UNIVERSIDAD DE CÓRDOBA

MEJORA GENÉTICA DE LA APTITUD PARA EL TROTE: DETECCIÓN DE SNPs DE GENES RELACIONADOS CON EL RENDIMIENTO DEPORTIVO Y EL TEMPERAMENTO DE UTILIDAD PARA LA SELECCIÓN ASISTIDA POR MARCADORES EN EL CABALLO TROTADOR ESPAÑOL

GENETIC SELECTION FOR TROTTING ABILITY: SNP DETECTION IN GENES ASSOCIATED WITH SPORTING PERFORMANCE AND TEMPERAMENT USEFUL FOR THE MARKER ASSISTED SELECTION IN THE SPANISH TROTTER HORSE

TESIS DOCTORAL

SARA NEGRO RAMA

Córdoba, 2017

TITULO: *Mejora genética de la aptitud para el trote: detección de SNPs de genes relacionados con el rendimiento deportivo y el temperamento de utilidad para la selección asistida por marcadores en el caballo trotador español*

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MEMORIA DE TESIS DOCTORAL PRESENTADA POR

SARA NEGRO RAMA

Para optar al Grado de Doctor con Mención Internacional
por la Universidad de Córdoba

DIRECTORES

Dra. Mercedes Valera Córdoba Dr. Antonio Molina Alcalá Dra. Marina Solé Berga

Córdoba, 20 de Junio de 2017

TÍTULO DE LA TESIS: **MEJORA GENÉTICA DE LA APTITUD PARA EL TROTE: DETECCIÓN DE SNPs DE GENES RELACIONADOS CON EL RENDIMIENTO DEPORTIVO Y EL TEMPERAMENTO DE UTILIDAD PARA LA SELECCIÓN ASISTIDA POR MARCADORES EN EL CABALLO TROTADOR ESPAÑOL.**

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DOCTORANDO/A: **SARA NEGRO RAMA**

INFORME RAZONADO DE LOS DIRECTOR/ES DE LA TESIS Y EL TUTOR (se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La Tesis Doctoral que se presenta está integrada por dos artículos publicados en revistas ISI, de gran impacto en el campo de la Ciencia Animal y dos artículos que actualmente se encuentran en fase de revisión en dos revistas indexadas. La Tesis se ha estructurado en tres capítulos:

Capítulo I: "**Caracterización genética de la raza del Caballo Trotador Español y diferenciación con el resto de razas de deporte españolas a partir de marcadores moleculares".** En el primer capítulo se ha abordado una caracterización, diferenciación y análisis de variabilidad genética en seis razas equinas de deporte españolas utilizando el panel de marcadores moleculares de tipo microsatélite para estudiar como los programas de mejora genética han contribuido a optimizar la selección y mejora de las razas equinas manteniendo a la vez la variabilidad dentro de cada raza. Este capítulo está integrado por 1 trabajo:

- **Negro S**, Solé M, Pelayo R, Gómez MD, Azor PJ, Valera M. *Molecular diversity between two cohorts of six Spanish riding-horse breeds: impact of selection in Crossbred vs Purebred populations*. Livestock Science, 193, 88-91. Doi: 10.1016/jlivsci.2016.09.013.
    - Impact Index: 1,377 (Journal Citation Report, 2016).
    - Subject and Quartile: Agriculture, Dairy, Animal Science, 2nd quartile.

Capítulo II: "**Evaluación del nivel de estrés durante las carreras de trote mediante nuevas técnicas no invasivas y búsqueda de genes asociados con este carácter en el Caballo Trotador Español".** En el segundo capítulo se evalúa el nivel de estrés durante las carreras en el Caballo Trotador Español a partir de la temperatura ocular con Termografía Infrarroja y de la frecuencia cardiaca, y se estudia su relación con el rendimiento deportivo y se determina el nivel umbral de estrés que conduce a los mejores resultados deportivos o por el contrario que lleva a distrés en esta raza. Posteriormente se estudia su asociación con diferentes polimorfismos de un solo

nucleótido (SNPs) en varios genes candidatos de genes de comportamiento (*BDNF, COMT, HTR1A, SLC6A4* y *TPH2*). Este capítulo está integrado por dos trabajos:

- **Negro S,** Bartolomé E, Molina A, Solé M, Gómez MD, Valera M. 2017. *Stress level effects on sport performance during trotting races in Spanish Trotter horse. Equine Vet J* (2017). EVJ-GA-17-138, under review.
    - Impact Index: 2,382 (Journal Citation Report, 2016).
    - Subject and Quartile: Veterinary Sciences, 1st quartile.

  Negro S., Valera M., Solé M., Bartolomé E., Sánchez M.J., Gómez, M.D., Molina, A. *Evidence for the effect of serotoninergic and dopaminergic gene variants on stress levels in horses participating in dressage and harness racing.* Anim. Genet., (2017). AnGen-17-06-0134, under review.
    - Impact Index: 1,815 (Journal Citation Report, 2016).
    - Subject and Quartile: Agriculture, Dairy, Animal Science, 1st quartile.

Capítulo III: **"Análisis genético cuantitativo del caballo trotador español y búsqueda de asociación de marcadores moleculares tipo SNP en genes candidatos".** En el tercer capítulo se ha contemplado el estudio de diferentes polimorfismos de un solo nucleótido (SNPs) en varios genes candidatos de rendimiento deportivo (*MSTN, DMRT3, COX4I2, PDK4* y *CKM*) y comportamiento (*BDNF, COMT, HTR1A, SLC6A4* y *TPH2*) en el caballo Trotador Español. Este capítulo está integrado por un trabajo:

- **Negro S.**, Valera M., Membrillo A., Gómez, M.D., Menendez-Buxadera, A., Anaya, G., Molina, A. *Quantitative analysis of short and long distance racing performance in young and adult horses and association analysis with functional candidate genes in Spanish Trotter Horses.* J. Anim. Breed. Genet. 133(5), 347–356. ISSN 0931-2668. (JBG12208).
    - Impact Index: 1,877 (Journal Citation Report, 2016).
    - Subject and Quartile: Agriculture, Dairy, Animal Science, 1st quartile.

Por lo tanto, por la presente consideramos que el trabajo realizado por D. Sara Negro Rama, bajo nuestra dirección y tutela, presenta unos elevados niveles de innovación y calidad y autorizamos su presentación y defensa como Tesis Doctoral por la Universidad de Córdoba.

Córdoba, 20 de Junio de 2017

Firma de los directores

Dra. Mercedes Valera Córdoba	Dr. Antonio Molina Alcalá	Dra. Marina Solé Berga
Catedrática, ETSIA, Universidad de Sevilla	Catedrático del Dpto. Genética Universidad de Córdoba	Investigadora Postdoctoral Universidad de Lieja

TÍTULO DE LA TESIS: **MEJORA GENÉTICA DE LA APTITUD PARA EL TROTE: DETECCIÓN DE SNPs DE GENES RELACIONADOS CON EL RENDIMIENTO DEPORTIVO Y EL TEMPERAMENTO DE UTILIDAD PARA LA SELECCIÓN ASISTIDA POR MARCADORES EN EL CABALLO TROTADOR ESPAÑOL.**

DOCTORANDA: **SARA NEGRO RAMA**

MENCIÓN DE DOCTORADO INTERNACIONAL

Esta Tesis reúne los requisitos establecidos en el artículo 35 de Normativa de Doctorado de la Universidad de Córdoba para la obtención del título de Doctor con Mención Internacional:

- Estancia internacional predoctoral de 3 meses (01/09/2014 AL 01/12/2014) en la Centro de Bioquímica Médica y Microbiología de la Universidad de Upsala (Suecia) bajo la supervisión del Dr. Leif Andersson.
- La Tesis cuenta con el informe previo de dos doctores expertos y con experiencia investigadora acreditada perteneciente a alguna institución de educación superior o instituto de investigación de fuera de España:

  Dra. Pilar Peral García. Department of Animal Science. National Scientific and Technological Research Council (CONICET). Argentina.

  Dra. Llibertat Tusell Palomero. Department GenPhySE. INRA. Toulouse, Francia.

Parte de esta Tesis se ha redactado en español y parte en inglés y será presentada en estos dos idiomas. Para facilitar la lectura de la Tesis, aunque al final de cada capítulo aparecen las referencias correspondientes, al final de la memoria de la Tesis se vuelven a recoger junto con las referencias de la Introducción general y Discusión general en el apartado REFERENCIAS BIBLIOGRÁFICAS.

”Dime y lo olvido,
enséñame y lo recuerdo,
involúcrame y lo aprendo”.

‘Tell me and I forget,
teach me and I remember,
involve me and I learn.’

(*Benjamin Franklin*)

A mis padres y mis hermanos,

por mostrarme un amor incondicional con cada abrazo,

por ofrecerme todo su apoyo en este recorrido,

y sobre todo por creer en mí.

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Aprovecho para agradecer, de forma destacada, la labor de la Asociación de Criadores y Propietarios de Caballos Trotadores (ASTROT) en el marco del Programa de Mejora del Caballo Trotador Español, que con su buen hacer está favoreciendo la mejora genética de la Raza. Así mismo, agradecer la colaboración de la Federación Balear de Trote, de los técnicos, de los ganaderos, de los conductores que han contribuido a generar los datos de rendimientos reflejados en los valores genéticos.

ÍNDICE

ÍNDICE DE CONTENIDOS

ABREVIATURAS

AÁ – Caballo de Raza Anglo-árabe
ASTROT- Asociación de Criadores y Propietarios de Caballos Trotadores
BDNF - Factor neurotrófico derivado del cerebro
BLUP - Best linear unbiased prediction
CDE - Caballo de Deporte Español
CKM - Creatina quinasa muscular
COMT - Catecol-O-transferasa
COX4I2 - Citocromo C oxidasa subunidad 4, isoforma 2
CTE - Caballo Trotador Español
DMRT3 - Factor de transcripción 3 relacionado con mab-3 y el doble sexo
FC - Frecuencia Cardiaca
FBT - Federación Balear de Trote
HPA - Eje Hipotálamo-Pituitario-Adrenal
HTR1A - Receptor 5-hidroxitriptamina (serotonina) 1A
LG - Libro Genealógico
MAPAMA - Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente
MAS - Selección Asistida por Marcadores
MERAGEM - Mejora de Razas y Genética Molecular
MSTN - Miostatina
PDK4 - Piruvato desqhidrogenasa quinasa isoenzima 4
PRÁ - Caballo de Pura Raza Árabe
PRE - Caballo de Pura Raza Español
PRMe - Caballo de Pura Raza Menorquina
RRM - Modelo de Regresión Aleatoria
SLC6A4 - Transportador de serotonina
SNP - Polimorfismo de un solo nucleótido
TO - Temperatura Ocular
TPH2 - Triptófano hidroxilasa 2
TPK - Tiempo por kilómetro
VGED – Valor genético estimado deregresado

RESUMEN / SUMMARY

RESUMEN

El Caballo Trotador Español (CTE), *Raza Equina Integrada*, es la cuarta raza en importancia en función del número de efectivos registrados en nuestro país. Su impacto económico anual es de aproximadamente de 34 millones € al año. Su Programa de Mejora se aprobó en 2005 con el objetivo principal de mejorar el rendimiento del animal en carreras de trote, y siendo uno de sus principales criterios de selección la variable tiempo por kilómetro (TPK). El actual sistema de valoración, el BLUP (modelo animal de repetibilidad multivariable) asume que el potencial genético del animal es el mismo a lo largo de su vida o para cualquier tipo de carrera (distancia). Este sistema clásico precisa de una exhaustiva toma de datos a lo largo de la vida del animal para conseguir valores fiables, provocando que se clasifiquen como "Mejorantes" por primera vez animales con edades avanzadas. Por otra parte, la selección de los animales en base a su nivel genético determina a largo plazo un incremento del nivel de parentesco con los animales con mayor potencial genético, lo que a la larga puede traer consecuencias negativas en la población, tales como disminución de su variabilidad genética y el aumento de consanguinidad.

Por ello, el objetivo que se persigue en la presente Tesis Doctoral es en primer lugar detectar si el impacto de la selección ha reducido la diversidad genética del CTE. A la vez, incluir en el sistema de valoración el nivel de estrés, validando la termografía infrarroja de fondo de ojo como herramienta de evaluación objetiva y no invasiva y ver si está relacionada con el rendimiento deportivo. Finalmente, para disminuir la edad para la obtención de animales con prueba de descendencia superada sería útil identificar marcadores moleculares en genes candidatos que estuvieran relacionados tanto con el rendimiento deportivo así como con el estrés del animal para la selección precoz de los animales de esta raza. Para la selección de animales a genotipar se utilizará una estrategia de búsqueda de animales extremos (alto potencial genético y bajo potencial genético para la aptitud deportiva para el trote) con el fin de incrementar la potencia (o capacidad de detección de asociaciones estadísticamente significativas), utilizando para ello los valores genéticos deregresados y una nueva metodología de valoración genética mediante RRM (modelo de regresión aleatoria) para el rendimiento deportivo que permita estimar la evolución del valor genético (potencial genético) a la largo de la vida del animal, así como para carreras de corta distancia y carreras de larga distancia.

Para la evaluación del grado de diferenciación y el impacto sobre la variabilidad molecular a lo largo de generaciones en el CTE, se compararon las poblaciones equinas de deporte de libro genealógico (LG) abierto (la del CTE junto al Anglo-árabe o AÁ y el Caballo de Deporte Español o CDE) y LG cerrado (Pura Raza Árabe o PRÁ, Pura Raza Español o PRE, y Pura Raza Menorquín o PRMe) trabajando con un total de 12.632 animales y con dos generaciones (2.530 caballos del el 1989 al 2000, y 10.102 del 2001 al 2012), a través el análisis de 17 marcadores moleculares tipo microsatélite.

En primer lugar, nuestros resultados han demostrado que el CTE presenta alta variabilidad genética, fruto por un lado de su origen multipoblacional y por otro del carácter abierto de su LG, con valores de los diferentes parámetros utilizados (número efectivo de alelos, heterocigosidad…) entre los más altos descritos en las poblaciones equinas.

Por otra parte, para evaluar el nivel de estrés en el CTE y determinar si afecta al rendimiento deportivo en el CTE, se ha medido la temperatura ocular (TO) con termografía infrarroja de fondo de ojo y junto a la frecuencia cardiaca (FC) en 130 animales 2h antes y justo después de la carrera para determinar si hay asociación con los resultados deportivos utilizando un GLM; el umbral de estrés por regresión segmentada, y las condiciones óptimas de TO basal que maximicen el rendimiento deportivo mediante el modelo de superficie de respuesta seguido de un análisis de regresión robusta. Nuestros resultados han determinado por primera vez el umbral de estrés que potencie el mayor rendimiento deportivo en el CTE (incremento de TO o ΔTO = -0,97), observándose una disminución del TPK a 77,3s cuando el valor de TO basal alcanza los 37,6ºC y ΔTO es de 7,6%. Posteriormente, se genotiparon 135 CTE junto a 135 PRE que participaban en doma para genes candidatos de comportamiento (*BDNF*, *COMT*, *HTR1A*, *SLC6A4* y *TPH2*) y ver su posible asociación con el estrés durante competición utilizado un modelo mixto unificado que tiene en cuenta la estructura poblacional y el parentesco entre los animales a analizar. Nuestros resultados han mostrado una asociación significativa con los genes *SLC6A4* (transportador de serotonina), para la TO en las tres fases de la toma de medida en la carrera, y *COMT* (relacionado con la degradación de catecolaminas), para la TO en fase de recuperación o 2h después de la carrera.

Para la selección de los animales a genotipar para el análisis de asociación de determinados genes candidatos (*COX4I2*, *CKM*, *DMRT3*, *MSTN* y *PDK4)* se realizó la puesta a punto del modelo de RRM y se estimaron los parámetros genéticos utilizando 334.516 resultados de carrera para la variable tiempo por kilómetro (TPK) pertenecientes a 5.958 CTE. La heredabilidad de la variable TPK varió en función de la distancia de 0,16 a 0,40, existiendo una tendencia a disminuir con el aumento de la distancia y la edad, confirmando la idoneidad de su utilización para la evaluación genética de los animales a genotipar. A partir de las valoraciones genéticas obtenidas en función de la edad del animal (jóvenes: de 2 a 4 años, y adultos: de 5 a 8 años) y de la distancia recorrida (1600m y 2600m) se obtuvieron los valores genéticos estimados deregresados. Así se genotiparon 321 animales con el mayor y el menor potencial para el trote (5% superior e inferior en el ranking) buscando asociación mediante regresión logística y robusta con los SNPs de los genes *COX4I2*, *CKM*, *DMRT3*, *MSTN* y *PDK4*. Nuestros resultados han determinado una asociación estadísticamente significativa con los genes *PDK4* y *CKM* (relacionados con la con la resistencia cardiorrespiratoria), el primero con el mayor potencial de los caballos para carreras cortas, y el segundo para carreras largas o de resistencia. Mientras que el gen *DMRT3* (relacionado con la coordinación de las extremidades) está asociado con un mayor potencial en los caballos jóvenes y adultos para las carreras de velocidad y de resistencia.

Por tanto con esta Tesis, se ha mostrado que la introducción continua de sementales de otras poblaciones está compensando la posible pérdida de variabilidad determinada por la selección hacia la aptitud deportiva de Trote. Para la valoración del CTE, se ha confirmado que el rendimiento deportivo está influenciado por el nivel basal de estrés y que la mejor manera de medirlo es con técnicas objetivas y no invasivas como la temperatura del fondo de ojo mediante termografía infrarroja. Además se ha detectado el nivel umbral de estrés que conlleva a los mejores resultados deportivos, o por el contrario pone en peligro el bienestar del animal. Por último, el uso de herramientas moleculares a partir de la asociación encontrada tanto para el rendimiento deportivo como para el nivel de estrés podrían acelerar los procesos de selección con la creación de test genéticos para la preselección de potros al reducir principalmente costes de entrenamiento, y al permitir un incremento del progreso genético con una disminución del intervalo generacional.

SUMMARY

The Spanish Trotting Horse (CTE), an *Integrated Equine Breed*, is the fourth breed in importance according to the number of effectives registered in our country. Its annual economic impact is approximately of 34 million euros per year. Its Breeding Program was approved in 2005 with the main objective of improving the animal performance in trotting races, being one of its main selection criteria the variable time per kilometre (TPK). The actual genetic evaluation, the BLUP (multivariate repeatability animal model) assumes that the genetic potential of the animal is the same throughout its life for any type of race (distance). This classic system requires an exhaustive data collection throughout the life of the animal in order to obtain reliable breeding values, leading to the classification of "breeding stock" for the first time in animals of advanced age. On the other hand, the selection of animals based on their genetic level determines an increment, in long-term, of the kinship level between the animals with the highest genetic potential, which could have negative consequences on the population, like a decrease of their genetic variability and the increase of inbreeding.

Therefore, the aim of this Doctoral Thesis is first to detect if the impact of selection has reduced the genetic diversity of the CTE. At the same time, the stress level should be included in the genetic evaluation, validating the measure of eye temperature with infrared thermography as a reliable and non-invasive tool and see if it is related to sporting performance. Finally, in order to decrease the age for obtaining animals that get proved progeny testing, it would be useful to identify molecular markers in candidate genes related to sporting performance as well as stress level for the early selection of the animals belonging to this breed. For the genotyping, the selection of the top and bottom 5% of the animals in the genetic ranking of sporting performance will be made in order to increase the power or detection capacity of statistically significant associations. For that purpose, the deregressed estimated breeding values based on the new methodology of RRM (random regression model) for the sporting performance will be used. This method allows estimating the evolution of the breeding value (genetic potential) throughout the life of the animal, as well as for short and long distance races.

For the evaluation of the degree of differentiation and the impact of selection on molecular diversity over time in the CTE, the Spanish riding-horse populations were compared: crossbreds (the CTE with the Anglo-Arab or AÁ and the Spanish Sport Horse or CDE) and purebreds (Arab Purebred or PRÁ, Pura Raza Español or PRE, and Menorca Purebred or PRMe) working with a total of 12,632 animals of two generations (2,530 horses from 1989 to 2000 and 10,102 from 2001 to 2012), using 17 microsatellite molecular markers.

First, our results have shown that CTE presents high genetic variability, due to its multipoblational origin and being an open population, being the values of the different parameters used (effective number of alleles, heterozygosity ...) among the highest described in horse populations.

On the other hand, to evaluate the stress level in the CTE and to determine if it affects the sporting performance in the CTE, the eye temperature (TO) using infrared thermography and the heart rate (HR) have been assessed in 30 animals 2h before and just after the race. A GLM determined association of TO and HR with sporting performance, a Segmented Regression Model estimated the threshold level of stress and a response surface model and ridge regression determined the optimum conditions of basal TO which maximize sporting performance. Our results have determined for the first time the threshold level of stress which potentiates the highest sporting performance in the CTE (increase of TO or ΔTO = -0.97), observing a decrease of TPK to 77.3s when the value of basal TO and ΔTO reached values of 37.6ºC and is 7.6%, respectively. Subsequently, 135 CTE were genotyped together with 135 PRE, participating in dressage, for behaviour candidate genes (*BDNF*, *COMT*, *HTR1A*, *SLC6A4* and *TPH2*) to analyse their possible association with stress during competition using a unified mixed model (counting for population structure and individual relatedness). Our results have shown a significant association with the *SLC6A4* (serotonin transporter) gene, for TO in the three phases of the race, and *COMT* (related to catecholamine degradation) gene, for recovery TO or 2h after the race.

The RRM model was used to estimate the genetic parameters of the variable TPK using 334,516 race results belonging to 5,958 CTE. These values were used for the selection of the animals to be genotyped for the association analysis of certain candidate genes (*COX4I2*, *CKM*, *DMRT3*, *MSTN* and *PDK4*) related to sporting performance. The heritability of the TPK variable varied from 0.16 to 0.40 according to the race distance, with a tendency to decrease with the increasing of the distance and age, confirming the suitability of its use for the genetic evaluation and genotyping. Based on the genetic evaluations obtained according to the age of the animal (young horses: from 2 to 4 years old, and adults: from 5 to 8 years old) and the race distance (1600m and 2600m), the deregressed estimated genetic values were obtained. Thus, 321 animals with the highest and lowest trotting potential (5% higher and lower in the ranking) were genotyped. Association analyses were performed using logistic and ridge regression models, for the SNPs of the genes *COX4I2*, *CKM*, *DMRT3*, *MSTN* and *PDK4*. Our results have determined statistically significant associations with the *PDK4* and *CKM* genes (related to cardiorespiratory resistance), the first with the horses with the greatest potential for short distance races, and the second for long or endurance races. While the *DMRT3* gene (related to the coordination of the limbs) has been associated to the young and adult horses with the highest potential for short and long distance races.

Therefore, with this thesis, it has been shown that the continuous introduction of stallions from other populations is compensating the possible loss of variability determined by the selection for trotting aptitude. For the genetic evaluation of the CTE, it has been confirmed that the sporting performance is influenced by the basal stress level and that the best way to measure it is with reliable and non-invasive techniques as the eye temperature using infrared thermography. In addition, the threshold level of stress that leads to the best sporting results, or on the contrary, compromises the welfare of the animal has been detected. Finally, the use of molecular tools based on the

association found for sporting performance and for stress level could accelerate the selection processes. This would allow the use of genetic tests for the pre-selection of foals by reducing mainly the training costs, and allowing an increased genetic progress with a decrease in the generation interval.

INTRODUCCIÓN

JUSTIFICACIÓN, HIPOTESIS DE PARTIDA Y OBJETIVOS

INTRODUCCIÓN: JUSTIFICACIÓN, HIPOTESIS DE PARTIDA Y OBJETIVOS

El Caballo Trotador Español

Los caballos de trote son la segunda población equina más importante a nivel mundial, después del Pura Sangre Inglés. Son caballos ligeros que han sido seleccionados buscando un movimiento de trote controlado en varios países tanto en Europa, como en EE.UU. y/o Canadá (Thiruvenkadan et al., 2009). El trote es una disciplina en la que el caballo debe correr empleando un aire simétrico de dos tiempos en el cual los bípedos diagonales se mueven sincronizados, de manera que cuando un bípedo inicia la flexión, el otro inicia la extensión (Agüera and Sandoval, 1999), sin romper el aire durante la carrera para no ser automáticamente descalificado (Burns et al., 2004). Aunque existen carreras de "trote montado", las más frecuentes son las de "trote enganchado", en las que el conductor permanece detrás del animal montado sobre un carro muy ligero de dos ruedas, conocido con el nombre de "sulky" (Thiruvenkadan et al., 2009). También existen diferencias entre carreras en función del tipo de salida empleado, existiendo carreras con salida de "handicap", en las que los animales parten de una distancia prefijada respecto al primer ejemplar en salir, en función de su rendimiento deportivo previo (a mayor rendimiento, mayor penalización en la distancia de salida) con el objetivo de intentar igualar el potencial de los caballos. El otro tipo de salida es la de "autostart", en la que los ejemplares inician la competición en iguales condiciones y a una velocidad constante que es marcada por un vehículo provisto de unos brazos mecánicos que precede al grupo de competidores, manteniendo la velocidad constante hasta el punto de salida (Gómez et al., 2010b).

Actualmente el Caballo Trotador Español (CTE) se encuentra reconocido como una Raza Equina Integrada, según el Catálogo Oficial de Razas de Ganado de España (Real Decreto 2129/2008, de 26 de diciembre, *por el que se establece el Programa nacional de conservación, mejora y fomento de las razas ganaderas*) siendo la cuarta raza en importancia en función del número de efectivos registrados en nuestro país (tras el Pura Raza Español, el Pura Raza Árabe y el Pura Sangre Inglés). Proviene del cruce de yeguas trotonas con caballos de Pura Sangre Inglés de origen danés, holandés y español, pudiendo considerarse una metapoblación de animales con una mayor o menor influencia de trotadores italianos, americanos o franceses. Esta hipótesis está corroborada por (Azor et al., 2007), que confirma que los antecedentes genéticos de la población actual de CTE no están basados en la población nativa de caballos de las Islas Baleares, su localización geográfica principal y donde se encuentra el núcleo de desarrollo principal de las carreras de trote y la mayor concentración de hipódromos..

