Estructura y diversidad genética en poblaciones fragmentadas de herrerillo común (*Cyanistes caeruleus*)

Esperanza Socorro Ferrer

Tesis doctoral

Toledo, 2015

UCLM
UNIVERSIDAD DE CASTILLA-LA MANCHA

CSIC
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

Estructura y diversidad genética en poblaciones fragmentadas de herrerillo común (*Cyanistes caeruleus*)

Fotografía cubierta: Emili Casals y Carme Jurado

Departamento de Ciencias Ambientales. Área de Zoología.
Facultad de Ciencias Ambientales y Bioquímica
Universidad de Castilla-La Mancha

Estructura y diversidad genética en poblaciones fragmentadas de herrerillo común (*Cyanistes caeruleus*)

Memoria presentada por la Licenciada Esperanza Socorro Ferrer para optar al grado de Doctora por la Universidad de Castilla-La Mancha.

Trabajo dirigido por el Dr. Joaquín Ortego Lozano de la Estación Biológica de Doñana y el Dr. Juan José Sanz Cid del Museo Nacional de Ciencias Naturales.

Toledo, 2015

El Doctorando	Vº Bº del Director	Vº Bº del Director
Esperanza Socorro Ferrer	Joaquín Ortego Lozano	Juan José Sanz Cid

Esta Tesis ha sido financiada con los proyectos de investigación CGL2010-21933-C02-01 del Ministerio de Ciencia e Innovación y POIC10-0269-7632 de la Junta de Comunidades de Castilla la Mancha y del Fondo Social Europeo. Durante la realización de esta Tesis disfruté de una beca predoctoral de Formación de Personal Investigador (FPI) de la Junta de Comunidades de Castilla la Mancha y del Fondo Social Europeo.

A mi madre Esperanza y a mi hermana Dary.

A Tito.

ÍNDICE

RESUMEN

El estudio de los factores que determinan la estructura y diversidad genética de las poblaciones naturales es fundamental para entender sus trayectorias demográficas y evolutivas y poder así predecir sus respuestas a cambios ambientales futuros. En esta tesis doctoral se ha estudiado la estructura genética y la relación entre diversidad genética y eficacia biológica medida en términos de prevalencia de parásitos, supervivencia y desarrollo de ornamentos en poblaciones mediterráneas de herrerillo común (*Cyanistes caeruleus*), empleando para ello marcadores microsatélites clasificados como neutros y funcionales en función de si están localizados o no en regiones del genoma que son transcritas activamente a ARN. Los resultados de los análisis de estructura genética mostraron una cierta limitación al flujo genético entre las poblaciones estudiadas. Las hembras presentaban distancias de dispersión mayores que los machos, un patrón que se veía reflejado en la presencia de estructura genética en machos pero no en hembras. Los análisis de genética del paisaje también revelaron una estructuración genética de las poblaciones, aunque ésta fue sutil, posiblemente debido a que la fragmentación del hábitat podría forzar a los individuos a dispersarse a mayores distancias atravesando hábitats desfavorables para llegar a nuevos lugares de reproducción. Además, la estructuración genética fue detectada fundamentalmente para marcadores neutros, lo cual podría ser debido a que los marcadores funcionales estuvieran sometidos a selección estabilizante y, por lo tanto, reflejaran en menor medida procesos neutrales de diferenciación genética. La heterogeneidad espacial y la fragmentación de las poblaciones parecen también haber generado diferencias en las prevalencias de malaria aviar entre las distintas zonas de estudio. La probabilidad de infección por malaria aviar estuvo relacionada de modo no linear con la heterocigosidad estimada en marcadores neutrales, de modo que los individuos con niveles intermedios de heterocigosidad presentaban mayores probabilidades de estar parasitados. Esta relación no fue significativa para el conjunto de marcadores funcionales, ni difirió entre zonas de estudio con diferente grado de presión de parasitismo. Sin embargo, el contexto ambiental sí influyó en la dirección e intensidad de la relación entre diversidad genética y probabilidad de supervivencia interanual. En particular, observamos que la precipitación acumulada está positivamente correlacionada con la intensidad de selección en heterocigosidad. Además, la selección de individuos heterocigotos y la moderada heredabilidad de la heterocigosidad parecen haber generado un aumento progresivo de la diversidad genética en la población, lo que sugiere una respuesta micro-evolutiva a la selección. Finalmente, observamos una relación positiva entre la expresión de caracteres sexuales secundarios y la heterocigosidad de los machos estimada en marcadores funcionales. Esto indica que el mecanismo por el cual las hembras seleccionan pareja podría estar mediado por la mayor capacidad de machos con alta diversidad genética para desarrollar ornamentos, algo que podría facilitar la elección por

