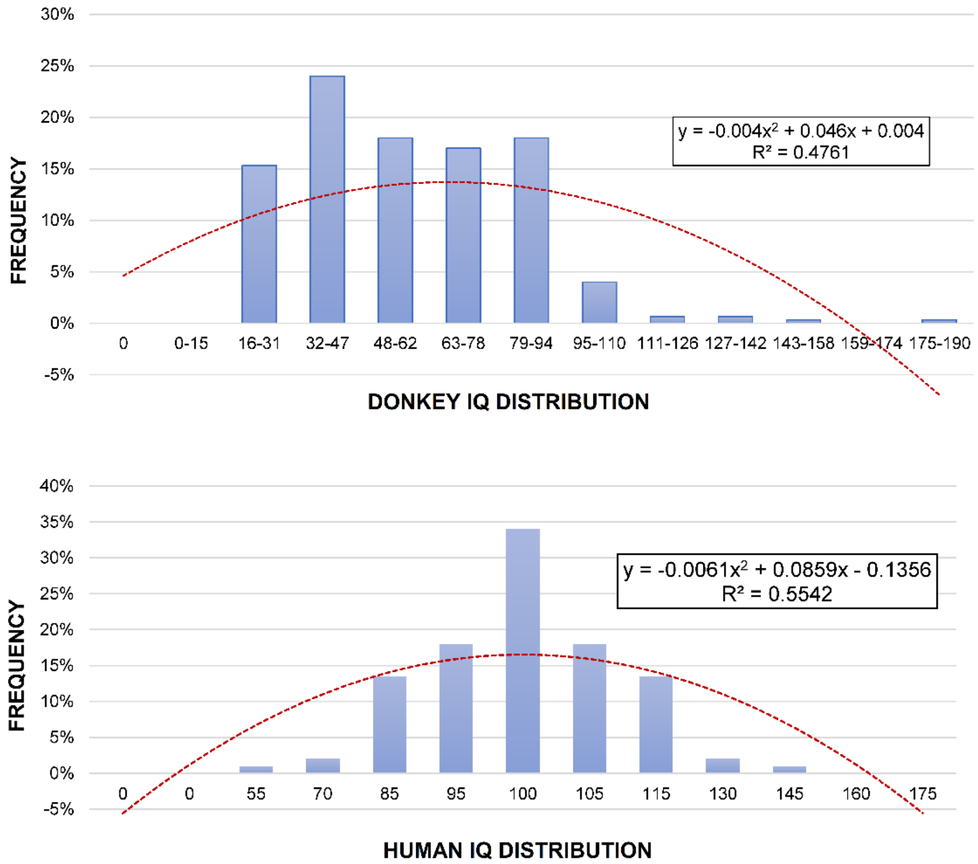
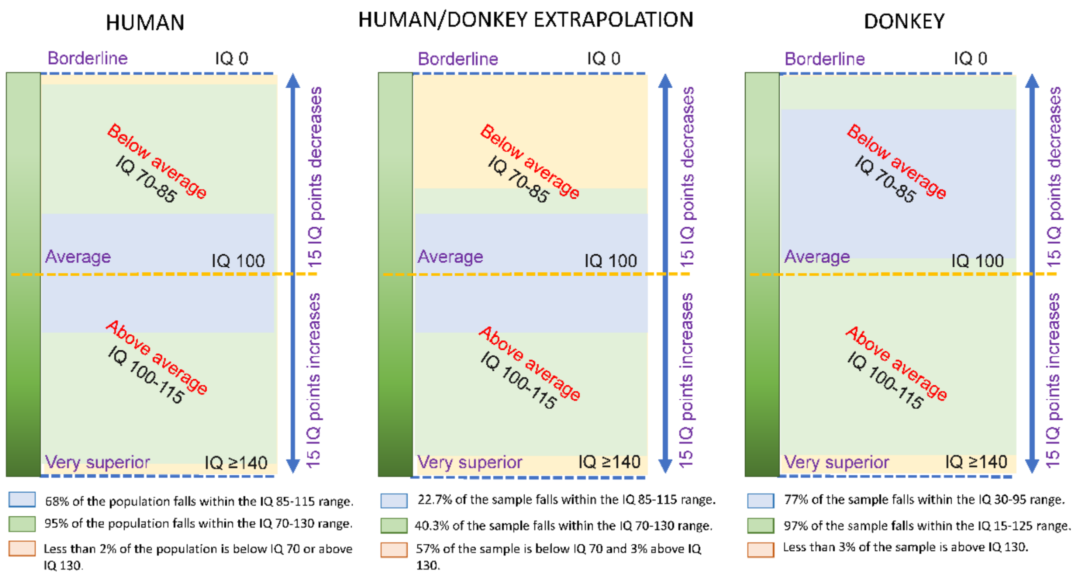


Figure 3. Distribution of human and donkey's IQ and Human-donkey IQ extrapolation, frequency representation and scale description.



Supplementary Table S1. Description of the thirteen intelligence and cognition traits and the definition of their scales studied in donkeys.

Intelligence Cluster			
Trait	Definition	Scale	Description
Concentration	The animal collaborates during the assessment session and does not get distracted by the environment.	1	Distracted
		2	Poor
		3	Inconstant
		4	Intermediate
		5	Concentrated
Curiosity	The animal is interested in the novel stimuli being presented and moves towards them.	1	Never (0%)
		2	Rarely (5-10%)
		3	Sometimes (50%)
		4	Frequently (70%)
		5	Always (100%)
Memory	The animal remembers the stimuli being presented.	1	Scattered
		2	Poor short-term memory
		3	Average short-term memory
		4	Average long-term memory
		5	Good long-term memory
Stubbornness	The donkey rejects following the requests of the assessor.	1	Stubborn (Cautious)
		2	Indifferent
		3	Moaner
		4	Reluctant
		5	Obedient
Docility	The donkey easily follows the orders of the instructor.	1	Stubborn
		2	Indifferent
		3	Moaner
		4	Reluctant
		5	Obedient
Alertness	The animal shows a vigilant or alert status focusing on the stimulus around.	1	Untamed
		2	Unwilling
		3	Reticent
		4	Adaptable
		5	Docile
Cognition Cluster			
Dependence	The donkey is comfortable when separated from the main herd	1	Dependant
		2	Restless
		3	Stable
		4	Adapted
		5	Calm
Trainability	Ability of the animal to be trained into the fulfilment of the tests	1	Never (0%)
		2	Rarely (5-10%)
		3	Sometimes (50%)
		4	Frequently (70%)
		5	Always (100%)
Cooperation	The donkey cooperates with its handlers during the daily tasks	1	Never (0%)
		2	Rarely (5-10%)
		3	Sometimes (50%)
		4	Frequently (70%)
		5	Always (100%)
Emotional stability	The animal is not predictable form one to another stimulus	1	Unpredictable
		2	Surprising
		3	Stable
		4	Balanced
		5	Predictable
Perseverance	The animal is patient when completing several sequential tests.	1	Impatient
		2	Generally impatient but easily handled
		3	Patient but pushes the operator occasionally
		4	Patient without pushing the operator
		5	Awaits the operator's orders
Get In/Out of Stables	The animal shows no problem when leaving or entering its housing facilities.	1	Never (0%)
		2	Rarely (5-10%)
		3	Sometimes (50%)
		4	Frequently (70%)
		5	Always (100%)
Ease at Handling	The animal shows sympathy towards humans.	1	Mistrustful towards humans in general
		2	Mistrustful towards unknow people
		3	Comfortable with familiar people, but mistrustful to unknown people
		4	Comfortable with the human presence
		5	Increased sympathy for human presence

Supplementary Table S3. Descriptive statistics for the estimates of Predicted Breeding Values (PBV) for intelligence and cognition behavioural traits sorted by sex in Andalusian donkeys.

Jacks (N=272)									
Cluster	Cognitive process	Minimun	Maximun	Mean	SEM	SD	Variance	Kurtosis	Standard error
Intelligence cognitive process related traits	Concentration	-1.25	0.43	-0.03	0.01	0.22	0.05	6.30	0.29
	Curiosity	-0.61	0.29	-0.03	0.01	0.12	0.02	3.06	0.29
	Memory	-0.28	0.08	-0.01	0.00	0.04	0.00	10.16	0.29
	Stubbornness	-0.58	0.25	-0.02	0.01	0.11	0.01	5.26	0.29
	Docility	-0.37	0.17	-0.01	0.01	0.08	0.01	2.09	0.29
	Alertness	-0.42	0.15	-0.01	0.00	0.08	0.01	4.61	0.29
General cognitive processes related traits	Dependence	-0.78	0.41	-0.02	0.01	0.18	0.03	3.28	0.29
	Trainability	-0.71	0.33	-0.04	0.01	0.15	0.02	3.67	0.29
	Cooperation	-0.39	0.21	-0.01	0.01	0.08	0.01	3.62	0.29
	Emotional stability	-0.63	0.32	-0.02	0.01	0.11	0.01	7.47	0.29
	Perseverance	-0.36	0.16	-0.01	0.00	0.07	0.00	4.63	0.29
	Get In/Out of Stables	-1.60	0.46	-0.03	0.02	0.28	0.08	5.46	0.29
	Ease at Handling	-0.47	0.16	-0.03	0.00	0.08	0.01	6.07	0.29
Jennies (745)									
Cluster	Cognitive process	Minimun	Maximun	Mean	SEM	SD	Variance	Kurtosis	Standard error
Intelligence cognitive process related traits	Concentration	-1.25	0.50	-0.01	0.01	0.16	0.03	7.51	0.18
	Curiosity	-0.58	0.32	-0.01	0.00	0.09	0.01	5.66	0.18
	Memory	-0.24	0.11	0.00	0.00	0.03	0.00	11.75	0.18
	Stubbornness	-0.50	0.34	-0.01	0.00	0.08	0.01	6.17	0.18
	Docility	-0.32	0.24	-0.01	0.00	0.06	0.00	3.67	0.18
	Alertness	-0.59	0.18	-0.01	0.00	0.08	0.01	10.90	0.18
General cognitive processes related traits	Dependence	-0.78	0.44	-0.01	0.00	0.13	0.02	6.89	0.18
	Trainability	-0.77	0.39	-0.02	0.00	0.12	0.02	5.20	0.18
	Cooperation	-0.27	0.18	0.00	0.00	0.06	0.00	4.02	0.18
	Emotional stability	-0.59	0.37	-0.01	0.00	0.09	0.01	7.50	0.18
	Perseverance	-0.33	0.16	0.00	0.00	0.05	0.00	9.18	0.18
	Get In/Out of Stables	-1.07	0.48	-0.01	0.01	0.18	0.03	8.34	0.18
	Ease at Handling	-0.46	0.19	-0.01	0.00	0.06	0.00	8.90	0.18

Supplementary Table S4. Summary of the dispersion measures ("accuracies") of the PBV, through Bayesian methods for the thirteen cognitive processes studied in Andalusian donkeys.

Cluster	Cognitive process	SD	Mean	Variance	SEM
Intelligence	Concentration	6.348	0.086	40.298	0.026
	Curiosity	6.511	0.087	42.390	0.027
	Memory	5.145	-0.120	26.474	0.017
	Stubborness	8.328	0.111	69.359	0.045
	Docility	6.455	0.091	41.668	0.027
	Alertness	3.925	0.060	15.403	0.010
Cognition	Dependence	4.774	-0.109	22.794	0.015
	Trainability	5.049	-0.140	25.494	0.016
	Cooperation	7.085	0.101	50.194	0.032
	Emotional stability	6.170	0.082	38.066	0.024
	Perseverance	5.633	0.084	31.736	0.020
	Get In/Out of Stables	4.843	0.067	23.457	0.015
	Ease at Handling	7.451	0.097	55.515	0.036

Supplementary Table S5. Descriptive statistics summary for IQ related parameters in Andalusian donkeys for the thirteen cognitive processes studied.

Item	Mean	SEM	SD	Kurtosis
Chronological age (in months)	84.10	3.55	61.46	-0.57
Mental age (in months)	39.17	1.66	28.71	1.90
IQ (%)	63.91	3.31	57.34	66.08
Concentration score	3.80	0.06	1.03	0.62
Mental age for concentration (in months)	20.89	1.95	33.72	7.31
Dependence score	4.33	0.06	1.09	2.09
Mental age for dependence (in months)	21.30	1.42	24.51	40.41
Trainability score	3.80	0.06	1.04	-0.06
Mental age for trainability (in months)	31.08	2.19	37.87	7.74
Curiosity score	4.10	0.05	0.93	1.47
Mental age for curiosity (in months)	54.75	3.13	54.28	0.71
Memory score	4.11	0.06	1.04	0.81
Mental age for memory (in months)	19.95	1.69	29.35	9.28
Cooperation score	4.13	0.06	1.08	0.17
Mental age for cooperation (in months)	36.31	2.38	41.17	5.29
Emotional stability score	3.78	0.06	0.98	0.28
Mental age for emotional stability (in months)	62.11	3.20	55.41	0.81
Stubbornness score	3.67	0.07	1.17	0.06
Mental age for stubbornness (in months)	62.69	3.27	56.62	0.73
Docility score	3.99	0.05	0.94	-0.50
Mental age for docility (in months)	53.75	2.92	50.56	1.80
Alertness score	4.74	0.03	0.57	9.52
Mental age for alertness (in months)	23.50	2.89	50.10	6.18
Perseverance score	4.64	0.04	0.76	6.52
Mental age for perseverance (in months)	17.03	2.09	36.28	7.38
Get In/Out of Stables score	4.58	0.05	0.79	4.22
Mental age for get in/out of stables (in months)	83.83	3.55	61.56	-0.57
Ease at handling score	4.03	0.07	1.12	0.18
Mental age for ease at handling (in months)	22.01	1.72	29.73	7.00

Chapter 7

Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M..; McLean, A.K.; Pizarro Inostroza, G.; Delgado Bermejo, J.V.

Genetic parameter estimation and implementation of the genetic evaluation for gaits in a breeding program for assisted-therapy in donkeys

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ORIGINAL ARTICLE

Genetic parameter estimation and implementation of the genetic evaluation for gaits in a breeding program for assisted-therapy in donkeys

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Abstract

Genetic analyses in donkeys are likely to face compromises in terms of sample size and population structure. This study aims at implementing a suitable model to estimate breeding values and genetic parameters for gaits in Andalusian donkeys. Empirical observation revealed that ambling donkeys (showing a slightly uneven, non-isochronous 1–2, 3–4 lateral sequence gait) did not walk (i.e. presented an isochronous, even 1-2-3-4 sequence gait) and vice versa. However, the two donkey groups could trot, equally. In this study, 2700 gait records were registered from 300 donkeys. The sample included 1350 gait records from 169 ambling/trotting donkeys and 1350 gait records from 131 walking/trotting donkeys. Fixed effects included year, season, sex, farm/owner, husbandry system, weather, ground type and appraisers. Weight and age were included as covariates. MTDFREML software was used to estimate (co)variance components, genetic parameters and predict breeding values and their accuracies in both sets, separately. Gaits' heritability ± SE estimates were 0.56 ± 0.155, 0.53 ± 0.317 and 0.67 ± 0.166 for amble, walk and trot, respectively. Genetic correlations were 0.31 ± 0.216, 0.42 ± 0.115 and 0.28 ± 0.178, for amble and walk, amble and trot and walk and trot, respectively. Not all gaits are suitable to treat every human sensomotor condition. We developed a locomotion selection index, assessing the relative loss/gain in index accuracy when each gait modality was excluded to develop different gait specific therapeutic lines to genetically select the best performing donkeys from each gait modality. Our results suggest that gait genetic lines could be developed and may be potential selection criteria to consider in assisted-therapy donkey breeding programs.

Keywords Donkey · Restricted maximum likelihood · Genetic parameters · Amble · Walk · Trot

Introduction

The smooth riding characteristics of donkeys were already reported in text fragments by Al-Maqrīzī dating back to the thirteenth Century. He would report the custom of riding on donkeys was widespread among Egyptian notables (Alkhateeb-Shehada 2008). The Andalusian donkey breed is believed to be closely related to or even the direct descendent of the 'White' Egyptian donkey breed, also known as Hassawi riding donkeys (Porter et al. 2016). Unfortunately, functional traits in donkeys have been overlooked over time.

The technological improvement in agricultural machinery and the modernisation of transport facilities and networks ended relegating the role of these valuable animals to an afunctional secondary place within society. Donkeys can perform all the gaits that other affine species such as the horse develop. However, these gaits should be considered

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analogous variations as donkeys are conditioned by their anatomical and physiological characteristics (Navas et al. 2016).

These facts together with the close bonds that they form with humans, the application of each gait modality in the treatment of specific human conditions and their kinetic versatility are key advantages when setting the base for their sustainable functional future. Genetic analyses for gaits and functional skills have long and deep been studied in horses by several authors such as Vicente et al. (2014b). However, genetic analyses in donkeys are likely to face compromises in terms of sample size and population structure.

The objective of this study was to estimate (co)variance components and genetic parameters, and to predict breeding values and their accuracies for amble, walk and trot gait modalities in donkeys using MTDFREML software. Then, we computed different possible combinations of these gait modalities in selection indexes to find the best fitting selection methods when the breeding goal was locomotion, aiming at developing different therapeutic kinetic lines, considering the gait modalities for which every donkey assessed may be better suited.

Materials and methods

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 guide for the care and use of laboratory and farm animals in research. The Spanish Ministry of Economy and Competitivity through the Royal Decree Law 53/2013 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.

Study sample and study background

We studied a sample of 300 stud-book registered Andalusian donkeys (78 jacks and 222 jennies). Empirical observation revealed that ambling donkeys (showing a slightly uneven, non-isochronous 1–2, 3–4 lateral sequence gait) did not walk (i.e. presented an isochronous, even 1-2-3-4 sequence gait) and vice versa. However, the two donkey groups could trot, equally (Table 1). For this reason, two different kinds of donkeys were studied, ambling/trotting donkeys and walking/trotting donkeys. Ambling/trotting donkeys were those that could amble and trot, but could not walk, while walking/trotting donkeys were those that could walk and trot, but could not amble, respectively. The sample included 169 ambling/trotting donkeys (52 jacks and 117 jennies) and 131 walking/trotting donkeys (26 jacks and 104 jennies). The mean age of ambling/trotting donkeys was 97.63 ± 61.43 months while the mean age of walking/trotting donkeys was 73.39 ± 61.43 months (Fig. 1). Empirical visual observation and video recordings highlighted the fact that 100% ambling jacks produced ambling offspring in all cases, while the offspring of ambling jennies and walking jacks and jennies could either amble or walk, equally (Table 1). Parentage tests for each offspring had previously been performed with microsatellite molecular markers to ensure the reliability of the information in the pedigree as a way to counteract the small size of the sample tested. 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. The pedigree of the donkeys in the sample was traced back six generations providing indirect information from 724 connected ancestors (71% of the historical population registered) and accounting for an average inbreeding of 1%.