Las carreras de trote son gestionadas por la Asociación de Criadores y Propietarios de Caballos Trotadores (ASTROT), que tiene como objetivo principal

fomentar la cría de animales y la práctica del deporte de carreras de caballos trotadores en España. Es también la encargada del control y desarrollo del programa de mejora específico para esta Raza en España, en colaboración con el personal del grupo de investigación MERAGEM, ostentando el grupo PAIDI AGR-273 de la Universidad de Sevilla la Dirección técnica del Esquema. Este Programa de Mejora fue aprobado oficialmente mediante Resolución de la Dirección General de Ganadería a fecha 1 de septiembre de 2005. El objetivo principal es conseguir un animal capaz de destacar en las carreras de trote en las que participe, a nivel nacional e internacional, que posea una conformación que favorezca esta funcionalidad y un temperamento que le haga ansiar la consecución de la victoria (MAPAMA, *Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente*). Los trotones con el mejor rendimiento de carreras son aquellos con mejores resultados para el tiempo por kilómetro, el porcentaje de primeros puesto por año, las ganancias anuales y el mejor tiempo por hipódromo y tipo de salida (Langlois, 1982). Estos caracteres presentan un parámetro genético de heredabilidad de una magnitud media a alta (h^2 = 0,14-0,46, (Gómez et al., 2010b) suficiente para su inclusión en el programa de mejora de los équidos para esta disciplina. Toda esta información junto con los registros informáticos de la Federación Balear de Trote (FBT), encargada de la recogida sistemática de los resultados de las pruebas para esta disciplina, se emplea para la Valoración Genética de estos animales.

Importancia económica del sector ecuestre ligado a las carreras de trote

El Sector Ecuestre en España genera un importante impacto económico (5.303,6 millones de €), representando el 0,51% del PIB del país. El estudio realizado del Impacto Económico del sector equino en España realizado por Deloitte (2013), indica que el mantenimiento del caballo de carreras significa el 12’5% del impacto económico en el sector de carreras. Considerando que dicho estudio en el sector carreras incluye costes de las actividades relacionadas en días de carreras, premios a propietarios y financiación y que el trote no obtiene ni la misma financiación ni las mismas dotaciones en premios de otro tipo de carreras realizadas en el estado, se estima que el porcentaje aproximado del mantenimiento del caballo de carreras de trote en este apartado significa el 20% del conjunto. Por dicho motivo se puede estimar que el impacto económico del caballo de carreras de trote en la economía de las Islas Baleares es de aproximadamente de 33.877.800€ al año. Añadir que en el 2016 la FBT, mediante los acuerdos internacionales con operadores de apuestas, ha invertido directamente al sector de carreras 2.288.095€ en premios, ha realizado un gasto superior a los 100.000€ en el sector audiovisual y ha ingresado a la Comunidad Autónoma las pertinentes retenciones tributarias estipuladas sobre premios concedidos, aparte de numerosa actividad directa e indirecta añadida.

Por otro lado, la práctica reproductiva más frecuente de esta raza es la inseminación artificial utilizando principalmente de Caballos Trotadores procedentes de Francia y América (Gómez et al., 2010b), a la vez que es común la castración para la mejora del rendimiento deportivo. En este sentido, en España se emplean grandes

cantidades de dinero para la importación de material genético de alta calidad, la inseminación y castración como gastos de cría (Deloitte, 2013).

Asimismo, el mercado ecuestre para las competiciones deportivas de trote posee una proyección mundial en ascenso siendo Francia, Estados Unidos, Italia y Suecia los países más importantes dentro de este sector.

Problemática actual y justificación del estudio

Hay numerosos factores ambientales tanto extrínsecos como intrínsecos que afectan al rendimiento deportivo. Entre estos factores se encuentran el tiempo de transporte hasta el lugar de la competición, las horas de entrenamiento, el número de animales participantes de la propia carrera, el hipódromo, la distancia total de la carrera, etc. Por ello, dentro del programa de mejora se lleva a cabo una recogida de todos estos factores, los cuales se incluyen como efectos ambientales en los modelos de valoración genética que se están empleando actualmente de forma rutinaria. Este es el modelo animal de repetibilidad multivariable (BLUP) basado en los resultados de competición (Arnason, 1999; Rohe et al., 2001; Gómez et al., 2010b) y utilizado para las variables descritas anteriormente. Sin embargo, es difícil hacer una recogida sistemática de otros factores que también tienen un gran impacto sobre el rendimiento deportivo, como es el caso de los parámetros fisiológicos que están relacionados con el nivel de estrés que sufren los caballos. Realizar una correcta evaluación del nivel de estrés de los animales que están participando es muy complicado, en primer lugar debido a que todos los factores mencionados anteriormente pueden afectar a este nivel de estrés y en segundo lugar porque hasta el momento no existía un método objetivo y no invasivo de análisis que nos permita obtener valores con la suficiente precisión y fiabilidad. No obstante, en los últimos años se ha puesto a punto una nueva metodología denominada termografía infrarroja de fondo de ojo de gran objetividad y poco invasiva (el simple hecho de su medida no induce a un incremento del propio estrés en el animal), que ya se utiliza con regularidad para controlar el nivel de estrés o de bienestar en otras especies como el vacuno o el cerdo (Stewart et al., 2008; Weschenfelder et al., 2013). En caballos se han realizado estudios utilizando esta metodología para medir el estrés en caballos participantes en disciplinas de Salto y Doma Clásica (Valera et al., 2012; Bartolomé et al., 2013b; Sánchez et al., 2016), pero nunca antes se había probado en las carreras de trote. Por lo que sería interesante aplicar esta metodología al Caballo Trotador Español junto con el análisis de otros parámetros fisiológicos como es la frecuencia cardiaca, a pesar de que hasta ahora no ha se haya reconocido como una medida fiable en otros animales en competición.

Por otra parte, para hacer una valoración genética del potencial deportivo de los animales deben obtenerse tanto los controles de rendimiento del animal como los de sus hijos y/o familiares próximos, especialmente hijos. Por lo tanto, para obtener valoraciones con suficiente fiabilidad es necesario muchas participaciones en carreras, así como la existencia de parientes cercanos compitiendo, lo que hace que los animales

con la categoría genética de “Mejorantes” sean caballos relativamente viejos (8-10 años de edad). No obstante, el gran desarrollo de la genética molecular en las últimas décadas, ha puesto a disposición de los programas de mejora una metodología de apoyo a esta selección basada en la utilización de marcadores moleculares relacionados con la aptitud deportiva. Esta metodología denominada *selección asistida por marcadores* (MAS), presenta innumerables ventajas a la selección clásica (permite la preselección incluso antes de nacer el animal, permite la evaluación en ambos sexos y no exige el sacrificio del animal entre otras), pero requiere la evaluación previa de la asociación genética en la población a utilizar (Dekkers and Hospital, 2002). Esta comienza por la detección de la variabilidad genética para dicho marcador en la población, y termina con el estudio de asociación con el parámetro o parámetros relacionados con la aptitud deportiva que se quiere mejorar. Por otra parte el hecho de permitir la preselección precoz de animales evitaría el elevado coste del entrenamiento a animales cuyo potencial genético no es el más adecuado para este tipo de disciplinas.

Por todo ello sería necesario hacer una búsqueda de genes candidatos que estuviesen asociados con el temperamento o estrés y el rendimiento deportivo en una población representativa de CTE, evaluando la posibilidad de utilizarlos en la valoración genética de esta raza.

HIPÓTESIS DE TRABAJO

Por lo tanto, **la Hipótesis** a contrastar en la presente tesis es si es posible analizar el tipo de impacto del estrés sufrido por los animales durante las competiciones sobre su rendimiento deportivo, por medio de métodos objetivos como la termografía infrarroja. En segundo lugar, si existen marcadores moleculares en genes candidatos funcionales relacionados con el estrés y con el rendimiento deportivo que puedan ser utilizados para mejorar la fiabilidad y la precocidad de las valoraciones genéticas en la raza del Caballo Trotador Español.

OBJETIVOS

El objetivo general que se persigue en la presente Tesis Doctoral es validar la utilidad de la termografía infrarroja de fondo de ojo como herramienta de evaluación objetiva y no invasiva del nivel de estrés y ver la relación que existe con el rendimiento deportivo en carreras de trote; y buscar marcadores de genes candidatos que estén relacionados con el estrés del animal así como con el rendimiento deportivo para la selección precoz de los animales. Para la consecución de la misma, se pretende desarrollar los siguientes objetivos específicos:

1. Realizar una caracterización del perfil genético del caballo CTE y su grado de diferenciación genética con el resto de las razas deportivas de caballos españolas. Este objetivo se ha abordado en el CAPÍTULO I.

2. Poner a punto una metodología de Termografía Infrarroja de fondo de ojo para la evaluación del nivel de estrés de los animales de la raza del CTE durante las carreras de trote y evaluar el impacto de dicho estrés sobre el rendimiento deportivo de los animales de esta raza. Este objetivo se ha abordado en el ARTÍCULO 1 DEL CAPÍTULO II.

3. Seleccionar un grupo de marcadores moleculares en genes candidatos (*BDNF*, *COMT*, *HTR1A*, *SLC6A4* y *TPH2*) y analizar su posible asociación con el nivel de estrés sufrido por el animal durante las carreras de trote, y su comparación con el de otras disciplinas menos exigentes físicamente como la doma clásica. Este objetivo se ha abordado en el ARTÍCULO 2 DEL CAPÍTULO II.

4. Seleccionar de un grupo de marcadores moleculares en genes candidatos (*CKM, COX4I2, DMRT3, MSTN* y *PDK4*) que puedan estar relacionados con el rendimiento deportivo de la raza del CTE en carreras de trote. Este objetivo se ha abordado en el CAPÍTULO III.

CAPÍTULOS

CAPITULOS

CAPÍTULO I: CARACTERIZACIÓN GENÉTICA DE LA RAZA DEL CABALLO TROTADOR ESPAÑOL Y DIFERENCIACIÓN CON EL RESTO DE RAZAS DE DEPORTE ESPAÑOLAS A PARTIR DE MARCADORES MOLECULARES

Este primer capítulo está integrado por un artículo científico:

- **Negro S**, Solé M, Pelayo R, Gómez MD, Azor PJ, Valera M. *Molecular diversity between two cohorts of six Spanish riding-horse breeds: impact of selection in Crossbred vs Purebred populations*. Livestock Science, 193, 88-91. Doi: 10.1016/jlivsci.2016.09.013.

ARTÍCULO 1

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Short communication

Molecular diversity between two cohorts of six Spanish riding-horse breeds: Impact of selection in Crossbred vs Purebred populations

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ABSTRACT

The genetic structure and level of diversity were assessed across 6 riding-horse breeds raised in Spain and born between 1989 and 2012 (Anglo-Arab, AA; Arab Purebred, PRA; Spanish Purebred, PRE; Menorca Purebred, PRMe; Spanish Sport horse, CDE; Spanish Trotter horse, TRO). The impact of selection on molecular diversity over time looking at 2 different cohorts in Crossbreds (AA, CDE and TRO) vs Purebreds (PRA, PRE and PRMe) has been also analysed. A total of 17 microsatellite loci were analysed in 2,530 horses from the cohort 1 (C1, born between 1989 and 2000) and 10,102 horses from the cohort 2 (C2, born between 2001 and 2012). The mean number of alleles per locus was the highest in C2 of TRO horses (11.76) and the lowest in C1 of PRMe breed (7.71). The lowest global values for the expected heterozygosity were found within Purebreds (0.71–0.76) compared to Crossbreds (0.76–0.78). For C2, the effective population size values based on linkage disequilibrium (from 4.9 in PRA to 668.3 in PRE) were higher than those computed for C1 (from 3.3 in PRA to 204.1 in CDE). Genetic distances and structure analysis showed that a significant amount of genetic variation is maintained due to an increase in the genetic uniformity across generations in Pure and Crossbreds. PRA, CDE and AA breeds seem to be the most genetically related, and for PRE, PRMe and TRO, both cohorts appeared closely related. In all populations, the genetic variability and effective population size have increased over time. Thus breeding policies have been properly managed to preserve diversity levels. Regarding the intense impact of selection in Purebreds across generations shown through the differences in the structure of the populations, no loss of genetic variability is expected in a short-term, although an extremely high degree of similarity and homogeneity between individuals of Purebreds compared to Crossbreds was shown.

1. Introduction

Currently, there are 6 Spanish horse breeds, Anglo-Arab (AA), Arab Purebred (PRA), Spanish Purebred (PRE), Menorca Purebred (PRMe), Spanish Sport horse (CDE) and Spanish Trotter horse (TRO), which participate in the main equestrian competitions (Dressage, Endurance, Eventing, Harness Racing and Show Jumping) in Spain. Their breeding programs are focused on improving their aptitude for these disciplines, while avoiding any loss of genetic variability. As a result of the human selection processes, most horse breeds today are closed populations (Purebred), with high phenotypic and genetic uniformity of individuals within the breed and/or bloodlines, but with a great deal of variation among breeds (Petersen et al., 2013). However, some studbooks allow for admixtures between different breeds, and they could therefore be considered Crossbreds.

Thus, genetic characteristics within horse breeds are expected to differ depending on differences in the definition of the breed, the historical census size, the relationship between relatives, the diversity of the breeding stock, the time elapsed since breed establishment, the selective pressures applied by breeders or the selected genome areas considered (Petersen et al., 2013). However, the population structure does not depend on breeder's selection alone - it could also be affected by preferential mating within groups of animals for different aims. Both intensive selection and assortative mating within breeding stocks could result in a loss of biodiversity, which is necessary for adaptation to changes and controlling increases in inbreeding (De Cara et al., 2013).

Molecular tools are especially useful to measure genetic differentiation and distance between breeds. However, the differential impact of selection between horse populations using molecular data has not been evaluated, although several studies have been performed to assess molecular diversity in a specific or a limited number of horse breeds (Achmann et al., 2004; Plante et al., 2007; Leroy et al., 2009; Prystupa

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et al., 2012; Berber et al., 2014). Therefore, this study aimed to research into the impact of selection on molecular diversity over time by analysing 2 different cohorts in different types of populations (Crossbred vs Purebred).

2. Material and methods

Six different Spanish riding-horse populations were analysed and the animals of each population were classified into 2 different cohorts (C1: $Cohort_{1989–2000}$ and C2: $Cohort_{2001–2012}$), according to their year of birth and taking into account an average generation interval of 11 years (Table 1). DNA was extracted from blood samples using the QIAamp 96 DNA Blood Kit (Qiagen, Hilden, Germany). All the animals were genotyped for a set of 17 microsatellite markers (Table S1).

To assess molecular genetic diversity across populations and cohorts, the mean number of alleles per locus (MNA), the observed heterozygosity (H_o), the unbiased expected heterozygosity (H_e, corrected for sample size) and Wright's F_{IS} were computed. Genetic differentiation among populations and cohorts was studied from Wright's F_{ST}. A dendogram was constructed from the distance matrix using Nei genetic distances, corrected for sample size (Nei, 1972), and was visualized using the Phylip 3.5 package (Felsenstein, 1993). The Microsatellite analyser (MSA) 4.05 program (Dieringer and Schlötterer, 2003) was used to compute the parameters. In addition, the effective population size based on the linkage disequilibrium method (N_{eLD}) was calculated (for each population and cohort, corrected for sample size), with NeEstimator v2.3 Program (Do et al., 2014).

The population structure was analysed using a Bayesian model-based clustering approach with admixture model implemented in Structure 2.2 (Falush et al., 2007). The best K-value, corresponding to the number of subpopulations, was calculated from ΔK, and based on second order rate changes for likelihood with respect to K (Evanno et al., 2005). Ten runs were performed for each K (from K=1 to K=8) with a 10^5 initial burn-in period, followed by 10^6 Markov chain Monte Carlo iterations.

3. Results

The genetic diversity within the 6 populations based on 17 microsatellite loci is presented in Table 1. The sample size for the global populations was around 2000–2500 individuals; in C1 the sample size was between 60 individuals for PRA and 811 for PRE, and in C2 the sample size was higher, between 1187 for TRO and 2349 for PRA. In general, the lowest global values for observed and expected heterozygosity were found within the Purebreds (0.62–0.76) compared to Crossbreds (0.69–0.78). Four populations (AA, CDE, PRA and PRE) showed increased H_o values in C2 compared to C1, whereas TRO breed showed no differences within cohorts and PRMe breed presented reduced values in the C2 (0.71) compared to C1 (0.73). H_e values were higher in C1 compared to C2 for all the populations. Global F_{IS} values within populations varied between −0.01 in PRMe and 0.19 in PRA, and values decreased in C2 for all breeds, except for PRMe.

Table 2 gives the estimates of effective population size (corrected for sample size) based on linkage disequilibrium (N_{eLD}) per cohort and population. For C2, estimates were higher than those computed for C1. The genetic differentiation (F_{ST}) between breeds is presented in Table S2. A neighbour-joining dendrogram between both cohorts of each population is shown in Fig. S1.

The representation of the proportion of individuals assigned at the best K-value (calculated from ΔK) for C1 and C2 are presented in Fig. S2, corresponding to the Purebred and Crossbred populations, respectively.

Table 2
Effective sample size based on Linkage disequilibrium (N_{eLD}) per cohort and horse breed population.

Breed	Cohort	N_{eLD} (95% CIs)	Breed	Cohort	N_{eLD} (95% CIs)
Purebreds			*Crossbreds*		
PRA	C1	3.3 (3.2–3.5)	AA	C1	16.2 (14.9–17.7)
	C2	4.9 (4.0–5.6)		C2	28.5 (27.0–30.1)
PRMe	C1	67.3 (62.0–73.1)	CDE	C1	204.1 (183.2–228.4)
	C2	120.8 (112.2–129.9)		C2	335.0 (310.9–360.9)
PRE	C1	72.7 (68.2–77.4)	TRO	C1	12.5 (11.8–13.3)
	C2	668.3 (601.0–745.3)		C2	33.3 (31.5–35.2)

CIs = confidence intervals; Arab Purebred = PRA; Spanish Purebred = PRE; Menorca Purebred = PRMe; Anglo-Arab = AA; Spanish Sport horse = CDE; Spanish Trotter horse = TRO; C1= $Cohort_{1989–2000}$ and C2= $Cohort_{2001–2012}$.

Table 1
Genetic diversity within the 6 Spanish riding-horse breed populations (12,632 individuals) based on 17 microsatellite loci.

	Breed	Code	N	Studs	AR	MNA	H_o	H_e	F_{IS}***
Purebreds	Arab Purebred	PRA_{C1}	60			8.59	0.61	0.79	0.22
		PRA_{C2}	2349			11.47	0.62	0.76	0.19
		PRA_{Global}	2409	794	7.78	11.47	0.62	0.76	0.19
	Menorca Purebred	$PRMe_{C1}$	442			7.71	0.73	0.72	−0.02
		$PRMe_{C2}$	1237			8.18	0.71	0.71	0.00
		$PRMe_{Global}$	1679	652	2.16	8.24	0.72	0.71	−0.01
	Spanish Purebred	PRE_{C1}	811			10.47	0.61	0.74	0.18
		PRE_{C2}	1997			10.35	0.66	0.70	0.06
		PRE_{Global}	2808	2112	11.35	11.06	0.64	0.71	0.11
Crossbreds	Anglo-Arab	AA_{C1}	89			9.76	0.70	0.80	0.12
		AA_{C2}	1232			11.53	0.71	0.77	0.09
		AA_{Global}	1321	414	1.33	11.82	0.71	0.78	0.09
	Spanish Sport horse	CDE_{C1}	483			9.76	0.71	0.77	0.08
		CDE_{C2}	2100			10.47	0.74	0.76	0.03
		CDE_{Global}	2583	796	0.17	10.76	0.73	0.76	0.04
	Spanish Trotter horse	TRO_{C1}	645			10.76	0.69	0.79	0.12
		TRO_{C2}	1187			10.76	0.69	0.75	0.09
		TRO_{Global}	1832	795	2.34	11.12	0.69	0.76	0.10
	Total		12632						

N = sample size; AR = percentage of average relatedness; MNA = mean number of alleles per locus; H_o = observed heterozygosity; H_e = expected heterozygosity (corrected for sample size); F_{IS} = heterozygote deficiency coefficient (****P value* < 0.001); C1 = $Cohort_{1989-2000}$ and C2 = $Cohort_{2001-2012}$. Global = C1+C2.

4. Discussion

The global mean number of alleles per locus denoted wide genetic variability, except in PRMe. However, the PRMe is an "endangered" autochthonous breed, with a low census (3199 of living animals), which may have suffered a recent bottleneck (Solé et al., 2013). So this could explain the low number of alleles and the loss of genetic diversity observed in the short-term. In general, the values presented for the level of global expected heterozygosity in the populations studied (0.71–0.78) were among the highest values reported for other horse populations using the same or similar loci (0.40–0.79, Achmann et al., 2004; Plante et al., 2007; Leroy et al., 2009; Prystupa et al., 2012; Berber et al., 2014).

As a result of intensive selection across generations, a higher degree of similarity and homogeneity between individuals in Purebreds than in Crossbreds is expected (Amador et al., 2013). Additionally, in mixed populations, it is assumed that inbreeding remains stable as a consequence of the balance between migration and drift (Cervantes et al., 2009), as it is observed in AA and CDE breeds with heterozygosity levels increasing over time. Thus, it seems that open population management and introgression of other breeds have had a major influence on its variability (Cervantes et al., 2009). In addition, there were certain differences between cohorts, especially for PRA, AA and CDE breeds, which reflect the increase in their genetic variability over generations. In AA and CDE, the difference could be due to the incorporation of foreign breeds. Nevertheless, the higher differences in census size could also influence the differential variability observed between cohorts.

Regarding the F_{IS} values, especially the Purebreds presented high positive values (over 0.11). It is known that PRE is under selection pressure for functional and morphological traits, and the selection of PRA horses for different economically relevant traits is increasing. Moreover, the values for TRO breed were also high (over 0.09), as the use of semen from selected foreign studs is frequent and this breed is under high selection pressure for racing performance (Gómez et al., 2010). In all these cases, the F_{IS} values found indicate some kind of preferential mating within groups of animals according to different selection objectives, although the increase of F_{IS} among cohorts is not higher.

The effective population size (N_e) computed using genealogy could be biased if pedigree depth is shallow. In this sense, molecular marker information could be a good alternative for characterizing N_e in livestock populations (Goyache et al., 2011). Thus, in order to compare the F_{IS} values across cohorts, the effective sample size based on Linkage disequilibrium (corrected for sample size) was used (N_{eLD}). The values found for PRA, AA and TRO populations (under 34) were less than the values found in other horse populations (over 40; Druml et al., 2007; Goyache et al., 2011). The excess of homozygosity observed in these breeds, especially in C1 (F_{IS} over 0.12), could explain the small effective population size observed. However, in all the populations, the values obtained for C2 increased compared to those obtained in C1. Therefore, the differences observed between both periods among and within each breed could be explained by the use of different lineages and/or changes in their mating policies over time by the implementation of the breeding programs in the last decade. For instance, the maintenance of diversity while selection is carried out for economically important traits has proved to be applicable in PRMe population, despite the lower census size (Solé et al., 2013). Or in the case of PRE, where N_e reached from 72.2 to 668.3 in the last generation, even though it is known that this breed is under selection pressure for functional and morphological traits, it has, at the same time, maintained a high diversity of ancestral genes making it possible to effectively manage the actual genetic variability. This could be caused by the new mating policies applied by the breeders in this breed which promote the selection using different criteria (functionality or morphology) in the same breed, the avoidance of inbreeding and the less abusive use of founders for mating purposes on Purebreds.

Regarding genetic differentiation, the F_{ST} estimates between populations indicated that PRA breed is shown to be the one most closely linked to AA and TRO. This comes as no surprise, as the Spanish breeds derived from PRA are AA and CDE, amongst others (Cervantes et al., 2009). According to the results of the neighbour-joining tree, PRA has evolved deeply compared to the other breeds, as the 2 cohorts appeared to be far apart, this could be explained by the improvement in the selection criteria based on the 2 different fold objectives for conformation and endurance performance traits (Cervantes et al., 2009). The obtained results indicate that different founder lines of PRA had been involved in forming AA and CDE breeds, but they have been replaced by foreign Arabian over time, due to this, Spanish Purebred has been gradually selected more for conformation and morphology than sporting performance. Moreover, CDE which originated from foreign sport breeds (Cervantes et al., 2009), has shown the greatest degree of similarity with other breeds studied (F_{ST} below 0.05), reflecting the influences of a wide variety of sport breeds over time. This can also be observed in the neighbour-joining tree, where CDE appeared close to the middle of the tree. Here, for PRE, PRMe and TRO breeds, both cohorts appeared close together but in separate branches, which indicate that selection objectives probably have changed over time.

Bayesian clustering methods have proved to be powerful analytical tools for identifying genetic structure in data sets (Druml et al., 2007). In the present study, the results from the Structure analysis showed that the best K-value changed from K =5 in C1 to K =6 in C2, which evidences an increase in variability between generations probably due to the improvement in breeding policies to manage and preserve the levels of diversity in the breeds studied. Thus, currently, there is no kind of subdivision or substructure within the breeds studied in the short-term. Nevertheless, the proportion of individuals assigned to each of the clusters differs within the cohorts studied, especially in Crossbreds. In general, both cohorts of each Purebred showed as high degree of similarity and homogeneity between individuals as expected, except in PRA breed, thus confirming its influences on the foundation of other breeds like AA, CDE or TRO.

As regards the intense impact of selection and the improvement in selection aims on Purebreds over generations, it can be stated that no loss of genetic variability is expected in the short-term for the use of stallions at different studs as an appropriate strategy to monitor its mating systems. A greater degree of similarity and homogeneity between individuals for these populations has also been observed (except in PRA for its differential influence over the Crossbreeds studied) compared to Crossbreds, where above-average diversity values were shown to be increasing over time. In all the populations, the genetic variability and effective population size have increased over time, which show that the breeding policies have managed to preserve diversity levels through strategies based on arrange contributions from one generation to the next to yield the maximum gain, while restricting the weighted global coancestry between individuals (Solé et al., 2013).

Conflict of interest statement

All authors declare that there are no known conflicts of interest associated with this publication.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.livsci.2016.09.013.