parte de la hembra de una pareja con mayor calidad genética. En conjunto, los resultados derivados de esta tesis doctoral destacan la importancia de integrar diferentes tipos de marcadores moleculares y datos ecológicos obtenidos a diferentes escalas espacio-temporales para mejorar el conocimiento de los factores que determinan la estructuración genética de las poblaciones naturales, así como de las consecuencias que la diversidad genética tiene en diferentes componentes de la eficacia biológica.

Introducción y objetivos

INTRODUCCIÓN

La diversidad genética es la responsable fundamental de que los individuos presenten diferencias fenotípicas y de que las poblaciones y especies tengan capacidad para responder a la selección y adaptarse a los cambios ambientales (Frankham *et al.* 1996). La evolución es el motor del cambio genético que actúa en el seno de las poblaciones. Pero ¿qué procesos son los responsables del cambio evolutivo? La **selección natural y sexual** es uno de estos procesos, a través del cual ciertos individuos presentan una mayor eficacia biológica, de modo que las variantes genéticas más favorables se acumulan progresivamente a lo largo de generaciones. La selección natural actúa sobre la variabilidad existente en la población pero **las mutaciones** constituyen realmente el proceso fundamental que genera dicha variación genética (Muller 1964). Otra fuerza evolutiva destacable es la **deriva genética**, que altera la frecuencia de los alelos de la población por procesos azarosos. Debido a que la deriva genética se trata exclusivamente del efecto acumulativo de fenómenos aleatorios, ésta no puede dar lugar a adaptación, únicamente a que las frecuencias alélicas de las poblaciones cambien. Este proceso es especialmente importante en poblaciones pequeñas porque induce la pérdida progresiva de variabilidad genética tanto a nivel individual como poblacional (Kimura 1955; Willi *et al.* 2006). En contraposición a la deriva genética se encuentra el **flujo genético**, que genera el intercambio de alelos entre poblaciones, produciendo cambios en sus frecuencias e incrementando la diversidad genética poblacional (Felsenstein 1976). El flujo genético puede variar considerablemente entre poblaciones de una misma especie dependiendo de distintos factores. En este sentido, la fragmentación del hábitat puede provocar una reducción del flujo genético y generar una pérdida de diversidad genética, particularmente en aquellas poblaciones de pequeño tamaño más proclives a sufrir las consecuencias de la deriva genética (Willi *et al.* 2006). Por lo tanto, el estudio de la variación genética en poblaciones con diferente grado de aislamiento es necesario para comprender su capacidad para responder a la selección y adaptarse a cambios ambientales en el futuro (Davis *et al.* 2005).

Estructura genética poblacional

Los individuos no están distribuidos en el espacio de forma aleatoria, sino que generalmente se agregan en poblaciones ocupando hábitats adecuados que presentan una distribución parcheada dentro de la matriz heterogénea que caracteriza la mayoría de los paisajes. Además, esta distribución no es estática y puede verse modificada por diferentes procesos que tienen lugar a distintas escalas espaciotemporales. La heterogeneidad espacial, ya sea producto de la actividad del hombre o de procesos naturales, juega un papel importante en los procesos ecológicos y evolutivos. En tiempos recientes, uno de

los factores que más ha contribuido a la alteración de la distribución de los organismos es la fragmentación del hábitat. Los organismos que antes se distribuían a lo largo de poblaciones más o menos continuas ahora a menudo forman poblaciones aisladas como consecuencia de la destrucción de porciones importantes del hábitat original por parte del hombre (Fahrig 2003; Foley *et al.* 2005). Las barreras que limitan el movimiento de los individuos pueden ser tanto físicas (ej. cultivos, infraestructuras humanas, etc.) como comportamentales (ej. riesgo de depredación, reluctancia a cruzar hábitats subóptimos) (Pierson *et al.* 2010). Por consiguiente, la dispersión entre fragmentos es un mecanismo clave que determina la persistencia a largo plazo de muchas poblaciones, e identificar los elementos del paisaje que impiden o favorecen el flujo genético entre ellas se ha convertido en un objetivo prioritario en genética de la conservación.