Table 1 Summary of the frequencies for slow gaits found in the Andalusian donkey sample

Item	N = 300
Animals with unknown sire	117
Animals with unknown dam	116
Animal with both unknown parents	111
Ambling males	26
Walking males	52
Ambling females	105
Walking females	117
Ambling offspring from ambling dam	24
Ambling offspring from ambling sire	32
Walking offspring from ambling sire	0
Walking offspring from ambling dam	11
Ambling offspring from walking sire	17
Ambling offspring from walking dam	25
Walking offspring from walking sire	40
Walking offspring from walking dam	54

Accessed from Navas and Delgado (2016)

Record description and scales

Animals belonged to 22 different farms located in Andalusia (southern Spain). The donkeys were recorded on four randomly chosen days from June to November per

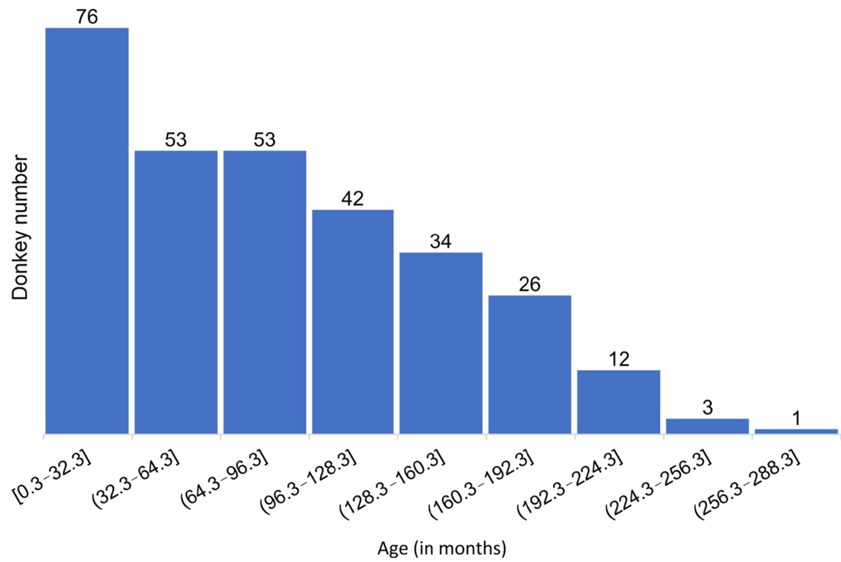

Fig. 1 Age distribution of the sample of Andalusian donkeys (N = 300)

year from 2013 to 2015. The 2700 records included direct information on the performance of 300 donkeys when developing two gait modalities, slow-moving gaits (walk or amble), and a fast-moving gait (trot). By slow gaits we refer to the movement patterns that the donkeys use to move without exerting an extra effort aimed at increasing their speed. All donkeys were scored by three trained appraisers. Another appraiser simultaneously videotaped (1080 p, 50 Hz, shutter speed: 1/250 s) the experiences to assess the donkey's performance after the field experiences. The donkeys were led on a neck collar and lead rope, while the 3 trained appraisers watched them in a straight line from the side. Each donkey was assessed according to a 1 to 5 linear scale. A score number of 1 was assigned to gaits that lacked uniformity (likely meaning lacked balance) and cadence or harmony and were poorly developed, as the limbs involved did not move in synchrony. Animals scoring a 5 moved at a harmonic, rhythmic and smooth pace and their body reflected such synchrony. On the one hand, the field experiences revealed that donkeys that ambled did not walk and vice versa. On the other hand, no donkey reported the intermedium scores of the scale (2 or 4) for slow gaits (i.e. their amble or walk score was either 1, 3 or 5). Based on these two findings, we decided to reduce the scale into a 0 to 3 scale to fit the variation found in the population sample. When donkeys were assessed for ambling, a score of 0 was given to those donkeys presenting a walking gait, as they were unable to amble. In the same way, when donkeys were assessed for walking, a score of 0 was given to those who ambled, as ambling donkeys did not walk. Then a score of 1 was assigned to donkeys whose slow gaits lacked uniformity and cadence or harmony and were poorly developed, as the limbs involved did not move in synchrony. By contrast, animals scoring a 3 moved at a harmonic, rhythmic and smooth pace and their body reflected such synchrony.

Statistical analysis

First, a Shapiro–Wilk test was applied to check the fitness of the variables in the model to a normal distribution. Second, as the elements in the model did not fit to a normal distribution ($P < 0.001$), a Kruskal–Wallis H was performed in order to study the potentially existing differences between levels of the same factor. Then a Spearman's rho test was used to compute the correlations between factors affecting locomotion traits. One-way ANOVA and a posthoc Tukey test were performed using the Compare Means procedure from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016) to compute the fraction of the variance explained by each factor separately. R^2 and Reduced R^2 were computed for the whole model using the General Linear Model (GLM) procedure from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016). ε^2 and ω^2 were computed to assess the partial size of the variance explained by the items in the model for each trait because of the small sample size using $\varepsilon^2 = \frac{SS_b - df_b MS_w}{SS_t}$ and $\omega^2 = \frac{SS_b - df_b MS_w}{SS_t + MS_w}$, respectively. ε^2 and ω^2 use unbiased measures of the variance components and report the least mean root square errors in cases in which there is a small sample size (Okada 2013).

Genetic model, phenotypic and genetic parameters

Each gait was scored once in the lifetime of the individual, but independently by three appraisers. Therefore, the statistical model used in the analysis of gaits was a bivariate animal model with multiple observations. The fixed effects comprising the mixed model consisted of the year (2013, 2014 and 2015); season (summer/spring and autumn/winter); the farm (22 farms/owners); husbandry system (intensive, semi-intensive, semi-extensive, Official Morphological Contest and extensive; see Table 2); weather (sunny or cloudy); ground type (concrete or soil) and the appraiser (3 judges). Body weight was estimated as Delgado et al. (2014) and included as a linear covariate. The age of the animals expressed in months was included as a linear and quadratic covariate. In matrix notation, the mixed bivariate model used was:

$$\begin{aligned}\text{Yijklmnopqrs} = \mu &+ \text{Yeai} + \text{Seaj} + \text{Sexk} + \text{Farl} + \text{Sysm} \\ &+ \text{Wean} + \text{Groo} + \text{Aprp} + \text{b1} + \text{b2Aq} \\ &+ \text{b3Aq} + \text{Animalr} + \text{eijklmnopqr}\end{aligned}$$

where Yijklmnopqr is the separate score of slow gaits (amble or walk) and trot traits for a given donkey; μ is the overall mean; Yeai is the fixed effect of the ith year of assessment (i = 2013, 2014, 2015); Seaj is the fixed effect of the jth season of evaluation (j = summer/spring, autumn/winter); Sexk is the fixed effect of the kth sex (k = jackstock, jenny); Farl is the fixed effect of the lth farm/owner (l = 1–22); Sysm is the fixed effect of the mth husbandry system (m = intensive, semi-intensive, semi-extensive, contest, extensive); Wean is the fixed effect of the nth weather (n = sunny, cloudy); Groo is the fixed effect of the oth ground type (o = concrete, soil); Aprp is the fixed effect of the pth judge (p = judge 1, judge 2, judge 3); b1 is the linear regression coefficient on the body weight of the donkeys, b2Aq and b3Aq are the linear and quadratic regression coefficients on age when the tests took place (Aq), Animalr is the random additive genetic effect of the rth donkey, and eijklmnopqr is the random residual effect.

Genetic analyses

The objective of the first stage of our study was to obtain estimates of genetic parameters and breeding values for gait modalities in Andalusian donkeys. To this aim, we carried out univariate and bivariate analyses and mixed model procedures using an Animal Model (BLUP) by Restricted Maximum Likelihood, with the MTDFREML software package (Boldman et al. 1995), iterating until a convergence criterion of 10^{-12} was obtained. Univariate analyses were carried out to compute heritabilities, while bivariate analyses were used to estimate correlations. The analyses were run including the relationship matrix of animals with direct records related through at least one known ancestor. This matrix comprised the 1017 donkeys in the historical pedigree. After convergence was reached, we directly estimated predicted breeding values, their accuracies and reliabilities, and the standard errors of genetic correlations using the MTDFREML software.

Index selection

Aiming at selecting the best performing, best balanced or more harmonic donkeys for each gait modality, we assessed all the possible combinations of the three gait modalities through standard selection index procedures as suggested by Van Vleck (1993). We based on the estimated phenotypic relationship between each of the three gait modalities to quantify their weight when the breeding goal was locomotion. We assumed three records were available per animal. In matrix notation, the weights to be applied on the selection index combining the partial scores of each modality were obtained as $b = P^{-1}g$, where b is the vector of weights to be applied to each gait modality, P is the phenotypic (co) variance matrix, and g is the vector of genetic (co)variances of every gait modality with each other. MatLab r2015a (Inc. 2015) was used to compute all selection index combinations. After solving for b, the variance of the selection index was obtained as, $\sigma_{\mathrm{i}}^2 = b'Pb$. Aiming at the development of gait specific therapeutic genetic lines, we computed the repercussion that removing each of the gaits may have on the accuracy of selection for the i$^{\mathrm{th}}$ gait modality trait. Selection index accuracy was estimated as $r_{API} = \sqrt{\frac{\sigma_{\mathrm{I}}^2}{\sigma_{\mathrm{Ai}}^2}}$, where r_{API} is the accuracy obtained from direct selection for the ith gait modality trait and σ_{Ai}^2 is the corresponding additive genetic variance. We assessed the relative weight given to each of the three gait modalities included in the complete selection index by constructing a reduced selection index where each of those gait modalities was removed. Then, we calculated the reduction or gain occurring in weighted average accuracy comparing to the complete selection index comprising the three gait modalities (Cameron 1997). With this, we aimed to develop different gait specific therapeutic lines to genetically select the best performing donkeys according to the gait modality for which they may be better suited, as they may indirectly be better suited to treat certain sensomotor conditions.

Results

Statistical analysis

A summary of the descriptive statistics for the slow gait (amble and walk) and fast gait modality (trot) traits and the fixed effects and covariates comprising the model is shown in Table 3. Shapiro–Wilk Test and the deviation kurtosis values ranging from − 1.86 to 0.92 on all the fixed effects showed they significantly (P < 0.001) did not fit to a normal distribution. The variability observed for the two traits analysed was from moderate to high, with

Table 2 Description of the levels included in the husbandry system fixed effect

Husbandry system	Live in reduced space facilities	Live in wider extension territories	Minimum punctual handling (sanitary inspection and stud book inclusion)	Daily human contact and regular handling	Donkey is familiar with the owners' requests	Unknown conditions
Intensive	X			X	X	
Semi-intensive		X		X	X	
Semi-extensive		X			X	
Contest					X	X
Extensive		X	X			

Table 3 Descriptive statistics for fixed effects and covariates for kinetic traits in Andalusian donkeys (N = 900)

Factor type	Factor	Minimum	Maximum	Mean	SEM	SD	Variance	Skewness[a]	Kurtosis[a]	CV (%)
Fixed effect	Year	1.00	3.00	1.97	0.02	0.65	0.43	0.03	−0.65	0.33
	Season	1.00	2.00	1.59	0.02	0.49	0.24	−0.38	−1.86	0.31
	Sex	1.00	2.00	1.74	0.02	0.44	0.19	−1.10	−0.80	0.25
	Farm/Owner	1.00	22.00	7.34	0.19	5.54	30.65	1.16	0.92	0.75
	System	1.00	5.00	2.58	0.03	0.97	0.94	0.48	0.37	0.38
	Weather	1.00	2.00	1.27	0.02	0.45	0.20	1.02	−0.96	0.35
	Ground	1.00	2.00	1.72	0.02	0.45	0.20	−1.00	−1.00	0.26
	Judge	1.00	3.00	2.00	0.03	0.82	0.67	0.00	−1.50	0.41
Covariate	Weight	71.75	501.19	267.32	3.06	91.88	8442.33	0.11	−0.01	0.34
	Age (in months)	0.27	270.40	84.08	2.05	61.43	3773.67	0.51	−0.57	0.73
Kinetic traits	Amble	0.00	3.00	1.55	0.05	1.41	1.98	−0.11	−1.87	0.91
	Walk	0.00	3.00	1.10	0.04	1.31	1.70	0.47	−1.59	1.19
	Trot	1.00	5.00	4.62	0.02	0.61	0.37	−1.79	4.59	0.13

[a]Standard error for Skewness statistic was 0.082 and standard error for Kurtosis statistic was 0.163 for all factors assessed

a coefficient of variation of 25.23% for the sex effect and 75.42% for the farm/owner effect. R^2 and Reduced R^2 were 0.521 and 0.502; 0.489 and 0.469; and 0.271 and 0.242, for amble, walk and trot, respectively. ε^2 and ω^2 ranged from 0, for the appraiser effect for the three gaits, to 0.937, 0.941 and 0.960 for the effect of age for the amble, walk and trot gaits, respectively.

Genetic model, phenotypic and genetic parameters

The estimates for heritability, genetic, phenotypic and environmental variance obtained through REML methods are shown in Table 4. The genetic (r_G) and phenotypic correlation (r_P) estimated were positive and moderate to high (Table 5).

Selection index

The accuracy of selection was 0.7701, 0.7295 and 0.8638 for amble, walk and trot, respectively (Tables 6 and 7). When we assessed the index weights per genetic standard deviation

Table 4 Estimated components of variance, heritability (h^2) and standard error (SE) for walk, amble and trot obtained from multivariate analyses through REML methods in Andalusian donkeys

Modality	Trait	σ_a^2	σ_e^2	σ_p^2	$h^2 \pm SE$
Slow gaits	Amble	0.3819	0.2952	0.6771	0.56 ± 0.155
	Walk	1.0789	0.9572	2.0360	0.53 ± 0.317
Fast gaits	Trot	0.4139	0.2348	0.6163	0.67 ± 0.166

Table 5 Estimated heritabilities (h^2) (diagonal), phenotypic (r_P) (above diagonal) and genetic (r_G) (below diagonal) correlations for slow gaits (walk/amble) and trot obtained in bivariate analyses through REML methods in Andalusian donkey

Modality	Traits	Amble	Walk	Trot
Slow gaits	Amble	0.56 ± 0.155[a]	0.42 ± 0.332[b]	0.90 ± 0.100[b]
	Walk	0.31 ± 0.216[c]	0.53 ± 0.317[a]	0.53 ± 0.318[b]
Fast gaits	Trot	0.42 ± 0.115[c]	0.28 ± 0.178[c]	0.67 ± 0.166[a]

[a]$h^2 \pm SE$
[b]$r_P \pm SE$
[c]$r_G \pm SE$

Table 6 Summary of the selection index parameters and partial accuracy of selection (r_{APi}) for slow gaits (walk/amble) and trot in Andalusian donkeys, and percentage of relative loss/gain in accuracy of the index selection if each trait were removed from the index when the selection goal is locomotion

Item	Direct selection goal: locomotion		
	Slow gait modalities		Fast gait modality
	Amble	Walk	Trot
Vector of selection index weight/selection index (b)	0.6706	0.5445	0.8641
Variance of the selection index $\left(\sigma_I^2\right)$	0.2265	0.5741	0.3088
Partial accuracy of selection (r_{APi})	0.7701	0.7295	0.8637
Vector of standardized index weights[a]	1.7556	0.5047	2.0877
Relative loss/gain in selection accuracy when excluded (%)	1.6925[b]	3.0204[c]	7.1971[b]

[a]Index weight standardized per additive genetic standard deviation unit

[b]Relative loss in selection accuracy

[c]Relative gain in selection accuracy

Table 7 Summary of the reduced selection indexes where each of the gait modality traits is removed, and reduction observed in weighted average accuracy relative to the optimum index

Item	Amble	Walk	Trot	Weighted average accuracy (r_{APi})
Selection Index (b)	0.6706	0.5445	0.8641	79.8367
r_{APi}	0.7701	0.7295	0.8638	
Selection Index (b)		0.5439	0.7094	78.4855
r_{APi}	Excluded	0.7295	0.8273	
Selection Index (b)	0.6687		0.8483	82.2481
r_{APi}	0.7701	Excluded	0.8638	
Selection Index (b)	0.5776	0.5385		74.0908
r_{APi}	0.7524	0.7286	Excluded	

unit, the results were positive and strong. Table 6 shows a summary of the parameters related to the index weights per unit of genetic standard deviation of the three modalities, as well as the relative loss/gain in accuracy of selection index if each modality were individually removed from the index to assess the relative partial weight of each modality. Using the estimates obtained from REML analyses we computed a weighted average accuracy of 78.49 when selecting for locomotion including the three gait modalities. The low potential loss in accuracy resulting from excluding amble or trot from the selection index indicates that both modalities are traits to retain when selection is for locomotion, with a relative loss in accuracy of 1.6925 and 7.1971 respectively. In the same way, the potential gain in accuracy when excluding the walk modality from the direct selection goals implied an increase in the accuracy of selection of 3.0204 (Tables 6 and 7).

Predicted or estimated breeding values and prediction accuracy

The results for the estimates of predicted breeding values (PBV) ranged between −2.505 and 2.469 for the amble gait modality, -1.840 and 2.835 for the walk gait modality, -3.160 and 0.934 for the fast gait modality (trot). The accuracy (rTi) ranged from 0 to 0.940 and the reliability (R_{AP}) ranged from 0 to 0.884 for all gait modalities. The standard error of prediction ranged from 0.210 to 1.120 for all gait modalities. A summary of the descriptive statistics of predicted breeding values (PBV), standard error of prediction (SEP), accuracy (rTi) and reliability (RAP) for the slow gait (amble and walk) and fast gait (trot) modalities sorted by sex is shown in Table 8.

Discussion

Even though donkeys describe almost the same gaits as other equids, they present slight variations to adapt to their anatomic and physiological characteristics (Navas et al. 2016). Mutations in DMRT3 affect locomotion as it appears to configure the spinal circuits controlling gait patterns in vertebrates. Andersson et al. (2012) addressed the effect of the DMRT3 mutation on the diversification of the horse, as the altered gait characteristics of a number of breeds apparently require this mutation to occur.

The amble or stepping pace is faster and smoother than the walk or single-foot, but not as energetically efficient. In donkeys, ambling is a lateral gait as the feet on the same side of the donkey move forward, but one after the other, usually following a footfall pattern of right rear, right front,

Table 8 Descriptive statistics of predicted breeding values, standard error of prediction (SEP), accuracy (rTi) and reliability (R_{AP}) for the slow gait (amble and walk) and fast gait (trot) modalities sorted by sex

Sex	Modality	Gait	Parameter	Minimum	Maximum	Mean	SEM	SD	Skewness[a]	Kurtosis[a]
Jacks (N = 272)	Slow gait	Amble	PBV	−2.505	2.170	0.010	0.659	−0.630	2.032	−2.505
			SEP	0.240	0.630	0.496	0.119	−0.615	−1.207	0.240
			rTi	0	0.920	0.480	0.307	−0.207	−1.217	0
			R_{AP}	0	0.846	0.324	0.287	0.448	−1.355	0
		Walk	PBV	−1.733	2.835	0.062	0.659	1.086	2.935	−1.733
			SEP	0.430	1.060	0.845	0.187	−0.608	−1.216	0.430
			rTi	0	0.910	0.469	0.301	−0.206	−1.218	0
			R_{AP}	0	0.828	0.310	0.275	0.451	−1.348	0
	Fast gait	Trot	PBV	−1.410	0.804	−0.034	0.309	−0.984	3.544	−1.410
			SEP	0.210	0.650	0.499	0.142	−0.659	−1.171	0.210
			rTi	0	0.940	0.501	0.320	−0.203	−1.217	0
			R_{AP}	0	0.884	0.353	0.313	0.451	−1.352	0
Jennies (N = 745)	Slow gaits	Amble	PBV	−2.008	2.469	0.019	0.532	−0.004	4.125	−2.008
			SEP	0.270	0.660	0.515	0.134	−0.794	−1.167	0.270
			rTi	0	0.900	0.352	0.375	0.365	−1.604	0
			R_{AP}	0	0.810	0.264	0.323	0.702	−1.294	0
		Walk	PBV	−1.840	2.458	0.016	0.514	0.820	4.556	−1.840
			SEP	0.490	1.120	0.877	0.208	−0.798	−1.149	0.490
			rTi	0	0.890	0.344	0.366	0.367	−1.599	0
			R_{AP}	0	0.792	0.251	0.308	0.709	−1.273	0
	Fast gait	Trot	PBV	−3.160	0.934	−0.016	0.304	−2.366	20.069	−3.160
			SEP	0.210	0.690	0.517	0.161	−0.829	−1.111	0.210
			rTi	0	0.940	0.369	0.393	0.366	−1.603	0
			R_{AP}	0	0.884	0.290	0.355	0.703	−1.290	0

[a]Standard error for Skewness statistic was 0.148 and 0.090 and standard error for Kurtosis statistic was 0.294 and 0.179 for jacks and jennies, respectively for all factors assessed

left rear, left front. A common trait of the ambling gaits is that usually only one foot is completely off the ground at any one time. Ambling can turn into a 2-beat gait at higher speeds (for instance, the trot). Among faster gaits, trot was assessed while canter or gallop were discarded, as they are not normally described under regular domestic conditions in donkeys.

Studies on the genetic background behind special gaits (Andersson et al. 2012) enable the genetic quantification for their incidence in previously phenotypically characterized species like donkeys (Navas and Delgado 2016). Scoring gaits using continuous linear scales between two extremes, results in much better distribution properties, translating into a better selection accuracy as reported in horses for similar locomotion traits (Rustin et al. 2009).