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Supplementary material

Table S1. Descriptive characteristics of the 17 microsatellite marker loci for all the studied breeds

Name	Chrom	Primers	Size (bp)	Reference
AHT4	24	5'-AACCGCCTGAGCAAGGAAGT-3' 5'-CCCAGAGAGTTTACCCT-3'	148-164	Binns *et al.*, 1995
AHT5	8	5'-ACGGACACATCCCTGCCTGC-3' 5'-GCAGGCTAAGGGGGCTCAGC-3'	130-146	Binns *et al.*, 1995
ASB17	2	5'-GAGGGCGGTACCTTTGTACC-3' 5'-ACCAGTCAGGATCTCCACCG-3'	91-109	Breen *et al.*, 1997
ASB2	15	5'-CCTTCCGTAGTTTAAGCTTCTG-3' 5'-CACAACTGAGTTCTCTGATAGG-3'	222-254	Breen *et al.*, 1997
ASB23	3	5'-GAGGTTTGTAATTGGAATG-3' 5'-GAGAAGTCATTTTTAACACCT-3'	128-154	Irvin *et al*, 1998
CA425	28	5'-AGCTGCCTCGTTAATTCA-3' 5'-CTCATGTCCGCTTGTCTC-3'	230-250	Eggleston-Stott *et al.*, 1997
HMS1	15	5'-CATCACTCTTCATGTCTGCTTGG-3' 5'-TTGACATAAATGCTTATCCTATGGC-3'	173-189	Guérin *et al.*, 1994
HMS2	10	5'-ACGGTGGCAACTGCCAAGGAAG-3' 5'-CTTGCAGTCGAATGTGTATTAAATG-3'	218-238	Guérin *et al.*, 1994
HMS3	9	5'-CCAACTCTTTGTCACATAACAAGA-3' 5'-CCATCCTCACTTTTTCACTTTGTT-3'	150-170	Guérin *et al.*, 1994
HMS6	4	5'-GAAGCTGCCAGTATTCAACCATTG-3' 5'-CTCCATCTTGTGAAGTGTAACTCA-3'	153-169	Guérin *et al.*, 1994
HMS7	1	5'-CAGGAAACTCATGTTGATACCATC-3' 5'-TGTTGTTGAAACATACCTTGACTGT-3'	165-183	Guérin *et al.*, 1994
HTG10	21	5'-CAATTCCCGCCCCACCCCCGGCA-3' 5'-TTTTTATTCTGATCTGTCACATTT-3'	93-113	Marklund *et al.*, 1994
HTG4	9	5'-CTATCTCAGTCTTCATTGCAGGAC-3' 5'-CTCCCTCCCTCCCTCTGTTCTC-3'	127-41	Marklund *et al.*, 1994
HTG6	15	5'-CCTGCTTGGAGGCTGTGATAAGAT-3' 5'-GTTCACTGAATGTCAAATTCTGCT-3'	84-106	Marklund *et al.*, 1994
HTG7	4	5'-CCTGAAGCAGAACATCCCTCCTTG-3' 5'-ATAAAGTGTCTGGGCAGAGCTGCT-3'	120-130	Marklund *et al.*, 1994
LEX33	4	5'-TTTAATCAAAGGATTCAGTTG-3' 5'-TTTCTCTTCAGGTGTCCTC-3'	203-217	Coogle *et al.*, 1996
VLH20	30	5'-CAAGTCCTCTTACTTGAAGACTAG-3' 5'-AACTCAGGGAGAATCTTCCTCAG-3'	86-106	Van Haeringen *et al.*, 1994

Table S2. F_{ST} estimates between the populations (above diagonal) of the 6 Spanish riding-horse breeds studied.

	Breed	PRA	PRE	PRMe	AA	CDE	TRO
Purebreds	**PRA**	0	0.080	0.070	0.031	0.044	0.069
	PRE			0.077	0.042	0.042	0.083
	PRMe				0.062	0.051	0.086
Crossbreds	**AA**					0.009	0.042
	CDE						0.045
	TRO						0

Arab horse = PRA; Spanish Purebred horse = PRE; Menorca Purebred horse = PRMe; Anglo-Arab horse = AA; Spanish Sport horse = CDE; Spanish Trotter horse = TRO.

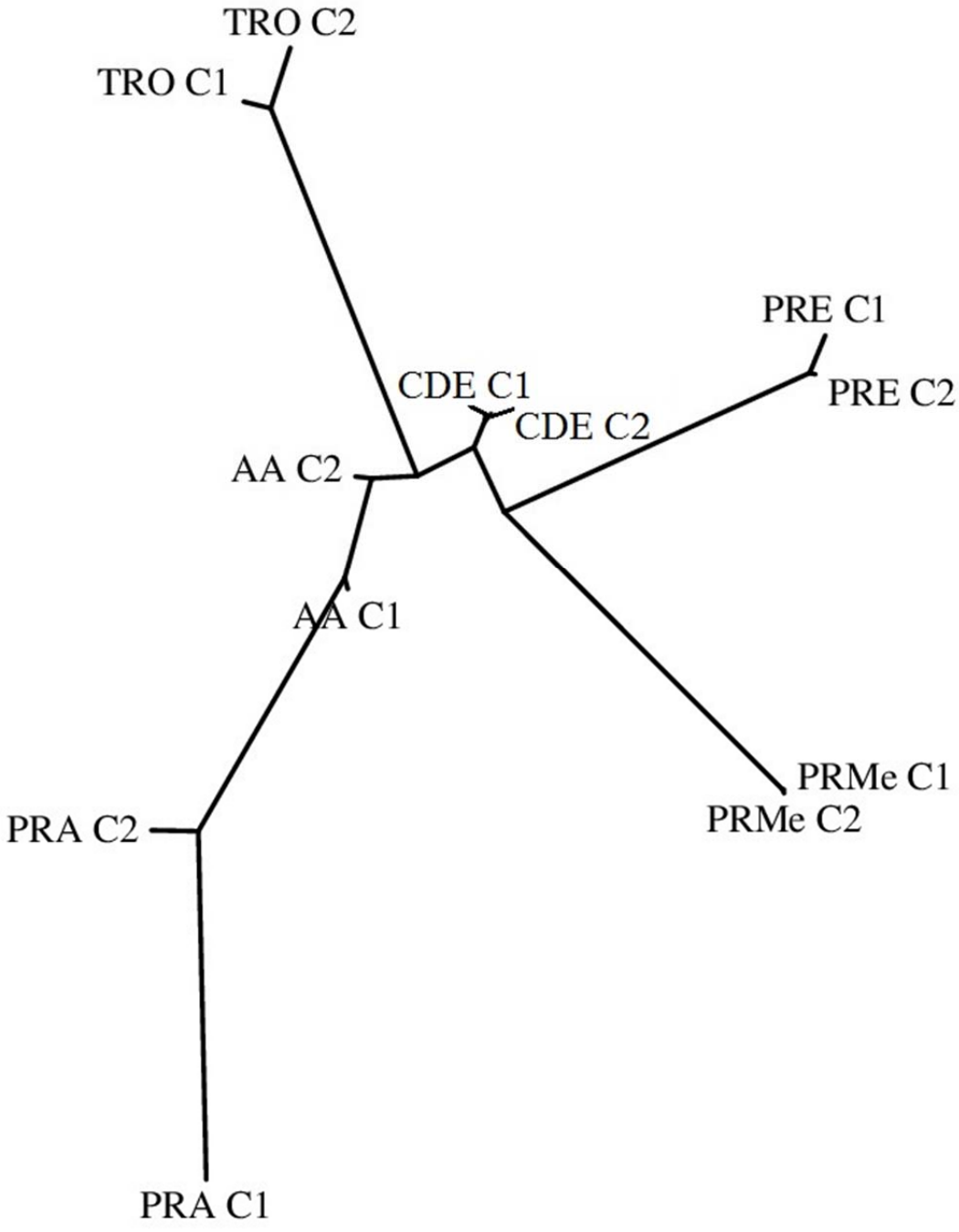

Figure S1. Neighbour-joining tree obtained from the Nei genetic distances between the two cohorts of each of the 6 Spanish riding-horse populations studied (Phylip) (1000 bootstrap). Arab horse = PRA; Spanish Purebred horse = PRE; Menorca Purebred horse = PRMe; Anglo-Arab horse = AA; Spanish Sport horse = CDE; Spanish Trotter horse = TRO; C1 = $Cohort_{1989\text{-}2000}$ and C2 = $Cohort_{2001\text{-}2012}$.

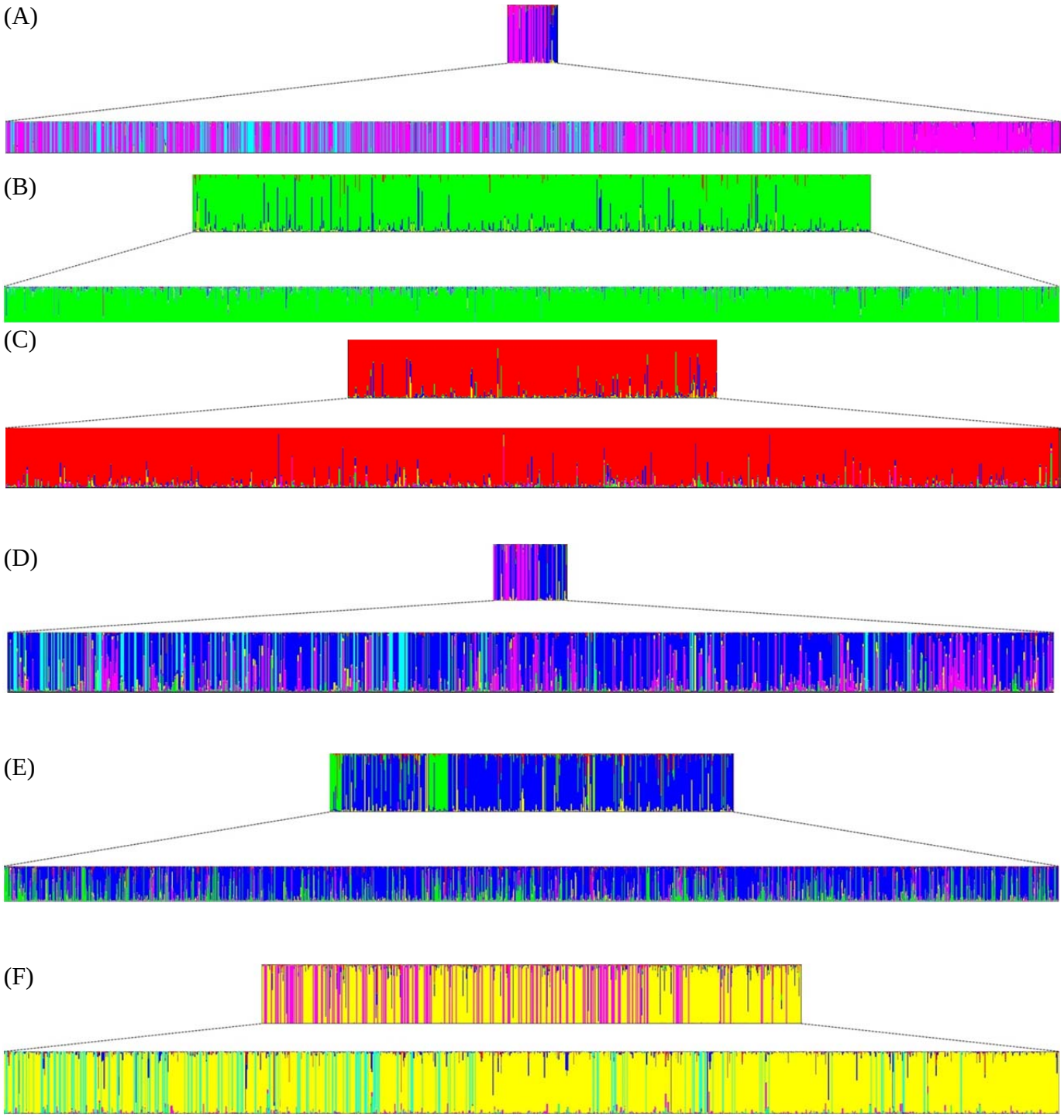

Figure S2. Proportion of individuals assigned at the best-K (K = 5, for the cohort 1 at the top and K = 6 for the cohort 2 at the bottom) corresponding to the Purebred riding-horse populations: (A) Arab Purebred, PRA; (B) Spanish Purebred, PRE; and (C) Menorca Purebred, PRMe; and to the Crossbred populations: (D) Anglo-arab, AA; (E) Spanish Sport horse, CDE; and (F) Spanish Trotter horse, TRO.

CAPÍTULO II: EVALUACIÓN DEL NIVEL DE ESTRÉS DURANTE LAS CARRERAS DE TROTE MEDIANTE NUEVAS TÉCNICAS NO INVASIVAS Y BÚSQUEDA DE GENES ASOCIADOS CON ESTE CARÁCTER EN EL CABALLO TROTADOR ESPAÑOL

Este segundo capítulo está integrado por dos artículos científicos:

- **Negro S.,** Bartolomé E, Molina A, Solé M, Gómez MD, Valera M. 2017. *Stress level effects on sport performance during trotting races in Spanish Trotter horse. Equine Vet J* (2017). EVJ-GA-17-138, under review.
- **Negro S.**, Valera M., Solé M., Bartolomé E., Sánchez M.J., Gómez, M.D., Molina, A. *Evidence for the effect of serotoninergic and dopaminergic gene variants on stress levels in horses participating in dressage and harness racing.* Anim. Genet., (2017). AnGen-17-06-0134, under review.

ARTÍCULO 2

Stress level effects on sport performance during trotting races in Spanish Trotter horses

Journal:	*Equine Veterinary Journal*
Manuscript ID	Draft
Wiley - Manuscript type:	General Article
Discipline:	Study design and data analysis, Physiology
Body System/Disorder:	Not applicable
Abstract:	Background: The influence of usual stress level is questioned as negative effect on equestrian competitions, specifically in trotter racing. Objectives: The main aims were to measure stress levels in Spanish Trotter Horse (STH) using a reliable non-invasive system and to determine the threshold level of stress that lead to the best results, showing when it becomes distress. Study design: Longitudinal study. Methods: 130 STH were evaluated, measuring their performance (based on racing time per kilometre, TPK) and their stress (based on eye temperature (ET) with infrared thermography and heart rate (HR)) in different competitions. ET and HR were collected 2hours before the race (ETB, HRB) and just after the race (ETJA, HRJA), and the increases of ET (ΔET) and HR (ΔHR) were estimated. A GLM evaluated the effects of ET, HR, age and sex on TPK; a Segmented Regression Model (SRM) estimated the breakpoint where the relationship between TPK and ΔET changes; and a response surface plot illustrated the effects of ΔET and ETB on TPK. Results: Statistically significant differences associated with performance level were found for ΔET and ETB. The SRM indicated that when the animal was more stressed before the race than just after finishing it (ΔET<0), it showed the worst competition results, and from a certain point dividing the regression curve, reached at ΔET=-0.97%, horse's performance started to improve. When comparing all race and ET variables, TPK was optimum (77.27s) when ETB and ΔET reached values of 37.61ºC and 7.57%, respectively. Main limitations: Increasing sample size is recommended to verify the obtained results before their application in the breeding program. Conclusions: The stress levels of the horse before the race influenced its competition results, and ΔET during competitions till a threshold point is related to an improvement in performance results.

Stress level effects on sport performance during trotting races in Spanish Trotter horses

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Keywords: heart rate; infrared thermography; performance level; trotter racing; welfare

Word count: 4975

Ethical animal research

Research ethics committee oversight not currently required by this journal.

Authors' declaration of interests

No competing interests have been declared.

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Authorship

S. Negro, A. Molina and M. Valera designed the research. S. Negro, E. Bartolomé, M. Solé and M.D. Gómez contributed to data collection. S. Negro, E. Bartolomé and A. Molina analyzed the data. S. Negro, E. Bartolomé, A. Molina, M. Solé, M.D. Gómez and M. Valera contributed to the interpretation and edition of the manuscript. All authors approved final version of the manuscript.

Summary

Background: The influence of usual stress level is questioned as negative effect on equestrian competitions, specifically in trotter racing.

Objectives: The main aims were to measure stress levels in Spanish Trotter Horse (STH) using a reliable non-invasive system and to determine the threshold level of stress that lead to the best results, showing when it becomes distress.

Study design: Longitudinal study.

Methods: 130 STH were evaluated, measuring their performance (based on racing time per kilometre, TPK) and their stress (based on eye temperature (ET) with infrared thermography and heart rate (HR)) in different competitions. ET and HR were collected 2hours before the race (ET_B, HR_B) and just after the race (ET_{JA}, HR_{JA}), and the increases of ET (ΔET) and HR (ΔHR) were estimated. A GLM evaluated the effects of ET, HR, age and sex on TPK; a Segmented Regression Model (SRM) estimated the breakpoint where the relationship between TPK and ΔET changes; and a response surface plot illustrated the effects of ΔET and ET_B on TPK.

Results: Statistically significant differences associated with performance level were found for ΔET and ET_B. The SRM indicated that when the animal was more stressed before the race than just after finishing it (ΔET<0), it showed the worst competition results, and from a certain point dividing the regression curve, reached at ΔET=-0.97%, horse's performance started to improve. When comparing all race and ET variables, TPK was optimum (77.27s) when ET_B and ΔET reached values of 37.61ºC and 7.57%, respectively.

Main limitations: Increasing sample size is recommended to verify the obtained results before their application in the breeding program.

Conclusions: The stress levels of the horse before the race influenced its competition results, and ΔET during competitions till a threshold point is related to an improvement in performance results.

Introduction

Harness racing has a great economic importance in equine industry being the second international equestrian discipline, only after gallop racing [1]. The Spanish Trotter Horse (STH) is a trotter breed of increasing importance in Spain. Their breeding program, officially approved in 2005, includes as main breeding goal the improvement of racing performance results. According to McBride and Mills [2], to achieve an optimal performance during competition, the animal requires being in peak physical condition and having a correct psychological state of mind. During any competition, the horse has to face different environmental stimuli that potentially threaten its internal equilibrium (stressors), hence developing a stress response that helps it to cope properly with these external factors. However, this stress response could have either a positive or negative influence on its welfare and sport performance [3]. During normal exercise activity, acute stress response has positive and desirable physiological functions that might be beneficial for the adaptation of the horse to exercise demands [4]. However, when this stress response exceeds the thresholds of tolerance due to maintenance of the stressful situation over time, a distress response can be exhibited by the animal. This has both physical and psychological consequences that could compromise horse adaptation to exercise during competition [5]. In this sense, the type of stress response is a critical factor in horse sport performance as it can either enhance or decrease the sporting ability of the animal.

Recent developments in reliable non-invasive techniques as infrared thermography (IRT) could allow measure stress levels in horses during competitions [6-8]. IRT equipment is portable, simple to use and animal restraint is minimal or unnecessary. It has certain advantages over other non-invasive methods by offering insight into the metabolic consequences of stress and enabling measurement of short-term acute stress [9-11]. Additionally, the analysis of heart rate (HR) involves measurement of physiological stress parameters with an advantage over other approaches that require blood sampling [12-14]. Thus, the main aim of this study was to assess the influence of stress level of STH on their

performance during competition through reliable non-invasive techniques. Besides, the type of stress response in STH was studied to determine the threshold level from which the positive effects of stress become negative influencing performance. And finally, some recommendations are given for the implementation of these results on STH breeding program.

Material and methods

Animals

A total of 130 STH animals (71 sires, 13 geldings and 46 mares) aged between 2 and 10 years-old were evaluated. Measurements (sport performance and stress level) were taken during 4 competitions held in October 2014 and 2015, in the hippodromes of Son Pardo and Manacor (Mallorca, Spain). Thus they showed similar environmental conditions with temperature ranging 20-30ºC, and humidity ranging 40-65%. Animals were randomly chosen within the participants of each race considered.

Data collection

Eye temperature and heart rate

The stress level of the animals included in this study was assessed by eye temperature (ET) and heart rate (HR) measurements. They were collected at two different stages: 2h before the race (ET_B and HR_B, respectively) and just after the race (ET_{JA} and HR_{JA}, respectively). Considering that all horses arrived at the same time (approximately) to the finish line, it was not technically feasible taking ET and HR measures in all participants at the same time. Thus, the time spent in taking the measure from the end of the race was also registered as $Time_{increm}$ and included in the statistical model.

In addition, eye temperature increase (ΔET) between ET_B and ET_{JA} and heart rate increase (ΔHR) between HR_B and HR_{JA} were also estimated.

ET images were taken with a FLIR i7 camera (FLIR Systems AB, FLIR Systems, Inc., Sweden), following the indications of Bartolomé *et al.* [7]. Heart rate was quantified as heart beats per minute

(bpm) and assessed with a portable pulsometer (Equine Healthcheck; Polar Electro®, Kempele, Finland).

Performance level and competition data

Records from STH competitions were collected, using only trotting races with autostart starting method in the analyses.

The performance level was evaluated based on the racing time per kilometre (TPK), as recommended [15].

Statistical analyses

All physiological parameters included in this study (ET and HR measurements) satisfied assumptions of Gaussian normal distribution.

A statistical description of ET, HR and TPK measurements was provided according to age, with a Tukey post-hoc multiple comparison test for least square means (LS means) calculated for each stress parameter considered (ET_B, ET_{JA}, HR_B, HR_{JA}, ΔET, ΔHR) and for the performance trait (TPK).

The effects of ET, HR, age and sex on TPK were evaluated using a General Linear Model procedure (proc GLM). The fixed model used was:

$$\boldsymbol{Y}_i = \mu + ET_{Bi} + HR_{JAi} + \Delta ET_i + sex_i + age_i + age_i \cdot \Delta ET_i + Time_{increm i} + EBV_i + e_i$$

where Y_i is the observation x of the dependent variable TPK for each individual in each race (i), μ is a common intercept, ET_B is the eye temperature taken 2hours before the race, HR_{JA} is the heart rate measured just after the race and ΔET is the eye temperature increase between ET_{JA} and ET_B. The analysis included 3 fixed effects: *sex* (3 classes; sire, mare and gelding), *age* (4 classes: 2-3, 4-5, 6-7 and 8-10 years-old), the $Time_{increm}$ (6 classes: 5-6, 7-8, 9-10, 11-12, 13-15, 16-20 min) and the interaction *age*-ΔET. $Time_{increm}$ is the time spent in taking the measure just after race. *EBV* is the estimated breeding value of TPK trait, calculated as described Gómez *et al*. [16]. This parameter was

considered to correct for environmental effects affecting trotting performance and consider just racing performance ability.

The effect of ΔET on performance level was analysed using a piece-wise linear or segmented regression model (following previous GLM model), since this provides a useful method for determining the threshold level of stress. This analysis estimates the breakpoint between two fitted regression lines [17], assuming an abrupt change in linear relationship between performance level (measured with the residual predicted TPK, an estimation of the predicted potential of each individual once the influence of the environmental effects have been removed from the model) and ΔET changes.

Finally, a three-dimensional response surface analysis was carried out to illustrate the main and interactive effects of the independent factors, ΔET and ET_B, on the dependent factor racing TPK. The response surface was fitted by a second degree polynomial regression, with age as covariate. A ridge analysis was also conducted to search for the region of optimum response (minimum TPK).

All statistical analyses were carried out using SAS, Statistical Analysis Systems Institute v. 9.2 package [18].

Results

The results of descriptive statistics analysis for variables measuring stress and performance level in trotting races are summarised in Table 1. Trotter horses obtained LS means of ET_B and HR_B from 35.4°C to 36.7ºC and from 40.2bpm to 44.2bpm, respectively; and LS means of ET_{JA} and HR_{JA}, from 36.6ºC to 38.7ºC and from 102.7bpm to 111.3bpm, respectively. The ΔET's means oscillated from 2.9% to 5.4%, the ΔHR's from 152.4% to 169.4% and the TPK's from 78.5s to 81.4s. All stress variables (ET_B, ET_{JA}, HR_B, HR_{JA}, ΔET, except ΔHR) showed a decreasing tendency with age; so the older the animal, the lower the measure.

On the other hand, statistically significant differences were found between age groups and ET variables except for ΔET. For ET_B and ET_{JA}, 2-3 age-group showed higher statistically significant ET values than the other groups. According to TPK, the same decreasing tendency with age was showed, younger animals (2-3 and 4-5) showed higher statistically significant values than older ones (6-7 and 8-10).

The GLM analysis showed the main parameters affecting TPK results during competitions (Table 2). Statistically significant differences were found for the age (p<0.05), $Time_{increm}$ (p<0.05), ΔET (p<0.05) and ET_B (p<0.001).

The segmented regression analysis for performance level and ΔET was shown in Fig 1. It represented the residual predicted TPK versus the ΔET. The breakpoint (c=-0.97%) between the two regression lines (Y_1=-0.96+-1.31·x; Y_2=-0.96 +-1.31·x+1.24·(x–c)) is the optimal ΔET and corresponds to the model with the lowest residual squared error for ΔET. The slope decreased abruptly until the estimated break point of -0.97±1.27%, decreasing slightly after this point to minimize the residual predicted TPK.

Fig 2 showed the response surface analysis for stress and performance levels, and the relationship among ET_B, ΔET and TPK. Both ΔET and ET_B had a great influence on TPK as statistically significant differences were shown (p<0.05). The response surface comprised values of ET_B from 32.3ºC to 38.6ºC, ΔET from -4.2% to 12.5% and TPK from 74s to 87s. The optimum conditions were determined by ridge analysis, which computed the estimated ridge of minimum response (min TPK) for an increasing radius to 0.8, setting TPK, ET_B and ΔET to 77.27s, 37.61ºC and 7.57%, respectively.

Discussion

Previous studies reported the influence of environmental factors on racing performance [16, 19-20]. These factors might produce a stress response on the horse that could either limit or enhance

performance, affecting EBV obtained for trotter horses. In the present study, changes in ET and HR were monitored (Table 1). They were measured at two stages of the race, obtaining differences of 1-2ºC and 50-60bpm for ET and HR, respectively, when comparing the horse response before competition and inmediately after race.

When accounting to age, TPK results showed that the younger the animal, the greater the LS mean TPK spent on finishing the race, hence the worse the competition results. It supports previous findings on stress parameters describing that age was a key factor that would influence animal's previous experience and training, consequently improving their sportive results with time [21-22]. This support Posta *et al.* [23] results about being preferable to make genetic selection based on performance at older ages.

The ET_B measures mainly the stress related to the animal itself and hence to its natural tendency to become easily stressed or not on a new environment. This measure would be more related with intrinsic factors (breed, age...) rather than with factors directly related with competition. According to the breed, Hausberger *et al.* [24] found Quarter Horse, Haflinger or French Trotter breeds with less neophobic reactions than Selle Français, Thoroughbred or Arab breeds. Therefore, considering that STH is a composite breed with influence from other trotter horse breeds such as the French Trotter [16], a lower ET_B LS mean value was expected in trotting races (35.9ºC) compared to those reported, for example, in show jumping competitions (36,2ºC; [6]). In this discipline the breed studied, the Spanish Sport Horse, is also a composite breed formed mainly by Thoroughbreds and Selle-Français [25], reported as more emotional horses than the trotters.

The ΔET measures the stress produced during the "activation" of the horse to compete on the race. Thus, our results indicated that the stress generated to get ready and perform adequately influenced performance results. According to Kuipers and Keizer [26], each exercise induces an acute disturbance of homeostasis in cells and organs, which may result in decreased mechanical output and fatigue. Furthermore, as competition is a mixture of various stressors, just being at the competition

center before competing induced a classic physiological stress response in horses [27] that could affect race performance.

In addition, the GLM analysis between TPK, ET and HR (Table 2) on STH found statistically significant differences on ET_B, ΔET and age according to TPK. Kinnunen *et al.* [28] studied the regulation of HR in trotters during training and competition periods obtaining significant differences for HR variability during competitions, indicating an increased stress load. However, in this study no significant differences were found for HR according to TPK results. This could be due to HR remains constant during competition once the first big increase takes place after the physical effort developed at the beginning of the race, showing no significant differences between TPK results.

In general, results obtained for ET were different to those obtained for other equestrian disciplines studied with this technique, such as show jumping [6-7] or dressage [8].