La mayoría de estudios sobre genética poblacional han demostrado que la diferenciación genética entre poblaciones está relacionada con la distancia geográfica que las separa, un patrón que se conoce como "**aislamiento por distancia**" (IBD en sus siglas en inglés) (Wright 1945; Slatkin 1993). Sin embargo, la distancia geográfica es uno de los muchos factores que pueden potencialmente influir en la conectividad genética de las poblaciones. En los últimos años, las investigaciones sobre genética del paisaje han contribuido a mejorar nuestro conocimiento sobre la conectividad de las poblaciones en relación a la estructura del paisaje y la resistencia que diferentes componentes ambientales ofrecen al flujo genético. Esto ha dado lugar al término "**aislamiento por resistencia**" (IBR, en sus siglas en inglés); (McRae & Beier 2007), que hace referencia a la probabilidad de que un individuo se disperse de una población a otra sopesando todas las posibles rutas a tomar en base a la resistencia que el entorno que las separa ofrece a la dispersión. Finalmente, la mayoría de las especies se encuentran distribuidas en paisajes con una considerable heterogeneidad ambiental, de modo que poblaciones localizadas en distintos ambientes (altitud, hábitat, clima, etc.) pueden desarrollar adaptaciones locales que generen una disminución del flujo genético entre poblaciones sometidas a distintas presiones evolutivas, independiente de la distancia geográfica que las separa (Nosil *et al.* 2005; Wang & Bradburd 2014). Este proceso se conoce como **"aislamiento por ambiente"** (IBE, en sus siglas en inglés) y hace referencia al aumento de la diferenciación genética de las poblaciones con las diferencias ambientales que existen entre ellas (Sexton *et al.* 2014; Wang & Bradburd 2014). Diferentes estudios han descrito procesos de aislamiento genético entre poblaciones en base a numerosos factores ambientales, incluyendo diferencias de hábitat (Porlier *et al.* 2012), altitud (Murphy *et al.* 2010; Bradburd *et al.* 2013) o clima (Wang 2012). Por lo tanto, la composición y estructura del paisaje pueden afectar a la conectividad de las poblaciones a través de interacciones mucho más complejas que la distancia geográfica. Sin embargo, todos estos

procesos (IBD, IBR y IBE) no son excluyentes y la diferenciación genética de las poblaciones puede ser originada por una combinación de todos ellos (Wang & Bradburd 2014). Por consiguiente, su consideración simultánea en los estudios de genética del paisaje puede mejorar notablemente el conocimiento sobre los mecanismos que determinan la estructuración genética de las poblaciones naturales (Pflüger & Balkenhol 2014).

Diversidad genética y eficacia biológica

Comprender cómo la variación genética se relaciona con la eficacia biológica es esencial para entender una parte importante de los procesos evolutivos. La correlación entre eficacia biológica y el grado de endogamia de los individuos o su diversidad genética ha sido estudiada en una gran variedad de organismos (Keller & Waller 2002; Chapman *et al.* 2009). Las relaciones entre diversidad genética y eficacia biológica surgen como consecuencia de la mayor probabilidad que tienen los individuos endogámicos de expresar alelos deletéreos recesivos o la ventaja de los heterocigotos en genes que presentan sobredominancia (Charlesworth & Charlesworth 1987; Falconer & Mackay 1996). De esta manera, numerosos trabajos han mostrado que una mayor diversidad genética individual está relacionada con un mayor éxito reproductor (Kempenaers 2007) medido en términos de fertilidad (Gage *et al.* 2006), fecundidad (Ortego *et al.* 2007; Szulkin *et al.* 2007) o número de descendientes (Olano-Marin *et al.* 2011a). Asimismo, se ha visto que una baja diversidad genética puede disminuir la resistencia a parásitos y enfermedades (Casinello *et al.* 2001; Acevedo-Whitehouse *et al.* 2006; Reid *et al.* 2007) y comprometer la viabilidad del individuo (Szulkin *et al.* 2007; Olano-Marin *et al.* 2011b; Richner & Nunziata 2014). Otros rasgos indirectamente asociados con la eficacia biológica también han sido relacionados con una mayor diversidad genética, incluyendo el estatus social del individuo (dominancia: Tiira *et al.* 2006; agresividad: Tiira *et al.* 2003) y el desarrollo de conspicuas señales de atracción sexual (Aparicio *et al.* 2001; Marshall *et al.* 2003; Van Oosterhout *et al.* 2003; Bolund *et al.* 2010; Leclaire *et al.* 2011; revisado en Kempenaers 2007).