Specific studies in horses (Rustin et al. 2009) have reported the importance of factors such as the appraiser, age, location (date and place of appraisal), environment and handling on kinetic patterns (Vicente et al. 2014b). Many of such effects are gathered in the five levels comprising the husbandry system fixed effect described in Table 2. Parallelly, Navas and Delgado (2016) reported the existence of a moderate sexual dimorphism for slow gait (amble or walk) with a 13% higher percentage of jacks presenting ambling gaits than jennies. Differences were also detected depending on the season of qualification as shown in horses (Suontama et al. 2013). The sharp change between seasons in the area in which the study took place made the four regular seasons turn into two seasonal categories, a hot (summer/spring) and a cold season (autumn/winter).

Kruskal–Wallis H tests reported the combination of fixed effects to be highly statistically significant ($P < 0.001$), except for ground type for all gait modalities, weather for walk and trot gait modalities, and sex for trot gait modality which were very significant ($P < 0.01$). Sex and farm/owner effects were significant ($P < 0.05$) for amble gait traits. The effect of the appraiser was not significant ($P > 0.05$) for all gait modalities. The bivariate correlations found almost all fixed effects and covariates were highly statistically significant ($P < 0.001$) for all gait modalities.

The inexistent effect of the three appraisers reflects the fact that they were thoroughly trained to score each animal using the same methods simultaneously, and thus, it did not affect the percentage of unexplained variance by additive effects (only statistically non-significant effect for all three gait modalities). This made homogenizing the classification criteria easier and indirectly indicated that the criteria followed to select the staff in charge of the valuations were strict enough as to provide quality appraisers.

A wide range of gait heritability values can be found in literature for horses (Ducro et al. 2007). Our results are around the mean value for the same parameters reported for horses. Slightly higher values were reported for Swedish Warmblood horses, 0.75 and 0.77 (Gerber Olsson et al. 2000), and slightly to moderately lower values (0.25–0.39) were reported for Finnhorse and Standardbred foals by Schroderus and Ojala (2010) and (0.18–0.27) for Hispano-Árabe horses (Gómez et al. 2016) for movement traits (walk and trot, respectively).

Our similar results denote that the application of Andalusian donkeys for mule production may have resulted in an indirect selection for the reproduction of animals whose locomotive characteristics were better for the performance of the hybrid offspring. Extreme values occasionally shown for horses may rely on the influence of different training procedures on the performance of the animals. The moderate genetic correlations found between the slow gaits (amble and walk) and the fast gait (trot) may base on the existing pleiotropy affecting the ability of donkeys to perform different gaits as suggested by some authors (Andersson et al. 2012). The phenotypic correlation obtained by our analyses was from slightly to moderately higher resembling those results obtained by other authors (Ducro et al. 2007; Vicente et al. 2014a). The lack of antagonism between gaits at a genetic and phenotypic level, enables the inclusion of the three traits in a combined selection index (Tables 5, 6 and 7).

Like therapy horses (Uchiyama et al. 2011), walking donkeys may promote sensomotor inputs similar to those produced by human walking being recommended to treat ambulatory difficulties, while ambling donkeys smoothly sustain rhythm for relatively longer periods of time, good for treating severe motor disabilities. With the breeding goal of locomotion in mind, we followed standard selection index procedures to configure therapeutic lines based on selecting either ambling/trotting or walking/trotting animals that may better suited for treating certain human condition better than others. For example, if we want to obtain donkeys better suited for severe sensomotor disabilities, that is outstandingly performing at ambling rather than at walking (better suited for ambulatory difficulties), we would assign ambling a higher weighting. This weighting value is then multiplied by the observed value in each individual animal and then the score for each of the characteristics is summed for each individual. This result is the index score and can be used to compare the worth of each donkey being selected. Therefore, only those with the highest index score are selected for breeding via artificial selection. With these methods, we select for traits simultaneously rather than sequentially. Thereby, no useful traits are being excluded from selection at any one time and so none will stagnate or reverse while you concentrate on improving another property of the donkey. This becomes of especial relevance in endangered species, as instead of discarding the animals we select them for different purposes.

Weightings assigned to each trait are inherently quite hard to calculate precisely and so require some elements of trial and error before they become optimal to the breeder. Thus, we computed how removing each of the gait modalities from the selection index would affect index accuracy to ensure selection for ambling/trotting donkeys instead of walking/trotting donkeys and vice versa, did not affect selection practices (Tables 6 and 7).

In spite of its demographic bottlenecks, the Andalusian donkey still maintains considerable levels of genetic variability for gait traits (Navas et al. 2017). Given the favourable existing genetic relationships between the traits involved, gaits can play an important role in a selection program aimed at improving the suitability of donkeys for new functional niches. 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 gaits 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). Defining breeding objectives is the key element of any breeding program (Van Vleck 1993), and the need to include functional traits in the breeding goals while maintaining selection for morphological and phaneroptical characteristics is of prominent importance in highly standardized donkey breeds. 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 form occurring. The incorporation of genetic markers in the functional selection of donkeys for locomotion is a still a developing possibility. Nonetheless, harmonically gaited donkeys selection becomes a worth considering selection criteria as the balanced movement of the donkeys may result in an improvement on the abilities of disabled patients (Voznesenskiy et al. 2016). Hence, the remarkable 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.

Conclusions

High levels for genetic parameters resembling the ones obtained for horses in literature were obtained for the different gait modalities described by donkeys. Such values enable the potential inclusion of locomotion traits within breeding programs seeking the genetic progress of donkey breeds, such as the Andalusian donkey. The statistically non-significant effect of the appraisers suggested the success of a highly uniform scoring procedure among appraisers. Genetic correlations were high and positive for all trait combinations, thus enabling the combined selection for both gaits, with low detrimental effect for either one. Selection for certain gaits in donkeys may have traditionally been carried out indirectly, thus the routine application of the assessment including a greater number of animals is required to standardize the valuation methodology implemented in this study, a difficult task to achieve, considering the existing extinction risk of donkey breed endangered populations. In addition, given the favourable genetic relationships existing between the traits involved, gaits can play an important role in a selection program aimed at improving the suitability of donkeys for the treatment of specific motorial disabilities within assisted therapy programs. However, the specific nature and magnitude of the existing genetic relationships of the functional traits assessed in this study may make interesting to consider the possibility of developing and maintaining specialized lines relying on the ability of the donkeys to develop certain gait patterns (amble or walk and trot) within the Andalusian donkey breeding program, as these different patterns may be especially suitable for the treatment of different human motor disabilities.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Chapter 8

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Modeling for the Inheritance of Endangered Equid Multiple Births and Fertility: Determining Risk Factors and Genetic Parameters in Donkeys *(Equus asinus)*

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

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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 fertilized 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 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. These estimates enable the potential selection against/for these traits, offering a new perspective for donkey breeding and conservation.

Keywords: Donkey; twinning; heritability; Gibbs sampling; risk factors.

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% ended from 3 months gestation to term. In the remaining cases, either one (21%) or both twins (14.5 %.) were born alive or survived after birth complications. However, the foals are usually born stunted or emaciated, which does not allow them to survive further from 2 weeks 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.

Furthermore, 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 12 months 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 standardized reproduction techniques in horses and other equids (Hearn and Summers, 1986) such as artificial insemination with frozen semen (mainly attributed to the high immune response in the endometrium of jennies, which are more likely to get acute endometritis postinsemination with frozen semen than mares, due to anatomic, histologic and physiologic differences of the reproductive tracts of both species) and embryo transfer still represent a challenge in donkeys (Miró and Papas, 2017; Rota et al., 2017; Saragusty et al., 2017).

Under this context, embryo vitrification and freezing 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 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 are anecdotal as fertility, in general, has a very low heritability. These studies are even more limited when we focus on studying equids such as horses (Bresińska et al., 2004; Wolc et al., 2006) or donkeys, for which no study has been reported.

The present paper describes a retrospective study over a period of 38 years (the birth year of the oldest animal registered in the studbook was 1980) that aimed to investigate the frequency of multiple pregnancies in the historical population of Andalusian donkeys and the influence that non-genetic factors such as farm, husbandry system, location, year of birth, birth season, birth month or age. Second, we estimated the genetic parameters of fertility and multiple births through the analysis of cumulative foal number born per animal, maximum foal number per birth and multiple birth number per animal using Gibbs sampling. Last, we predicted breeding values for all the traits as a way to assess the potential implementation of a bidirectional breeding strategy. 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.

Materials and methods

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 ($P \leq 0.01$ Shapiro-Francia W' test for normality), we used minimum, Q1, median, Q3 and maximum to describe the age range in our sample. Minimum age in the range was six months, Q1 age was six years, the median age was ten years, Q3 age was 14 years, and the maximum age was 29 years. 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 recognized as apt for reproduction and included in the main section of studbook of the breed what takes place when the animals turn 3 years 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 in the pedigree of the breed are routinely genetically tested through microsatellite genotyping and parentage tests for the resulting offspring of each mating. Parentage tests for each offspring had previously been performed with microsatellite molecular markers to ensure the reliability of the information in the pedigree as a way to counteract the small size of the sample tested. 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.

Birth-related traits

First, we studied multiple birth occurrence assessing three different traits for each jack or jenny. To obtain this information, we contrasted the registries of the pedigree file with interviews with the 145 owners whose animals participated in the study. We decided to interview the owners 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. Second, 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 summarized 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 lifetime 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 the births occurring in the 91% of Andalusian donkey population and their characteristics. For genetic analyses, the unit of analysis that we considered was the lifetime parentship record of each animal separately to avoid the possibly occurring unmodeled 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.

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 stud-book 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 10 minutes. 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, semiextensive, semiintensive 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.

Records description and scales

We organized 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 stud book 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.

Previous statistical analysis (screening)

The average number of foals born per year was 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.

A Shapiro-Francia W' test was applied to the data to check the fitness degree of the variables in the model to a normal distribution. Second, the high statistical significance of all the elements in the model ($P<0.001$), revealed that the data significantly deviated from a normal distribution (Figure 1). Kurtosis values supported these results (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 1). 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.

Simultaneously, 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.

If we test multiple comparisons (hypotheses), the likelihood of incorrectly rejecting a null hypothesis increases, that is rejecting the existence statistically of significant differences between two or more groups (for instance, making a Type I error). Bonferroni corrections for multiple comparisons were used.

Once we test for the differences in the distribution of the levels for each category, an independent-sample median test was carried out to assess the differences in the median between levels within the same factor.

After conducting a Kruskal-Wallis H with three or more groups (k), we computed the strength effect of the factors on the variables tested. 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 research described and reported by (Li et al., 2019).

Partial eta-squared (ηp^2), defined as the ratio of variance associated with an effect, plus that effect and its associated error variance, 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 1). 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 1). 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).

Categorical regression (CATREG) was used to describe how the variables in our study depended on the factors considered (Tables 2 and 3).

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).

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 (Equation 1). As results for adjusted R-squared for non-normal data may be misleading, Akaike's Information Criterion and Bayesian Information Criterion 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). The formula for the Bayesian information criterion (BIC) is similar to the formula for AIC, but with a different penalty for the number of parameters. With AIC the penalty is 2k, whereas with BIC the penalty is ln(n) k. In regression contexts (Yong, 2005), such as the one in our study, AIC is asymptotically optimal for selecting the model with the least mean squared error and the rate to which it converges is the optimum, under the assumption that the true model is not in the candidate set, while BIC is not asymptotically optimal under the assumption. 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 less than 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 greater than 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:

$$Y_{ijklmop}=\mu+a_{ij}+ Sea_k+Sex_l+Far_m+Sys_n+ Loc_o+b1A_q+b2A^2_q+e_{ijklmnop} \qquad (1)$$

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; a*ij* 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 *l*th sex (*l*=jack, jenny); Far_m is the fixed effect of the *m*th farm/owner (*m*=1-92); Sys_n is the fixed effect of the *n*th husbandry system (*n*=intensive, semi-intensive, semi-extensive, extensive); Loc_o is the fixed effect of the *o*th 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:

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

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:

$$\begin{bmatrix} X'X & X'Z \\ Z'X & Z'Z + A^{-1}k \end{bmatrix} \begin{bmatrix} \beta \\ \alpha \end{bmatrix} = \begin{bmatrix} X'y \\ Z'y \end{bmatrix} \quad (3)$$

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.

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.

Results

Pedigree knowledge

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). Navas et al. (2017) 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 2.98 years older than breeding jennies on average. The average age of parents when their offspring was born was 8.08 years (8.03 for jennies and 8.16 for jacks). The average generation interval was 7.40 years (Navas et al., 2017).

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).

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.

Statistical analyses

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 1. 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 1). 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 1).

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=46363.500, P<0.001 and U=50364.000, 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= 47730.000, 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, what 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). Determining the quantitative values for the variable categories, alternating least squares (als) iterative prediction method is used. The value determination after optimal scaling can be saved as a new variable set. With the results from CATREG, it is still required to verify the statistical significance of the predictors. Consequently, CATREG is equivalent to an standard linear regression when the qualitative predictors are substituted by the transformed (quantified) values (Çilan and Can, 2014).

CATREG bases on an optimal scaling method for both linear and nonlinear transformation of variables in regression analysis. Optimal scaling transformations were carried out as described by (Van der Kooij and Meulman, 2007). According to these authors the original 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 (toward zero) of the estimates of standard errors and thus, F-tests and P-values that is likely to occur. CATREG optimal linear regression analysis involves minimizing the sum of squared differences between a response (dependent) variable and a weighted combination of predictor (independent) variables. Variables are typically quantitative, with (nominal) categorical data recoded to binary or contrast variables. As a result, categorical variables serve to separate groups of cases, and the technique estimates separate sets of parameters for each group. The estimated coefficients reflect how changes in the predictors affect the response. Prediction of the response is possible for any combination of predictor values. We present the summary results with the significant variables in Tables 2 and 3. The standardized coefficients (β) are listed in Table 3. 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.

There was a small to moderate monotonic (whether linear or not) significant ($P<0.05$) correlation between age and the three variables tested (Table 1). 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) standardized coefficients are shown in Table 3. CATREG (β) standardized 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, there was not any statistical difference between the values of the dependent variables for each of the twelve levels of birth month and thirty-two levels of the birth year, respectively, thus it was not included in the CATREG analysis. Partial eta values ranged from 0.117 to 0.146 reporting a moderate association between 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 ($P<0.05$). Thus, there was a statistical difference between the values of that dependent variable for each of the four levels of birth season. 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 standardized coefficient for the birth season and multiple birth number per animal was non-significant. However, CATREG standardized 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 the values of the dependent variables for each of the two levels of sex. 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 standardized 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 standardized 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 standardized coefficient for owner/farm were all highly statistically significant (P<0.001). CATREG standardized 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), thus there were highly significant statistical differences between the values of the dependent variables for each of the 11 levels of the location factor. 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 standardized 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 standardized 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, thus there were highly significant statistical differences between the values of that dependent variable for each of the four levels of the husbandry system factor. 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 standardized 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 standardized coefficients (β) in Table 4. Since we used the stepwise method, there was no multicollinearity problem. The standardized solution for the regression equations can be found in Table 4 as well.

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 2).

Genetic model, variance components, genetic and phenotypic correlations, predicted Breeding Values and prediction accuracy (distribution and correlation).

We show the estimates for heritability, genetic and phenotypic variance estimated with Gibbs sampling in Table 5. Table 6 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 7.

Covariate and 'fixed' effects posterior means

We show 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.

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 literature, ranging from 5.3% to almost 70% (Quaresma, 2015) so that our results fall within the range reported for other donkey breeds.

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 captive mammalian 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 would report 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 2) 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. However, still there seem 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.

Using Gibbs sampling methods, as we consider the relationship among the individuals present in the pedigree, 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 3), 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 5). The heritability estimates reported in our study overcome those reported in literature for the highest margin of heritabilities for stallion fertility varying from 0.03–0.15 for foaling rate per breeding season (Giesecke et al., 2010). 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.

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).

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 having 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 standardized 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 favourable 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 7). 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 form 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.

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 enable 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 specialized 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.

Conflict of interest statement

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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Table 1. *Summary of the results for the Kruskal-Wallis H test and their partial eta-squared coefficients (ηp²) 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	Multiple birth number per animal for a given donkey
Year of birth	χ^2	41.548	30.787	22.313
	df	31	31	31
	p-value	0.098	0.477	0.873
	Levels	1984-2017	1984-2017	1984-2017
	Mean rank	345.15-404.00	345.15-404.00	345.15-404.00
	ηp²	0.234	0.184	0.177
Month of birth	χ^2	16.085	15.128	7.729
	df	11	11	11
	p-value	0.138	0.177	0.737
	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	January, February, March, April, May, June, July, August, September, October, November, December
	Mean rank	345.22-424.28	350.00-405.01	321.01-425.48
	ηp²	0.146	0.134	0.117
Season of birth	χ^2	7.750	7.201	4.014
	df	3	3	3
	p-value	0.050	0.066	0.260
	Levels	Winter / Spring / Summer / Autumn	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	382.89 / 387.63 / 360.80 / 408.18
	ηp²	0.099	0.093	0.102
Sex	χ^2	12.348	3.676	5.630
	df	1	1	1
	p-value	0.001	0.050	0.018
	Levels	Jack / Jenny	Jack / Jenny	Jack / Jenny
	Mean rank	418.85 / 371.89	396.39 / 378.85	412.39 / 373.89
	ηp²	0.124	0.074	0.227
Farm/Owner	χ^2	302.220	321.748	151.075
	df	91	91	91
	p-value	<0.001	<0.001	<0.001
	Levels	1-92	1-92	1-92
	Mean rank	162.00-732.00	350.00-744.75	241.50-709.50
	ηp²	0.626	0.558	0.330
Husbandry system	χ^2	24.169	5.027	0.249
	df	3	3	3
	p-value	<0.001	0.170	0.969
	Levels	Intensive / Semiintensive / Semiextensive / Extensive	Intensive / Semiintensive / Semiextensive / Extensive	Intensive / Semiintensive / Semiextensive / Extensive
	Mean rank	370.390 / 385.340 / 397.550 / 317.960	406.390 / 388.060 / 385.490 / 363.450	402.820 / 385.680 / 381.130 / 385.740
	ηp²	0.176	0.076	0.033
Location	χ^2	67.358	42.013	31.193
	df	10	10	10
	p-value	<0.001	<0.001	<0.001
	Levels	1-11	1-11	1-11
	Mean rank	222.75-620.50	350.00-613.17	241.50-513.62
	ηp²	0.291	0.229	0.113
Age (in years)	Spearman's rho	-0.137	0.085	0.339
	p-value	<0.001	0.019	<0.001

Table 2. *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

Table 3. *Standardized 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	Standardized Coefficients (β)	Significance	Standardized Coefficients (β)	Significance	Standardized 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

Table 4. *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
$Z'Y_{maxmulhis}$=βfarm*Zfarm+βlocation*Zlocation+βbirthseason*Zbirthseason + βsex*Zsex + βage*Zage		$Z'y_{maxmulhis}$= Z score for each variable (maximum foal number per birth, multiple birth number per animal for a given donkey and cumulative foal number born per donkey). β=standardized coefficient for 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 for maximum foal number per birth. $\beta_{Farm}Z_{Farm}=0.592(Z_{Farm})$ $\beta_{Birthseason}Z_{Birthseason}=0.086(Z_{Birthseason})$ $\beta_{Location}Z_{Location}=0.246(Z_{Husbandrysystem})$ $\beta_{Age}Z_{Age}=0.163(Z_{Age})$
Multiple birth number per animal for a given donkey	$Z'y_{mul}$=0.921(ZFarm) + 0.307(ZLocation)	$Z'y_{mul}$= Z score for multiple birth number per animal for a given donkey. $\beta_{Farm}Z_{Farm}=0.921(Z_{Farm})$ $\beta_{Location}Z_{Location}=0.307(Z_{Location})$
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 cumulative foal number born per donkey. $\beta_{Farm}Z_{Farm}=0.478(Z_{Farm})$ $\beta_{Sex}Z_{Sex}=0.435(Z_{Sex})$ $\beta_{Birthseason}Z_{Birthseason}=0.098(Z_{Birthseason})$ $\beta_{Location}Z_{Location}=0.159 (Z_{Location})$ $\beta_{Age}Z_{Age}=0.207(Z_{Age})$