A decreasing tendency was observed on stress parameters with age, so that the older the animal, the lower the ET, HR or ΔET values shown during competition and hence the lower the stress perceived by the horse. These results were consistent with those obtained by Valera *et al.* [6], where ET decreased for 6 years-old animals compared to 4-5 years-old horses participating in show jumping competitions. Becker-Birck *et al.* [20] also found that age was a key factor on stress response during competition, as well as on the recovery time of the horse, as the age has an emotional component that decreases with the experience. Therefore, the higher ET shown by 4 years-old horses could be due to the stress of the novel situation, whereas the lower ET shown by 8-10 years-old horses could be due to their previous experience at competitions and likely habituation to the events.

In order to check the evolution of ET according to performance level, a segmented regression analysis was developed (Fig 1), showing that the lower the TPK (the better the race results), the higher the ΔET (the higher the stress measured just after the race and the lower the stress measured 2h before the race; ΔET>0). This makes ET a potential helper to improve competition results also considering that ET showed a genetic basis with heritabilities between 0.14-0.50 according to

Sánchez *et al.* [8]. Segmented regression lines were fitted to identify the point (ET level) when physiological stress turns into deleterious distress effects, causing a negative influence on trotter metabolism and welfare and consequently on their results. Furthermore, accounting for TPK breeding value within the genetic model, used to fit the segmented regression lines, assures the correction of the results by the influence of the genetic effect of TPK, already corrected by the environmental factors. Thus, this would increase the accuracy of the estimation of the break point from which the physiological stress developed by the horse to accomplish the race, becomes harmful. This break point was found at a ΔET of -0.97% (ET measured just after the race is lower than ET measured 2h before the race). So, from that point on, horse performance improves. Therefore, the higher the ET activation developed during the race (higher ET_{JA} with respect to ET_B) the faster the horse and thus, the better the results achieved (less TPK). These results were supported by previous findings which show that the regulation of the hypothalamus–pituitary–adrenal axis (HPA) during exercise reflects the intensity of the physical activity in horses [29-31]. During normal exercise activity, the sympathoadrenal axis and the HPA functions are activated, producing the so-called acute stress response that has positive and desirable physiological functions that might be beneficial for the adaptation of the horse to exercise demands. Healthy sport horses recover quickly once the threatening stimuli from the competition day are finished. However, maintaining the stressful stimuli from the beginning and during prolonged periods can cause deleterious effects indicating that the animal is not coping with the stress experienced during exercise (distress). Bartolomé *et al.* [7] found an influence of ET and age on competition results for show jumping horses, describing ET as an adequate tool to assess the activation of HPA during exercise, responsible for acute stress response.

Furthermore, a response surface design followed by a minimum ridge analysis between TPK, ΔET and ET_B (Fig 2) highlighted the optimal ET conditions that minimized the TPK and maximized performance results. The estimated ridge of minimum response for an increasing radius to 1.0 set TPK, ET_B and ΔET to 75.50s, 38.15ºC and 8.42%, respectively. However, taking into consideration

the range obtained for ET_B in this study (Table 1), the TPK could be minimized just on 2-3 years-old horses (1.54% of the population), as they were the only ones that showed an ET_B of 38.15ºC. Thus, by reducing the ratio to 0.8, shorter values of ET_B as 37.61ºC, presented in 12.31% of the population, may allow TPK be optimized to a minimum of 77.27s when the value of ΔET is 7.57%. These results highlights that the stress developed before competition is higher on younger horses and also helps them to perform better during the race. However, previous studies reported that younger trotters showed slower finish time (higher TPK) compared to older ones [32].

Our findings highlight that racing performance was influenced by the level of physiological stress, because ET, but not HR, influence competition results as it is supported by previous studies in other disciplines like show jumping [7]. Furthermore, this study goes one step further highlighting an elliptical behaviour of ET during race, which would increment till a certain point from which the animals would suffer the consequences of distress, diminishing its performance and worsening its racing results. Therefore, we can conclude that ET measured using IRT could be considered a suitable tool to assess the stress level of the horse during races and the physiological level when this stress becomes distress, compromising welfare during competition. It should be taken into consideration that the stress measured by IRT is partly influenced by horse's emotionality and temperament, as it would determine the stress response of the horse over new stimuli.

Most breeding programs consider character and temperament as important traits [33]. The STH breeding program also includes a mention to breed for a horse with a temperament that shows its willing to win the race [16]. Thus, the STH breeding program could be implemented with ET as another selection criterion that would help breeders to select better horses for racing performance, accounting for the minimum ET_B from which horses' performance gets worse. However, further studies are required to confirm these hypotheses and to include this parameter in the Breeding Program.

TABLES

Table 1. Tukey post-hoc multiple comparison adjustment for least square (LS) means for traits measuring stress (ET and HR) and performance level (TPK) of Spanish Trotter Horses during trotting races, according to age.

Traits		Age Groups (years-old)	LS Means ± s.e.	CL (95%)	
Stress level	**ET_B (ºC)**	2-3	36.73 ± 0.21[b]	36.31	37.14
		4-5	35.97 ± 0.19[a]	35.60	36.35
		6-7	35.38 ± 0.29[a]	34.80	35.96
		8-10	35.57 ± 0.25[a]	35.08	36.06
		All	35.93 ± 0.11	35.71	36.15
	ET_{JA} (ºC)	2-3	38.67 ± 0.26[c]	38.14	39.19
		4-5	37.68 ± 0.24[b]	37.20	38.16
		6-7	37.39 ± 0.38[ab]	36.64	38.14
		8-10	36.63 ± 0.31[a]	36.01	37.25
		All	37.60 ± 0.15	37.31	37.89
	HR_B (bpm)	2-3	44.20 ± 1.92[a]	40.39	48.00
		4-5	40.46 ± 1.74[a]	37.00	43.91
		6-7	41.11 ± 2.71[a]	35.73	46.48
		8-10	40.17 ± 2.30[a]	35.62	44.72
		All	41.41 ± 0.96	39.51	43.32
	HR_{JA} (bpm)	2-3	111.31 ± 2.29[a]	106.77	115.85
		4-5	106.89 ± 2.12[a]	102.70	111.09
		6-7	102.70 ± 3.26[a]	96.24	109.15
		8-10	104.28 ± 2.68[a]	98.97	109.58
		All	107.59 ± 1.12	105.38	109.80
	ΔET (%)	2-3	5.43 ± 0.66[a]	4.12	6.75
		4-5	4.53 ± 0.60[a]	3.33	5.72
		6-7	5.39 ± 0.95[a]	3.51	7.28
		8-10	2.90 ± 0.78[a]	1.35	4.46
		All	4.67 ± 0.33	4.03	5.31
	ΔHR (%)	2-3	152.38 ± 12.24[a]	128.14	176.63
		4-5	164.80 ± 11.17[a]	142.69	186.91
		6-7	154.10 ± 17.55[a]	119.35	188.85
		8-10	169.38 ± 14.51[a]	140.66	198.11
		All	167.33 ± 5.92	155.62	179.05
Performance level	**TPK(s)**	2-3	81.40 ± 0.38[b]	80.64	82.17
		4-5	80.58 ± 0.33[b]	79.94	81.23
		6-7	78.49 ± 0.52[a]	77.45	79.53
		8-10	78.65 ± 0.46[a]	77.73	79.57
		All	80.25 ± 0.21	79.83	80.66

ET_B and ET_{JA}(ºC)= eye temperature measured 2 hours before and just after the race, respectively, measured in celsius degrees; HR_B and HR_{JA}(bpm)= heart rate measured 2 hours before and just after the race, respectively, measured in beats per minute; ΔET (%) = eye temperature increase between ET_B and ET_{JA} measured in percentage; ΔHR = heart rate increase between HR_B and HR_{JA} measured

in percentage; TPK (s) = time per kilometre, measured in seconds; s.e. = standard error; CL (95%) = confidence level of 95%; a,b,c Different letters indicate statistical significant differences ($p<0.05$) between age LS means calculated within parameters.

Table 2. General linear model analysis for factors influencing time per kilometre (TPK) measurement for the Spanish Trotter Horse competing in trotting races.

Trait	Factors	d.f.	F	p-value
TPK	**ET_B**	1	10.37	**
	HR_{JA}	1	0.87	n.s.
	ΔET	1	4.72	*
	ΔET*Age	3	1.78	n.s.
	EBV	1	0.07	n.s.
	$Time_{increm}$	14	1.77	*
	Sex	2	0.07	n.s.
	Age	3	2.61	*

ET_B = eye temperature measured 2 hours before the race; HR_{JA}= heart rate measured just after the race; ΔET = eye temperature increase between ET_B and ET_{JA}; EBV = estimated breeding value of TPK trait; ΔET*Age = interaction between eye temperature increase and age; $Time_{increm}$ = time spent in taking the measure from the end of the race. *$p < 0.05$; **$p< 0.01$; n.s. = not significant.

296 **FIGURE LEGENDS**

297 Fig 1: Segmented regression analysis and break point for the residual predicted of time per kilometre
298 (TPK) according to the eye temperature increase (ΔET) in the Spanish Trotter Horse participating in
299 trotting races.

300 Fig 2: Response surface plot describing the relationship among eye temperature measured 2h before
301 the race (ET_B), eye temperature increase (ΔET) and the predicted value of time per kilometre (TPK)
302 in the Spanish Trotter Horse participating in trotting races.

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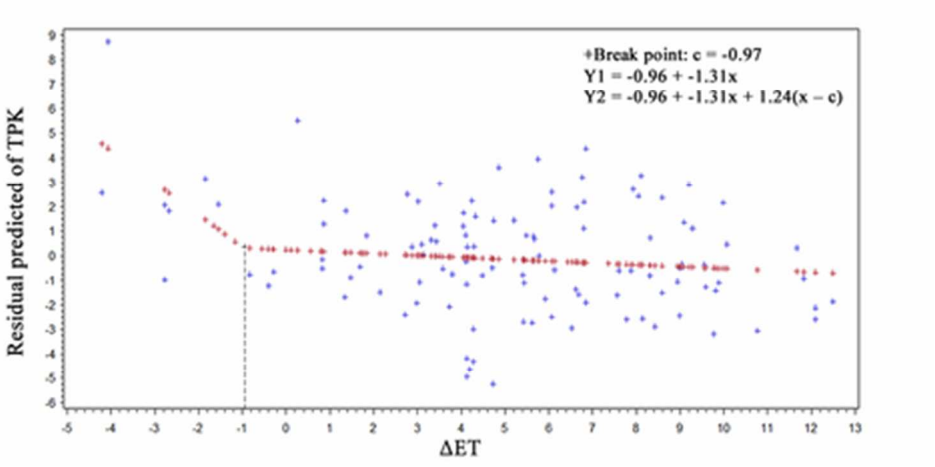
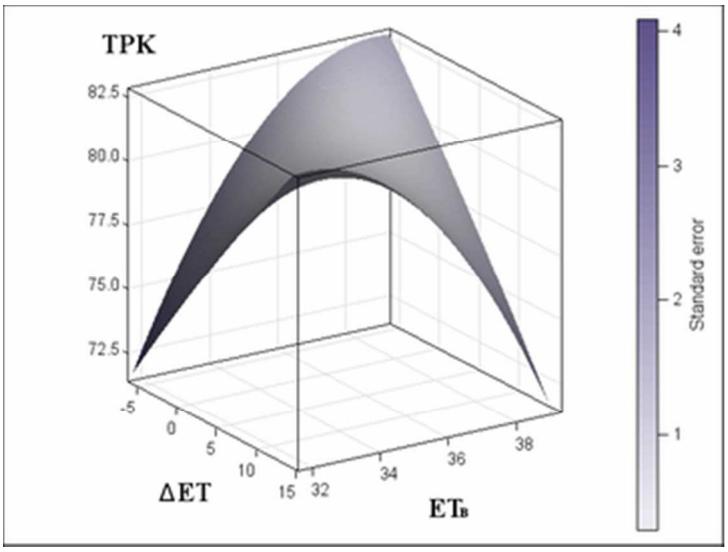


Segmented regression analysis and break point for the residual predicted of time per kilometre (TPK) according to the eye temperature increase (ΔET) in the Spanish Trotter Horse participating in trotting races.

40x19mm (300 x 300 DPI)



Response surface plot describing the relationship among eye temperature measured 2h before the race (ET_B), eye temperature increase (ΔET) and the predicted value of time per kilometre (TPK) in the Spanish Trotter Horse participating in trotting races.

30x22mm (300 x 300 DPI)

ARTÍCULO 3

ANIMAL GENETICS
Immunogenetics, Molecular Genetics and Functional Genomics

Evidence for the effect of serotoninergic and dopaminergic gene variants on stress levels in horses participating in dressage and harness racing

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Keywords:	neurotransmitter, reactivity, infrared thermography, SNP, dressage ability, trotter racing

Evidence for the effect of serotoninergic and dopaminergic gene variants on stress levels in horses participating in dressage and harness racing

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Summary

Eye temperature assessed with infrared thermography is an adequate tool for stress level assessment in sport horses' competitions having a moderate heritability. Serotonin and dopamine signal transduction-linked gene variants have been associated with anxiety-related traits in several species. This study examined the association between ten variants in *BDNF*, *COMT*, *HTR1A*, *TPH2* and *SLC6A4* genes (and the haplotypes at *SLC6A4* gene) with stress level (measured with eye temperature and heart rate), in 270 animals 135 Spanish Trotter Horses (STH) participating in trotting races and 135 PRE in dressage. Association analyses were performed using a unified mixed model (counting for population structure and individual relatedness). The *g.43865600G>A* intronic variant located 11.0 kb downstream from the transcription start site of *SLC6A4* gene was associated with an increase in eye temperature before competition with a relative contribution of this variant of 38.8% (*P*=0.001), 31.8% just after (*P*=0.001) and 29.8% after the competition (*P*=0.003). In STH, this variant showed the same association with eye temperature before (*P*=0.001, contribution 27.2%), just after (*P*=0.0003, 29.0%) and after the competition (*P*=0.002, 17.5%); and the *c.*111G>A* variant located at the 3'UTR region of *COMT* gene was associated with eye temperature 2h after the competition (*P*=0.001, 22.3%). These results showed that *SLC6A4* and *COMT* variants are associated with stress level measured as eye temperature increase during competitions and may be promising tools for genetic testing against resistance at high stress levels in trotter horses.

Keywords: neurotransmitter, reactivity, infrared thermography, SNP, dressage ability, trotter racing

Main text

Sport horse disciplines, as trotter racing or dressage, require not only a set of physiological and mechanical, but also behavioural characteristics (McBride and Mills, 2012). During any competitions, the sympathoadrenal (ANS) and the hypothalamus–pituitary–adrenal (HPA) axis functions are activated, as environmental stimuli potentially threaten horse internal equilibrium, and this could have either a positive or a negative influence on horse's welfare and sporting performance (Bartolomé & Cockram 2016). Recent developments in reliable non-invasive techniques as eye temperature (ET) analysis with infrared thermography (IRT) could allow measure stress levels in horses during competitions (Valera *et al.* 2012; Bartolomé *et al.* 2013; Sánchez *et al.* 2016; Negro *et al.* 2017). However, there is little knowledge about genetic architecture of horse behaviour. The aim of this study was to test for the first time the association of ten variants, two of them described for the first time in *Equus caballus*, in five candidate genes with stress levels, measured with a reliable non-invasive technique in two Spanish horse breeds participating in dressage and trotter racing disciplines. To examine the association of *BDNF, COMT, HTR1A, SLC6A4* and *TPH2* variants and the haplotypes at *SLC6A4* gene (Table S1) with the stress levels, 270 horses were genotyped (135 STH and 135 PRE). STH measurements were taken during four trotting races held in October 2014 and 2015 (hippodromes of Son Pardo and Manacor, Mallorca, Spain), whereas PRE records were taken during final dressage competitions between 2012 and 2015 (equestrian centres located in Toledo, Zaragoza and Seville, Spain). The stress level was evaluated with ET assessed by IRT and with HR measurements, collected at three stages of the competition: 2h before, just after and 2h after the competition. ET images were taken with a FLIR i7 camera (FLIR Systems AB, FLIR Systems, Inc., Sweden), following the methodology of Bartolomé *et al.* (2013). And HR was assessed with a portable pulsometer (Equine Healthcheck; Polar Electro®, Kempele, Finland). Genomic DNA was extracted from fresh

blood or hair samples using standard laboratory protocols. Primers for PCR amplification are indicated in Table S2. The PCR reaction (annealing temperature of 65º C) and sequencing was performed using the same conditions as in Negro *et al.* (2016). Association analysis was performed for the different measures of ET and HR following the code implemented by Yu *et al.* (2006) using SAS 9.2 (Statistical Analysis System, Inc. USA).The mixed model equation for Q + K method is an expanded version of a traditional mixed model.

The population structure (Q matrix) was obtained using a Bayesian model-based clustering approach with admixture model implemented in Structure 2.2 (Falush *et al.* 2007). Kinship matrices were built for the global population and for each breed using the complete information from the official studbooks, using ENDOG v.4.6 (Gutiérrez & Goyache 2005). Furthermore, the percentage of variance absorbed for each gene variant (V_a), was measured on each breed for the different measures of ET and HR, as the subtraction between the percentage of variance absorbed for the predictive model and the percentage of variance absorbed for the model excluding the SNP questioned. Finally, the Benjamini-Hochberg correction was used to control false discovery rate (FDR) of *P*-values at a significance level of 5%.

In the present study, changes in ET and HR were monitored for PRE and STH horses (Table S3). When trotter racing measurements were compared to those of dressage, higher values for ET and HR were shown in all stages, suggesting the existence of different stress perception patterns for each discipline. Additionally, in STH, ET has been shown as a suitable tool to assess not only the stress level during trotting races, but also the physiological level when this stress becomes distress (Negro *et al.* 2017), thus compromising horse welfare during competition. Genotyping for the variants of *BDNF, COMT, HTR1A, SLC6A4* and *TPH2* genes (Table S1) using a unified mixed model (Yu *et al.* 2006) from the two breeds studied (PRE and STH), globally and separately, showed association of ET measurements and two of the

variants analysed of *SLC6A4* and *COMT* genes. Thus the *g.43865600G>A* intronic variant located 11.0 kb downstream from the transcription start site of *SLC6A4* gene was associated with an increase in eye temperature before competition with a relative contribution of this variant of 38.8% (*P*=0.001), 31.8% just after (*P*=0.001) and 29.8% after the competition (*P*=0.003) (Table 1). The percentages of variance explained by this gene marker (V_a) were 2.0, 0.9 and 2.3% for before, just after and 2h after the competition, respectively. In STH (Table 2), this variant showed the same association with eye temperature before (*P*=0.001, contribution 27.2%, V_a=4.6%), just after (*P*=0.0003, 29.0%, V_a=0.6%) and after the competition (*P*=0.002, 17.5%, V_a=1.0%). However, in PRE this marker was not statistically significant for any stage, explaining only 0.29, 0.08 and 0.09% of the additive genetic variance, respectively. Two new variants, *g.43865368C>T* and *g.43864387C>T*, have been discovered "downstream" of the polymorphism described above at *SLC6A4* gene, which has not been previously published in *Equus caballus*. They were located at positions 232 and 1213 (Genbank ref MF043946-MF043951) (Table S1). The Serotonin transporter gene (*SLC6A4*) is related to modulation of anxiety (Hori *et al.* 2016; Piszczek *et al.* 2015). In STH, the *c.*111G>A* variant located at the 3'UTR region of *COMT* gene was associated with eye temperature after the competition (*P*=0.001, 22.3%, V_a=0.8%). Catechol-O-methyltransferase (*COMT*) plays a role in some aspects of emotional and social behaviour, such as anxiety, aggression, pain sensitivity, etc. (Tunbridge *et al.* 2006).

Therefore, our results seem to be in line with the highly plausible assumption that the genes of dopaminergic (*COMT* gene) and serotoninergic (*SLC6A4* gene) systems can affect the emotional response and behavioural traits in horses and influence their performance. Thus, this would help STH breeders to use genetic testing to control the stress levels in horses in order to obtain the best results in competitions. No significant associations with the genes

studied were found for PRE. However, further studies are required to confirm these hypotheses.

Acknowledgments

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Table 1 Determination of association for SNPs in the mixed model for simultaneous correction of global population structure and unequal relatedness among sampled individuals.

Table 2 Determination of association for SNPs in the mixed model for simultaneous correction of PRE and TRO population structures and unequal relatedness among sampled individuals.

Supplementary material

Table S1 Description of BDNF, COMT, HTR1A, SLC6A4 and TPH2 polymorphisms analyzed and allelic frequencies of the SNP under study comparing different stress levels for Pura Raza Español (PRE) and Spanish Trotter Horse (STH) populations.

Table S2 Description of the PCR primers at *BDNF,COMT*, *HTR1A*, *SLC6A4* and *TPH2* polymorphisms analyzed in the study indicated.

Table S3 Descriptive statistics for measurements the stress level of Spanish Trotter Horses (STH) during trotting races and of Pura Raza Español (PRE) horses during dressage competitions.

Table 1 Determination of association for SNPs in the mixed model for simultaneous correction of global population structure and unequal relatedness among sampled individuals.

	Gene (reference allele)	ET_B		HR_B		ET_{JA}		HR_{JA}		ET_A		HR_A	
		B	*P*	B	*P*	B	*P*	B	*P*	B	*P*	B	*P*
GLOBAL	COMT (A)	0.16	n.s.	-0.06	n.s.	0.28	0.17	0.88	n.s.	0.23	n.s.	0.00	n.s.
	BDNF (A)	-0.09	n.s.	-0.22	n.s.	-0.16	0.35	-1.23	n.s.	-0.14	n.s.	0.00	n.s.
	HTR1A (C)	-0.07	n.s.	0.60	n.s.	-0.04	0.87	-2.77	n.s.	0.05	n.s.	0.00	n.s.
	TPH2 G)	0.04	n.s.	-1.39	n.s.	-0.01	0.98	1.49	n.s.	0.00	n.s.	0.00	n.s.
	g.43865600G>A (A)	0.59	***	0.55	n.s.	0.65	***	1.64	n.s.	0.41	**	0.00	n.s.
	g.43865368C>T (T)	-0.20	n.s.	-0.84	n.s.	-0.25	0.15	-0.85	n.s.	-0.12	n.s.	0.00	n.s.
	g.43864387C>T (T)	-0.11	n.s.	-1.01	n.s.	0.03	0.93	0.09	n.s.	0.00	n.s.	0.00	n.s.
	g.43864288A>C (C)	0.05	n.s.	-0.59	n.s.	0.11	0.47	-1.70	n.s.	0.00	n.s.	0.00	n.s.
	g.AG43864196_7TC (AC)	0.01	n.s.	0.09	n.s.	0.28	0.12	-0.26	n.s.	0.00	n.s.	0.00	n.s.
	g.43864091C>A (A)	-0.20	n.s.	0.41	n.s.	-0.25	0.27	1.81	n.s.	0.00	n.s.	0.00	n.s.
	SLC6A4_Haplotype	0.00	n.s.	0.01	n.s.	-0.00	0.78	-0.08	n.s.	-0.31	n.s.	0.03	n.s.

ET_B, ET_{JA} and ET_A= eye temperature measured approx. 2 hours before, just after and approx. 2 hours after the competition, respectively; HR_B, HR_{JA} and HR_A= heart rate measured approx. 2 hours before, just after and approx. 2 hours after the race, respectively. Significance level:*: P< 0.05, **: P< 0.01***: P< 0.001 in bold after applying FDR (Benjamini & Hochberg) correction for multiple testing. B: coefficients of the number of copies of the reference allele.

Table 2 Determination of association for SNPs in the mixed model for simultaneous correction of PRE and TRO population structures and unequal relatedness among sampled individuals.

	Gene (reference allele)	ET_B		HR_B		ET_{JA}		HR_{JA}		ET_A		HR_A	
		B	*P*	B	*P*	B	*P*	B	*P*	B	*P*	B	*P*
Pura Raza Español (PRE)	COMT (A)	-0.20	n.s.	0.12	n.s.	-0.06	n.s.	0.44	n.s.	0.00	n.s.	0.00	n.s.
	BDNF (A)	-0.19	n.s.	0.67	n.s.	0.00	n.s.	-2.30	n.s.	-0.02	n.s.	0.00	n.s.
	HTR1A (C)	-0.24	n.s.	1.70	n.s.	-0.27	n.s.	-3.77	n.s.	-0.03	n.s.	0.00	n.s.
	TPH2 G)	-0.10	n.s.	-0.40	n.s.	-0.08	n.s.	4.47	n.s.	0.00	n.s.	0.00	n.s.
	g.43865600G>A (A)	0.19	n.s.	-0.99	n.s.	-0.06	n.s.	1.13	n.s.	0.05	n.s.	0.00	n.s.
	g.43865368C>T (T)	0.01	n.s.	0.26	n.s.	-0.03	n.s.	-1.65	n.s.	0.03	n.s.	0.00	n.s.
	g.43864387C>T (T)	-0.14	n.s.	-1.51	n.s.	0.01	n.s.	1.73	n.s.	0.00	n.s.	0.00	n.s.
	g.43864288A>C (C)	0.22	n.s.	-0.68	n.s.	0.13	n.s.	-1.02	n.s.	0.04	n.s.	0.00	n.s.
	g.AG43864196_7TC (AC)	-0.10	n.s.	0.39	n.s.	-0.05	n.s.	-1.41	n.s.	0.13	n.s.	0.00	n.s.
	g.43864091C>A (A)	-0.18	n.s.	1.00	n.s.	-0.25	n.s.	1.70	n.s.	-0.05	n.s.	0.00	n.s.
	SLC6A4_Haplotype	0.01	n.s.	0.01	n.s.	0.00	n.s.	-0.16	n.s.	0.00	n.s.	-0.00	n.s.
Spanish Trotter Horse (STH)	COMT (A)	0.00	n.s.	-0.20	n.s.	0.79	n.s.	1.53	n.s.	0.97	***	-3.44	n.s.
	BDNF (A)	0.09	n.s.	-2.10	n.s.	-0.38	n.s.	1.06	n.s.	-0.45	n.s.	0.00	n.s.
	HTR1A (C)	0.00	n.s.	-0.57	n.s.	0.19	n.s.	-1.71	n.s.	0.18	n.s.	0.26	n.s.
	TPH2 G)	0.00	n.s.	-2.22	n.s.	0.06	n.s.	-1.54	n.s.	-0.26	n.s.	0.00	n.s.
	g.43865600G>A (A)	0.90	***	1.68	n.s.	1.19	***	1.97	n.s.	0.76	**	-2.11	n.s.
	g.43865368C>T (T)	-0.40	n.s.	-2.14	n.s.	-0.49	n.s.	-0.07	n.s.	-0.36	n.s.	0.00	n.s.
	g.43864387C>T (T)	0.00	n.s.	-0.54	n.s.	0.00	n.s.	0.00	n.s.	0.00	n.s.	8.12	n.s.
	g.43864288A>C (C)	0.00	n.s.	-0.47	n.s.	0.07	n.s.	0.00	n.s.	0.00	n.s.	0.00	n.s.
	g.AG43864196_7TC (AC)	0.00	n.s.	-0.28	n.s.	0.67	n.s.	1.25	n.s.	0.00	n.s.	0.00	n.s.
	g.43864091C>A (A)	0.00	n.s.	-5.43	n.s.	-0.25	n.s.	0.00	n.s.	0.00	n.s.	0.00	n.s.
	SLC6A4_Haplotype	0.01	n.s.	0.02	n.s.	0.03	n.s.	-0.02	n.s.	0.00	n.s.	-0.05	n.s.