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

Table 5. *Estimated components of variance, heritability (h²) 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 6. *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.*

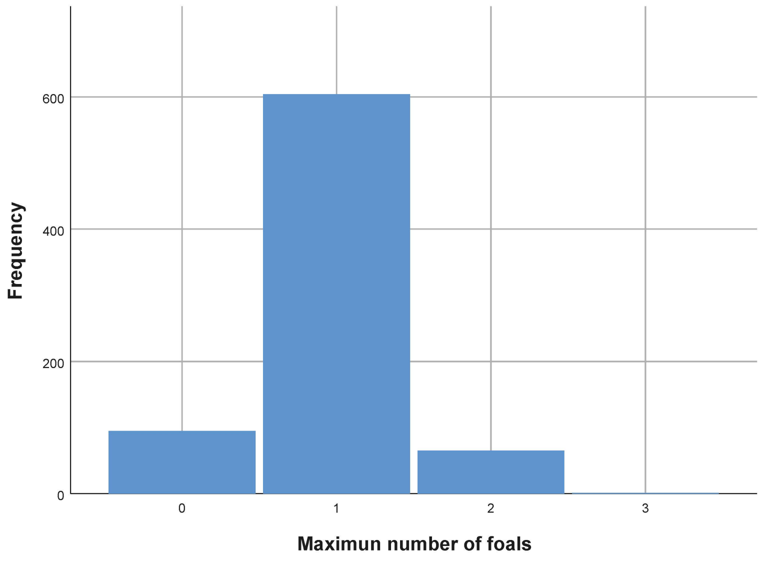
Traits	Maximum foal number per birth	Multiple birth number per animal for a given donkey	Cumulative foal number born per donkey
Maximum foal number per birth	-	0.607±0.054	0.206±0.063
Multiple birth number per animal for a given donkey	0.846±0.152	-	0.530± 0.045
Cumulative foal number born per donkey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

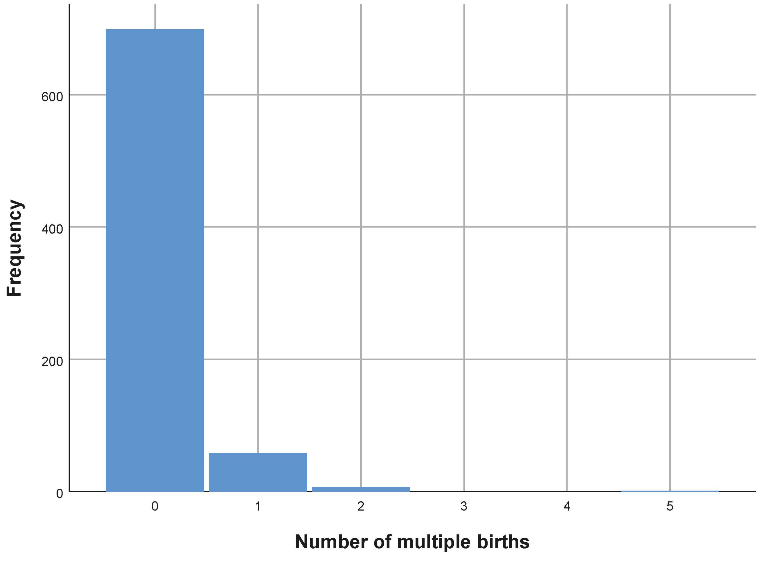
Table 7. *Descriptive statistics of predicted breeding values (PBVs) for maximum foal number per birth, multiple birth number per animal for a given donkey and cumulative foal number born per donkey for all the donkeys included in the pedigree sorted by model and sex.*

Sex	Trait	Mean	SEM	95% Confidence Interval for Mean	Std. Deviation	Median	Minimum	Maximum	Skewness	Kurtosis
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

Figure captions

Figure 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.


Frequency
600
400
200
0
0
1
2
3
Maximun number of foals


Frequency
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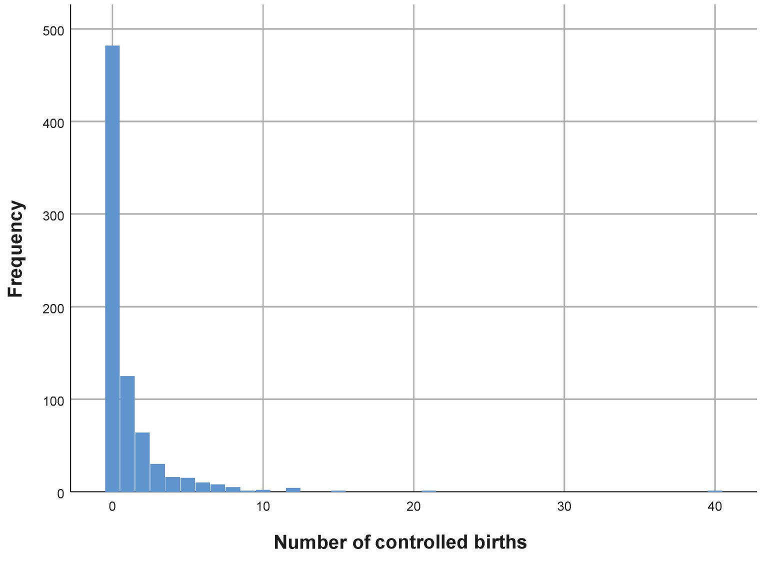
400
200
0
0
1
2
3
4
5
Number of multiple births


Frequency
500
400
300
200
100
0
0
10
20
30
40
Number of controlled births

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

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Supplementary Table S1. Questions asked to the owner interviewed during the survey carried out regarding the fertility and prevalence of multiple births in donkeys.

Question	Question type/Answer
Block 1. Questions aimed at characterizing the farms	
1. How many animals are there in your farm?	Open question, depended on the farm.
2. How many animals have been born at your farm?	Open question, depended on the farm. We confirmed the foaling record per each animal registered in the studbook.
3. Where is your farm located?	Open question, depended on the farm.
4. What is the sex of the animals?	Male Female
5. What is the age of your animals?	Open question, depended on the animals.
6. What husbandry system would you consider could better describe your farm? (see Table 2, for descriptions on the husbandry system categories)	Intensive Semi-intensive Semi-extensive Extensive
7. Has any of the jennies in your farm given birth to multiple foals: twins, triplets, quadruplets, etc.?	Yes No
8. Did the offspring resulting survive?	Yes. 7.1. How long? No
9. Has any of the jennies in your farm suffered miscarriage or abortion? Did that abortion or miscarriage involve two or more embryos?	Yes No
10. Has any of the matings involving one of your jacks ended in a jenny giving birth to multiple foals?	Yes No
11. Do you sell semen from your jacks?	Yes No
Block 2. Excluding question. Only the owners, who affirmatively responded to the question in this block, were considered for the estimation of genetic parameters (92 out of 145 owners interviewed).	
12. Was the theriogenologist or veterinarian requested for diagnosis and was an official diagnose issued?	Yes No
Block 3. Prevention and care practices.	
13. Did the jennies presenting multiple gestations carried two or more embryos in more than one occasion? How many times? How many embryos were implanted and how many of them survived?	Yes No
14. Does the occurrence of multiple births prevent you from using a jenny or jack for breeding?	Yes No
15. Have you requested the actions of a veterinarian for the treatment of these conditions or to interrupt multiple gestations?	Yes No
16. Do you apply any treatment or preventive measure against multiple pregnancies?	Yes No
17. Have you ever used a traditional treatment or preventive measure? Was it effective?	Yes No
18. Is there any factor that you have regarded to potentially influence multiple gestation at your farms? Which one/s?	Yes No

The same questions were performed for each owner regarding each animal that has historically been under their care.

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

Husbandry system	Reduced space facilities	Access to wider extension territories	Handled just for minimum punctual sanitary inspection and stud book inclusion	Regular reproductive care provided to your donkeys	Daily human contact and handling by the owner
Intensive	X			X	X
Semiintensive		X		X	X
Semiextensive		X		X	
Extensive		X	X		

The information provided by the owners was later contrasted with the data provided by the Union of Andalusian Donkey Breeders (UGRA).

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

Descriptive statistics / Items	Mean	Std. Error	95% Confidence Interval for Mean		5% Trimmed Mean	Median	Variance	Std. Deviation	CV	Minimum	Maximum	Range	Interquartile Range	Skewness	Std. Error	Kurtosis	Std. Error
			Lower Bound	Upper Bound													
Birth month	5.060	0.111	4.840	5.270	4.910	5.000	9.501	3.082	0.609	1.000	12.000	11.000	4.000	0.570	0.088	-0.431	0.177
Birth year	22.160	0.227	21.710	22.600	22.540	23.000	39.313	6.270	0.283	1.000	32.000	31.000	8.000	-0.766	0.088	0.392	0.177
Birth season	2.180	0.035	2.110	2.250	2.140	2.000	0.943	0.971	0.445	1.000	4.000	3.000	2.000	0.426	0.088	-0.792	0.177
Sex	1.760	0.015	1.730	1.790	1.790	2.000	0.181	0.425	0.241	1.000	2.000	1.000	0.000	-1.242	0.088	-0.459	0.177
Owner/Farm	26.030	0.774	24.510	27.550	24.290	21.000	458.078	21.403	0.822	1.000	91.000	90.000	24.000	1.203	0.088	0.685	0.177
Location	4.600	0.105	4.390	4.810	4.560	4.000	8.513	2.918	0.643	1.000	11.000	10.000	7.000	0.078	0.088	-1.458	0.177
Husbandry system	2.940	0.023	2.900	2.990	2.960	3.000	0.392	0.626	0.213	1.000	4.000	3.000	0.000	-0.407	0.088	0.822	0.177
Herd*Year Interaction	234.650	4.546	225.730	243.580	235.790	239.000	15807.245	125.727	0.536	1.000	441.000	440.000	212.000	-0.094	0.088	-1.124	0.177
Age (in years)	10.762	0.197	10.375	11.148	10.568	10.463	29.675	5.447	0.506	0.518	29.362	28.844	7.927	0.425	0.088	-0.215	0.177
Maximum foal number per birth	0.960	0.017	0.93	1	0.96	1.000	0.213	0.462	0.481	0	3	3	0	-0.054	0.088	2.035	0.177
Multiple birth number per animal for a given donkey	0.100	0.013	0.07	0.13	0.04	0.000	0.135	0.368	3.680	0	5	5	0	5.469	0.088	47.932	0.177
Cumulative foal number born per donkey	1.030	0.089	0.86	1.21	0.66	0.000	6.098	2.469	2.397	0	40	40	1	7.214	0.088	89.613	0.177

TYPE OF ITEM	
	Fixed effects
	Interactions
	Covariates
	Traits/Variables

SKEWNESS	
	If skewness is less than −1 or greater than +1. the distribution is highly skewed.
	If skewness is between −1 and −½ or between +½ and +1. the distribution is moderately skewed.
	If skewness is between −½ and +½. the distribution is approximately symmetric.

KURTOSIS	
	A normal distribution has kurtosis exactly 3 (excess kurtosis exactly 0). Any distribution with kurtosis ≈3 (excess ≈0) is called mesokurtic.
	A distribution with kurtosis <3 (excess kurtosis <0) is called platykurtic. Compared to a normal distribution. its central peak is lower and broader. and its tails are shorter and thinner.
	A distribution with kurtosis >3 (excess kurtosis >0) is called leptokurtic. Compared to a normal distribution. its central peak is higher and sharper. and its tails are longer and fatter.

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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).

Birth year	N	Historical foal number born per donkey	Maximum foal number per birth	Multiple birth number per animal for a given donkey
1984	1	732	728.5	737.5
1985	2	564.75	539.25	393.25
1986	1	397.5	350	241.5
1987	6	336.75	413.08	307.83
1988	2	222.75	350	241.5
1989	7	445.29	404.07	460.86
1990	8	397.5	350	418.75
1991	6	453.25	413.08	521.67
1992	6	339.25	350	307.83
1993	3	281	350	342.67
1994	5	327.6	350	330.5
1995	7	247.71	350	385.07
1996	14	346.5	377.04	457.29
1997	20	396.75	368.93	385.48
1998	22	396.82	367.2	399.91
1999	23	410.74	399.37	379.17
2000	24	396.25	383.06	353.06
2001	23	365.8	382.91	328.89
2002	44	427.64	411.69	417.74
2003	51	348.35	380.32	399.45
2004	46	336.72	350	366.62
2005	45	403.6	392.06	375.49
2006	46	350.93	374.68	348.63
2007	39	414.27	379.12	394.24
2008	50	368.64	372.71	381.59
2009	59	390.31	383.18	359.02
2010	47	359.04	382.9	380.41
2011	33	385.09	396.86	395.26
2012	43	404.23	385.21	395.43
2013	27	383.44	378.04	402.07
2014	43	420.14	394.01	387.01
2015	12	395	413.08	390.83

Birth month	N	Historical foal number born per donkey	Maximum foal number per birth	Multiple birth number per animal for a given donkey
January	124	359.89	374.68	383.23
February	57	390.58	369.92	371.13
March	63	384.26	405.1	401.37
April	112	387.2	373.66	376.33
May	121	424.28	403.45	383.54
June	89	383.87	397.51	376.94
July	42	371.46	377.04	371.8
August	34	346.1	350	321.01
September	31	362.71	374.42	425.48
October	34	345.22	373.34	406.6
November	27	397.61	379.24	389.72
December	31	385.26	374.42	414.9

Birth season	N	Historical foal number born per donkey	Maximum foal number per birth	Multiple birth number per animal for a given donkey
Winter	608	371.85	373.36	384.61
Spring	450	401.73	392.53	384.61
Summer	352	372.93	382.51	364.11
Autumn	717	366.49	375.43	408.01

Farm	N	Historical foal number born per donkey	Maximum foal number per birth	Multiple birth number per animal for a given donkey
1	32	397.03	361.83	353.89
2	1	48	350	686.5
3	19	414.32	389.84	424.42
4	6	162	413.08	292.08
5	36	416.08	371.03	368.54
6	7	445.29	408.71	328.21
7	25	397.5	350	310.64
8	11	427.91	384.41	417.27
9	6	732	728.5	576.5
10	79	401.73	354.79	431.7
11	34	243.31	350	290.97
12	4	393.75	444.63	428.63
13	4	397.5	350	393.25
14	7	445.29	408.71	488
15	20	380.03	350	411.3
16	41	295.21	350	428.54
17	12	310.13	350	391.58

18	3	397.5	350	241.5
19	11	364.36	387.73	325.27
20	15	732	728.5	622.77
21	27	433.56	421.3	468.31
22	7	445.29	404.07	492
23	55	333.95	350	299.88
24	3	397.5	350	241.5
25	10	434.25	391.1	338.8
26	6	222.75	350	241.5
27	57	420.97	376.56	346.86
28	4	481.13	444.63	528.13
29	6	281	350	366.25
30	4	48	350	416.88
31	4	397.5	350	476.75
32	2	564.75	539.25	545
33	15	416.8	450.93	409.9
34	7	247.71	350	405.29
35	5	324.6	425.7	302.2
36	4	397.5	350	504.5
37	1	397.5	350	639.5
38	1	732	761	760.5
39	4	477.38	539.25	652.13
40	12	397.5	350	401.04
41	2	397.5	350	241.5
42	7	397.5	350	241.5
43	3	620.5	613.17	443.83
44	4	481.13	444.63	341
45	3	164.5	350	342.67
46	3	397.5	350	389.83
47	4	397.5	350	393.25
48	5	187.8	350	241.5
49	3	397.5	350	241.5
50	3	397.5	350	342.67
51	12	397.5	350	400.5
52	6	281	350	491
53	3	281	350	374.17
54	1	397.5	350	241.5
55	3	392.5	476.17	389.83
56	7	395.36	404.07	432.07
57	1	397.5	350	241.5
58	5	464.4	425.7	424.1
59	3	397.5	350	475.33
60	1	397.5	350	241.5
61	3	397.5	350	241.5
62	5	48	350	241.5

63	3	397.5	350	409.83
64	2	732	744.75	735.75
65	2	397.5	350	241.5
66	5	464.4	425.7	442.5
67	9	397.5	350	405.17
68	1	397.5	350	241.5
69	3	397.5	350	342.67
70	1	397.5	350	241.5
71	1	397.5	350	241.5
72	3	509	476.17	475.33
73	1	48	350	241.5
74	3	509	476.17	414.5
75	5	327.6	350	490.4
76	1	732	728.5	709.5
77	1	397.5	350	241.5
78	2	397.5	350	241.5
79	7	347.57	350	298.36
80	2	397.5	350	241.5
81	2	397.5	350	393.25
82	4	397.5	350	241.5
83	1	48	350	241.5
84	2	397.5	350	440.5
85	1	397.5	350	241.5
86	3	164.5	350	241.5
87	1	397.5	350	241.5
88	2	222.75	350	440.5
89	2	222.75	350	241.5
90	1	397.5	350	241.5

Husbandry system	N	Historical foal number born per donkey	Maximum foal number per birth	Multiple birth number per animal for a given donkey
Extensive	14	371.46	377.04	398.54
Semiintensive	131	372.23	382.53	373.08
Semiextensive	505	400.92	387.74	383.32
Intensive	115	317.96	363.45	391.02

Sex	N	Historical foal number born per donkey	Maximum foal number per birth	Multiple birth number per animal for a given donkey
Jack	14	418.85	396.75	411.3
Jenny	131	371.89	378.74	374.23

Location	N	Historical foal number born per donkey	Maximum foal number per birth	Multiple birth number per animal for a given donkey
1	223	402.07	413.66	377.16
2	6	499.5	509	476.17
3	56	297.43	247.45	356.76
4	148	401.8	373.81	411.63
5	13	513.62	394.04	437.35
6	60	376.93	402.58	368.93
7	6	241.5	397.5	350
8	237	365.97	377.65	371.45
9	2	241.5	222.75	350
10	11	447.41	458.32	421.77
11	3	443.83	620.5	613.17

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

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Research in Veterinary Science

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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.

With herd*birthyear interaction								Without herd*birthyear interaction							
Variable	Multiple R	R Square	Adjusted R Square	Apparent Prediction Error	Expected Prediction Error	Akaike's Information Criterion (AIC)	Bayesian Information Criterion (BIC)	Variable	Multiple R	R Square	Adjusted R Square	Apparent Prediction Error	Expected Prediction Error	Akaike's Information Criterion (AIC)	Bayesian Information Criterion (BIC)
Cumulative foal number born per animal	0.777	0.604	0.424	0.396	0.706	1954.161	4264.82	Cumulative foal number born per animal	0.966	0.933	0.933	0.067	0.129	1877.002	2563.704
Maximum foal number per birth	0.716	0.512	0.421	0.488	0.113	1070.884	3330.503	Maximum foal number per birth	0.919	0.844	0.406	0.156	0.198	880.087	1515.75
Multiple birth number per animal	0.980	0.961	0.803	0.039	1.838	974	3233.62	Multiple birth number per animal	1.000	1.000	1.000	0.000	6.177	498.602	1134.265

Chapter 9

Navas González, F.J.; Jordana Vidal, J.; Camacho Vallejo, M.E.; León Jurado, J.M.; de la Haba Giraldo, M.R.; Barba Capote, C.; Delgado Bermejo, J.V.

Risk factor meta-analysis and Bayesian estimation of genetic parameters and breeding values for hypersensibility to cutaneous habronematidosis in donkeys

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Research paper

Risk factor meta-analysis and Bayesian estimation of genetic parameters and breeding values for hypersensibility to cutaneous habronematidosis in donkeys

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ABSTRACT

Cutaneous habronematidosis (CH) is a highly prevalent seasonally recurrent skin disease that affects donkeys as a result from the action of spirurid stomach worm larvae. Carrier flies mistakenly deposit these larvae on previous skin lesions or on the moisture of natural orifices, causing distress and inflicting relapsing wounds to the animals. First, we carried out a meta-analysis of the predisposing factors that could condition the development of CH in Andalusian donkeys. Second, basing on the empirical existence of an inter and intrafamilial variation previously addressed by owners, we isolated the genetic background behind the hypersensibility to this parasitological disease. To this aim, we designed a Bayesian linear model (BLM) to estimate the breeding values and genetic parameters for the hypersensibility to CH as a way to infer the potential selection suitability of this trait, seeking the improvement of donkey conservation programs. We studied the historical record of the cases of CH of 765 donkeys from 1984 to 2017. Fixed effects included birth year, birth season, sex, farm/owner, and husbandry system. Age was included as a linear and quadratic covariate. Although the effects of birth season and birth year were statistically non-significant ($P > 0.05$), their respective interactions with sex and farm/owner were statistically significant ($P < 0.01$), what translated into an increase of 40.5% in the specificity and of 0.6% of the sensibility of the model designed, when such interactions were included. Our BLM reported highly accurate genetic parameters as suggested by the low error of around 0.005, and the 95% credible interval for the heritability of ± 0.0012. The CH hypersensibility heritability was 0.0346. The value of 0.1232 for additive genetic variance addresses a relatively low genetic variation in the Andalusian donkey breed. Our results suggest that farms managed under extensive husbandry conditions are the most protective ones against developing CH. Furthermore, these results provide evidence of the lack of repercussion of other factors such as age or sex. Potentially considering CH hypersensibility as a negative selection aimed goal in donkey breeding programs, may turn into a measure to improve animal welfare indirectly. However, the low heritability value makes it compulsory to control environmental factors to ensure the effectiveness of the breeding measures implemented to obtain individuals that may genetically be less prone to develop the condition.

1. Introduction

Cutaneous habronematidosis (CH) is an Equidae specific skin disease that occurs when stomach worm larvae from the spirurid species comprising the superfamily Habronematidae (*Habronema* or *Draschia*, for instance) are deposited on injured or irritated skin tissue or mucous membranes (Giangaspero and Traversa, 2017). Although donkey cutaneous habronematidosis (summer sores) would not be scientifically

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described until a few decades ago (Mohamed et al., 1989), current research suggests this dermatological condition causes more severe lesions in donkeys than it does in other equids such as horses and their hybrids (White, 2013). Traditional nomenclature ("Summer or Jack sores") not only highlights a higher disease incidence and severity reported in donkeys (White, 2013), but also the progressively increasing incidence of this disease when weather conditions become warmer in late spring or early summer (late April through June, generally after March rainy periods), partially regressing or even disappearing in winter (Gerry, 2007).

A higher predisposition to develop cutaneous habronematidosis has been suggested for grey or diluted coat equines (Pusterla et al., 2003; Caro et al., 2014), such as the Andalusian donkey. However, neither breed, sex nor age different predilections seems to exist in horses (Reed et al., 2009), and no statistically proven information has been reported for donkeys up to the date. Moist body orifices and areas (eyes, lip commissures, ears, ventral abdomen, prepuce, penis and urethral process) are more commonly affected as they are more likely to attract the attention of parasite carriers such as flies. Areas on the limbs, especially from the fetlock to the coronary band, are frequently prone to mild cuts, scrapes, and trauma and thus can also be susceptible to summer sores. In addition, biting flies prefer to alight on shaded parts of animals lower on their bodies (Mohamed et al., 1989; Schuster et al., 2010; Pugh et al., 2014). The results can range from annoying and unsightly to fatal. Young foals, thin-skinned and poor body condition animals are especially hypersensible to the action of carrier flies (Giangaspero and Traversa, 2017). In the particular case of donkeys, these parts are so thin that are easily harmed by the larvae, which cause discomfort and distress as they progress in their life cycle, what becomes a critical point for the welfare of the species.

Although equids are the final host of the parasites responsible for this condition, the cutaneous myiasis caused by the larvae of these gastrointestinal parasites occurs because of an abnormal step in the normal life cycle of the parasites (Fig. 1). These misplaced larvae cannot grow into their adult forms in such locations, but still induce a severe local inflammatory reaction characterized by intense swelling, ulceration, redness, and itching. Donkeys produce self-inflicted injuries during the subsequent rubbing and scratching to alleviate the itching produced by the simultaneous action of carrier or vector parasites, such as flies, and the action of the larvae, what apart from irritating the animals, damages the skin and makes it easier for the larvae to access the stomach through the mouth (Pugh et al., 2014).

The selection of other species against their enhanced hypersensibility to gastrointestinal parasites has been suggested as an alternative to develop the sustainable control of parasite infections (Gutiérrez-Gil et al., 2010; Kornaś et al., 2015). Apparently, some equids tend to be more predisposed to suffer from cutaneous habronematidosis than others, exhibiting clinical signs on consecutive years, whereas other individuals on the same premises never develop this condition (Pugh et al., 2014). Despite CH is a highly prevalent condition, with 94.5% of the Andalusian donkeys affected at least once in their lives, there is a simultaneous inexistence of studies testing for the conditioning factors that may be involved or the genetic background existing behind cutaneous habronematidosis hypersensibility in donkeys. The present model not only computes the strength of the effects of highly predisposing factors on the appearance of this skin condition, which may enable enhancing the implementation of prophylactic measures, but also isolates the additive genetic component laying underneath CH hypersensibility. This way, we approach the hypothetical possibility of the implementation of a selective breeding plan for the individuals, which may indirectly reduce the incidence of cutaneous habronematidosis. Breeding for less CH sensitive donkeys together with the implementation of proper husbandry techniques may translate into the avoidance of detrimental repercussions for donkey welfare derived from the development of this disease.

Basing on the empirical observation of a potential different intra and interfamilial affectation among the individuals, the first aim of this study was the isolation and study of the strength of potential

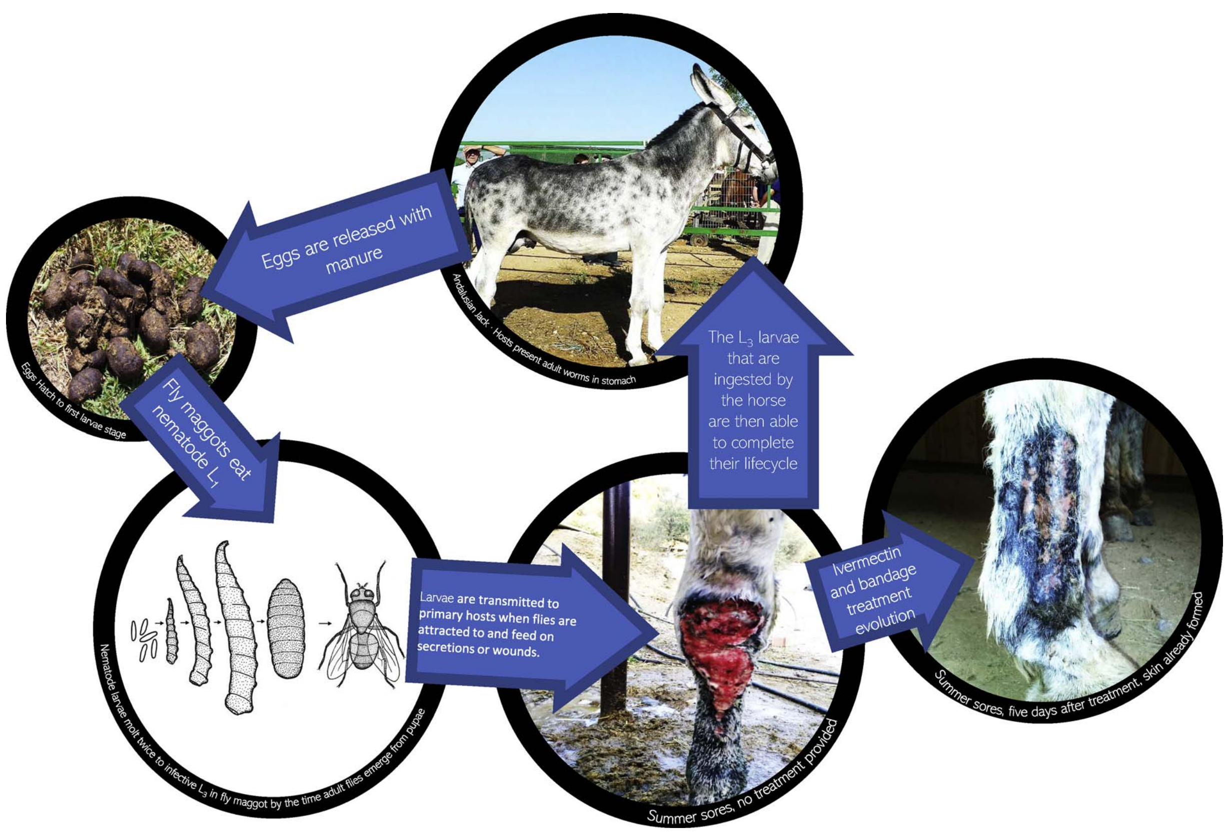

Fig. 1. Cutaneous habronematidosis cycle in donkeys.

predisposing environmental factors influencing the hypersensibility to this parasite in naturally infected donkeys. Then, we quantified the magnitude of the genetic background behind the limitedly variable phenotypes for the CH hypersensibility trait and its inheritance as a binary trait. Second, we developed a model that may enable the possibility of the inclusion of CH hypersensibility traits within the breeding programs of standardized donkey breeds. Third, considering the model that we had previously developed, we estimated the genetic parameters for CH hypersensibility and the predicted breeding values of the individuals in the historical population of Andalusian donkeys (kindship matrix) through Bayesian analyses as the basis for a selective breeding program aiming at reducing the hypersensibility of donkeys to this cutaneous myiasis.

2. Material and methods

2.1. Study sample and study background

We used a sample of 765 Andalusian donkeys (181 jacks and 584 jennies), registered in the stud-book, and with a mean age of 10.76 ± 5.45 years. All tests were carried out using a pedigree file provided by the Union of Andalusian Donkey Breeders (UGRA) including 1 017 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 time that the study took place. Parentage tests for each offspring registered in the studbook had been performed with 24 microsatellite molecular markers to test for the reliability of the pedigree (Navas et al., 2017b). Our sample gathered above 75% of the historical population of the breed. Pedigree of the sample was traced back six generations providing indirect information from 956 connected ancestors (94% of the historical population). 94.5% of the Andalusian donkeys in the sample had been affected by the condition at least once in their lives.

2.2. Survey 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, as this is the time of the year during which the animals are more likely to become affected by this condition. The owners were interviewed regarding the specific clinical status of all the animals that had historically been present at their farms since the 1980s until 2017 and were registered in the stud-book 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 20 questions that were asked by the same interlocutor and each interview lasted for a mean time of 10 min. Despite the lack of incidence of the condition in their farms stated by the owners, all the questions were asked indistinctly. A description of the questions and options asked to the owners is shown in Tables 1 and 2. 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 evolution of the incidence of this condition 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.

Table 2
Summary of the results for the Kruskal-Wallis H test for fixed effects and the covariate included in the model to test for cutaneous habronematidosis trait in Andalusian donkeys.

Factor	Item	Hypersensibility to cutaneous habronematidosis			
Year of birth	χ^2	22.773			
	df	31			
	p-value	0.857			
	Levels	1984–2017			
	Mean rank	345.15–404.00			
Season of birth	χ^2	2.979			
	df	3			
	p-value	0.395			
	Levels	Winter	Spring	Summer	Autumn
	Mean rank	387.76	376.86	383.14	391.53
Sex	χ^2	9055			
	df	1			
	p-value	< 0.01			
	Levels	Jack	Jenny		
	Mean rank	365.96	388.28		
Farm/Owner	χ^2	313.314			
	df	90			
	p-value	< 0.001			
	Levels	1–91			
	Mean rank	21.50–404.00			
Husbandry system	χ^2	23.164			
	df	3			
	p-value	< 0.001			
	Levels	Intensive	Semi intensive	Semi extensive	Extensive
	Mean rank	294.71	395.4	378.25	400.67
Age (in years)	χ^2	25.470			
	df	27			
	p-value	0.548			
	Levels	1–29			
	Mean rank	276.17–403.50			

2.3. Records description and scales

The questions were organized into three blocks (Supplementary Table S1). The first block aimed at describing the farms of the owners' interviewed in order to statistically assess the possible effects that may condition the incidence of cutaneous habronematidosis. The owners were asked the questions included in Supplementary Table S2 to classify the husbandry system under which their farms were managed. The

Table 1
Descriptive statistics for fixed effects and covariate included in the model to test for hypersensibility to cutaneous habronematidosis in Andalusian donkeys (N = 765).

Item	Factor	Minimum	Maximum	Mean	SEM	Variance	Kurtosis[a]	CV (%)
Fixed effects	Year of birth	1	32	22.16	0.23	39.31	0.39	28.30
	Season of birth	1	4	2.18	0.03	0.94	−0.79	44.56
	Sex	1	2	1.76	0.01	0.18	−0.46	24.12
	Farm/Owner	1	91	26.03	0.774	458.08	0.68	82.22
	Husbandry system	1	4	2.94	0.023	0.39	0.82	21.28
Covariable	Age (in years)	0.52	29.36	10.76	0.19	29.67	0.21	50.62
Trait	Hypersensibility to summer sores	0	1	0.95	0.01	0.05	13.37	24.12

[a] Standard error for Kurtosis statistic was 0.177 for all factors assessed.

second block comprised a single question related to whether the diagnosis by a veterinarian had been requested. This question was excluding as only the owners affirmatively responding to it were included in the statistical and genetic analyses. Third block consisted of questions regarding the assertive diagnosis of the lesions that had previously been suspected to be caused by cutaneous habronematidosis, the sanitary status of the animals, and the care and preventive measures that were taken in each case. When the animals had never presented any signal of cutaneous habronematidosis through their lives, they were given a score of 0. However, a score of 1 was provided to the animals on which, not only the lesions had been observed through their lives, but for which the veterinarian had confirmed the presence of larvae by cytology or biopsy, the results of histologic examination were consistent with a diagnosis of habronematidosis, and the treatment with ivermectin or moxidectin had been effective.

2.4. Previous meta-analysis (screening)

First, a descriptive statistics analysis (Table 1) and a Shapiro-Wilk test were applied to the data to check the fitness degree of the variables in the model to a normal distribution. Second, the fact that the elements in the model were all below 0.05 ($P \le 0.024$), revealed that the data significantly deviated from a normal distribution. Thus, we carried out a cross-sectional study employing Chi-square analysis to determine whether the categorical independent effects of birth year, birth season, sex, farm/owner and husbandry system may randomly influence the dependent variable of hypersensibility to cutaneous habronematidosis. A Kruskal-Wallis H test was performed to study the potentially existing differences between levels of the same factor (Table 2).

Cramer's V was computed to measure the strength of association between each independent factor from the first set with the dependent variable of hypersensibility to cutaneous habronematidosis using the Crosstabs procedure from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016) (Table 3). We assessed all possible double and multiple interactions between all fixed effects. However, the only statistically significant interactions found were the double interactions between birth year and farm/owner, and birth season and sex, respectively, which were included in the model.

Then a Spearman's rho test was used to compute the correlations between risk factors and cutaneous habronematidosis hypersensibility (Table 4).

2.5. Model-testing statistical analysis

Direct binomial logistic regression was performed to predict the probability that an observation falls into one of two categories (binary trait) of a dichotomous dependent variable (1 = hypersensibility to cutaneous habronematidosis; 0 = resistance to cutaneous habronematidosis) basing on the independent categorical or continuous variables and the interactions that the previous univariate meta-analysis had reported to be statistically significant (at least $P < 0.05$ for interactions, and at least $P < 0.01$ for the rest of variables) (Faraway, 2016). The final model contained five independent variables (birth year, birth season, sex, farm/owner, and husbandry system), one covariable (age), two double interactions (between birth season and sex, and between birth year and farm/owner) and included 765 donkeys. Direct logistic regression was performed using the Binary logistic regression procedure from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016). Then, we computed Cox and Snell R^2 and Nagelkerke R^2 for the whole model to assess the percentage of variance present in the cutaneous habronematidosis hypersensitive variable. That is to say, to evaluate the goodness of fit of the logistic regression model, as these pseudo-R^2 can be used to report information about the power of explanation of the model for the variable being tested (Smith and McKenna, 2013).

Table 3
Chi-square statistical significance and strength of the fixed effects, covariate and interactions included in the model to test for hypersensibility to cutaneous habronematidosis in donkeys (N = 765).

Parameter	Item	χ^2	p-value	Cramer's V
Fixed effects	Birth year	22.773	0.857	0.173
	Birth season	2.979	0.395	0.062
	Sex	9.055	< 0.01**	0.109
	Farm/owner	313.314	< 0.001***	0.640
	Husbandry system	23.1694	< 0.001***	0.174
Covariate	Age (in years)	25.470	0.548	0.906
Interaction	Birth season · Sex	11.854	< 0.01**	N/A
	Birth year · Farm/owner	624.311	< 0.001***	N/A

Levels of significance are indicated by ** and *** for $P < 0.01$, very statistically significant and $P < 0.001$, highly statistically significant, respectively.
N/A: Not applicable. Cramer's V cannot be computed for a nonparametrical interaction.

2.6. Genetic model, phenotypic and genetic parameters

As only one measure per animal was considered (binary trait, 0 = the animal had been historically unaffected and 1 = the animal had been affected at some point in its life), the model used in the analysis of hypersensibility to cutaneous habronematidosis was a simple Animal Model with single records. The fixed effects that were submitted to the above described statistical procedures and comprised the mixed model consisted of the birth year (from 1985 to 2017); birth season (summer, spring, autumn and winter); sex (jack or jenny); the farm (91 farms/owners) and husbandry system (intensive, semi-intensive, semi-extensive and extensive). The interactions between birth year and farm/owner and between birth season and sex. The age of the animals expressed in years was included as a linear and quadratic covariate. In matrix notation, the mixed multi-trait model used was:

$$Y_{ijklmop} = \mu + Yea_i + Sea_j + Sex_k + Far_l + Sys_m + Yea * Far_n + Sea * Sex_o + b1A_p + b2A_p + e_{ijklmnop}$$

where $Y_{ijklmnop}$ is the separate score for cutaneous habronematidosis hypersensibility for a given donkey; μ is the overall mean; Yea_i is the fixed effect of the ith birth year (i = 1984–2017); Sea_j is the fixed effect of the jth birth season (j = summer, spring, autumn, winter); Sex_k is the fixed effect of the kth sex (k = jack, jenny); Far_l is the fixed effect of the lth farm/owner (l = 1–91); Sys_m is the fixed effect of the mth husbandry system (m = intensive, semi-intensive, semi-extensive, extensive); $Yea * Far_n$ is the interaction between birth year and farm/owner; $Sea * Sex_o$ s the interaction between birth season and sex; b1 and b2 are the linear and quadratic regression coefficients on age when the tests took place (A_p), and $e_{ijklmnop}$ is the random residual effect. 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., 2017a).

2.7. Genetic assessment software

We used Bayesian methods with the Multiple Trait Gibbs Sampling for Animal Models (MTGSAM) software by Van Tassell and Van Vleck (1996) to obtain estimates of variance components and heritability (Table 5) for hypersensibility to habronematidosis in Andalusian donkeys. A single chain of 550,000 cycles was obtained, 50,000 of which were discarded (burn-in), and thinning intervals of 200 cycles were used to retain sampled values which reduced the lag correlation among thinned samples. The convergence criteria used implied the change in the Log-likelihood of the function in successive iterations and were less than 10^{-10}. Gibbs sampling procedures enable building and saving a random number or the total number of samples of variances obtained in the iterative process (1151 solutions in our case). Then, for each saved sample of variances, the genetic parameters could be calculated and assessed to obtain descriptive statistics such as mean, standard

Table 4
Spearman's correlation coefficients and significance level for fixed effects and covariate with the cutaneous habronematidosis hypersensibility trait in Andalusian donkeys.

Factors	Birth year	Birth season	Sex	Farm/Owner	Husbandry system	Age (in years)
Cutaneous habronematidosis hypersensibility	−0.046	0.002	0.109**	−0.127**	0.049	−0.001

** Denotes a statistically highly significant correlation of $P < 0.001$.

Table 5
Estimated components of variance, heritability (h^2), standard error of the heritability (SE), and 95% Credible intervals for hypersensibility to cutaneous habronematidosis obtained from multivariate analyses through Gibbs sampling methods in Andalusian donkeys.