ET_B, ET_{JA} and ET_A= eye temperature measured approx. 2 hours before, just after and approx. 2 hours after the competition, respectively; HR_B, HR_{JA} and HR_A= heart rate measured approx. 2 hours before, just after and approx. 2 hours after the race, respectively. Significance level:*: P< 0.05, **: P< 0.01***: P< 0.001 in bold after applying FDR (Benjamini & Hochberg) correction for multiple testing. B: coefficients of the number of copies of the reference allele.

Supplementary material

Table S1 Description of BDNF, COMT, HTR1A, SLC6A4 and TPH2 polymorphisms analyzed and allelic frequencies of the SNP under study comparing different stress levels for Pura Raza Español (PRE) and Spanish Trotter Horse (STH) populations.

Gene (Target variant)	Variant Name	Polym. (DNA)	Polym. (cDNA)	Position (Chr.)	Reference		PRE			STH		
							Low stressed (32.1-36.0ºC)	High stressed (36.0-39.4ºC)	Total	Low stressed (32.2-37.7ºC)	High stressed (37.8-42.1ºC)	Total
COMT-3'UTR (A)	$COMT^{3'UTR}$	g.989906 G>A	c.*111G>A	Exon 4 (ECA8)	(Dall'Olio *et al.* 2009)	G	0.51	0.54	0.53	0.87	0.77	0.82
						A	0.49	0.46	0.47	0.13	0.23	0.18
BDNF (A)	$BDNF^{A87A}$	g.94041447 G>A	c.261G>A	Exon 1 (ECA7)	-	G	0.64	0.61	0.62	0.78	0.84	0.81
						A	0.36	0.39	0.38	0.22	0.16	0.19
HTR1A (C)	$HTR1A^{E257D}$	g.9478637 G>C	c.771G>C	Exon 1 (ECA21)	Hori *et al.* (2016)	G	0.88	0.94	0.91	0.88	0.87	0.88
						C	0.12	0.06	0.09	0.12	0.13	0.12
TPH2 (G)	$TPH2^{H447R}$	g.671744 A>G	c.1340A>G	Exon 11 (ECA28)	-	A	0.91	0.94	0.92	0.92	0.93	0.92
						G	0.09	0.06	0.08	0.08	0.07	0.08
SLC6A4 (A)	rs68968521	g.43865600 G>A	-	Intron 10 (ECA11)	-	G	0.86	0.90	0.84	0.88	0.70	0.83
						A	0.14	0.10	0.16	0.12	0.30	0.17
SLC6A4 (T)	g.727C>T*	g.43865368 C>T	-	Intron 10 (ECA11)	-	C	0.79	0.75	0.77	0.71	0.77	0.74
						T	0.21	0.25	0.23	0.29	0.23	0.26
SLC6A4 (T)	g.1708C>T*	g.43864387 C>T	-	Intron 10 (ECA11)	-	C	0.93	0.95	0.94	0.91	0.97	0.94
						T	0.07	0.05	0.06	0.09	0.03	0.06
SLC6A4 (C)	rs68968520	g.43864288 A>C	-	Intron 10 (ECA11)	-	A	0.62	0.60	0.61	0.36	0.38	0.37
						C	0.38	0.40	0.39	0.64	0.62	0.63
SLC6A4 (AC)	$SLC6A4^{T539A-I}$	g.AG43864196_7TC	c.AC1615-1616GT	Exon 11 (ECA11)	Momozawa *et al.* (2006)	GT	0.24	0.27	0.25	0.37	0.25	0.31
						AC	0.76	0.73	0.75	0.63	0.75	0.69
SLC6A4 (A)	rs396986545	g.43864091 C>A	-	Intron 11 (ECA11)	-	C	0.72	0.74	0.76	0.97	0.98	0.98
						A	0.28	0.26	0.24	0.03	0.02	0.02

The Low and High stressed groups were made according to ET_{JA} (eye temperature measured just after the competition) values. * Two new SNP variants described for the first time in *Equus caballus* (Genbank ref: MF043946-MF043951)

Table S2 Description of the PCR primers at *BDNF*,*COMT*, *HTR1A*, *SLC6A4* and *TPH2* polymorphisms analyzed in the study indicated.

Gene	Genbank accession	PCR primers (5' – 3')
COMT (ECA8)	AB17828	F: TGACAGTCTGGATGTGGGACTAG R: TGTGAGAAAGGGATGCTCATGG
TPH2 (ECA28)	AB264323	F: CGAAGACCACGTGCTTGCAG R: CAGATGCAGTTTGGTTAGGGA
HTR1A (ECA21)	AB264325	F: CGACTACGTGAACAAGAGGACACC R: CGCGGGCTACTCCTTTATCATC
BDNF (ECA7)	AB264324	F: GCACTTAGAACAGTACCTGGCACACAGC R: CTGACAATGCTTTCTGGTCCTCTGGG
SLC6A4 (ECA11)	NC_009154	F: ATCCAGAGGCCCTTTTGAGTTT R: AACAGGAACATTCTCGCCTCTT F: ATTCTCTGTCGAGTGGGTTCAC R: CAGGGGAGCGATGTGATAAAGA

Table S3 Descriptive statistics for measurements the stress level of Spanish Trotter Horses (STH) during trotting races and of Pura Raza Español (PRE) horses during dressage competitions.

Traits		PRE (n = 135)			STH (n = 135)		
		Mean ± s.e.	Min	Max	Mean ± s.e.	Min	Max
Stress level	**ET_B**	35.28 ± 0.10	31.15	42.26	36.13 ± 0.15	32.30	39.70
	ET_{JA}	36.05 ± 0.10	32.07	39.35	37.54 ± 0.19	32.30	42.10
	ET_A	36.02 ± 0.06	34.00	37.70	36.21 ± 0.14	32.10	38.55
	HR_B	37.84 ± 0.41	24.00	54.00	41.39 ± 0.99	30.00	90.00
	HR_{JA}	68.83 ± 1.35	40.00	122.00	107.47 ± 1.17	84.00	138.00
	HR_A	38.11 ± 0.53	10.00	56.00	51.15 ± 1.34	30.00	90.00

ET_B, ET_{JA} and ET_A= eye temperature measured approx. 2 hours before, just after and approx. 2 hours after the competition, respectively; HR_B, HR_{JA} and HR_A= heart rate measured approx. 2 hours before, just after and approx. 2 hours after the race, respectively; s.e. = standard error.

CAPÍTULO III: ANÁLISIS GENÉTICO CUANTITATIVO DEL CABALLO TROTADOR ESPAÑOL Y BÚSQUEDA DE ASOCIACIÓN DE MARCADORES MOLECULARES TIPO SNP EN GENES CANDIDATOS

Este tercer capítulo está integrado por un artículo científico:

- **Negro S.**, Valera M., Membrillo A., Gómez, M.D., Solé, M., Menendez-Buxadera, A., Anaya, G., Molina, A. *Quantitative analysis of short and long distance racing performance in young and adult horses and association analysis with functional candidate genes in Spanish Trotter Horses.* J. Anim. Breed. Genet. 133(5), 347–356. ISSN 0931-2668. (JBG12208).

ARTÍCULO 4

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ORIGINAL ARTICLE

Quantitative analysis of short- and long-distance racing performance in young and adult horses and association analysis with functional candidate genes in Spanish Trotter horses

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Keywords
Deregression; endurance ability; equine; expected breeding value; SNP; sprinting ability.

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Summary

The association of five candidate genes with sporting performance in young and adult Spanish Trotter horses (STHs) was performed according to a previous selection based on quantitative analysis of the trait time per kilometre (TPK). A total of 334 516 records of TPK from 5958 STHs were used to estimate the estimated breeding values (EBVs) at different age groups (young and adults horses) throughout the range of distances (1600–2700 m) using a bicharacter random regression model. The heritability estimated by distance ranged from 0.16 to 0.40, with a different range for the two age groups. Considering the animals with the best and the worst deregressed EBV, 321 STHs were selected for SNP genotyping in *MSTN*, *COX4I2*, *PDK4*, *DMRT3* and *CKM* genes. An association analysis based on ridge and logistic regression revealed that the young trotters with genotype GG in *PDK4* ($p < 0.05$) and AA of *DMRT3* ($p < 0.001$) SNPs show the best potential in short-distance races, while those carrying the genotype AA in *DMRT3* ($p < 0.001$) and CC in *CKM* ($p < 0.05$) genes seem to be the best in long-distance races. Adult trotters with genotype AA in *DMRT3* also display greater speed ($p < 0.05$) and endurance ($p < 0.001$).

Introduction

Trotter racing is an economically important equine sporting event, which ranks second only to thoroughbred racing (Thiruvenkadan *et al.* 2009). In Spain, trotting races have been held since the beginning of the 20th century and approximately 1500 races take place annually, with around 5000 horses taking part (Gómez *et al.* 2010b). The racing performance of trotter horses is evaluated through a combination of different traits that estimate racing ability (Thiruvenkadan *et al.* 2009). In Spain, as in other European countries, genetic evaluation of racing performance in trotters is routinely performed by a repeatability multivariate BLUP animal model using competition results from each individual race (Arnason *et al.* 1999; Bugislaus *et al.* 2005; Gómez *et al.* 2010b; Roehe *et al.* 2001). However, in practical terms, the animals compete at different ages and distances. Thus, in this case, the BLUP model evaluates a trait such as time per kilometre (TPK) as a traversal character, assuming that this trait is controlled by the same genes and with the same gene expression levels regardless of the length of the race and the age at which each animal is competing (Gómez *et al.* 2010a). However, there is evidence that gene expression varies with the developmental stages of the individual and different genes affect the traits, depending on the age of the animal, which changes the physiology and, consequently, the performance of trotters (Langlois & Vrijenhoek 2004; Bugislaus *et al.* 2006; Gómez *et al.* 2011). It has also been shown that the best animals

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for sprinting do not have to match the best animals for running long distances (endurance) (Gómez *et al.* 2010a). As a result, the use of quantitative analysis such as the random regression model (RRM), which have the ability to estimate genetic potential across different external factors such as race distance (Gómez *et al.* 2010a) or the animal's age (Gómez *et al.* 2011), has increased over the last few decades. However, to make a reliable estimate of genetic evaluation, each individual must be assessed in many trotting competitions for their own performance and that of their close relatives, and the breeding stock tested is normally made up of relatively old horses (10–12 years). Furthermore, racehorse training is a big business, so being able to make an early preselection of animals for trotter racing is likely to be of the utmost importance.

Useful markers to support the selection process are now becoming more widely available from the sequencing of the horse genome. Therefore, five candidate genes related to sporting performance previously analysed in other horse breeds for both short- and long-term races were selected: *myostatin* gene (*MSTN*; Hill *et al.* 2010a), *cytochrome C oxidase, subunit IV, isoform 2* gene (*COX4I2:* Gu *et al.* 2010), *pyruvate dehydrogenase kinase, isozyme 4* gene (*PDK4:* Hill *et al.* 2010c), *creatine kinase muscle* gene (*CKM:* Gu *et al.* 2010) and *doublesex and mab-3 related transcription factor 3* gene (*DMRT3:* Andersson *et al.* 2012). According to previous studies, these genes are related to physiological characters in animals for competition, which include coordination of limb movement (*DMRT3*), oxygen levels (*COX4I2*) and energy metabolism related to exercise intensity (*PDK4* and *CKM*) or skeletal muscle development (*MSTN*). The aim of this study then was to analyse the potential use of molecular genetics tools for sporting aptitude. For that a quantitative analysis of short- and long-distance racing performance in young and adult horses was performed to select the animals for the association analysis of SNP genotyping in *MSTN*, *COX4I2, CKM, PDK4* and *DMRT3* genes with racing performance breeding values for short- and long-distance races (sprint and endurance ability) with young and adult Spanish Trotter horses.

Materials and methods

Sport performance genetic analysis

Records from Spanish Trotter horses (STHs) competitions were collected from the *Official Trotting Federation* database. Only harness racing was included, because mounted races are not common in Spain. Therefore, the starting mode in all the races was autostart (mobile start at a constant speed) or handicap (assigning advantage to the horses with worst performance results in order to equalize the chances of winning).

For this study, the TPK was chosen as it is considered one of the best criteria to select animals for sporting performance in trotters (Gómez *et al.* 2010b). This is due not only to the fact that it represents the highest heritability of all analysed racing performances (Gómez *et al.* 2010a, 2011) as the only direct measure of speed, but it also reflects the speed capacity of the horses when many favourable environmental conditions are taken together, namely the sum of the effects of a number of genetic and environmental factors (Arnason 2001).

The combination of hippodrome-racing date (HDR) with less than 30 observations was deleted, and records with age over 8 years old were not considered as its effect was not possible to capture with small data. For the analysis, the age factor was divided into two groups: young horses (2–4 years old) and adult horses (5–8 years old), and they had to have over 5 race records in each age group to be included in the analysis.

The final database included 334 516 records from 5958 horses (2668 males and 3290 females), for races held from 1995 to 2013 from 599 HDR guided by 2293 drivers. The pedigree file to calculate the inverse of the relationship matrix was generated from this breed's studbook by including all the ancestors of the recorded animals until an average of fourth complete generations (the animals imported for breeding were registered with all their pedigree information) giving a total of 13 869 animals.

Norm reaction model

The dependent variable was the TPK, recorded for the 2 age groups as different traits. Accordingly, a bicharacter RRM was applied which allows us to estimate the (co)variance components for the different age groups and the full race distance (1600 to 2700 m). The next model was applied following models described by Gómez *et al.* (2010a, 2011):

$$\mathbf{y} = \mathbf{HDR_i} + \mathbf{S_l} + \mathbf{C_0} + \sum_{\mathbf{m=0}}^{\mathbf{q_f}} \boldsymbol{\beta}_{\mathbf{kp}} \boldsymbol{\lambda}_{\mathbf{tm}} + \sum_{\mathbf{m=0}}^{\mathbf{q_a}} \boldsymbol{\alpha}_{\mathbf{jp}} \boldsymbol{\lambda}_{\mathbf{tm}} + \sum_{\mathbf{m=0}}^{\mathbf{q_w}} \boldsymbol{\gamma}_{\mathbf{j^*p}} \boldsymbol{\lambda}_{\mathbf{tm}} + \sum_{\mathbf{m=0}}^{\mathbf{q_c}} \mathbf{v}_{\mathbf{np}} \boldsymbol{\lambda}_{\mathbf{tm}} + \mathbf{e}_{\mathbf{ijklnop}}$$

where **y** is the observation x of the dependent variable (TPK) recorded at age **p** of animal **j**, of sex **l,**

with starting method **o** in the combinations of hippodrome-racing date performed at distances **t** ($\mathbf{HDR_i}$ with 599 levels), trainer–driver **n**, $\mathbf{S_l}$ (2 sex levels) and $\mathbf{C_o}$ (2 starting mode levels). The regression of Legendre polynomials (order $\mathbf{m} = \mathbf{q_f} = 2$) of **y** on the distances **t** was also considered as fixed effects. Those effects considered as random were as follows: animal effects ($\boldsymbol{\alpha_j}$ and $\mathbf{j} = 13\,869$ animals in the pedigree), the permanent environment effect due to repetitions of the same variable in the animal (γ_{j^*} and $\mathbf{j}^* = 5958$ animals with data) and the trainer–driver effect (υ_n and $n = 16\,379$ levels). The variables $\lambda_{tm} = \phi_m(t)$ were as follows: elements of the K_i random regression matrix for the Legendre polynomial ϕ_m of order **m** and the variable scale **t** (distance) expressed in standardized form between −1 and +1. The order of adjustment of these polynomials was $\mathbf{q_a} = \mathbf{q_w} = \mathbf{q_c} = 1$ for the genetic effects of the animal, for the permanent environment of the animal with repeated data and for the effect of the trainer–driver, respectively. The residual variance (**e**) was considered heteroscedastic, for two levels (age groups). For a more detailed description of the model, see Appendix S1 of the Supporting Information.

The ASREML V3.0 software (VSN International, Harpenden, UK) was used. The solutions produced by the model for each animal contained **m** genetic random regression coefficients for age 1 and age 2 and were used to estimate the *EBV* for any point **t** along the full range of distances.

Finally, the 1600 and 2600 EBVs were used as a reference distance for short and long races (sprint and endurance ability), respectively, in young and in adult animals.

Deregressed estimated breeding values (DEBVs) were used as response variables to estimate SNP effects. DEBVs were obtained from the EBVs together with their reliabilities from animals and their sires and dams using a Fortran program implementing the Garrick *et al.* (2009) deregression method to remove parent average effects due to heterogeneous variance. The weight coefficients $(1 - h^2)/[(c + (1 - r^2)/r^2)h^2]$ were used, where $c = 0.8$ (the fraction of genetic variance not explained by markers) and r^2 the reliability of that EBV.

Association analysis

Samples selected for association analysis

A total of 321 samples were selected from 2469 available DNA samples of STHs in the Spanish Central Veterinary Laboratory (National reference DNA bank, Ministry of Agriculture, Algete, Madrid). The selection criterion was to present the highest reliabilities for the DEBV according to the norm reaction model in young and adult horses in short (1600 metres)- and long-distance races (2600 metres).

DNA extraction, PCR and sequencing

Genomic DNA had been extracted from fresh blood samples or hair samples using standard laboratory protocols. Primers for five gene fragments amplification were designed from GenBank sequences (Table 1). The PCR was performed using standard protocols. The thermal profiling consisted of a hot start step at 96°C for 3 min, followed by 35 cycles of 30 s at 96°C, 30 s at the annealing temperature of 71.9°C (*PDK4 and CKM* gene) or 63.9°C (*MSTN*, *COX4I2* and *DMRT3*), 2 min at 72°C and a final extension step of 10 min at 72°C. PCR products for Sanger sequencing were purified using SureClean kit (Bioline, Luckenwalde, Germany). The sequences obtained by ABI PRISM 3130 capillary electrophoresis equipment (Applied Biosystems, Foster City, CA, USA) were analysed with Sequencher™ v.4.1.4 software.

Table 1 Primer design in Sanger multiplex sequencing of performance gene SNP in the Spanish Trotter horse population

Gene	Chrom	SNP	PCR primers	GenBank accession
MSTN	18	g.66493737C>T	F: 5′AAGAGGTTATAGCTCAGAGTCCTGC3′ R: 5′ACTAGCAATTTCTTTTATTTTGGTTCCCC3′	GQ183900.1
COX4I2	22	g.22684390C>T	F: 5′CCCCCCCAAGACAGCCAGAACCCC3′ R: 5′CCTCACCGCCTCTCTCTGTTCCTTCCCC3′	NC_009165.2
PDK4	4	g.38973231A>G	F: 5′GCACTTAGAACAGTACCTGGCACACAGC3′ R: 5′CTGACAATGCTTTCTGGTCCTCTGGG3′	NC_009147.2
CKM	10	g.15884567A>G	F: 5′TGGGCTGTGTGGCCGGTGACGAGG3′ R: 5′CCTGCCCACGGAGGAGCAGAGCC3′	KF983328y-30
DMRT3	23	DMRT3_Ser301STOP	F: 5′AATCTTCCCCAACCAGAAGCCCACG3′ R: 5′ACAGGGTGACATCATTGGGCAGG3′	AAWR02015684.1

Statistical analysis for association study

Marker association and predictive model efficacy were evaluated with a ridge regression procedure (RRP):

$$\mathbf{Y} = \mu + \beta_{MSTN}X_1 + \beta_{COX412}X_2 + \beta_{CKM}X_3 + \beta_{PDK4}X_4 + \beta_{DMRT3}X_5$$

where **Y** is the vector of DEBV, μ is a common intercept, *βi coefficients* are the regression coefficients of the number of copies of the reference allele (A allele in *DMRT3*, T in *COX4I2*, C in *CKM*, A in *PDK4* and T in *MSTN* genes), and *X* is vector of incidence of markers.

Furthermore, the percentage of variance absorbed for each gene marker (percentage of the total additive genetic variance of the trait) was measured for each category of racing distance and trotter age following the Gianola *et al.* (2009) methodology.

Finally, a *logistic regression model* was used to obtain the receiver operating characteristic (ROC) curve (Brethour 2000). This showed the predictive power of this molecular multimarker tool through the probability that the classifier will rank a randomly chosen positive instance higher than a randomly chosen negative instance (Brethour 2000).

The parameter SGoF+ (Carvajal-Rodriguez & de Uña-Alvarez 2011) was used to control the false discovery rate (FDR) of the p-values at a significance level of 5%.

The statistical analysis was carried out using SAS 9.2 (Statistical Analysis System, Inc., Cary, NC, USA) software.

Results

The evolution of average time by distance is shown in Figure 1. The highest speeds were obtained for the shortest distances (<2000 m) in adult horses, whereas middle distance races (2000–2200 m) had the same average speed for both ages, and long races (>2200 m) showed similar average speed values, with a heterogeneous evolution.

Genetic parameters by race distance

Figure 2 shows, grouped by age (young, group 1 and adult, group 2), the heritability evolution and genetic correlations (between both age groups) for the TPK trait, along the full range of race distances. For age group 1, higher heritability values were obtained for shorter distances (h^2 = 0.401 for 1600 m). This value decreased with distance, with the lowest value at 2600 m (h^2 = 0.157). For group 2, the heritability levels ranged from 0.231 (for 1600 m) to 0.193 (for 2700 m). The heritability levels for medium-distance races (2100–2200 m) were very similar for both age groups (≈ 0.200). These differences were caused by the changes in genetic variances by distance, because the trajectory of the phenotypic variances by distance was similar for age 1 and 2. The Table S1 shows the genetic and phenotypic variance evolution for the full range of race distances analysed.

The genetic correlation at each distance between young and adult animals decreased as the distance

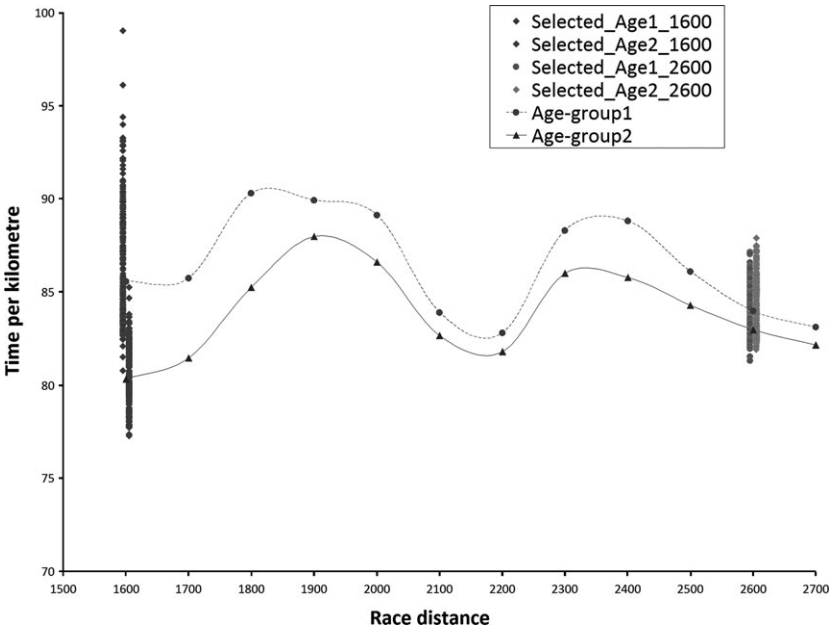

Figure 1 Evolution of speed (time per kilometre) by race distance and age group (1 = 2–4 years old and 2 = 5–8 years old) in Spanish Trotter horses, including the estimated speed average of the selected animals at both reference distances.

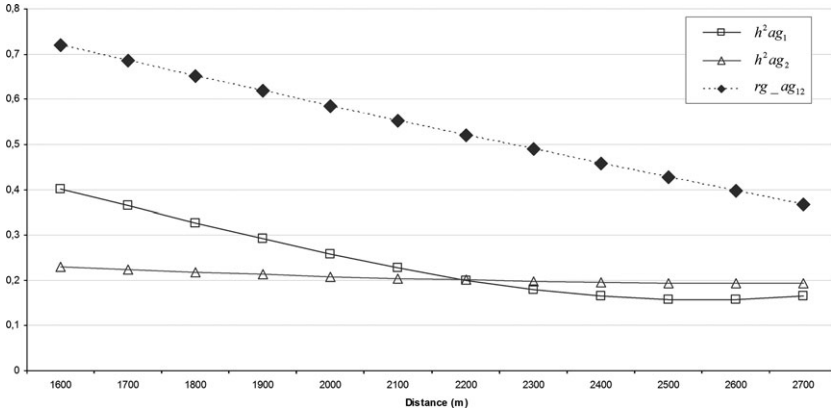

Figure 2 Evolution of heritability level for the time per kilometre grouped by age, along the trajectory of race distance, and the genetic correlation between both age groups at each distance in the Spanish Trotter horse. h^2ag_1 = heritability evolution in young animals. h^2ag_2 = heritability evolution in adult animals. rg_ag_{12} = genetic correlation for each distance between young and adult animals.

became greater (with a maximum of 0.719 at 1600 m and a minimum of 0.367 at 2700 m). The Table S2 shows the genetic correlations between distances for both age groups. These ranged from 0.301 to 0.986 for the group of young animals (above the diagonal) and from 0.888 to 0.990 for adults (below the diagonal). As was expected, the highest correlation values were obtained between the adjacent distances and the lowest ones between the most separate distances. It can be noted that, in young animals, the correlation is generally below 0.9 for distances which differ more than 600 m.

Figure S1 shows the genetic correlations between the speed at short- and long-distance reference points (1600 m and 2600 m, respectively) and the speed at the different distances, analysed according to age groups. In young animals, the loss of genetic correlation between speeds at 1600 m as the distance increases is more than evident, when it is compared with the evolution in adults (when the speed at 2600 m is analysed, the correlation is the opposite).

Genetic association analyses

Genotyping the selected fragment of each of the five candidate genes analysed (Table 1) confirmed their existence in the population of trotters analysed. However, the g.15884567A>G SNP previously described in the *CKM* gene (Gu *et al.* 2010) was not detected in this population. However, a new SNP marker (g.15884216C>G) was discovered 'upstream' of the polymorphism described above in the *CKM* gene, which has not been published before in *Equus caballus*. It was located at position 123 and referred to as KF983328-30 sequences (Table 1). The G allele (73.40%) in the g.15884216C>G marker of *CKM* and the G allele of g.38973231A>G in *PDK4* gene (83.64%) were the most frequent in all the groups of trotters (Table S3). The polymorphism g.66493737C>T of the *MSTN* gene (T allelic frequency of 97.95%) was relatively fixed and low variability could be appreciated in DMRT3_Ser301STOP mutation (A allelic frequency of 86.99%) (Table S3).