Trait	σ_a^2	σ_p^2	σ_e^2	$h^2 \pm$ SE	95% Credible intervals
Cutaneous habronematidosis hypersensibility	0.1232 ± 0.0053	0.4650 ± 0.0052	0.3418 ± 0.0052	0.0346 ± 0.0052	0.0346 ± 0.0012

Table 6
Descriptive statistics for the estimates of Predicted Breeding Values (PBV) for hypersensibility to cutaneous habronematidosis in the Andalusian donkey.

	N = 1017	Mínimum	Maximum	Mean	SEM	SD	Kurtosis	Standard error (SE)
PBV Males	272	−0.038	0.023	0.001	0.001	0.009	3.963	0.294
PBV Females	745	−0.037	0.025	0.001	0.001	0.005	9.951	0.179

deviation, variance and standard errors, which could provide us with a perspective of the existing variability. Bayesian approaches can summarize their uncertainty by giving a range of values on the posterior probability distribution that includes 95% of the probability, this is called a 95% credible interval. 95% credible interval for the heritability was computed with MTGSAM software (Table 5). Then, we computed predicted breeding values (PBV) and systematic deviation for all animals in the relationship matrix. Bayesian PBVs and their accuracies were directly computed with MTGSAM software as well (Table 6).

3. Results

3.1. Interview results

Out of the 145 owners interviewed, we considered the information from 91 farms/owners. These owners had affirmatively responded to the question in the second block as they were they only who had requested information concerning diagnosis by their veterinarians and therefore, were the only ones providing reliable information. Only 5 animals out of the 765 donkeys from which there was information (0.65% of the total sample) had been affected in several consecutive years, what could mainly be attributed to failures in the hygienical prevention measures implemented or problems on the treatment that they were provided with.

3.2. Statistical analysis

A summary of the descriptive statistics for the hypersensibility to cutaneous habronematidosis related trait, fixed effects and covariates is shown in Table 1. Shapiro-Wilk Test and the deviation kurtosis values ranging from −0.79 to 13.37 on all the factors showed that they highly significantly ($P < 0.001$) 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 21.28% for the husbandry system effect and 82.22% for the effect of the farm/owner. The results of the Kruskal-Wallis H test that was run to assess the differences among the levels of the different effects in the model are shown in Table 2. The results of Spearman's rho tests, which assess the correlations between the fixed effects, the covariate and the trait in the model are reported in Table 4. Chi-square test suggested that the effect of sex was very statistically significant ($P < 0.01$) and the effects of farm/owner and husbandry system were highly statistically significant ($P < 0.001$). However, the rest of effects resulted highly statistically non-significant. The statistical significance and strength of each of the effects on the hypersensibility to CH are shown in Table 3.

3.3. Covariate and fixed effects posterior means

The results for the estimates of non-genetic effects from the Bayesian quantitative genetic analysis, including age as a linear and quadratic covariate and the four fixed effects of sex, farm/owner, birth season and birth year are shown in Supplementary Table S3.

3.4. Genetic model, variance components, predicted breeding values and prediction accuracy

The estimates for heritability, genetic and phenotypic variance and 95% credible intervals obtained through Gibbs sampling are shown in Table 5. The results for the estimates of predicted breeding values (PBV) for both jacks and jennies are shown in Table 6.

4. Discussion

MTGSAM has scientifically been proved to be an effective tool for the estimation of genetic parameters and breeding values for binary traits, especially in those cases in which, although there is a long historical record, such traits were not recorded following quantitative procedures (Famula et al., 2007).

The traditional lack of attention paid to the donkey species may be the main reason of why no known study concerning the predisposing or conditioning factors involved, nor the genetic variation for hypersensibility of the donkey species to this disease has been published up to the date.

The effect of birth year, birth season and age did not statistically significantly affect the hypersensibility to cutaneous habronematidosis of the animals ($P = 0.857$, $P = 0.548$, and $P = 0.395$, respectively). However, these effects were kept in the model basing on the pseudo R square values obtained and the predictive power of the model when interactions including them were considered. The highly statistically significant, $\chi^2(126) = 200.071$, $P < 0.00003$ model presented in this paper was able to distinguish resistant individuals from those who were more likely affected by cutaneous habronematidosis efficiently. When interactions were not included, the model explained from 23.0% (Cox and Snell R^2) to 66.4% (Nagelkerke R^2) of the variance in the cutaneous habronematidosis status of the animals, and it was able to correctly classify 97.0% of cases into affected with a specificity of 57.1% and a sensitivity of 99.3%.

However, when the interactions between birth year and owner/farm and between birth season and sex were included in the model, this percentage considerably increased. The inclusion of interactions provided the model with the ability to explain between 33.1% (Cox and Snell R^2) and 95.6% (Nagelkerke R^2) of the variance in disease status. In the same way, it was able to correctly classify 99.7% of cases as affected individuals with a specificity of 97.6% and a sensitivity of 99.9%.

Cohen (1988), would report Cramer's V small effect associations range from 0.0 to 0.10, moderate effect associations from 0.3 to 0.5 and large effect associations from 0.5 to anything above considering a statistically significant measure of $P < 0.05$ to indicate a meaningful difference.

Sex presented a statistically significant small effect of 10.9% on the incidence of cutaneous habronematidosis. Jacks were slightly more prone to exhibit cutaneous habronematidosis than jennies, though their 0.126 times lower incidence was negligible. Therefore, our study agrees on the results found in a retrospective study in North America (Pusterla et al., 2003) which indicated that there was no sex predilection for this disease to occur. In the same way, this study reported cutaneous habronematidosis causal agents appear to present a higher predilection for grey coat animals, what may be the basis of the high prevalence found in the Andalusian donkey breed, because of its emblematic dapple-grey coat.

The effect of farm/owner was large (64.0%) and highly statistically significant (Cramer's V:0.64, $P < 0.001$), while husbandry system was slightly moderate (17.4%) and statistically significant ($P < 0.01$). Donkeys kept in farms managed under intensive systems statistically significantly showed 14.642% higher likelihood of presenting cutaneous habronematidosis than those in farms under extensive systems. This value was followed by 4.421% higher likelihood of those under semiextensive system conditions and 3.120% higher likelihood under semiintensive system conditions, respectively, when compared to animals managed under extensive systems. According to the definition of the husbandry systems found in Supplementary Table S2, factors such as the access to wider territory extensions and the supply with veterinary care resulted to be preventive factors. However, the regular contact with humans was not relevant, as highlighted by the slight differences found between the semiintensive and semiextensive husbandry system levels, for which human contact on a daily basis was the only difference.

The presence of hypersensible donkeys to cutaneous habronematidosis ranged from 19.244 to $6.595 \cdot 10^{-20}$ times higher, from the farms/owners whose donkeys presented a higher prevalence for cutaneous habronematidosis to those farms/owners in which there were neither incidence of cutaneous habronematidosis, nor any existing case had been registered in their historical record.

Belonging to certain farms, especially those managed under extensive systems, resulted to be the strongest preventive situation against being hypersensible to cutaneous habronematidosis, and therefore the most protective factor against the development of the disease ($P < 0.0001$).

Similarly to the papers reporting an inter-individual variation of less than 10% of the total variance (Kornaś et al., 2015), our results suggest a slightly higher genetic additive variance of 12.3%. Our data were corrected for known fixed effects such as farm/owner, husbandry system, birth season, birth year, the age covariate, and some interactions such as those between sex and birth season and birth year and farm/owner. However, additional environmental factors or interactions, or differences in the exposure of the animals to them, may have conditioned the historical development of this disease. These uncontrolled effects contribute to the increase of the residual variance found in the population or may be indirectly gathered in the previous effects considered (for example, feeding within husbandry system).

In turn, this residual variation prevents the correct estimation of within- and between-individual variation. However, the low standard error of prediction found suggests that we can consider this model to be highly accurate.

Low heritability estimates were found, i.e. less than 0.10, suggesting that only a slight proportion of the observed variation in cutaneous habronematidosis hypersensibility has a genetic basis. Our results match the results found for the genetic parameters associated with the hypersensibility against other nematodes in different species such as horses (Kornaś et al., 2015), sheep (Niks et al., 1993) and cattle (Morris, 2007). Simultaneously, the genetic parameters for hypersensibility to insect bites in equines has been historically reported to range from 0.359 to 0.07 (Eriksson et al., 2008; Schurink et al., 2009, 2011; Citek et al., 2017) in similar studies. These results evidence the need for prevention against intermediary hosts (carrier flies) and the careful treatment of the animals affected. Continuous treatment over the years should be implemented carefully, trying to avoid the appearance of parasite resistance to ivermectin. Some animals do not respond to the treatment and have a relapse, thus necessitating multiple doses or the use of moxidectin (Schumacher and Taintor, 2008; Elghryani and De Waal, 2016).

Restricting the dataset to only smaller sets of the population according to their age has been suggested to report increased values of the heritability of similar traits (Kornaś et al., 2015). However, age was included in our model as a covariable and the statistical results obtained revealed the lack of significance of the effect, what supports the incidence reported in the species for this condition (White, 2013). These results match the high incidence found in the historical population as, except for 27-year-old animals, the condition was present or have been present at least once in the life of the donkeys indistinctly, from their first year of age through their lives (Fig. 2). The prevalence per age level ranged from 67% to 100%. Pusterla et al. (2003), reported the age of horses affected by cutaneous myiasis to be around 7.3 years old, while this age was around 10.8 years old in Andalusian donkeys. Our results identified a low significant heritability ± SE (0.0346 ± 0.0052) of donkey hypersensibility to cutaneous habronematidosis, and only a 12.32% of the observed variation could be attributed to a genetic basis (as typical of diseases). Therefore, additional insights from other equid populations would be useful to confirm the potential of breeding strategies as part of integrated nematode management, as it has been addressed in other species such as sheep (Raadsma et al., 1989; Greeff and Karlsson, 2005).

In the case of cutaneous habronematidosis hypersensibility, the demographic bottlenecks suffered by the Andalusian donkey population may have affected the levels of genetic variability for related traits, as no selection has ever been carried (Navas et al., 2017a). Given the importance and donkey welfare repercussions of the trait involved in our study, genetic selection for hiposensible donkeys can play an important role in a selection program aimed at improving the welfare of the species. The potential opportunities arising from the incorporation of genomic information in the selection program should be investigated and implemented carefully in the future, as 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 cutaneous habronematidosis hypersensibility show a moderate variability, indicating a possibly effective negative selection based on genetic objective estimates. The low heritability values match the moderate existing phenotypic variability, resulting in a moderately narrow PBV distribution (Table 6). Defining breeding objectives is the key element of any breeding program (Van Vleck, 1993), and the need to include welfare related traits among the breeding goals of certain highly standardized donkey breeds, such as the Andalusian donkey,

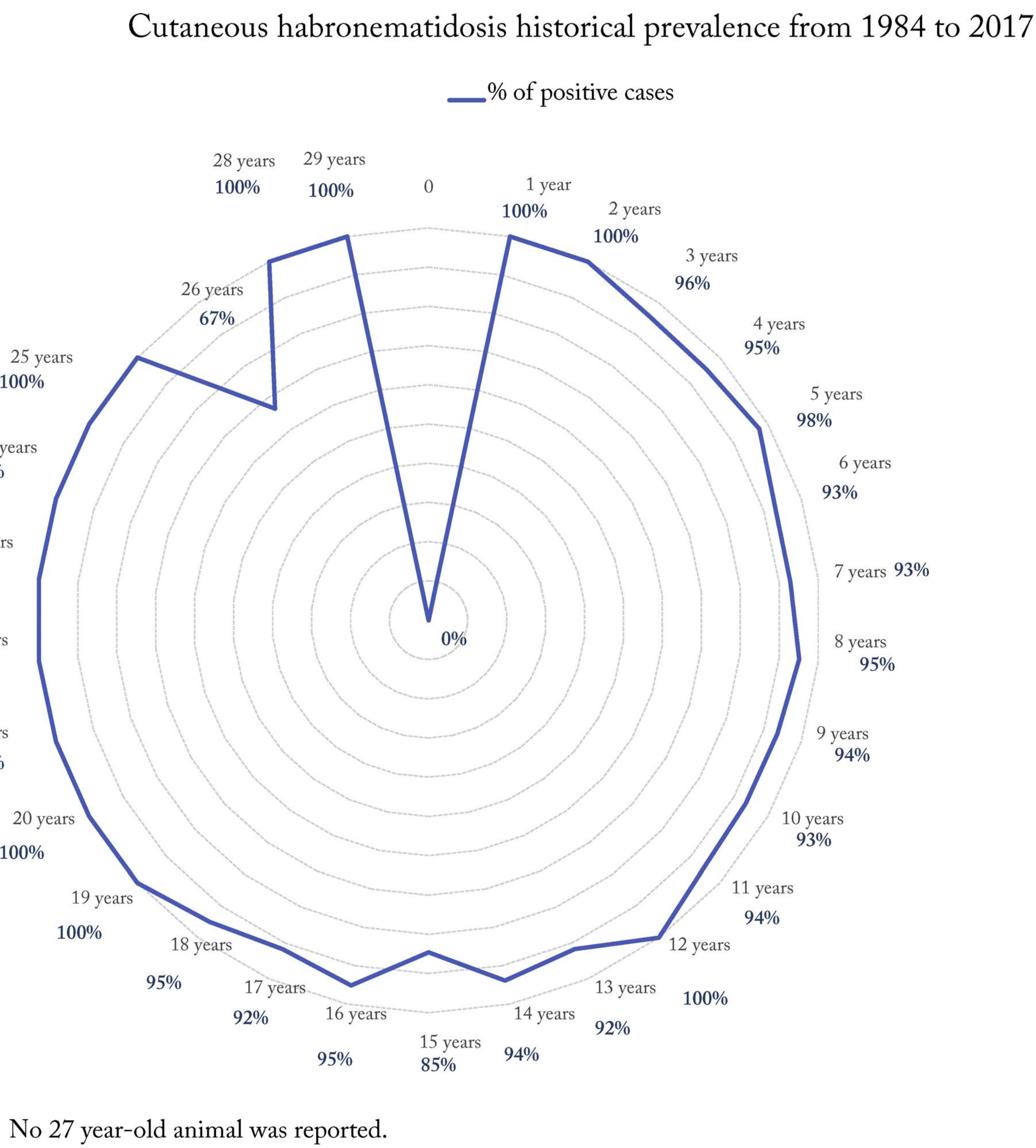

Fig. 2. Cutaneous habronematidosis historical prevalence from 1984 to 2017.

while maintaining selection for morphological and phaneroptical characteristics is of prominent importance. Implementing a systematic genetic evaluation procedure through the genetic information available that allows 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 be performed carefully considering the detrimental problems that are likely to appear because of an increase in inbreeding in breeds with such a low effective population number. In such breeds, the protection of genetic variability, minimizing inbreeding and avoiding population bottlenecks, becomes a primary concern.

The incorporation of genetic markers in the negative selection of donkeys according to their hypersensibility to certain diseases such as the one assessed in this study is still a possibility to be developed in the future. Nonetheless, the potential benefits for the health of donkeys and their welfare make it become a worth considering selection criteria to be achieved through the implementation of these validated assessment tools and new methodologies relying on larger studies carried out over several years.

5. Conclusions

The low levels for genetic parameters resemble the ones obtained in literature when assessing for the hypersensibility of other species to similar parasitoses. Although these values compromise the potential inclusion of disease hypersensibility related traits within breeding programs seeking the genetic progress of the breed, the benefits that could be obtained from negative selection against this prevalent condition may be worth considering. Farms in which the donkeys are handled under extensive conditions, where the animals are able to more openly react to the action of carrier vectors such as flies, stand out over those farms in which the combined presence of predisposing factors addressed by literature may contribute to the development of the disease. The low genetic component highlighted reveals prophylactic measures and prevention against the parasites is key for the protection of the donkeys from suffering from this skin condition, as ivermectin continuous treatment may cause resistance. Our model results highly suitable for the assessment of the background possibly contributing to the greater development of this disease as suggested by its high sensibility and specificity. Selection against disease hypersensibility parameters in donkeys is unlikely to have traditionally been carried out indirectly, therefore the routine application of the assessment including a greater number of animals is required to standardize the assessment methodology implemented in this study. However, considering the extinction risk that donkey breed endangered populations face generally, any measure aiming at the improvement of welfare in this species may be worth-considering.

Implications

The inexistence of genetic analyses for functional traits in donkeys can mostly be attributed to the existing limitations that researchers face when they study such populations. Such limitations generally concern donkey population structure and the size of the samples used. Cutaneous habronematidosis is a worldwide highly prevalent parasitosis that especially affects donkeys. Intra and interfamilial empirical differences have been suggested traditionally. Gibbs sampling is especially suitable for assessing traits that do not fit a normal distribution in small samples. Estimating the breeding values and genetic parameters for donkey cutaneous habronematidosis hypersensibility enable the possibility to set accurate conservation and breeding plans aiming at improving the welfare of donkey populations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetpar.2018.01.017.

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Conclusiones

Basándonos en los resultados obtenidos en la presente Tesis Doctoral, se puede concluir que:

- **Primer grupo de conclusiones.** (Primera publicación: "Navas, F.J.; Jordana, J.; León, J.M.; Barba, C.; Delgado, J.V. A model to infer the demographic structure evolution of endangered donkey populations. *animal* 2017, *11*, 2129-2138"):

1. La pérdida de diversidad genética desde las generaciones fundadores puede considerarse pequeña en la Raza Asnal Andaluza, lo que podría esperarse en razas similares con un fondo genético desconocido similar; sin embargo, la monitorización siempre es una decisión razonable.

2. La contribución excesiva de pocos antepasados al pool genético puede llevar a cuellos de botella más estrechos poblacionales en un futuro próximo. Los intervalos de generación encontrados pueden considerarse una ventaja para reducir el aumento de la endogamia manteniendo la diversidad genética existente de las razas de burro.

3. Nuestra mayor preocupación recae en la sostenibilidad productiva, ya que la conservación in situ se ve claramente afectada por la creciente demanda internacional, el aumento de los costos de alimentación y la disminución de los subsidios gubernamentales como principales contribuyentes a la pérdida de individuos descartados o exportados, cuya información genealógica no se considera nunca más.

4. Rastreando los 36 años de historia genética de la raza asnal andaluza, el bajo nivel de completitud del pedigrí, especialmente en la población actual, no permite la estimación fiable de los parámetros de variabilidad genética, como *fe*, *fa*, genomas fundadores equivalentes, y coeficientes de endogamia y parentesco.

5. El análisis de estos parámetros muestra que parte de la variabilidad genética de los fundadores se ha perdido en el transcurso de los años, y en especial el aumento del porcentaje de machos y hembras que exhiben altos valores de AR advierten que la amenaza de extinción aún se cierne sobre la raza. Además, el tamaño efectivo de la población es considerablemente menor que el rango del tamaño efectivo mínimo, que puede equilibrar la depresión de la endogamia, aproximándose al mínimo tamaño efectivo viable para la preservación de las especies amenazadas.

6. Aunque la tasa de endogamia en las poblaciones actuales e históricas fue aceptable (menos del 1%), su valor en el conjunto de población de contraste, difirió alarmantemente del valor recomendado (+0.23%) lo que sugiere que a medida que más información genealógica es conocida, mayor es el nivel de amenaza de extinción al que la raza está sometida en realidad.

7. Es necesario un manejo genético cuidadoso para minimizar las prácticas de endogamia y mejorar la variabilidad genética. Por lo tanto, medidas como el uso de la inseminación artificial y otras nuevas tecnologías de cría, como la vitrificación de embriones, deben implementarse para contener la tasa de endogamia y aumentar los tamaños efectivos de las poblaciones asnales a nivel mundial cuyo trasfondo genético es desconocido con frecuencia, evaluando el porcentaje de relaciones que comparten las parejas de reproductores en cada caso, seleccionando individuos para el apareamiento cuando estas relaciones se mantienen por debajo del 6% en la raza asnal andaluza, elegida para inferir el modelo general que se presenta en este estudio.

- **Segundo grupo de conclusiones.** (Segunda publicación: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; McLean, A.K.; Delgado Bermejo, J.V. Non-parametric analysis of the noncognitive determinants of response type and response intensity, mood and learning in donkeys. En consideración en *Journal of Veterinary Behavior: Clinical Applications and Research.*"):

1. La ubicación, sistema de explotación, explotación y los factores relacionados con las pruebas pueden condicionar las respuestas, su intensidad, el estado de ánimo y la capacidad de extinción/aprendizaje de los burros. Sin embargo, todavía hay un componente inherente notable modulado por la edad, el sexo o los antecedentes de los progenitores.

2. La prueba de condicionamiento operante multietapa aplicada permite cuantificar de manera eficiente y significativa varios factores relacionados con la cognición y el comportamiento del burro.

3. El etograma que describimos afronta al conocimiento popular sobre cómo las diversas señales del lenguaje corporal informan acerca de los sentimientos de los burros para así

establecer una descripción formal de los signos colaterales que los burros muestran regularmente al describir cierto estado de ánimo o patrones de temperamento de modo que dichos signos podrían ser la clave para la emisión de diagnósticos tempranos y el tratamiento de condiciones potencialmente mortales.

4. La aplicación de técnicas de refuerzo positivo y de tratos supera los resultados cognitivos obtenidos por refuerzo negativo, ya que no sólo permiten a los animales realizar ciertas tareas, sino que también les permiten hacerlo de una manera más cercana a la natural, generando menos problemas relacionados con el bienestar, que dado el riesgo de peligrosidad al que se expone la especie asnal puede ser vital para la preservación de aquellos nuevos nichos funcionales que promuevan la reintroducción de estos valiosos animales de nuevo en su relevante papel para desempeñar ciertas prácticas humanas.

- **Tercer grupo de conclusiones.** (Tercera publicación: "Navas González, F.**J.**; Jordana Vidal, J.; Pizarro Inostroza, G.; Arando Arbulu, A.; Delgado Bermejo, J.V. Can Donkey Behavior and Cognition Be Used to Trace Back, Explain, or Forecast Moon Cycle and Weather Events? *Animals* **2018**, *8*, 215."):

1. Las condiciones ambientales, estacionales, del momento (año) y ciclo lunar son factores de estrés potenciales o moduladores conductuales que afectan el comportamiento y las respuestas cognitivas de los burros, así como pueden tener efectos potenciales de larga duración que pueden ser rastreados hacia el pasado.

2. Los efectos de oscilación climática pueden afectar a los burros alterando sus biorritmos fisiológicos y produciendo modificaciones conductuales y cognitivas severas. Las desviaciones en los patrones de comportamiento o en las capacidades de los burros para realizar tareas complejas a las que pueden no estar acostumbrados, pueden convertirse en indicadores relevantes de bienestar, así como pueden permitir abordar qué técnicas o métodos son los más adecuados y por tanto se aplicarán en cada caso.

3. El comportamiento se convierte en una herramienta relevante al predecir las condiciones climáticas futuras, así como puede informar de la distorsión potencial que pueden causar, un hecho de importancia prominente para los veterinarios, practicantes y propietarios de burros, ya que puede permitirles anticipar tales situaciones con el fin de contrarrestar sus efectos.

- **Cuarto grupo de conclusiones.** (Cuarta publicación: "Navas, F.J.; Jordana, J.; León, J.M.; Arando, A.; Pizarro, G.; McLean, A.K.; Delgado, J.V. Measuring and modeling for the assessment of the genetic background behind cognitive processes in donkeys. *Research in Veterinary Science* **2017**, *113*, 105-114."):

1. La dificultad para encontrar y controlar modelos que evalúen el comportamiento animal aumenta especialmente cuando pretendemos hacerlo en las situaciones prácticas en el campo. Los niveles de significancia encontrados, demuestran que el modelo usado para evaluar el clúster relativo a las estrategias de afrontamiento es más exacto y conveniente que el usado para evaluar otros procesos cognitivos como los relativos a la cognición en general e inteligencia en particular. Esta situación no sólo permite una metodología de cuantificación más objetiva para los rasgos relacionados con las estrategias de afrontamiento, sino que también reporta resultados globales más fiables.

2. Las diferencias que aparecen debido a la influencia de los diferentes efectos fijos en los rasgos de comportamiento evaluados pueden atribuirse al hecho de que las pruebas utilizadas pueden, de hecho, evaluar la capacidad de ciertos propietarios para educar a sus burros en lugar de la propia capacidad cognitiva de los animales para desarrollar un determinado proceso.

3. Aunque el dimorfismo sexual es evidente en algunos de los procesos cognitivos, la variación se puede atribuir a las diferencias en los métodos de manejo y rutinas aplicadas a los garañones y las burras.

4. El sistema de cría aplicado puede ayudarnos a agrupar a los animales para salvar la posible distorsión de los resultados que puede producirse debido a la distribución desigual de los animales entre las granjas.

5. La fracción de varianza explicada por factores externos puede ser pequeña cuando los consideramos individualmente, pero puede mejorar cuando se suman sus pesos parciales. La varianza explicada por estos modelos multifactoriales permite comparativamente considerarlos eficientes para cuantificar los dieciséis procesos cognitivos en nuestro estudio, ya que proporcionan información muy útil para el diseño y facilitación de los modelos complejos utilizados en análisis de genética del comportamiento.

6. Ambas interacciones dobles y triples resultaron en su mayoría no significativas para los clústeres de inteligencia y cognición. Este hallazgo apoya el hecho de que, en los estudios conductuales, la dependencia de varios factores individualmente, puede ayudarnos a cuantificar los factores o efectos involucrados con mayor precisión que sus efectos conjuntos.

7. Nuestros resultados sugieren la idoneidad del sistema de toma de datos relativos a los procesos cognitivos propuesto para aplicarse en la selección genética de rutina de las razas asnales asnos.

8. Estos criterios de selección se implementarán en el futuro con el fin de hacer que el burro sea más competitivo comercialmente y útil, no sólo con el objetivo de salvar a los individuos, sino las razas enteras de la extinción.

- **Quinto grupo de conclusiones** (Quinta publicación: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; Arando Arbulu, A.; McLean, A.K.; Delgado Bermejo, J.V. Genetic parameter and breeding value estimation of donkeys' problem-focused coping styles. *Behavioural Processes* **2018**, *153*, 66-76."):

1. Se puede concluir que se obtuvieron heredabilidades moderadas para los rasgos relacionados con la reactividad, tales como el tipo de respuesta, estado de ánimo/emoción y grado/intensidad de la respuesta a través de la evaluación de pruebas de comportamiento y de la información de las hojas de campo asociadas.

2. La exactitud de estas estimaciones también fue alta, incluso más si se considera el número limitado de burros en el estudio, lo que puede resaltar la eficiencia de la prueba de comportamiento y el modelo diseñado para evaluar tales caracteres.

3. Es esencial tener en cuenta que este estudio es el primero en estimar una heredabilidad de los rasgos relacionados con el estilo de afrontamiento/reactividad medidos en una situación práctica relacionada con un programa de selección en razas de burro.

4. Los resultados indican que la selección para reducir la reactividad en los burros es alcanzable, aunque requiere una investigación más amplia incluyendo más animales, una tarea difícil de lograr si trabajamos a nivel de raza, teniendo en cuenta el riesgo de extinción existente al que estos animales están expuestos.

- **Sexto grupo de conclusiones.** (Sexta publicación: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; McLean, A.K.; Delgado Bermejo, J.V. Dumb or smart asses? Donkey's cognitive capabilities *(Equus asinus)* share the heritability and variation patterns of human's cognitive capabilities *(Homo sapiens)*. En consideración en *Journal of Veterinary Behavior: Clinical Applications and Research.*"):

1. Nuestros resultados sugieren que los burros podrían considerarse animales de alguna manera inteligentes cuando comparativamente los valoramos basándonos en una escala análoga humana. Sin embargo, no pretendemos afirmar que algunos burros podrían presentar un cociente intelectual más alto que algunos humanos comparados a través de la misma escala.

2. Los procesos cognitivos y los métodos para valorarlos ampliamente difieren de una especie a otra. Además, cuanto más complejo es el desarrollo cognitivo de la especie que se está valorando, más complejo deben ser estos métodos. Sin embargo, aun así, se

encuentra una notable variabilidad entre los burros, es decir, hay burros que son más inteligentes que otros, y la metodología actual permite cuantificar tales diferencias.

3. Los patrones de distribución y herencia fenotípicos notablemente similares descritos en los asnos pueden sugerir que la inteligencia se puede atribuir a un trasfondo científico similar o incluso estar sustentada por una estructura genética similar a la que se estudia ampliamente en los seres humanos. Tal hallazgo sienta la base para que la investigación futura profundice en el campo de la cognición animal. Nuestros resultados sugieren que los mecanismos heredables de la cognición del burro se podrían atribuir a un trasfondo genético similar al del ser humano.

4. Este estudio abre una puerta para la selección y la mejora de generaciones animales con un mejor rendimiento cognitivo. Nuestra metodología comprende un acercamiento novedoso a la controversia relativa a la inteligencia animal, usando un método estándar aplicado en humanos para computar el cociente de inteligencia de los individuos.

- **Séptimo grupo de conclusiones.** (Séptima publicación: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M..; McLean, A.K.; Pizarro Inostroza, G.; Delgado Bermejo, J.V. Genetic parameter estimation and implementation of the genetic evaluation for gaits in a breeding program for assisted-therapy in donkeys. *Veterinary research communications* 2018, *42*, 101-110."):

1. Se obtuvieron valores elevados para los parámetros genéticos parecidos a los obtenidos para caballos en bibliografía para las diferentes modalidades de marcha descritas por los burros. Estos valores permiten la inclusión potencial de rasgos de locomoción dentro de los programas de cría que buscan el progreso genético de las razas de burro, como el asno andaluz.

2. El efecto estadísticamente no significativo de los evaluadores sugirió el éxito de un procedimiento de puntuación altamente uniforme entre evaluadores.

3. Las correlaciones genéticas fueron altas y positivas para todas las combinaciones de rasgos, permitiendo así la selección combinada para ambos aires, con un bajo efecto perjudicial sobre cualquiera de los dos aires involucrados (ambladura o paso y trote).

4. La selección de ciertos aires en burros puede haberse llevado a cabo tradicionalmente de manera indirecta, por lo que se requiere la aplicación rutinaria de la evaluación incluyendo un mayor número de animales para estandarizar la metodología de valoración implementada en este estudio, una tarea difícil de lograr, teniendo en cuenta el riesgo de extinción que se cierne sobre las poblaciones de razas asnales amenazadas.

5. Dadas las favorables relaciones genéticas existentes entre los rasgos implicados, los aires pueden desempeñar un papel importante en un programa de selección destinado a mejorar la idoneidad de los burros para el tratamiento de discapacidades motoras específicas dentro programas de terapia asistida.

6. La naturaleza específica y la magnitud de las relaciones genéticas existentes entre los rasgos funcionales evaluados en este estudio pueden hacer interesante considerar la posibilidad de desarrollar y mantener líneas especializadas que difieran según la capacidad de los burros para desarrollar ciertos patrones de marcha (ambladura o paso y trote) dentro del Programa de cría del asno de raza Andaluza, ya que estos diferentes patrones pueden ser especialmente adecuados para el tratamiento de diferentes discapacidades motoras humanas.

- **Octavo grupo de conclusiones.** (Octava publicación: "Navas González, F.J.; Jordana Vidal, J.; Camacho Vallejo, M.E.; León Jurado, J.M.; de la Haba Giraldo, M.R.; Barba Capote, C.; Delgado Bermejo, J.V. Risk factor meta-analysis and Bayesian estimation of genetic parameters and breeding values for hypersensibility to cutaneous habronematidosis in donkeys. *Veterinary Parasitology* **2018**, *252*, 9-16."):

1. Los valores bajos para los parámetros genéticos se asemejan a los obtenidos en bibliografía al evaluar la hipersensibilidad de otras especies a parasitosis similares.

2. Aunque estos valores comprometen la inclusión potencial de rasgos relacionados con la hipersensibilidad de la enfermedad dentro de los programas de cría que buscan el progreso genético de la raza, los beneficios que podrían obtenerse de la selección negativa contra esta prevalente condición podrían hacer que esta fuera una estrategia a considerar.

3. Las explotaciones en las que los burros son manipulados bajo condiciones extensivas, donde los animales son capaces de reaccionar más abiertamente a la acción de vectores portadores como moscas, destacan sobre aquellas explotaciones en las que la presencia combinada de factores predisponentes abordados por bibliografía puede contribuir al desarrollo de la enfermedad.

4. El bajo componente genético detectado revela que la implementación de medidas profilácticas y preventivas contra estos parásitos es clave para la protección de los burros frente al padecimiento de esta condición de la piel, pues el tratamiento continuo de la ivermectina puede causar resistencia.

5. Nuestro modelo resulta altamente adecuado para la evaluación del trasfondo factorial que, posiblemente contribuya a un mayor desarrollo de esta enfermedad como sugiere su alta sensibilidad y especificidad.

6. Es improbable que la selección contra los parámetros de hipersensibilidad a la enfermedad en burros se haya llevado a cabo tradicionalmente de manera indirecta, por lo que se requiere la aplicación rutinaria de la evaluación, incluyendo un mayor número de animales, para estandarizar la evaluación por medio de la metodología implementada en este estudio. Sin embargo, teniendo en cuenta el riesgo de extinción que enfrentan las poblaciones amenazadas de razas de burro en general, cualquier medida destinada a mejorar el bienestar en esta especie puede ser una medida que merezca la pena considerar.

- **Noveno grupo de conclusiones.** (Novena publicación: "Navas González, F.J.; Jordana Vidal, J.; McLean, A.K.; León Jurado, J.M.; Barba, C.J.; Arando, A.; Delgado Bermejo, J.V. Modeling for the Inheritance of Endangered Equid Multiple Births and Fertility: Determining Risk Factors and Genetic Parameters in Donkeys *(Equus asinus)*. En consideración en *Reseach in Veterinary Science*"):

1. Los altos valores para los parámetros genéticos permiten la inclusión potencial de estos rasgos dentro de los programas de mejora que buscan el progreso genético de las razas de burro.

2. Las correlaciones genéticas positivas y moderadas permiten la selección combinada para el número máximo de ruchos por parto y el número histórico de ruchos nacidos por burro, con un bajo efecto perjudicial para cualquiera de los dos.

3. La selección a favor de las gestaciones múltiples o fertilidad en burros podría haberse llevado a cabo indirectamente tradicionalmente. Por lo tanto, se requiere la aplicación rutinaria de la evaluación, incluyendo un mayor número de animales para estandarizar la metodología de valoración implementada. Sin embargo, esta es una tarea difícil de lograr, teniendo en cuenta el riesgo actual de extinción de las poblaciones de razas de burro amenazadas.

4. Los rasgos funcionales relacionados con la fertilidad y la prolificidad pueden desempeñar un papel esencial en un programa de selección destinado a mejorar la idoneidad de los burros para su inclusión en programas de reproducción asistida por medio de vitrificación o congelación de embriones.

5. Los resultados actuales permiten una estrategia de selección bidireccional. Por un lado, la naturaleza específica y la magnitud de las relaciones genéticas existentes pueden hacer interesante considerar la posibilidad de desarrollar y mantener líneas especializadas que difieran en cuanto a la capacidad particular de cada burro para desarrollar gestaciones múltiples dentro del Programa de selección del asno Andaluz, aumentando así la productividad de las técnicas de reproducción asistida. Por otro lado, cuando la recolección de embriones no es el objetivo, la selección podría centrarse en la obtención de aquellos individuos que pueden ser menos propensos a desarrollar gestaciones

múltiples, por tanto, evitando los riesgos que implican dichas gestaciones múltiples, lo que al final se traduce en la mejora del bienestar reproductivo de los individuos.

Conclusions

Basing upon the results obtained in the present PhD Thesis, we can hereby conclude:

- **First group of conclusions.** (First publication: "Navas, F.J.; Jordana, J.; León, J.M.; Barba, C.; Delgado, J.V. A model to infer the demographic structure evolution of endangered donkey populations. *animal* 2017, *11*, 2129-2138"):

1. The loss of genetic diversity since the founder generations can be considered small in Andalusian donkeys, what could be expected in similar breeds with a similar unknown genetic background; however, monitoring is always a reasonable decision.

2. The excessive contribution of few ancestors to the gene pool may lead to narrower population bottlenecks in the near future. The generation intervals found may be considered an advantage to reduce the inbreeding increase maintaining the existing genetic diversity of the donkey breeds.

3. Our major concern falls on the productive sustainability as *in situ* conservation is clearly affected by a rising international demand, increasing feeding costs and a decrease in governmental subsides as the main contributors to the loss of discarded or exported individuals, whose genealogical information is no longer considered.

4. Tracking back 36 years of the Andalusian breed genetic history, the shallow level of pedigree completeness, especially in the current population, does not permit the reliable estimation of genetic variability parameters, like *fe, fa,* founder genome equivalent, inbreeding, and relatedness coefficients.

5. The analysis of these parameters shows part of founders' genetic variability has been lost in the course of years, and especially increased percentage of males and females exhibiting high AR values warn that the threat of extinction still looms over the breed.

Moreover, the effective population size is from considerably to slightly lower than the range of the minimum effective size, which may balance the inbreeding depression, approaching the estimated minimum viable effective size for the preservation of endangered species.

6. Although inbreeding rate in the current and historical populations was acceptable (under 1%), its value in the contrast population set, alarmingly differs from the recommended value (+0.23%) and indicates that the more genealogical information is known, the more endangered the breed reveals it actually is.

7. Careful genetic management is necessary to minimize inbreeding practices and enhance genetic variation. Thus, measures such as the use of artificial insemination and other new breeding technologies such as embryo vitrification, need to be implemented to contain the inbreeding rate and increase the effective population size of the worldwide donkey populations which frequently lack genetic knowledge, assessing the percentage of relationships that reproductive pairs share in each case, selecting individuals for mating when these relationships are kept below 6% in the Andalusian breed, studied to infer the general model presented.

- **Second group of conclusions.** (Second publication: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; McLean, A.K.; Delgado Bermejo, J.V. Non-parametric analysis of the noncognitive determinants of response type and response intensity, mood and learning in donkeys. Submitted to *Journal of Veterinary Behavior: Clinical Applications and Research.*"):

1. Location, management and farm characteristics and test related factors can condition the responses, their intensity, the mood and extinction/learning ability of donkeys. However, there is still a remarkable inherent component modulated by age, sex or parental background.

2. The multistage operant conditioning test applied enables efficiently and significantly quantifying several factors related to donkey cognition and behaviour.

3. The ethogram that we describe faces the popular knowledge on how different body language signals report a certain donkey's feelings and stablishing a formal description of the collateral signs that donkeys regularly display when describing certain mood or temperament patterns could be the key to the early diagnoses and treatment of potentially life-threatening conditions.

4. The application of luring/positive reinforcement techniques overcomes the cognitive results obtained by negative reinforcement as they do not only allow the animals to accomplish certain tasks but also let them do it in a closer to natural way, generating

less welfare related problems, which given the endangerment risk to which the donkey species is exposed may be vital for the preservation of new functional niches promoting the reintroduction of these valuable animals back to their relevant role in human practices.

- **Third group of conclusions.** (Third publication: "Navas González, F.**J.**; Jordana Vidal, J.; Pizarro Inostroza, G.; Arando Arbulu, A.; Delgado Bermejo, J.V. Can Donkey Behavior and Cognition Be Used to Trace Back, Explain, or Forecast Moon Cycle and Weather Events? *Animals* **2018**, *8*, 215."):

1. Environmental conditions, seasonal, timing (year) and moon cycle phases are potential stress factors or behavioural modulators that affect the behaviour and cognitive responses of donkeys, as well as may have potential long lasting effects which can be traced back.

2. Climate oscillation effects may affect donkeys altering their physiological biorhythms and produce severe behavioural and cognitive modifications. Deviations in behavioural patterns or on the abilities of the donkeys to perform complex tasks to which they may not be accustomed, may become relevant indicators of welfare as well as they may address the most suitable techniques or methods to be applied in each case.

3. Behaviour becomes a relevant tool when predicting future weather conditions as well as may report the potential distortion that they may cause, a prominent importance fact for veterinarians, practitioners and donkey owners, as it may allow them to anticipate such situations to counteract their effects.