The RRP used to analyse marker association with a multimarker model (Table 2) showed that *DMRT3* (with a contribution of 44.1%, $p < 0.001$) and *PDK4* (with a contribution of 25.9%, $p = 0.011$) markers were the best predictors (according to their contribution to the model) in the case of young horses in short-distance races. In adult horses, *DMRT3* also showed association for short-distance races (with a contribution of 53.8%, $p < 0.001$). In long-distance races, *DMRT3* (young horses: 58.1%, $p < 0.001$; adults: 54.4%, $p < 0.001$) and *CKM* (young horses: 33.4%, $p = 0.010$) markers were the best predictors.

The variance absorbed in the % of total additive variance of the trait by the markers analysed (Table 2) fluctuated in short-distance races between 0.01 and 0.30 in young horses, and between 0.01 and 0.89 in adults. For long-distance races, the variance oscillated between 0.01 and 1.67 in young horses, and between 0.01 and 0.68 in adults.

Finally, the ROC curves for speed and endurance potential are presented in Figure S2. The percentages of area under the curve in ROC space (prediction accuracy of the models with selected markers) were 68.2% and 60.1%, respectively, for young and adult horses for speed and 59.1% and 59.8% for endurance potential.

Discussion

According to previous studies, RRM is a promising tool because it allows us to estimate the animal's breeding value through all the stages of its trajectory in racing competitions (Gómez *et al.* 2011).

Table 2 Determination of β-coefficients and association levels for SNPs in the multimarker model through ridge regression procedure for the Spanish Trotter horse population

	Speed					Endurance				
	β	β'(%)	B	p-value	V_a (%)	β	β'(%)	B	p-value	V_a (%)
Young horses										
Intercept			7.46	<0.001				4.95	0.003	
MSTN(T)*	−0.08	14.6	−1.48	0.140	0.01	0.03	5.2	0.32	0.685	0.01
COX4I2(T)	−0.01	1.6	−0.05	0.878	0.01	0.01	1.0	0.02	0.937	0.01
CKM(C)	−0.08	13.8	−0.39	0.162	0.05	−0.17	33.4	−0.54[a]	**0.010**	0.92
PDK4(A)	0.14	25.9	1.01[a]	**0.011**	0.12	−0.01	2.3	−0.05	0.864	0.01
DMRT3(A)	−0.24	44.1	−1.88[b]	**<0.001**	0.30	−0.30	58.1	−1.16[b]	**<0.001**	1.67
Adult horses										
Intercept			3.68	0.123				2.90	0.088	
MSTN(T)	0.01	2.4	0.18	0.872	0.01	0.02	4.5	0.23	0.775	0.01
COX4I2(T)	−0.02	5.7	−0.12	0.707	0.01	0.02	4.6	0.07	0.776	0.02
CKM(C)	−0.09	23.3	−0.47	0.122	0.28	−0.10	28.1	−0.39	0.074	0.31
PDK4(A)	0.06	14.8	0.41	0.341	0.07	0.03	8.4	0.16	0.602	0.02
DMRT3(A)	−0.22	53.8	−1.60[a]	**<0.001**	0.89	−0.19	54.4	−1.14[a]	**<0.001**	0.68

a,b Values within a row with different superscripts differ significantly at $p < 0.05$. Bold values correspond to significant p-values after applying correction SGoF+ for multiple testing. *: Gene (reference allele) B: coefficients of the number of copies of the reference allele β: standardized coefficients β': relative contribution of each marker in the prediction of the model. V_a: percentage of the total additive variance of the trait.

Therefore, the heritability estimated by distance ranged from 0.16 to 0.40 and tends to decrease as racing distance increases for age 1 and 2, being reduced for adult trotters. These heritability levels were quite similar to the ones described in previous studies using random regression analysis for the same trait (0.12 to 0.34, Bugislaus *et al.* 2006; Gómez *et al.* 2010a).

The estimated breeding values (EBVs) at different ages in the genetic indexes can modify the selection to choose the early or late performance of the animals (Posta *et al.* 2009). Thus, the deregression of the EBVs obtained for young and adult horses in short- and long-distance races has been useful for the posterior selection of the top and bottom 5% of the animals in the genetic ranking for the association analysis.

This study focuses on the genetic association of SNPs with deregressed EBV of the variable TPK, as is the best trait for analysing trotter racing performance (Bugislaus *et al.* 2006). Also DEBVs are a right choice, as they allow us to know the genetic potential of the horses over different distances and ages.

Genetic association analysis (either GWAS or individual markers) often uses corrected means of progeny performances, such as daughter yield deviations (DYD) mainly in dairy species as pseudo-phenotypes (VanRaden & Wiggans 1991). In horses, own performances are available but performances from other relatives (other than sibs or half sibs) may play an important role in the EBV because the number of progeny remains low, even for stallions, so the use of DYD is unsuitable, and other pseudo-phenotypes need to be generated for association analysis. Compared with DYD, the use of deregressed proofs has the same objective: to summarize performances from ungenotyped progeny to reach the genetic value of the genotyped horses and to get rid at the same time of confounding effects (sex, age, year, hippodrome, assortative mating, etc.). So, DEBVs can be the right choice in order for the pseudo-phenotype to use all the performances included in the estimation of these EBVs and to benefit from the correction of fixed effects; another advantage is that the animals with no individual or progeny information are excluded, as these animals cannot usefully contribute to the molecular association.

In this study, the use of DEBVs is even more important because the RRM for obtaining the EBV allows us to know the genetic potential of the animals over distances in which they have never competed and, in addition, to use the results over all race distances to estimate the EBV at the required distances. Here, it allows the use of norm reaction models instead of classical repeatability animal models, and, in this methodology too, the EBVs are biased, due to the assumption that the type of response is the same for the whole range of race competitions (Gómez *et al.* 2010b). The potential to improve the EBV estimation using RRM for norm reaction analysis has been shown in trotter races (Gómez *et al.* 2010a). The variation of the genetic correlations between the speed at short and long distances (Figure S1) and the speed at the different distances analysed shows that there is a

marked loss of genetic correlation between the speed at the reference points and the rest of distances, and so the distance for the estimation of the EBV must also be considered. In the same way, the genetic correlations between young and adult horses at different distances (0.367 to 0.719, Figure 2) mean that it cannot be considered the same trait for genetic evaluations. A range of different factors have caused these differences: the physiological status of the animal (related to the animal's level of maturity and training and their previous experiences on the track), the preselection of young horses to participate in long distances, the driver–horse interaction in the more tactical (long-distance) or faster (short-distance) races and the stricter control of the young horses in the races (compared with adults) to avoid injuries and galloping.

In addition, genomewide association studies for racing performance (Schröder *et al.* 2012; Meira *et al.* 2014) and metabolic pathways involving speed and endurance in horses are being studied. Moreover, the use of the strategy of MAS or directed genomic selection to improve the genetic progress of equine populations, together with the search for molecular markers associated with sporting potential, is rapidly emerging as key components of cost-effective breeding programmes. This strategy is of greater interest to horse breeders since the cost of genotyping is low compared with the value of the animals, the generation interval is long, due to the late entrance in reproductive life of performer horses, and traits other than performance traits are still proving difficult to improve in classic breeding schemes (Dubois *et al.* 2008).

COX4I2 gene catalyses the electron transfer from reduced cytochrome C to oxygen in mitochondrial respiration and has been associated with the hypoxic response in limited oxygen environments (Fukuda *et al.* 2007). Previous studies in thoroughbreds identified a weak but significant association between g.22684390C>T polymorphism in an intronic region of *COX4I2* gene and racing performance (Gu *et al.* 2010). However, no significant associations were observed between this SNP of the equine *COX4I2* gene and the racing performance of Spanish Trotter horses.

The *CKM* gene plays a vital role in energy storage in tissues with fluctuating energy demand (Echegaray & Rivera 2001). The association between the *CKM* gene and racing ability has been supported by Gu *et al.* (2010), with the identification of the SNP g.15884567A>G. For long-distance races, CC in the *CKM* gene promotes better results. But recently, Pereira *et al.* (2015) have proven that this polymorphism is not associated with racing performance in Quarter Horses. In this study, a new SNP marker of the same gene, not previously described, has shown statistical significance in young horses for long-distance races by RRP (33.4%, $p = 0.010$), therefore denoting the importance of the product of this gene for the precocious selection of Spanish Trotter horses.

DMRT3 is linked to the growth of central circuit spinal interneurons during mammal development, and it is responsible for coordinating the movement of the limbs (Andersson *et al.* 2012). A nonsense mutation called DMRT3_Ser301STOP truncates the protein sequence lacking 174 amino acid residues. This is due to the presence of a SNP (C to A) that promotes synchronic gaits in different types of organisms, including horses (Andersson *et al.* 2012). In horses, this mutation contributes to the ability to perform alternate gaits (lateral or diagonal trot) and is abundant in all breeds of horses used for harness racing (Promevorá *et al.* 2014). In this study, most HBV animals had the AA genotype, which was associated with short-distance racing performance (in young horses with a contribution of 44.1%, $p < 0.001$; and adults, 53.8%, $p < 0.001$) and also with endurance in young (58.1%, $p < 0.001$) and adult horses (54.4%, $p < 0.001$). These data are compatible with previous results which conclude that racehorses with good limb coordination (AA in *DMRT3* gene) may be better suited for all types of trotting races (Andersson *et al.* 2012).

Myostatin, encoded by the *MSTN* gene, acts to limit skeletal muscle mass, by regulating the number and growth of the muscle fibres of skeletal muscle (Baron *et al.* 2011). The SNP g.66493737C> T, located in intron 1 of the *MSTN* gene, has been associated with speed rates in thoroughbreds (Hill *et al.* 2010a). The T allele is common among horses that compete in long-distance races, where greater endurance is required (Hill *et al.* 2010a). Furthermore, the study by Petersen *et al.* (2013) showed a significant association of this SNP with fibre-type proportions: the T allele led to a higher proportion of type 1 fibres (which are beneficial for endurance). In this study, most STH animals had the T allele of the *myostatin* gene (97.95%). Therefore, relatively low skeletal muscle mass (TT in *MSTN* gene) is required in all cases for trotter horses.

The PDK isoforms regulate alternatively carbohydrate metabolism, depending on the duration and intensity of exercise, and block the formation of the PDC resulting in the beta-oxidation of fatty acids to acetyl-CoA as the substrate for oxidative phosphorylation. The complex is negatively regulated by *PDK4*, resulting in decreased glucose oxidation concomitant with increased fatty acid oxidation (Hill *et al.* 2010b).

A link was found between the *PDK4* marker and elite racing performance in thoroughbreds, suggesting that this gene plays a key role in regulating strenuous exercise (Hill *et al.* 2010c). In this study, the *PDK4* G allele was associated with young horses for short-distance racing performance by RRP with a contribution of 25.9% ($p = 0.011$), thus proving the feasibility of an early selection.

The RRP offers advantages over single-marker analyses since the effects in a multimarker analysis are estimated within the context of other markers, and it is therefore a suitable method for implementing MAS or genomic selection. From the RRP results, the best predictors of the set of genes in STH for short-distance races would be *DMRT3* (in young and adult horses) and *PDK4* (in young horses), with a prediction potential of 68.2% and 60.1% for young and adult horses, respectively. For long-distance races, *DMRT3* (in young and adult horses) and *CKM* (in young horses) genes showed a prediction potential of 59.1% and 59.8% in young and adult horses, respectively, according to logistic regression. The relatively low additive variance produced for each individual marker (except for the *DMRT3* mutation) may have contributed to the high level of disequilibrium between allele frequencies. In populations in which allele frequencies were close to 0.5, the percentage of variance would significantly increase. This could be the case of *DMRT3* in the group of young horses for long-distance races, where the variance fluctuates between 1.67% and 3.69% (if p and q are equal to 0.5). Total additive variance oscillates from 0.49% to 2.62% (for short- and long-distance races, respectively) in the case of young trotters, and from 1.26% to 1.04% (for short- and long-distance races, respectively) in adults.

The efficacy of the multimarker model in ROC analysis suggests that this set of markers is reliable for selecting potential STHs for sporting purposes for both short- and long-distance races.

In conclusion, this research suggests that the young STH population carrying the genotypes GG in *PDK4* and AA in *DMRT3* genes showed the best genetic potential in short-distance races, while those carrying the genotypes AA in *DMRT3* and CC in *CKM* genes seem to be the best in long-distance races. Adult STHs with genotype AA in *DMRT3* also seem to be better at speed and endurance competitions. This would therefore allow us to consider the deregressed EBV of the trait TPK as the right choice for the selection of trotters and also to design a molecular tool for making a very early preselection of Spanish Trotter horses with a high potential for racing. Using a molecular tool such as SNaPshot assay may also increase the reliability of the genetic evaluations of animals by combining information from molecular genetics with sporting results. Due to the close interrelationships among different Trotter breeds, this molecular tool could also be applied to all the breeds if the association is retained.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Genetic correlations between the speed at 1600 and 2600 with the speed at the different distances analysed according to age group in Spanish Trotter Horse.

Figure S2 Receiver Operating Characteristic (ROC) curve analysis for the model's predictive ability evaluation for speed and endurance features in the young and adult STH population, respectively.

Appendix S1 Description of the Random Regression Model.

Table S1 Evolution of additive variance, total phenotypic variance and the heritability for the speed (TPK) along the trajectory of race distances analysed according to age group; also, the genetic correlation for the same distance between age groups in the Spanish Trotter Horse.

Table S2 Genetic correlations between the speed (TPK) at 1600 and 2600 with the speed at the different distances analysed according to age group (age 1 at the top, and age 2 at the bottom).

Table S3 Allelic frequencies (%) of SNP markers detected in five candidate genes for sporting performance in the Spanish Trotter Horse population.

Supporting information

Appendix 1. Description of the Random Regression Model

In this model, it is assumed that the variance of *y* is:

$$V(\boldsymbol{y}) = \lambda_{tm}(\mathbf{K}_{j:p} \otimes \mathbf{A})\lambda'_{tm} + \lambda_{tm}(\mathbf{K}_{w:p} \otimes \mathbf{I}_w)\lambda'_{tm} + \lambda_{tm}(\mathbf{K}_{c:p} \otimes \mathbf{I}_c)\lambda'_{tm} + \mathbf{R}$$

where $\boldsymbol{K_i}$ corresponds to the coefficients of random regression matrix, $\boldsymbol{i} = \boldsymbol{j}$ is the matrix of genetic effects (Go), $\boldsymbol{i} = \boldsymbol{w}$ is the matrix of individual permanent environment effects and $\boldsymbol{i} = \boldsymbol{c}$ is the matrix of environmental variance due to the effect of the trainer-rider. $\boldsymbol{A}$ is the numerator relationship matrix between animals; $\boldsymbol{I_w}$ and $\boldsymbol{I_c}$ are identity matrices with $\boldsymbol{q_w}$ *x* $\boldsymbol{j^*}$ levels for each age for the permanent environmental effect and $\boldsymbol{q_n}$ *x* $\boldsymbol{n}$ for the random effects due to the trainer-rider for each age. In this model the variance and covariance components of TPK for each age and each point in the trajectory of distances $\boldsymbol{t}$ are obtained by applying an additional procedure originally proposed by de Jong (1995):

For genetic components at distances **t** ($\boldsymbol{G_{ot}}$) for ages $\boldsymbol{p}$ = 1 and $\boldsymbol{p}$ = 2:

$$\mathbf{G}_{ot} = \begin{bmatrix} \mathbf{\Phi}_m \mathbf{K}_{j_1} \mathbf{\Phi}'_m & \mathbf{\Phi}_m \mathbf{K}_{j_{2,1}} \mathbf{\Phi}'_m \\ \mathbf{\Phi}_m \mathbf{K}'_{j_{1,2}} \mathbf{\Phi}'_m & \mathbf{\Phi}_m \mathbf{K}_{j_2} \mathbf{\Phi}'_m \end{bmatrix}$$

With the same notation for the individual permanent environmental effect ($\boldsymbol{P_{wt}}$) and for the driver effect ($\boldsymbol{P_{rt}}$), this produces:

$$\mathbf{P}_{wt} = \begin{bmatrix} \mathbf{\Phi}_m \mathbf{K}_{w1} \mathbf{\Phi}'_m & \mathbf{\Phi}_m \mathbf{K}_{w\,2,1} \mathbf{\Phi}'_m \\ \mathbf{\Phi}_m \mathbf{K}_{w1,2} \mathbf{\Phi}'_m & \mathbf{\Phi}_m \mathbf{K}_{w2} \mathbf{\Phi}'_m \end{bmatrix}$$

$$\mathbf{P}_{ct} = \begin{bmatrix} \mathbf{\Phi}_m \mathbf{K}_{c1} \mathbf{\Phi}'_m & \mathbf{0} \\ \mathbf{0} & \mathbf{\Phi}_m \mathbf{K}_{c2} \mathbf{\Phi}'_m \end{bmatrix}$$

By using the appropriate elements for $\boldsymbol{G_0}$, $\boldsymbol{P_w}$, $\boldsymbol{P_c}$ and Φ_m, the genetic (co)variance components and the environmental variance components can be obtained along all

points of the trajectory of distances ***t***. The heritability ($\boldsymbol{h^2}$) and the genetic correlation (r_g) for each trait, within and across the ages for each distance, are computed from these variance-covariance components, plus the corresponding residual variance.

Table S1 *Evolution of additive variance, total phenotypic variance and the heritability for the speed (TPK) along the trajectory of race distances analysed according to age-group, and the genetic correlation for the same distance between age groups in the Spanish Trotter Horse*

Distance	$VarAag_1$	$VarAag_2$	$VarPag_1$	$VarPag_2$	h^2ag_1	h^2ag_2	$rgag_{12}$
1600	5.359	1.490	13.378	6.461	0.401	0.231	0.719
1700	4.587	1.437	12.605	6.407	0.364	0.224	0.685
1800	3.900	1.389	11.918	6.359	0.327	0.218	0.651
1900	3.299	1.345	11.317	6.316	0.291	0.213	0.618
2000	2.784	1.308	10.802	6.278	0.258	0.208	0.585
2100	2.354	1.275	10.373	6.245	0.227	0.204	0.553
2200	2.011	1.248	10.029	6.218	0.200	0.201	0.521
2300	1.753	1.225	9.771	6.196	0.179	0.198	0.489
2400	1.580	1.208	9.599	6.179	0.165	0.196	0.458
2500	1.494	1.196	9.512	6.167	0.157	0.194	0.428
2600	1.493	1.190	9.512	6.160	0.157	0.193	0.397
2700	1.578	1.188	9.597	6.159	0.164	0.193	0.367

Table S2 *Genetic correlations between speed (TPK) at 1600 and 2600 with the speed at the different distances analysed according to age group (age 1 at the top, and age 2 at the bottom)*

Distance	1600	1700	1800	1900	2000	2100	2200	2300	2400	2500	2600	2700
1600	1.000	0.999	0.994	0.984	0.965	0.935	0.887	0.817	0.721	0.597	0.453	0.301
1700	0.999	1.000	0.998	0.992	0.977	0.952	0.910	0.846	0.755	0.637	0.498	0.349
1800	0.997	0.999	1.000	0.998	0.988	0.968	0.933	0.876	0.793	0.682	0.549	0.404
1900	0.993	0.997	0.999	1.000	0.997	0.983	0.956	0.908	0.834	0.732	0.606	0.467
2000	0.987	0.993	0.997	0.999	1.000	0.995	0.977	0.940	0.877	0.786	0.670	0.539
2100	0.979	0.986	0.992	0.996	0.999	1.000	0.993	0.969	0.920	0.843	0.740	0.620
2200	0.970	0.978	0.986	0.992	0.996	0.999	1.000	0.991	0.959	0.900	0.813	0.706
2300	0.958	0.968	0.977	0.985	0.991	0.996	0.999	1.000	0.988	0.950	0.884	0.795
2400	0.944	0.955	0.966	0.976	0.984	0.991	0.996	0.999	1.000	0.986	0.945	0.878
2500	0.927	0.941	0.953	0.965	0.975	0.984	0.991	0.996	0.999	1.000	0.986	0.945
2600	0.909	0.924	0.938	0.952	0.964	0.974	0.983	0.990	0.996	0.999	1.000	0.986
2700	0.888	0.905	0.921	0.936	0.950	0.963	0.974	0.983	0.990	0.996	0.999	1.000

Correlations lower than 0.9 are shaded.

Table S3 *Allelic frequencies (%) of SNP markers detected in five candidate genes for sporting performance in the Spanish Trotter Horse population*

			Total	Speed		Endurance	
			(n=321)	LBV	HBV	LBV	HBV
COX4I2	Alleles	C	57.63	59.10	51.61	60.32	57.89
		T	42.37	40.90	48.39	39.68	42.11
	Genotypes	CC	31.05	36.17	24.19	33.33	31.91
		CT	53.42	51.06	54.84	53.97	53.19
		TT	15.53	12.77	20.97	12.70	14.90
PDK4	Alleles	A	16.36	21.74	11.11	13.49	21.87
		G	83.64	78.26	88.89	86.51	78.13
	Genotypes	AA	1.70	4.35	0	1.59	0
		AG	28.70	34.78	22.22	23.81	14.30
		GG	69.60	60.87	77.78	74.60	85.70
MSTN	Alleles	C	2.05	6.38	0	0.82	2.08
		T	97.95	93.62	100	99.18	97.92
	Genotypes	CC	0	0	0	0	0
		CT	4.11	12.77	0	1.64	4.17
		TT	95.89	87.23	100	98.36	95.83
CKM (new) g.15884216C>G	Alleles	C	26.60	23.46	34.45	23.28	23.33
		G	73.40	76.54	65.55	76.72	76.67
	Genotypes	CC	19.80	10.26	16.95	14.04	6.82
		CG	29.70	28.20	35.59	17.54	34.09
		GG	50.50	61.54	47.46	68.42	59.09
DMRT3	Alleles	A	86.99	75.53	97.58	87.90	83.33
		C	13.01	24.47	2.42	12.10	16.67
	Genotypes	AA	76.26	59.57	95.16	75.81	68.75
		AC	21.46	31.92	4.84	24.19	29.17
		CC	2.28	8.51	0	0	2.08

LBV: Low Breeding Value

HBV: High Breeding Value

Figure S1 Genetic correlations between the speed at 1600 and 2600 with the speed at the different distances analysed according to age group in the Spanish Trotter Horse.

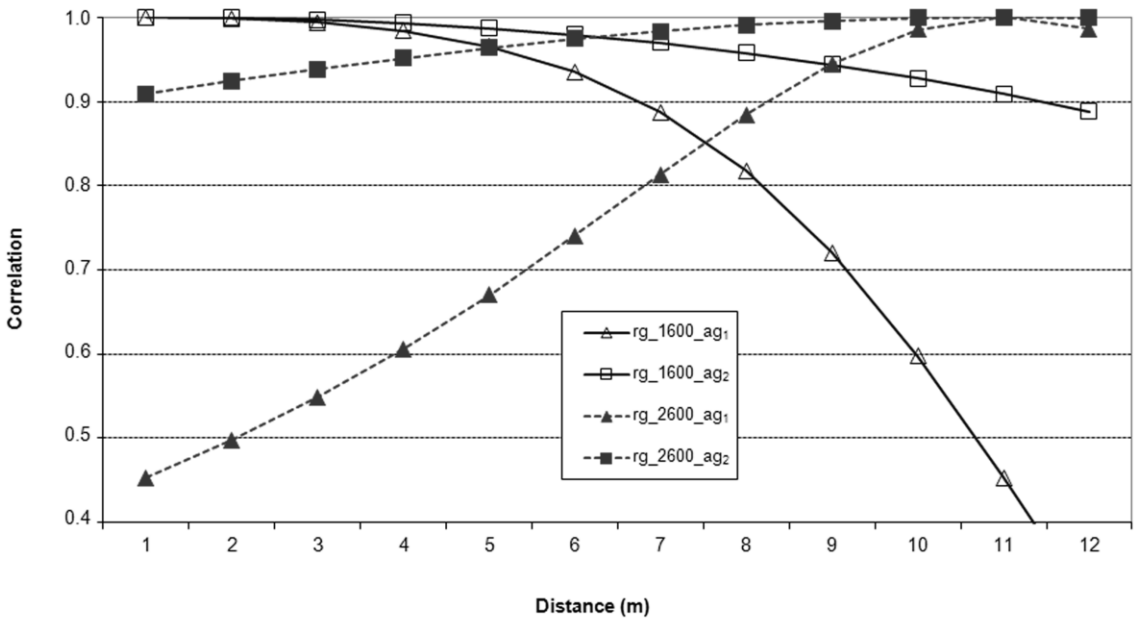
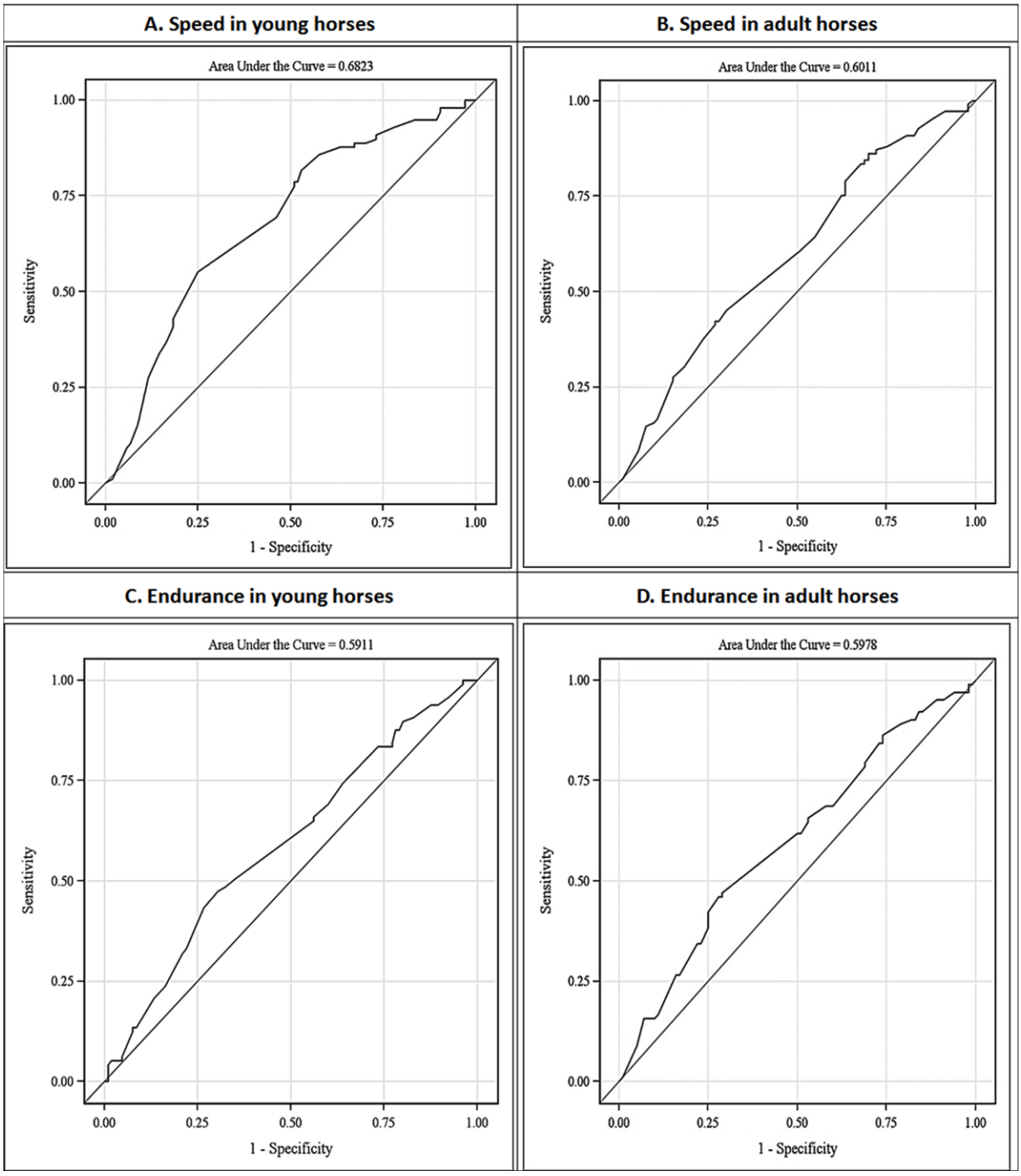


Figure S2 Receiver Operating Characteristics (ROC) curve analysis for model predictive ability evaluation for speed and endurance features in the young and adult STH population, respectively.



DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

La población fundadora del caballo doméstico ha sido seleccionada gradualmente hasta llegar a formar el gran abanico de poblaciones modernas especializadas o razas que existen actualmente destacando por su gran diversidad fenotípica y genética (Petersen et al., 2013a). Las diferencias en las poblaciones primitivas, debidas al perfil genético del fundador salvaje a partir del cual se fueron domesticando, fueron incrementándose durante siglos gracias a una combinación de la selección natural, una selección artificial más o menos intuitiva según los gustos y propósitos por los que se criaban (pe. para el sacrificio o como apoyo en la agricultura o en la guerra), el mayor o menor cruzamiento con reproductores de otras zonas con los que confluían etc. No obstante a partir del siglo XVIII se establece de forma sistemática la búsqueda de poblaciones homogéneas con una morfología y aptitud deseada, es decir, la selección de los animales más idóneos y su cría cerrada buscando el máximo parentesco con los animales más sobresalientes.

El perfil genético de las poblaciones equinas actuales difieren acorde a varios factores: su origen filogenético, el censo, el nivel de consanguinidad, el sistema de cría, o la presión de selección ejercida (Petersen et al., 2013b). A su vez, la estructura de la población también podría verse por la existencia de diferentes orientaciones productivas dentro de la raza que favorezcan el apareamiento preferencial dentro de grupos de animales con diferentes objetivos de selección, como ocurre en el caso del Pura Raza Árabe (PRÁ) que tiene dos objetivos de selección diferenciados, la funcionalidad para carreras de resistencia como las de raid y/o la morfología (Gleeson and Bishop, 2000). Tanto la selección artificial como el apareamiento dirigido dentro de las poblaciones reproductoras podrían determinar una pérdida de biodiversidad más o menos intensa en función del número efectivo de reproductores o del grado de flujo genético o de aislamiento entre las subpoblaciones existentes. No obstante, incluso en aquellas en las que por su bajo censo pueden presentar una mayor problemática de pérdida de variabilidad si se llevan a cabo esfuerzos selectivos, la utilización de metodologías adecuadas que ponderen el progreso genético deseado y la pérdida de variabilidad máxima permitida, es posible frenar su erosión genética. Un ejemplo lo tendríamos en el caso de la raza Pura Raza Menorquín (Solé et al. (2013), en la que a pesar de su bajo censo actual, de 1479 reproductores (MAPAMA), se están llevando a cabo pequeños esfuerzos selectivos buscando incrementar la diversidad genética de la raza al mismo tiempo que seleccionar por rasgos de importancia económica.

Como resultado de los procesos de homogeneización y de selección, la mayoría de las razas equinas en la actualidad son poblaciones cerradas o de pura raza (como son el PRÁ, el Pura Raza Español o PRE, y PRMe), con gran uniformidad fenotípica y genética entre individuos. Sin embargo, muchas razas equinas han sido creadas utilizando dos o más razas parentales, e incluso siguen permitiendo la participación de reproductores de dichas razas paternales (libro genealógico abierto), buscando tanto la

complementariedad de sus aptitudes como el llamado vigor híbrido (heterosis) que les permita la obtención de unos buenos rendimientos en competiciones hípicas (Hamann and Distl, 2008; Cervantes et al., 2009; Hellsten et al., 2009; Bartolomé et al., 2011) Ejemplos de razas con libros genealógicos que permiten el cruce entre diferentes razas los tendríamos en el caso del Anglo-árabe (AÁ), o el Caballo de Deporte Español (CDE).

En este sentido, el Caballo Trotador Español (CTE) es una raza moderna, de origen múltiple (Arnason, 2001; Ricard, 2005), y que sigue introduciendo genes de otras razas de trotadores mediante la importación de material genético procedente de otros países (en forma de reproductores vivos y/o semen fresco o congelado), principalmente del Caballo Trotador Americano (Standardbred) y el Trotador Francés (Bujosa, 2001), las razas de mayor importancia a nivel internacional junto al Trotador Sueco y el Trotador de Orlov. Actualmente existen diferentes razas equinas especializadas para la carreras de trote en distintos países (Thiruvenkadan et al., 2009). Prácticamente desde los inicios de la formación de esta raza, los criadores han seleccionado animales extranjeros para actuar como reproductores, en función de sus rendimientos funcionales para las carreras de trote en los países de origen, lo que ha contribuido al rápido progreso del rendimiento funcional de la población española. A pesar del origen multiracial del CTE, cabe destacar que en estudios previos realizados en base a la información molecular, no se han detectado diferencias moleculares entre las distintas poblaciones de caballos trotadores analizadas (Azor et al., 2007).

Con la finalidad de evaluar el grado de diferenciación y el impacto sobre la variabilidad del hecho de mantener o no un libro abierto en el **artículo 1** de la presente tesis doctoral se ha llevado a cabo la comparación de las poblaciones equinas de LG abierto y LG cerrado, a través el análisis de marcadores moleculares neutros de tipo microsatélite, que son especialmente útiles como alternativa al uso de la genealogía (Pirault et al., 2013).

Por ello, nuestro estudio incluye el análisis de la situación actual del CTE con respecto al resto de razas equinas españolas seleccionadas para el deporte en cuanto a su variabilidad y estructura genética con otras de LG abierto como el AÁ y el CTE, y con aquellas con cría en pureza (LG cerrado), como el PRÁ, PRE y PRMe. Para estudiar el impacto de la selección llevada a cabo se ha analizado así mismo la evolución de la variabilidad en cada raza entre dos generaciones consecutivas.

Nuestros resultados han demostrado que el CTE presenta alta variabilidad genética, fruto por un lado de su origen multipoblacional y por otro del carácter abierto de su libro genealógico, con valores de los diferentes parámetros utilizados (número efectivo de alelos, heterocigosidad …) entre los más altos descritos en las poblaciones equinas (0.40 - 0.79, (Achmann et al., 2004; Plante et al., 2007; Leroy et al., 2009; Prystupa et al., 2012; Berber et al., 2014). En el resto de razas cruzadas también se mantienen elevadas tasas de diversidad genética, mientras que las puras presentan una situación más o menos diferente en función de su censo efectivo. El caso extremo lo tendríamos en el caso del PRMe que por su bajo censo está considerada como una raza

en peligro de extinción (Solé et al., 2013), teniendo un nivel de variabilidad de 8,34 referido al número efectivo de alelos, en comparación las otras razas que tienen valores en torno a 11. No obstante, nuestros análisis también han detectado apareamiento diferencial buscando un mayor parentesco con animales sobresalientes de cada raza, determinando altos valores de consanguinidad en determinados individuos. En el caso del CTE ha contribuido también el uso frecuente de semen de muy pocos reproductores (o reproductores muy emparentados entre sí) (Gómez et al., 2010b). Este hecho, como veremos más adelante, determina una tendencia al incremento de la homogeneidad de los animales a pesar de que no se trata de una raza pura con libro cerrado.

La implementación y desarrollo de los programas de mejora equinos en la última década se ha centrado en el mayor uso de diferentes linajes y/o cambios en las políticas de apareamiento en su intento por evitar los apareamientos preferenciales utilizando los sementales más destacados. En aquellas razas en las que se han podido detectar ciertos problemas de depresión consanguínea o la posibilidad de que apareciesen por los niveles elevados de sus reproductores, se han establecido algunos mecanismos para corregir este problema. En el caso de las razas abiertas como el CTE estos niveles dependen en gran medida del flujo genético establecido por el uso de nuevos reproductores de las razas paternales. Esta evolución puede medirse indirectamente viendo la variación de tamaño efectivo de la población entre dos generaciones, que en nuestro caso fue calculado basándonos en el desequilibrio de ligamiento (Do et al., 2014). Nuestros resultados muestran que dicho tamaño se ha incrementado claramente en la segunda generación con respecto a la primera para todas las razas, incluida la del PRMe. Por lo que, aunque se trata de un proceso lento, se puede asegurar que se están tomando medidas efectivas para corregir la pérdida de variabilidad debido a la selección y al bajo tamaño poblacional de algunas de las poblaciones analizadas.

El análisis de la estructura poblacional a través de los F de Wright y la metodología de Druml et al. (2007) de asignación de individuos a cada raza empleando el software Structure 2.2 (Pritchard et al., 2000), mostró una clara diferenciación entre todas las razas y una ausencia de subdivisión dentro de estas (Figura S2, artículo 1), lo que está contribuyendo a un mayor control de la pérdida de variabilidad. La comparación entre ambas generaciones analizadas dentro de cada raza muestra que en el caso de las razas puras tienen un alto grado de similitud y homogeneidad, excepto en el caso del PRÁ. En esta raza, esto es debido a que se ha producido una mejora en los criterios de selección, basados en dos objetivos bien diferenciados, conformación y rendimiento deportivo para las carreras de resistencia (Cervantes et al., 2009).

En el caso de las razas de libro abierto, presentan diferencias en el perfil genético, principalmente debido al mayor o menor uso de las razas paternales originales en cada una de estas generaciones. En el caso del CTE hemos encontrado una situación intermedia, dado que las diferencias genéticas entre las razas que pueden incrementar o disminuir su participación en la formación de cada generación son muy inferiores a las existentes entre diferentes razas (son poblaciones de caballos trotadores que se están influenciando mutuamente).

Por tanto, en el CTE la introducción continua de sementales de otras poblaciones está compensando la posible pérdida de variabilidad determinada por la selección hacia la aptitud deportiva de Trote, aunque es evidente que el número de sementales es escaso y la relación de parentesco entre ellos elevada, por lo tanto hay que realizar monitorizaciones periódicas de la situación genética para determinar si es necesario el inicio de medidas correctoras.

VALORACIÓN GENÉTICA DEL CABALLO TROTADOR ESPAÑOL

Las valoraciones genéticas actuales del CTE incluidas en el Programa de Mejora se basan en un BLUP multivariable basado en un modelo animal con repetibilidad (Gómez et al., 2010b), semejante al que también es aplicado en otros países como Suecia (Arnason, 1999), Alemania (Rohe et al., 2001; Bugislaus et al., 2005) y Francia (Langlois and Vrijenhoek, 2004). Fruto de estas valoraciones y de la información generada, se lleva publicando, desde el año 2008 (Gómez, 2008), el Catálogo de Reproductores de Raza Trotador Español con el objetivo de poder ofrecer a los criadores de la raza una información objetiva y fiable que facilite la selección de los futuros reproductores. Los criterios que se utilizan para valorar la capacidad de rendimiento funcional de un animal durante el desarrollo de las carreras de trote son el porcentaje de primeros puestos anuales, con transformación raíz cuadrada (PPP), las ganancias anuales, con transformación logarítmica (GA), el tiempo por kilómetro (TPK) y el mejor tiempo anual por hipódromo y modo de salida (MTPAHM). La primera refleja el temperamento del animal, su espíritu y su deseo de ganar en la carrera (Thiruvenkadan et al., 2009), mientras que la cantidad total de dinero repartida en una carrera depende de la dificultad técnica o del nivel de los competidores en la misma (Tavernier, 1991). Por último, el TPK y el MTPAHM ponen en evidencia la capacidad deportiva de un animal (Arnason, 2001), aunque no están libre de la influencia del nivel relativo de un caballo en relación con el resto de competidores en una carrera (Thiruvenkadan et al., 2009). Estos parámetros presentan una alta correlación genética (Thiruvenkadan et al., 2009), entre el 0,44 y 0,99 en el caso del CTE (Gómez et al., 2010b). Las cuatro variables descritas anteriormente, tienen un nivel de heredabilidad (usando la metodología REML) en torno al 0,28, excepto para la variable PPP que es de 0,14 (Gómez et al., 2010b). Este rango bajo-moderado es semejante al obtenido en otras poblaciones de trotador (Arnason et al., 1989; Tavernier, 1989; Silvestrelli et al., 1995) y suficientemente amplio como para asegurar un progreso genético adecuado.

No obstante, este modelo de evaluación BLUP asume que el potencial genético del animal es el mismo (o se expresa de igual forma) a lo largo de la vida de los animales, aunque existen evidencias de que este hecho no es así tanto en otras especies como en el propio caballo. Uno de los factores que condiciona de forma considerable el rendimiento deportivo es la edad de participación, ya que determina tanto su experiencia deportiva, como su desarrollo físico, fisiológico y psicológico, todos ellos con cierta base genética. El otro es la longitud de la carrera o distancia, ya que diferentes factores

genéticos están implicados en el rendimiento de los animales en función de si las carreras son más o menos largas (Ricard et al., 2000).

En este sentido, nuestro grupo ha desarrollado una nueva metodología de norma de reacción, basada en la utilización de modelos de regresión aleatoria (RRM), que permiten estimar la evolución del potencial genético a lo largo de la vida del animal, así como para diferentes niveles ambientales como es el caso de la longitud de las carreras (Gómez et al., 2010a). Son modelos matemáticos que consideran que la expresión del genotipo sigue una función continua (que generalmente se ajusta a polinomios de diferente grado) a lo largo de la vida del animal, permitiendo la estimación de una función (polinomio) de valores genéticos a lo largo de un periodo de tiempo determinado. Esta metodología, que se desarrolló primero en especies como el vacuno o el ovino, asume que los diferentes genes involucrados en el fenotipo de un carácter a lo largo de un periodo de tiempo no son los mismos (o varía su expresión). Adicionalmente, la estimación de las covarianzas entre los diferentes puntos evaluados (las diferentes edades en que se controla el rendimiento) permite un incremento en la fiabilidad de las evaluaciones, ya que para estimar el valor genético a una determinada edad se utilizará la información de todos los controles de rendimiento obtenidos en las diferentes edades, en vez de un solo valor correspondiente a una variable resumen.

Tras el estudio aplicando esta metodología de RRM a las cuatro variables (GA, PPP, MTPAHM y TPK) para analizar el rendimiento deportivo, se obtuvo una mayor heredabilidad en relación a los modelos REML clásicos. Así la heredabilidad estimada para las ganancias anuales en función de la distancia de carrera osciló entre 0,08 y 0,10 para los caballos jóvenes y entre 0,10 y 0,14 para los animales adultos, presentando la misma trayectoria para ambos grupos de edad (Gómez et al., 2011). La heredabilidad estimada en función de la distancia de carrera para el tiempo por kilómetro osciló entre 0,12 y 0,34, presentando en este caso una evolución diferente para los dos grupos de edad analizados (Gómez et al., 2010a). La mayor heredabilidad de este TPK, determinó que fuese seleccionado para la evaluación genética de los animales genotipados en el **artículo 4** de la presente tesis. Éste ha tenido como objetivo buscar asociación de determinados genes con el potencial deportivo de los caballos CTE, diferenciando sus valores genéticos estimados en caballos de diferentes grupos de edad (jóvenes de 2 a 4 años, y adultos, de 5 a 8 años) en todo el rango de distancias de carreras (1600-2700m) (Figura 1, artículo 4). Con esta metodología, la curva de la heredabilidad estimada en función de la distancia ha variado de 0,16 a 0,40, existiendo una tendencia a disminuir con el aumento de la distancia de carreras para la edad 1 y 2, y a reducirse también en los caballos adultos con respecto a los jóvenes (Figura 2, artículo 4). Estos niveles de heredabilidad fueron bastante similares a los descritos en estudios previos utilizando esta misma metodología (0,12 a 0,34; (Bugislaus et al., 2006; Gómez et al., 2010a).

Nuestros resultados han demostrado que el uso rutinario de RRM para la valoración genética del CTE es una herramienta muy útil para la selección genética de los animales, porque permite estimar el valor genético a lo largo de todas las etapas de su trayectoria en las competiciones deportivas como ya demostró Gómez et al. (2010a).

De esta manera, se podrá ofrecer a los ganaderos una información más exacta y completa que les permita seleccionar a sus ejemplares en función de unos criterios de selección mucho más específicos.

ASOCIACIÓN DE GENES CANDIDATOS CON RENDIMIENTO DEPORTIVO

El desarrollo de nuevas metodologías de valoración permite la obtención de animales evaluados genéticamente a edades tempranas y con una mayor fiabilidad. Aun así, se requieren años para asegurar con suficiente fiabilidad que un reproductor tenga un gran potencial genético, y que este sea transmitido a la descendencia. Por ello, sería importante conseguir un incremento de la fiabilidad y de la precocidad con que se obtienen los animales mejorantes, lo que redundaría en una disminución del intervalo generacional y un incremento del progreso genético de la raza.

El rendimiento deportivo del caballo está influenciado por una compleja interacción entre el medio ambiente y un conjunto de genes más o menos amplio, dentro de los cuales se espera que existan algunos de un gran efecto (genes mayores, QTL), y otros muchos de un pequeño efecto a través de múltiples rutas metabólicas que inciden en una mejor o peor absorción de nutrientes, una mejor tasa respiratoria y circulatoria, y mejor temperamento etc. A nivel molecular los marcadores genéticos afectados pueden encontrarse en genes estructurales (pe los responsables de la producción de una determinada proteína), pero también en genes reguladores, o en genes que participen en vías metabólicas relacionadas con el carácter que nos interesa. Suelen ser mutaciones puntuales en un gen (segmentos de ADN polimórfico con una ubicación física identificable en un cromosoma y cuya herencia se puede rastrear). Actualmente los más utilizados son los polimorfismos de un solo nucleótido (SNPs), por ser la fuente más abundante de variabilidad genética entre individuos de cualquier especie, si bien cada vez más se están encontrando otros tipos de polimorfismos como serían los ROH, o los CNV que muestran asociación con caracteres relacionados con la salud, la aptitud reproductiva, o el potencial deportivo.

En el caso de los marcadores relacionados con el rendimiento deportivo equinos existen diversos estudios realizados en razas de caballos de carreras como el Pura Sangre Inglés (PSI) (Hill et al., 2010b; Schröder et al., 2011) y los trotones (Barrey, 2010; Andersson et al., 2012), en los que ya se han demostrado la asociación con mutaciones en determinados genes. Éstos codifican proteínas relacionadas con distintos procesos fisiológicos del desarrollo muscular en équidos como son el gen codificante de la miostatina (*MSTN*), o de la capacidad cardiaca o aeróbica como son el gen codificante de la piruvato deshidrogenasa quinasa isoenzima 4 (*PDK4*), el gen de la enzima citocromo C oxidasa (COX) o complejo IV (*COX4I2*), el gen que codifica para la creatina quinasa muscular (*CKM*); y por último el patrón de locomoción con el gen codificante del factor de transcripción 3 sexo específico y mab-3 (*DMRT3*).

En el **artículo 4** de la presente tesis se ha el realizado un análisis genético de asociación en el CTE del rendimiento deportivo con varios polimorfismos tipo SNP.

Éstos se encuentran en genes relacionados, unos con la extensión y maduración de la musculatura (*MSTN*, (Hill et al., 2010b), otros con la locomoción y la coordinación de las extremidades (*DMRT3*, Andersson et al., 2012), otros con los niveles de oxíogeno (*COX4I2*, (Gu et al., 2010)) y por último los relacionados con el sistema cardiorrespiratorio (*CKM*, (Gu et al., 2010); *PDK4*, (Hill et al., 2010a)). El rendimiento deportivo ha sido determinado a partir de los valores genéticos estimados deregresados del tiempo por kilómetro. Estos han sido utilizados para preseleccionar el grupo de animales a genotipar con el mayor y el menor potencial para el trote (5% superior e inferior en el ranking) tanto en carreras cortas (animales velocistas), como largas (animales resistentes), con vistas a incrementar la potencia de los test de asociación (obtener la menor tasa de falsos positivos posible con un número limitado de animales genotipados).

En este sentido, en el **artículo 4** hemos encontrado asociación con tres SNPs de los genes *CKM*, *PDK4* y *DMRT3*. Así el alelo C del *g.15884216C>G* (P <0,05) del gen *CKM* (relacionado con la resistencia cardiorrespiratoria), ha mostrado asociación con el mayor potencial de los caballos para carreras largas o de resistencia, con una contribución a la predicción del modelo o al mayor rendimiento deportivo del 33.4%. Este SNP no había sido descrito anteriormente o publicado en *Equus caballus*. Además, en este estudio se ha demostrado la asociación del alelo G del SNP *g.38973231A>G* en el gen *PDK4* (con una contribución del 25,9%) con el rendimiento deportivo de los animales que tienen mayor potencial para las carreras cortas o de velocidad. Este gen está relacionado con el metabolismo energético, cuya demanda incrementada resulta en el cambio hacia rutas metabólicas alternativas de oxidación de ácidos grasos en vez de utilización de glucosa, como ocurre en condiciones normales. En cuanto al gen *DMRT3*, la mutación *DMRT3_Ser301STOP* afecta al patrón de locomoción en los caballos, estimulando la capacidad del animal de presentar pasos alternados esenciales para las carreras de trote y presentando por lo tanto un efecto favorable en el rendimiento en carreras (Andersson et al., 2012). En nuestro estudio se ha obtenido una asociación del alelo A con un mayor potencial en los caballos jóvenes y adultos para las carreras de velocidad (con una contribución del 44,1% y 53,8% respectivamente) y también de resistencia (con una contribución del 58,1% en caballos jóvenes y del 54,4% en adultos). Sin embrago, aunque se ha demostrado que la mutación *g.66493737C>T* en el gen *MSTN* puede provocar el incremento de la musculatura asociándose esto con caballos PSI con mayor rendimiento deportivo en carreras de velocidad, este SNP estaba fijado en la población de trotador. Esto no es sorprendente dado que esta raza dispone de una musculatura ligera en comparación con el PSI.

Los resultados obtenidos en **Artículo 4**, apoyan la evidencia de que el uso de herramientas moleculares a partir de la asociación encontrada en los genes *PDK4*, *CKM* y *DMRT3* podrían acelerar los procesos de selección con la creación de test genéticos para la preselección de animales muy jóvenes que presentando los alelos adecuados (un potencial genético bueno) sean destinados al entrenamiento para esta disciplina, con un consiguiente ahorro económico e incremento en la tasa de éxito. De la misma forma, podría permitir a los criadores diferenciar los animales velocistas (mejores en distancias

cortas) de los de resistencia (mejores en distancias largas), y los precoces (mejores a edades tempranas) de los longevos (mejores a edades tardías), teniendo todo ello una clara repercusión positiva en el progreso genético de esta raza.

EVALUACIÓN DEL NIVEL DE ESTRÉS EN CARRERAS DE TROTE CON TERMOGRAFÍA INFRARROJA Y SU ASOCIACIÓN CON EL RENDIMIENTO DEPORTIVO

A pesar de los avances en los modelos de valoración genética para la evaluación de la aptitud deportiva en el CTE, y que en determinados animales se hayan alcanzado valores de hasta el 90% de fiabilidad (Sánchez, 2016), es importante seguir detectando posibles factores ambientales sistemáticos que estén condicionando la aptitud deportiva y por lo tanto que su inclusión en el modelo de valoración permita una mejora en la fiabilidad de está (Langlois and Blouin, 2007).

Entre estos factores se encuentra el estado fisiológico del animal (relacionado con el nivel de madurez y aprendizaje y sus experiencias previas en la pista), la interacción caballo-conductor durante el entrenamiento o la competición y el control (mayor en caballos jóvenes que en adultos) para evitar lesiones y romper el aire al galope. Sin embargo, además de unas buenas condiciones físicas se requiere un estado emocional adecuado (McBride and Mills, 2012), ya que durante la competición el caballo se enfrenta a diferentes estímulos ambientales que potencialmente amenazan su equilibrio interno (estresores). Esto provoca en él una respuesta fisiológica de estrés que podría tener tanto un impacto positivo como negativo sobre sus resultados finales. Aunque el estrés es una respuesta del organismo ante una situación de peligro, éste posee una naturaleza dual ya que, a corto plazo (estrés agudo), produce cambios adaptativos que ayudan al animal a responder ante el estímulo estresante (Moberg, 2000). Sin embargo, cuando la respuesta al estrés supera los umbrales de tolerancia debido al mantenimiento de esta situación a largo plazo (estrés crónico), puede comprometer su adaptación al ejercicio durante la competición (Aschbacher et al., 2012) y conducir a toda clase de fenómenos patológicos, ya que induce inmunodepresión en el animal (Herman and Cullinan, 1997).

Por lo tanto, el nivel de estrés que el animal sufre durante las carreras es un factor que, si bien diversos estudios han demostrado su influencia en su rendimiento deportivo (Kinnunen et al., 2006; Rivero et al., 2008), hasta ahora no se ha tenido en cuenta para su medición de forma rutinaria en competición. Su inclusión como un factor de corrección en los modelos de valoración genética de los équidos exige demostrar en una primera fase su relación con el rendimiento deportivo. Sin embargo, este análisis no se ha llevado a cabo aún debido en gran parte, a la dificultad asociada con su medición, pues la mayoría de los métodos presentan dos problemas fundamentales. En primer lugar, son invasivos, lo que puede generar un cierto estrés simplemente por su medida al exigir la contención del animal (aumento de la frecuencia cardíaca, cortisol plasmático y/o salivar o la presión arterial). En segundo lugar, son muy difíciles de usar durante la

competición, por la necesidad de utilizar instrumental específico de laboratorio para su detección o por requerir una situación ambiental concreta para su realización. Además, hay que tener en cuenta que durante las competiciones ecuestres los conductores, entrenadores y propietarios de los animales que van a competir son bastante reacios a que se les tomen determinados tipos de muestras a sus animales (invasivas y/o restrictivas), ya que piensan que éstas podrían alterar de alguna forma el estado del animal y, por tanto, afectar a sus resultados posteriores en pista.