- **Fourth group of conclusions.** (Fourth publication: "Navas, F.J.; Jordana, J.; León, J.M.; Arando, A.; Pizarro, G.; McLean, A.K.; Delgado, J.V. Measuring and modeling for the assessment of the genetic background behind cognitive processes in donkeys. *Research in Veterinary Science* **2017**, *113*, 105-114."):

1. The difficulty to find and control models to assess animal behaviour especially increases when we intend to do it under field practical situations. The levels of significance found, show that the model used to assess the coping style cluster is more accurate and suitable than the one used to test for intelligence and cognition traits. This situation not only enables a more objective quantification methodology for coping styles related traits but also reports more reliable global results.

2. The differences appearing because of the influence of the different fixed effects on the behavioural traits assessed may be attributed to the fact that the tests used may, in fact, evaluate the ability of certain owners to educate their donkeys rather than the inner cognitive capacity of the animals to develop a certain process.

3. Although sexual dimorphism is evident on some of the cognitive processes, the variation may be ascribed to differences in the handling methods and routines applied to jacks and jennies.

4. The husbandry system applied can help us group the animals to save the potential result distortion that may occur due to the unequal distribution of animals among the farms.

5. The fraction of variance explained by external factors may be low when we considered them individually, but it can improve when their partial weights are summarized. The variance explained by these multifactorial models permits comparatively considering them to be efficient to quantify the sixteen cognitive processes in our study, as they provide very useful information for the design and ease of the complex models used in behavioural genetic analyses.

6. Both double and triple interactions were mostly non-significant for intelligence and cognition clusters. This finding supports the fact that, in behavioural studies, the reliance on several factors individually, may help us quantify the factors or effects involved more accurately than their conjoint effects.

7. Our results suggest the suitability of the proposed cognitive recording system to be applied in the routinely genetic selection of donkeys.

8. These breeding criteria will be implemented in the future in order to make the donkey more commercially competitive and useful, not only aiming at saving animals but whole breeds from extinction.

- **Fifth group of conclusions.** (Fifth publication: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; Arando Arbulu, A.; McLean, A.K.; Delgado Bermejo, J.V. Genetic parameter and breeding value estimation of donkeys' problem-focused coping styles. *Behavioural Processes* **2018**, *153*, 66-76."):

1. It can be concluded that moderate heritabilities for reactivity related traits such as response type, mood/emotion and degree/intensity were obtained after the evaluation of the behavioural tests and of the information of the field sheets associated.

2. The accuracy of these estimates was high as well, even more when considering the limited number of donkeys in the study, what may highlight the efficiency of the behavioural test and model designed to assess such traits.

3. It is essential to note that this study is the first to estimate a heritability of coping style/reactivity related traits measured in a practical situation related to a selection programme in donkey breeds.

4. The findings indicate that selection for reduced reactivity and fearfulness in donkeys is achievable, although it requires more research including more animals, a difficult task to achieve if we work at a breed level, considering the existing extinction risk that they are exposed to.

- **Sixth group of conclusions.** (Sixth publication: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M.; McLean, A.K.; Delgado Bermejo, J.V. Dumb or smart asses? Donkey's cognitive capabilities *(Equus asinus)* share the heritability and variation patterns of human's cognitive capabilities *(Homo sapiens)*. Submitted to *Journal of Veterinary Behavior: Clinical Applications and Research.*"):

1. Our results suggest donkeys could be considered somehow intelligent animals when comparatively scoring them relying on an analogous human scale. However, we do not intend to assert that some donkeys may account for a higher IQ than humans compared through the same scale.

2. The cognitive processes and methods to score them widely differ from one species to another. Furthermore, the more complex the cognitive development of the species being tested is, the more complex these methods should be. However, still, a remarkable variation among donkeys is found, i.e., there are donkeys which are more intelligent than others, and the present methodology enables quantifying such differences.

3. The remarkably similar phenotypical distribution and inheritance patterns described in asses may suggest intelligence could be ascribed to a similar scientific background or even be supported by a similar genetic structure to the one widely studied in humans. Such finding lays the basis for future research to deepen in the field of animal cognition. Our results suggest that donkey cognition heritable mechanisms may be attributed to human's similar genetic background.

4. This study opens the door to selection and breeding for better cognitively performing animal generations. Our methodology comprises a novel approach to the animal intelligence controversy, using a standard human applied method to score individual intelligence quotient.

- **Seventh conclusion.** (Seventh publication: "Navas González, F.J.; Jordana Vidal, J.; León Jurado, J.M..; McLean, A.K.; Pizarro Inostroza, G.; Delgado Bermejo, J.V. Genetic parameter estimation and implementation of the genetic evaluation for gaits in a breeding program for assisted-therapy in donkeys. *Veterinary research communications* 2018, *42*, 101-110."):

1. High levels for genetic parameters resembling the ones obtained for horses in literature were obtained for the different gait modalities described by donkeys. Such values enable the potential inclusion of locomotion traits within breeding programs seeking the genetic progress of donkey breeds, such as the Andalusian donkey.

2. The statistically non-significant effect of the appraisers suggested the success of a highly uniform scoring procedure among appraisers.

3. Genetic correlations were high and positive for all trait combinations, thus enabling the combined selection for both gaits, with low detrimental effect for either one.

4. Selection for certain gaits in donkeys may have traditionally been carried out indirectly, thus the routine application of the assessment including a greater number of animals is required to standardize the valuation methodology implemented in this study, a difficult task to achieve, considering the existing extinction risk of donkey breed endangered populations.

5. Given the favourable genetic relationships existing between the traits involved, gaits can play an important role in a selection program aimed at improving the suitability of donkeys for the treatment of specific motorial disabilities within assisted therapy programs.

6. The specific nature and magnitude of the existing genetic relationships of the functional traits assessed in this study may make interesting to consider the possibility of developing and maintaining specialized lines relying on the ability of the donkeys to develop certain gait patterns (amble or walk and trot) within the Andalusian donkey breeding program, as these different patterns may be especially suitable for the treatment of different human motor disabilities.

- **Eighth conclusion.** (Eighth publication: "Navas González, F.J.; Jordana Vidal, J.; Camacho Vallejo, M.E.; León Jurado, J.M.; de la Haba Giraldo, M.R.; Barba Capote, C.; Delgado Bermejo, J.V. Risk factor meta-analysis and Bayesian estimation of genetic parameters and breeding values for hypersensibility to cutaneous habronematidosis in donkeys. *Veterinary Parasitology* **2018**, *252*, 9-16."):

   1. The low levels for genetic parameters resemble the ones obtained in literature when assessing for the hypersensibility of other species to similar parasitoses.

2. Although these values compromise the potential inclusion of disease hypersensibility related traits within breeding programs seeking the genetic progress of the breed, the benefits that could be obtained from negative selection against this prevalent condition may be worth considering.

3. Farms in which the donkeys are handled under extensive conditions, where the animals are able to more openly react to the action of carrier vectors such as flies, stand out over those farms in which the combined presence of predisposing factors addressed by literature may contribute to the development of the disease.

4. The low genetic component highlighted reveals prophylactic measures and prevention against the parasites is key for the protection of the donkeys from suffering from this skin condition, as ivermectin continuous treatment may cause resistance.

5. Our model results highly suitable for the assessment of the background possibly contributing to the greater development of this disease as suggested by its high sensibility and specificity.

6. Selection against disease hypersensibility parameters in donkeys is unlikely to have traditionally been carried out indirectly, therefore the routine application of the assessment including a greater number of animals is required to standardize the assessment methodology implemented in this study.

7. Considering the extinction risk that donkey breed endangered populations face generally, any measure aiming at the improvement of welfare in this species may be worth-considering.

- **Ninth conclusion.** (Ninth publication: "Navas González, F.J.; Jordana Vidal, J.; McLean, A.K.; León Jurado, J.M.; Barba, C.J.; Arando, A.; Delgado Bermejo, J.V. Modeling for the Inheritance of Endangered Equid Multiple Births and Fertility: Determining Risk Factors and Genetic Parameters in Donkeys *(Equus asinus)*. Submitted to *Research in Veterinary Science*"):

1. High values for genetic parameters enable the potential inclusion of these traits within breeding programs seeking the genetic progress of donkey breeds.

2. Positive and moderate genetic correlations enable the combined selection for maximum foal number per birth and historical foal number born per donkey, with low detrimental effect for either one.

3. 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.

4. 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.

5. The present results enable 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 specialized 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, what in the end translates in the improvement of the reproductive welfare of the individuals.

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Indicios de calidad

▪ Primera publicación:

- Título: *A model to infer the demographic structure evolution of endangered donkey populations.*
- Autores (por orden de firma): ***Navas, F.J.***; *Jordana, J.; León, J.M.; Barba, C.; Delgado, J.V.*
- Revista (año, volúmen, páginación): *animal* **2017**, *11*, 2129-2138.
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports (JCR), 2017.*
- Área temática en la Base de Datos de referencia: *Agriculture, Dairy and Animal Science.*
- Índice de impacto de la revista en el año de publicación del Artículo: *1,870.*
- Lugar que ocupa/Nº de revistas del Área temática: *10/60 (Q1/T1).*

▪ Segunda publicación:

- Título: *Non-parametric analysis of the noncognitive determinants of response type and response intensity, mood and learning in donkeys.*
- Autores (por orden de firma): ***Navas González, F.J.***; *Jordana Vidal, J.; León Jurado, J.M.; McLean, A.K.; Delgado Bermejo, J.V.*
- Revista (año, volúmen, páginación): *Journal of Veterinary Behavior: Clinical Applications and Research. Submitted on 29th May, 2019.*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports (JCR), 2017 datos año previo. Datos para 2018 no publicados.*
- Área temática en la Base de Datos de referencia: *Veterinary sciences.*
- Índice de impacto de la revista en el año de publicación del Artículo: *1.554*
- Lugar que ocupa/Nº de revistas del Área temática: *35/140 (Q1/T1).*

▪ Tercera publicación:

- Título: *Can Donkey Behavior and Cognition Be Used to Trace Back, Explain, or Forecast Moon Cycle and Weather Events?*
- Autores (por orden de firma): ***Navas González, F.J.****; Jordana Vidal, J.; Pizarro Inostroza, G.; Arando Arbulu, A.; Delgado Bermejo, J.V.*
- Revista (año, volúmen, paginación): *Animals* **2018**, *8*, 215.
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports (JCR), 2017 cuando el artículo fue enviado. Datos para 2018 no publicados.*
- Área temática en la Base de Datos de referencia: *Veterinary sciences.*
- Índice de impacto de la revista en el año de publicación del Artículo: *1,654*
- Lugar que ocupa/Nº de revistas del Área temática: *29/140 (Q1/T1).*

▪ Cuarta publicación:

- Título: *Measuring and modeling for the assessment of the genetic background behind cognitive processes in donkeys.*
- Autores (por orden de firma): ***Navas, F.J.****; Jordana, J.; León, J.M.; Arando, A.; Pizarro, G.; McLean, A.K.; Delgado, J.V.*
- Revista (año, volúmen, paginación): *Research in Veterinary Science* **2017**, *113*, 105-114.
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports (JCR), 2017.*
- Área temática en la Base de Datos de referencia: *Veterinary sciences.*
- Índice de impacto de la revista en el año de publicación del Artículo: *1,616*
- Lugar que ocupa/Nº de revistas del Área temática: *31/140 (Q1/T1).*
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▪ Quinta publicación:

- Título: *Genetic parameter and breeding value estimation of donkeys' problem-focused coping styles.*
- Autores (por orden de firma): ***Navas González, F.J.****; Jordana Vidal, J.; León Jurado, J.M.; Arando Arbulu, A.; McLean, A.K.; Delgado Bermejo, J.V.*
- Revista (año, volúmen, paginación): *Behavioural Processes* ***2018****, 153, 66-76.*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports (JCR), 2017 datos año previo. Datos para 2018 no publicados.*
- Área temática en la Base de Datos de referencia: *Zoology.*
- Índice de impacto de la revista en el año de publicación del Artículo: *1,746*
- Lugar que ocupa/Nº de revistas del Área temática: *40/163 (Q2/T1)*

- **Sexta publicación:**
    - Título: *Dumb or smart asses? Donkey's cognitive capabilities (Equus asinus) share the heritability and variation patterns of human's cognitive capabilities (Homo sapiens).*
    - Autores (por orden de firma): ***Navas González, F.J.****; Jordana Vidal, J.; León Jurado, J.M.; McLean, A.K.; Delgado Bermejo, J.V.*
    - Revista (año, volúmen, paginación): *Journal of Veterinary Behavior: Clinical Applications and Research. Submitted on 10th February, 2019.*
    - Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports (JCR), 2017 datos año previo. Datos para 2018 no publicados.*
    - Área temática en la Base de Datos de referencia: *Veterinary sciences.*
    - Índice de impacto de la revista en el año de publicación del Artículo: *1.554*
    - Lugar que ocupa/Nº de revistas del Área temática: *35/140 (Q1/T1).*

- **Séptima publicación:**
    - Título: *Genetic parameter estimation and implementation of the genetic evaluation for gaits in a breeding program for assisted-therapy in donkeys.*
    - Autores (por orden de firma): ***Navas González, F.J.****; Jordana Vidal, J.; León Jurado, J.M..; McLean, A.K.; Pizarro Inostroza, G.; Delgado Bermejo, J.V.*
    - Revista (año, volúmen, paginación): *Veterinary research communications* 2018, *42*, 101-110.
    - Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports (JCR), 2017.*
    - Área temática en la Base de Datos de referencia: *Veterinary sciences.*
    - Índice de impacto de la revista en el año de publicación del Artículo: *1,933*
    - Lugar que ocupa/Nº de revistas del Área temática: *21/140 (Q1/T1).*

- **Octava publicación:**
    - Título: *Risk factor meta-analysis and Bayesian estimation of genetic parameters and breeding values for hypersensibility to cutaneous habronematidosis in donkeys.*
    - Autores (por orden de firma): ***Navas González, F.J.****; Jordana Vidal, J.; Camacho Vallejo, M.E.; León Jurado, J.M.; de la Haba Giraldo, M.R.; Barba Capote, C.; Delgado Bermejo, J.V.*
    - Revista (año, volúmen, paginación): *Veterinary Parasitology* **2018**, *252*, 9-16.
    - Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports (JCR), 2017.*
    - Área temática en la Base de Datos de referencia: *Veterinary sciences.*
    - Índice de impacto de la revista en el año de publicación del Artículo: *2,422*
    - Lugar que ocupa/Nº de revistas del Área temática: *9/140 (D1/Q1/T1).*

- **Novena publicación:**
  - Título: *Modeling for Inheritance of Endangered Equid Multiple Births and Fertility: Determining Risk Factors and Genetic Parameters in Donkeys (Equus asinus).*
  - Autores (por orden de firma): ***Navas González, F.J.****; Jordana Vidal, J.; McLean, A.K.; León Jurado, J.M.; Barba, C.J.; Arando, A.; Delgado Bermejo, J.V.*
  - Revista (año, volúmen, páginación): *Research in Veterinary Science. Submitted on 5th June, 2019.*
  - Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports (JCR), 2017. Datos para 2018 no publicados.*
  - Área temática en la Base de Datos de referencia: *Veterinary Sciences.*
  - Índice de impacto de la revista en el año de publicación del Artículo: *1,616*
  - Lugar que ocupa/Nº de revistas del Área temática: *31/140 (Q1/T1).*

Producción científica

Otras aportaciones científicas derivadas directamente de la Tesis Doctoral:

Libros y capítulos de libro:

- **Navas, F.J.**; Delgado, J.V.; Vargas, J. *Current donkey production and functionality: Relationships with humans. Book 1*; UcoPress: Córdoba, Spain, 2016. ISBN: 978-84-9927-335-8.

- **Navas, F.J.**; Delgado, J.V.; Vargas, J. Funcionalidad y Producción Asnal Actual. Relaciones Con los Humanos. Libro 1; UcoPress: Córdoba, Spain, 2016. ISBN: 978-84-9927-255-9.

Otras Publicaciones en Revistas Indexadas en el JCR:

- McLean, A.K.; **Navas Gonzalez, F.J.** Can Scientists Influence Donkey Welfare? Historical Perspective and a Contemporary View. *Journal of Equine Veterinary Science* **2018**, 65, 25-32.

Podcasts:

- **Navas González FJ.** 2018. El burro y el hombre, historia y mito. En Ostáriz R, (Ed.). *El Libro Rojo.* iTunes: Ritxi Ostáriz. Emitido el 27/02/2018.

▪ Trabajos bibliográficos y de divulgación:

- **Navas González, F.J.;** Delgado Bermejo, J.V. Herencia fenotípica histórica y dimorfismo sexual en la raza asnal Andaluza/Phenotypical inheritance and sexual dimorphism in the Andalusian donkey breed. *FEAGAS* **2017**, *XXIII*, 88-98.

- **Navas, F.;** Miró-Arias, M.; Delgado, J. Preliminary assessment methodology of temperament in the Andalusian donkey breed. Actas Iberoamericanas de Conservación Animal AICA 2013, 3, 20-28.

- Delgado, J.V.; **Navas, F.J.;** Miranda, J.C.; Miró, M.; Arando, A.; Pizarro, M.G. Preliminary Body Weight Estimation Methodology and its Applicaction to the Andalusian Donkey Breed as an Energetic Producer. Actas Iberoamericanas de Conservación Animal 2014, 4, 207-209.

- **Navas, F.J.;** Miró-Arias, M.; Delgado, J. Preliminary proposal for a methodology of the assessment of body language with means of trainability in the Andalusian donkey breed. Actas Iberoamericanas de Conservación Animal AICA 2012, 2, 123-128.

▪ Contribuciones a Congresos:

a) Comunicaciones orales

- **Navas González, F.J.;** Arando, A.; Pizarro, G.; Miranda, J.C.; Miró-Arias, M.; Delgado, J.V. Estimación práctica de los parámetros zoométricos en la Raza Asnal Andaluza. En XVI Simposio sobre conservación y utilización de recursos zoogenéticos, 7-9 Octubre, 2015, Villavicencio (Colombia).

- **Navas González, F.J.**; León Jurado, J.M.; Delgado Bermejo, J.V. Metodología de cálculo del cociente intelectual para la cuantificación de la variabilidad fenotípica de los procesos cognitivos en animales. En SERGA/SPREGA, Proceedings del XI Congreso Ibérico sobre Recursos Genéticos Animales, 27-28 Septiembre, 2018, Beniaján, Murcia (España); p. 115.

b) Ponencias invitadas

- **Navas González, F.J.** Functional Genetics in Donkeys: Genetics workshop. En Proceedings del 5th Annual Donkey Welfare Symposium, University of California Davis, Davis, California (Estados Unidos), 4-6 Noviembre, 2017.

- **Navas González, F.J.** Functional Genetics in Donkeys: Disease hypersensibility & welfare potentialities. En Proceedings del 5th Annual Donkey Welfare Symposium, University of California Davis, Davis, California (Estados Unidos), 4-6 Noviembre, 2017.

- **Navas González, F.J.** Functional Genetics in Donkeys: Outlining the genetics behind cognition and intelligence. En Proceedings del 6th Annual Donkey Welfare Symposium, University of California Davis, Davis, California (Estados Unidos), 26-28 Octubre, 2018.

c) Proceedings

- Delgado Bermejo, J.V.; **Navas González, F.J.;** Miranda Alejo, J.C.; miró-Arias, M.; Arando Arbulu, A.; Pizarro Inostroza, G. Metodología preliminar de estimación del peso corporal y su aplicación a la raza asnal andaluza como productor energético. En Proceedings del XV Simposio Iberoamericano sobre la Conservación y Utilización de los Recursos Zoogenéticos, 8-10 Octubre, 2014, San Cristóbal de las Casas (Chiapas, México).

d) Posters

- **Navas González, F.J.;** Miró-Arias, M.; Delgado Bermejo, J.V. Metodología preliminar de evaluación del temperamento en su aptitud para el entrenamiento como base del estudio de la funcionalidad en la raza asnal Andaluza. En VIII Congresso Ibérico sobre Recursos Genéticos Animais, 13-15 Septiembre, 2012, Évora (Portugal).

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