En este sentido, siguiendo el modelo de otras especies, nuestro grupo de investigación está desarrollando nuevas metodologías más objetivas y no invasivas para evaluar el nivel de estrés del caballo en distintas disciplinas y razas. Se ha demostrado que la termografía infrarroja es una técnica capaz de detectar cambios en el flujo de sangre periférica a partir de los cambios resultantes asociados a la pérdida de calor y, por lo tanto, puede representar una herramienta útil para medir el estrés en los animales de forma no invasiva (Stewart et al., 2005). El equipo de termografía es portátil, fácil de usar y la restricción animal es mínima o innecesaria y tiene ciertas ventajas sobre otros métodos no invasivos al ofrecer una visión de las consecuencias metabólicas del estrés y permitir la medición del estrés agudo a corto plazo (Bartolomé and Cockram, 2016).

Así se han puesto a punto en el Pura Raza Español en concursos de doma clásica (Sánchez et al., 2016) o el Caballo de Deporte Español en pruebas de salto de obstáculos (Valera et al., 2012; Bartolomé et al., 2013b). Con la finalidad de evaluar el nivel de estrés y determinar si afecta al rendimiento deportivo en el CTE, en el **artículo 2** se ha puesto a punto esta metodología para medirlo durante las carreras de trote, determinando por primera vez el umbral de estrés que potencie el mayor rendimiento deportivo, o por el contrario conlleve a distrés o riesgo del bienestar, y por tanto a peores resultados deportivos.

Por ello hemos realizado la evaluación del nivel de estrés con la medición de la temperatura ocular (TO) con termografía infrarroja de fondo de ojo, utilizando como referencia la medida simultánea de la frecuencia cardiaca (FC), que a diferencia de otras metodologías no requiere muestras sanguíneas (Munsters et al., 2012).

Siguiendo las referencias de estudios anteriores en competiciones ecuestres, la toma de datos se ha realizado en tres momentos distintos de la competición: 2 horas antes de que el animal comience la carrera, para medir el estrés de reposo/anticipación; justo al terminar la carrera, para medir el estrés relacionado con el ejercicio; y 2 horas después de la competición, para medir su capacidad de recuperación. La primera fase determina el nivel de estrés basal que el animal tiene antes de iniciar la competición, midiendo principalmente el estrés relacionado con el propio animal y, por lo tanto, su tendencia natural a ser fácilmente estresado o no en un nuevo entorno. Esta medida estaría más relacionada con factores intrínsecos (raza, edad, etc.) que con factores directamente relacionados con la competición. Según la raza, Hausberger et al. (2004) encontró que las razas de caballo Cuarto de Milla, Haflinger o el Trotador francés presentan menos reacciones neofóbicas que el caballo de Silla francés, el PSI o la razas de caballos árabes. La TO medida justo tras finalizar la carrera está más relacionada con

el estrés producido en la "activación" del caballo debido al esfuerzo físico desarrollado durante la competición. Esto se debe a que el caballo percibe la carrera como un factores estresante positivo preparando su sistema circulatorio para el esfuerzo que determina esta (Bartolomé and Cockram, 2016). La TO tomada 2h después de la competición, mide la capacidad de recuperación del caballo tras un gran esfuerzo, que podría provocar distrés si se mantienen los estresores, causando efectos deletéreos que indican que el animal no tiene capacidad para superar el estrés experimentado durante la competición (Aschbacher et al., 2014). Por otro lado, el incremento de TO o ΔTO sobre los niveles basales iniciales mide el estrés producido durante la "activación" del caballo para competir en la carrera.

Nuestros resultados indicaron que el estrés generado para prepararse para la competición puede afectar al rendimiento deportivo de una forma positiva, o negativa según su intensidad. Así un nivel muy bajo precompetición imposibilita una correcta preparación del animal, mientras que unos niveles demasiado elevados bloquearían su capacidad de incrementar su actividad a lo largo de la prueba (Bartolomé and Cockram, 2016).

En nuestro estudio, la variable que se ha elegido para definir el rendimiento deportivo y asociarla con el nivel de estrés (TO y FC) es el TPK, por las ventajas descritas anteriormente. Y se ha analizado la toma de datos en dos de las fases descritas anteriormente, la basal y justo al terminar la carrera, difiriendo en 1-2ºC para la TO, y de 50-60 ppm para la FC. En general, los resultados obtenidos para TO fueron diferentes a los obtenidos en el CDE en salto de obstáculos (Valera et al., 2012; Bartolomé et al., 2013b) o el PRE en la doma clásica (Sánchez et al., 2016). Como se describió anteriormente, hay diferencias en cuanto a temperamento según la raza (Hausberger et al., 2004), y teniendo en cuenta que CTE es una raza compuesta con influencia de otras razas de caballos trotones como el Trotador francés (Muggeo, 2003), se esperaba un valor medio de TO basal más bajo en carreras de trote (35,9ºC) en comparación con los datos presentados, por ejemplo, en Salto de obstáculos (36,2ºC (Valera et al., 2012), donde la raza estudiada, el Caballo de Deporte Español, es también una raza compuesta formada principalmente por el PSI y el caballo de Silla francés (Bartolomé et al., 2011), con mayor reacción emocional que los primeros.

El análisis por grupos de edades (2-3 años, 4-5 años, 6-7 años y 8-10 años) mostró que cuanto más joven es el animal, mayores niveles de estrés presenta (TO y FC) en todas las fases de la competición analizadas. Esto es debido a que la edad es un factor clave en la magnitud de la respuesta al estrés, tanto durante la competición, como después de su finalización (Becker-Birck et al., 2010; Bartolomé et al., 2013a), ya que condiciona en parte el nivel de experiencia del animal y, por tanto, su reactividad ante estímulos potencialmente estresantes (a mayor experiencia, menor reactividad y viceversa).

El estudio de asociación entre el rendimiento deportivo (TPK) y las medidas del nivel de estrés (TO y FC) ha determinado diferencias estadísticamente significativas de la TO basal, ΔTO y la edad con respecto a TPK. Sin embargo, no se han encontrado

diferencias significativas para FC, en contraposición a los resultados de Kinnunen et al. (2006) en carreras de trote del Standardbred. El análisis de regresión segmentada (Figura 1, artículo 2) para analizar la evolución de TO en función del rendimiento deportivo y determinar el umbral en el que el estrés fisiológico se transforma en distrés, mostró que cuanto mayor es el ΔTO (el nivel de estrés justo tras la carrera es más alto que el basal, ΔTO> 0) menor es el TPK (mejores resultados deportivos). Esto indicaría que el nivel de estrés TO dentro de unos límites fisiológicos sirve para preparar mejor al animal para la competición. Finalmente para determinar las condiciones óptimas de TO basal que maximizan el rendimiento deportivo se ha representado, mediante el modelo de superficie de respuesta seguido de un análisis de regresión robusta, la relación entre las variables TPK, ΔTO y TO basal (Figura 2, Artículo 2), pudiéndose observar una disminución del TPK a 77,3s cuando el valor de TO basal alcanza los 37,6ºC y ΔTO es de 7,6%.

En definitiva, nuestros hallazgos confirman que el rendimiento deportivo está influenciado por el nivel de estrés fisiológico, y que la mejor forma de evaluarlo actualmente es midiendo la TO con la termografía. Además, este estudio va más allá destacando un comportamiento elíptico de TO durante la carrera, que se incrementaría hasta un cierto punto desde el cual los animales sufrirían distrés, disminuyendo su rendimiento y por tanto empeorando sus resultados deportivos. La inclusión de esta variable como factor de corrección dentro de los modelos de evaluación genética de los reproductores para las carreras de trote en el CTE, podría mejorar la precisión de los valores genéticos al verse optimizados los modelos de valoración genética. Esta nueva metodología puede contribuir a que los ganaderos realicen una selección de animales con un nivel de estrés basal bajo y menor que el obtenido tras las carreras de trote. De la misma forma pueden ser utilizados como test específicos que sirvan para la preselección de los potros a someter a entrenamiento, aunque para ello sean necesarios estudios adicionales ya que no está demostrada la heredabilidad del carácter a edades tempranas ni su correlación genética con edades posteriores.

GENES ASOCIADOS CON EL ESTRÉS EN CARRERAS DE TROTE

Como se ha descrito anteriormente, el desarrollo paralelo de la Genética Molecular y la Genética Cuantitativa ha potenciado los estudios de identificación de variabilidad genética asociada con caracteres de interés en equinos. Por lo que en este caso, sería interesante analizar la asociación entre genes relacionados con el temperamento/comportamiento y el nivel estrés en carreras de trote.

Estudios en otras especies y en equinos ha mostrado que polimorfismos tipo SNPs en genes relacionados con neurotransmisores u hormonas afectan a caracteres relacionados con el comportamiento (Reif and Lesch, 2003; Takeuchi and Houpt, 2003; Hong et al., 2011). Dentro de estos genes destacan el *BDNF* (factor neurotrófico derivado del cerebro) que actúa como factor de crecimiento de la familia de las neurotrofinas asociadas al factor de crecimiento nervioso; los genes *TPH2* (triptófano

hidroxilasa), *HTR1A* (Receptor 1A de serotonina) y *SLC6A4* (transportador de serotonina) que intervienen en la síntesis del neurotransmisor serotonina; los genes *COMT* (catecol-O-metiltransferasa), *DRD4* (receptor de dopamina D4) y *TH* (Tiroxina Hidroxilasa, relacionados con la síntesis y degradación de dopamina.

Sin embargo, poco se conoce sobre la arquitectura genética del comportamiento del caballo o cómo interactúan los genes con los factores ambientales para generar la combinación compleja de actitudes y acciones que comprenden el temperamento y el comportamiento (Benjamin et al., 1996). En équidos de deporte, la vía fisiológica de respuesta al estrés es el eje hipotálamo-hipofisario (HPA), que desencadena la producción de cortisol por un lado, y de catecolaminas (adrenalina y noradrenalina) por otro (Nagatomi et al., 1999; Gleeson and Bishop, 2000). El primero, que representa la respuesta crónica al estrés, se genera en la corteza de la glándula adrenal y se libera como consecuencia de la estimulación de ésta por parte de la hormona adrenocorticotropa. Las catecolaminas, que representan la respuesta aguda al estrés, se generan en la médula adrenal y son producidas por el sistema nervioso simpático (McKeever et al., 2002). Éstas median una gran cantidad de procesos fisiológicos que dan lugar a la respuesta frente al estímulo estresante, entre ellos, el aumento de la frecuencia y contracción cardíaca, una contracción esplénica (para aumentar el volumen sanguíneo) y la disminución de la circulación sanguínea hacia los vasos sanguíneos periféricos (McKeever, 1993).

Por ello, en el **artículo 3** de la presente tesis ha aportado evidencias sobre la posible asociación de varios de esos genes candidatos (*BDNF*, *COMT*, *HTR1A*, *SLC6A4* y *TPH2*) con el nivel de estrés (medido con TO y FC) en el CTE para carreras de trote. Así cuando se compararon las mediciones de TO y FC obtenidas en el CTE en trote con el PRE en Doma, siempre se mostraron valores más altos para el CTE en las tres etapas de toma de muestra (2h antes, justo después y 2h después de la competición), lo que sugiere la existencia de diferentes patrones de percepción de estrés para cada disciplina. Esto es debido por un lado a que el PRE está considerado una raza con un temperamento más dócil y a que la actividad física que se le exige durante un concurso de doma no es comparable al sobreesfuerzo físico que se le exige a un caballo de carreras, lo que puede implicar diferencias en la reacción emocional, las fibras musculares esqueléticas, la absorción de oxígeno y la FC.

El análisis de asociación en el CTE sólo determinó asociación estadísticamente significativa con un SNP del gen *SLC6A4* y otro del gen *COMT* (Tabla 2, artículo 3). La variante intrónica *g.43865600G> A* en el gen *SLC6A4* estaba asociada con una mayor TO, en las tres fases de toma de muestra en la competición (con una contribución relativa de esta variante a la predicción del modelo rondaba del 30-40%). Por su parte la variante *c.*111G>A* en la región 3'UTR del gen *COMT* mostró asociación con ET 2h después de la carrera (con una contribución de 22.3%). La secreción de catecolaminas como dopamina, norepinefrina y epinefrina está regulada por la enzima catecol-O-metiltransferasa (*COMT*), que se encarga de degradarlos y mantener así los niveles de catecolaminas en el cerebro. Por tanto, se ha mostrado evidencia de que las variantes en

este gen están relacionadas con aspectos del comportamiento emocional y social, como la ansiedad, agresión, sensibilidad al dolor, etc. (Stein et al., 2005; Tunbridge et al., 2006). A su vez, Maron et al. (2005) demostraron que un desequilibrio de la actividad serotoninérgica producida por variantes genéticas del gen *SLC6A4* (que disminuye la tasa de reabsorción de serotonina de la hendidura sináptica) podría estar relacionado con síntomas de ansiedad y pánico. En estudios previos en humanos, se ha demostrado que existe una interacción entre estos dos genes, *SLC6A4* y *COMT*, y ambos relacionados con situaciones cotidianas que provocan estrés (Mandelli et al., 2007). Además, según estudios previos en caballos, el nivel medio-alto de dolor, provocando mayor estrés, está relacionado con altas concentraciones de catecolaminas (Zierz and Wintzer, 1996), mientras que bajas concentraciones de serotonina parecen contribuir a la hiperactividad del eje HPA que induce o facilita el estrés o incluso estados agresivos (Westergaard et al., 2003).

PERSPECTIVAS DE FUTURO DEL PROGRAMA DE MEJORA DEL CABALLO DE TROTADOR ESPAÑOL

La integración de un conjunto de marcadores específicos de los caracteres asociados con el rendimiento deportivo junto con genes relacionados con el nivel de estrés en competición, tiene el fin último de implantarse como herramienta de rutina para realizar una preselección de los animales de la raza para la mejora funcional de la misma de una manera muy eficiente, económica y precoz, a la vez que evitaría el elevado coste del entrenamiento a animales cuyo potencial genético no es el más adecuado para este tipo de disciplinas con el consiguiente ahorro en tiempo y dinero para los criadores. En el caso de que se aplicara el análisis para estos marcadores a mayor número de individuos de la raza para validar la asociación, se podría considerar realizar un test genético aplicable a todos los reproductores de la raza, dentro de una estrategia de selección asistida por marcadores. Sin embargo, esta decisión debe ser tomada por parte de los criadores y ganaderos de esta Raza, en función de la orientación de la cría y las perspectivas de futuro que persigan, contando con el visto bueno de las administraciones autonómicas y/o nacionales, responsables finales de la gestión de esta población.

Otra alternativa para el avance en la evaluación genética y la mejora del CTE sería la aplicación de la Selección Genómica (utilización de miles de marcadores que saturan el genoma) (Hayes et al., 2009). La aplicación de la valoración genómica equina representa una nueva oportunidad en el campo de investigación para la mejora de la salud, el bienestar y el rendimiento deportivo del caballo En la especie equina la secuenciación del genoma se completó muy tardíamente, en el año 2007 (Wade et al., 2009), apareciendo el primer chip de genotipado equino, *EquineSNP50 Beadchip de Illumina* (de 54.602 SNPs), en el año 2008 y el *EquineSNP70 de GeneSeek* (65.000 SNPs) en el año 2014. Actualmente se tiene prácticamente concluido el desarrollo de un microarray de genotipado con una mayor cobertura, el 670K SNP Chip (670.796 SNPs) de Affimetrix (Schaefer et al., 2017). Estas técnicas nos permiten conocer la

distribución y posición de los genes en cada uno de los cromosomas y secuenciar en muy poco tiempo cientos de miles de marcadores. No obstante, en la especie equina aún no se ha realizado ninguna valoración genómica a nivel internacional, si bien se han dado los primeros pasos de estandarización del control de rendimientos para abordar dicha valoración en los próximos años a través de diferentes reuniones llevadas a cabo dentro de InterStallion, en los que está participando miembros del grupo Meragem como representantes españoles del sector equino.

CONCLUSIONES

CONCLUSIONES

Durante el desarrollo de la presente tesis doctoral se ha podido llegar a las siguientes conclusiones:

[CAPITULO I]

1. A pesar del impacto que ha tenido el esquema de selección en el Caballo Trotador Español (CTE), al igual que ha sucedido con el resto de razas equinas españolas de deporte, no se ha detectado pérdida de variabilidad genética, manteniéndose e incluso incrementándose su tamaño efectivo gracias al desarrollo de políticas de cría en unos casos, y a la importación de semen de otras poblaciones, como es el caso del CTE.

[CAPITULO II]

2. Según nuestros resultados, la utilización de técnicas de medida de la temperatura del fondo de ojo mediante termografía infrarroja en el CTE, permiten estimar el nivel de estrés de forma objetiva y no invasiva en condiciones de competición.
3. Se ha demostrado que el nivel de estrés basal del animal, antes de iniciar la carrera de trote, influye en su rendimiento deportivo, habiéndose podido determinar el umbral del nivel de estrés que conlleva a los mejores resultados deportivos, o por el contrario pone en peligro el bienestar del animal.
4. Se ha desarrollado una herramienta molecular, que consiste en el análisis por PCR y secuenciación de 2 puntos polimórficos en los genes *COMT* y *SLC6A4* relacionados con el nivel de excitabilidad de los animales, pudiéndose utilizar para realizar una selección de los animales con un nivel de estrés óptimo para un mejor rendimiento deportivo.

[CAPITULO III]

5. En esta Tesis Doctoral se ha desarrollado una herramienta molecular, consistente en el análisis por PCR y secuenciación de 4 puntos polimórficos en los genes *MSTN*, *CKM*, *PDK4* y *DMRT3* para determinar el potencial deportivo del CTE según la edad y el tipo de carreras en las que participa (de resistencia o velocidad).

[CONCLUSIÓN GENERAL]

6. El uso de las metodologías planteadas en la presente Tesis Doctoral, como herramientas moleculares de apoyo en el Programa de Mejora del CTE, podría permitir la preselección de potros con gran potencial genético para la aptitud del trote y un nivel de estresabilidad adecuado. Esto permitirá un gran ahorro económico a los criadores de CTE, al reducir principalmente costes de entrenamiento y al permitir un incremento del progreso genético y una disminución del intervalo generacional.

CONCLUSIONS

During the development of the present Doctoral Thesis it has been possible to become with the following conclusions:

[CHAPTER I]

1. In spite of the impact of selection in the Spanish Trotter Horse (CTE), as it occurs in other Spanish riding-horse breeds, no loss of genetic variability has been detected, and even the effective population size increased due to the development of the breeding policies in some cases, or to the use of semen from foreign populations, as it is the case of the CTE.

[CHAPTER II]

2. According to our results, the use of technics as the infrared thermography to measure eye temperature in the CTE allows to estimate the stress level with reliability and in a non-invasive way under competition conditions.
3. It has been proved that the animal's basal stress level, before starting the trotting race, influences its sporting performance, being determined the threshold level of stress which leads to the best sporting results in the CTE or, on the contrary, causes a negative influence on its welfare.
4. A molecular tool have been developed, based on the PCR and sequencing analysis of 2 polymorphisms in the *COMT* and *SLC6A4* genes, related to the excitability level of the animals, which could be used to select the animals with an optimal stress level for the best sporting performance.

[CHAPTER III]

5. In this Thesis, a molecular tool have been developed, based on the PCR and sequencing analysis of 4 polymorphisms in the *MSTN*, *CKM*, *PDK4* and *DMRT3* genes to determine the sporting potential of the CTE according to its age or the type of races in which participates (long or short distance races).

[GENERAL CONCLUSION]

6. The use of the methodologies considered in the present Doctoral Thesis, as supporting tools within the breeding program of the CTE, could allow the preselection of foals with high sporting potential for the trotting ability and an adequate stress level. This will determine great economic savings for the breeders, by mainly reducing the training costs, and allowing an increment of the genetic process and a decrease in the generation interval.

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LISTADO DE PUBLICACIONES

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En esta sección se presenta un listado en el que se recogen todas las publicaciones a las que ha dado lugar esta Tesis Doctoral, a nivel nacional e internacional.

PUBLICACIONES EN REVISTAS ISI

AUTORES: **Negro S.,** Valera M., Membrillo A., Gómez, M.D., Menendez-Buxadera, A., Anaya, G., Molina, A.
TÍTULO: Quantitative analysis of short and long distance racing performance in young and adult horses and association analysis with functional candidate genes in Spanish Trotter Horses.
REFERENCIA: Journal of Animal Breeding and Genetics (2016) 133(5), 347–356.
ISSN: 0931-2668 (online)
IF=1.877 (1º cuartil) en Agriculture, Dairy, Animal Science

AUTORES: **Negro S.,** Solé, M., Pelayo, R., Gómez, M.D., Azor P.J., Valera M. (2016)
TÍTULO: Molecular diversity between two cohorts of six Spanish riding-horse breeds: impact of selection in Crossbred vs Purebred populations.
REFERENCIA: Livestock Science (2016) 193, 88-91.
ISSN: 1871-1413 (online)
IF=1.377 (2º cuartil) en Agriculture, Dairy, Animal Science

AUTORES: **Negro S.;** Bartolomé E.; Molina A.; Solé M.; Gómez M.D.; Valera M.
TÍTULO: Stress level effects on sport performance during trotting races in Spanish Trotter horse.
REFERENCIA: Equine Veterinary Journal (2017) EVJ-GA-17-138, under review.
ISSN: 0425-1644 (online)
IF=2.382 (1º cuartil) en Veterinary Sciences

AUTORES: **Negro S.;** Valera M.; Solé M.; Bartolomé E.; Sánchez M.J.; Gómez, M.D.; Molina, A.
TÍTULO: Evidence for the effect of serotoninergic and dopaminergic gene variants on stress levels in horses participating in dressage and harness racing.
REFERENCIA: Animal Genetics (2017) AnGen-17-06-0134, under review.
ISSN: 1365-2052 (online)
IF=1.779 (1º cuartil) en Agriculture, Dairy, Animal Science

LIBROS COMPLETOS

AUTORES: Sánchez M.J.; Solé M.; Gómez M. D.; Molina A.; **Negro S.;** Medina C.; Valera M.
LIBRO: Catálogo de Reproductores del Caballo Trotador Español, 2016. 2016. 132.
EDICIÓN: Grupo de investigación MERAGEM
PUBLICACIÓN (ISSN/ISBN): 978-84-617-4950-8

AUTORES: Solé M.; Molina A.; **Negro S.;** Gómez M. D.; Medina C.; Valera M.

LIBRO: Catálogo de Reproductores del Caballo Trotador Español, 2015. 2015. 132.
EDICIÓN: Grupo de investigación MERAGEM
PUBLICACIÓN (ISSN/ISBN): 978-84-608-3659-9.

AUTORES: Gómez M. D.; Molina A.; Negro S.; Medina C.; Valera, M.
LIBRO: Catálogo de reproductores Caballo Trotador Español, 2013. 2013. 62.
EDICIÓN: Grupo de investigación MERAGEM
PUBLICACIÓN (ISSN/ISBN): 978-84-695-9516-9

CONGRESOS INTERNACIONALES

AUTORES: **Negro S.;** Molina A.; Valera M.; Bartolomé E.
TÍTULO: Stress level effects on sporting performance in Spanish Trotter horses. The
CONGRESO: 68th Annual Meeting of EAAP.
ENTIDAD ORGANIZADORA: EAAP
PUBLICACIÓN (ISSN/ISBN): Book of Abstracts of the 68th Annual Meeting of the European Association for Animal Production.
TIPO DE PARTICIPACIÓN: Ponencia oral, Abstract nº 26807
LUGAR DE CELEBRACIÓN: Tallin, Estonia
FECHA: 28-01 de Septiembre 2017.

AUTORES: **Negro S.;** Solé M.; Sánchez M.J.; Bartolomé E.; Molina A.; Valera M.
TÍTULO: Association analysis of SLC6A4 variants with welfare-behavioural traits in PRE dressage competitions.
CONGRESO: 67th Annual Meeting of EAAP.
ENTIDAD ORGANIZADORA: EAAP
PUBLICACIÓN (ISSN/ISBN): Book of Abstracts of the 67th Annual Meeting of the European Association for Animal Production.
TIPO DE PARTICIPACIÓN: Póster Abstract nº 23892
LUGAR DE CELEBRACIÓN: Belfast, UK
FECHA: 29-02 de Septiembre 2016

AUTORES: **Negro, S.;** Valera, M.; Membrillo, A.; Gómez, M.D.; Menéndez-Buxadera, A.; Anaya, G.; Molina, A.
TÍTULO: Association analysis of SNPs in MSTN, PDK4 and DMRT3 genes with sporting performance in Trotters
CONGRESO: 66th Annual Meeting of EAAP.
ENTIDAD ORGANIZADORA: EAAP
PUBLICACIÓN (ISSN/ISBN): Book of Abstracts of the 66th Annual Meeting of the European Association for Animal Production.
TIPO DE PARTICIPACIÓN: Póster Abstract nº 20282
LUGAR DE CELEBRACIÓN: Varsovia, Polonia
FECHA: 31-04 de Septiembre 2015

CONGRESOS NACIONALES

AUTORES: **Negro, S.;** Valera, M; Molina, A.; Gómez, M.D.; Membrillo, A.

TÍTULO: Nuevas metodologías de mejora genética equina: marcadores moleculares para la selección asistida en el Caballo Trotador Español.
CONGRESO: III Congreso Científico de Jóvenes investigadores en Formación de la Universidad de Córdoba"
ENTIDAD ORGANIZADORA: Universidad de Córdoba
PUBLICACIÓN (ISSN/ISBN): Libro de actas
TIPO DE PARTICIPACIÓN: Póster
LUGAR DE CELEBRACIÓN: Córdoba
FECHA: 09-10 de Abril 2013

AUTORES: Bartolomé, E.; Sánchez, M.J.; Gómez, M.D.; Cervantes, I.; Solé, M.; **Negro, S.;** Anaya G.J.; Azor, P.J.; Molina, A.; Valera, M.
TÍTULO: Programas de mejora de las razas equinas españolas.
CONGRESO: 14ª Feria Nacional de Ganadería y la Subasta Nacional de Ganado Selecto
ENTIDAD ORGANIZADORA: Comité Organizador de FEGASUR
PUBLICACIÓN (ISSN/ISBN): Libro de actas
TIPO DE PARTICIPACIÓN: Póster
LUGAR DE CELEBRACIÓN: Jerez de la Frontera, Cádiz
FECHA: 9-12 de Noviembre de 2012

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