Idealmente, la diversidad genética individual debería ser determinada mediante el cálculo de los coeficientes de endogamia usando información del pedigrí de las poblaciones. Sin embargo, esta información es extremadamente complicada de conseguir en poblaciones naturales (Pemberton 2004), especialmente en aquellas que son abiertas y en especies donde las fertilizaciones extra-pareja son comunes (Griffith *et al.* 2002). Debido a estas limitaciones, la utilización de marcadores moleculares para determinar de manera indirecta el grado de endogamia de los individuos y su diversidad genética se ha convertido en una herramienta alternativa ampliamente utilizada. Sin embargo, muchos estudios han puesto en duda la capacidad de unos pocos marcadores moleculares para estimar la diversidad

genética del conjunto del genoma o el grado de endogamia de los individuos (Balloux *et al.* 2004; Väli *et al.* 2008; Szulkin *et al.* 2010), cuestionándose si las relaciones entre heterocigosidad y eficacia biológica (HFC en sus siglas en inglés) reflejan depresión por endogamia u otros fenómenos. Como resultado, se han formulado tres hipótesis para explicar la existencia de las HFC. Por un lado, la hipótesis de *efecto general* sugiere que las HFC surgen como consecuencia de que la heterocigosidad medida con los marcadores moleculares estaría correlacionada con la heterocigosidad del conjunto del genoma y reflejaría el grado de endogamia de los individuos (David 1998). No obstante, diferentes estudios han indicado que sólo bajo determinados contextos que generen varianza en diversidad genética y desequilibrio de identidad (ID, en sus siglas en inglés) es posible detectar las HFC debidas a un efecto general (Balloux *et al.* 2004; Szulkin *et al.* 2010). La varianza en diversidad genética se puede producir cuando las poblaciones presentan una cierta frecuencia de cruces endogámicos, están sometidas a deriva genética o cuellos de botella (Cristescu *et al.* 2009) o cuando han experimentado cierta mezcla de alelos entre individuos inmigrantes y locales (Tsitrone *et al.* 2001). Por otro lado, la hipótesis de *efecto local* sugiere que es el efecto de algunos marcadores ligados a genes con funciones que afectan a la eficacia biológica y sometidos a selección balanceante (sobredominancia) los que generan las HFC (Hansson & Westerberg 2002). La tercera hipótesis, denominada de *efecto directo*, propone que las HFC surgen debido a que la diversidad genética en los marcadores utilizados afecta directamente a la eficacia biológica, algo que solo puede darse si dichos marcadores son funcionales *per se* (David 1998).

Muchos estudios han sugerido que el contexto ambiental es uno de los factores más importantes que puede determinar la detección de las relaciones entre diversidad genética y eficacia biológica (Hoffman & Hercus 2000; Da Silva *et al.* 2006; Annavi *et al.* 2014). En este sentido, algunos estudios han observado que las HFC son más fuertes o sólo se manifiestan en presencia de determinados agentes estresantes como son, por ejemplo, la limitación de recursos (Lesbarèrres *et al.* 2005), el estrés térmico (Scott & Koehn 1990), condiciones ambientales severas (Marr *et al.* 2006; Forcada & Hoffman 2014) o la presencia o abundancia de parásitos (Coltman *et al.* 1999; Voegeli *et al.* 2012). La gran variabilidad en condiciones ambientales experimentadas por distintas poblaciones o entre distintos años dentro de una misma población podría ser responsable de los resultados contradictorios encontrados en diferentes estudios sobre HFC (Audo & Diehl 1995; Chapman *et al.* 2009). Por este motivo, estudiar la relación entre diversidad genética y eficacia biológica considerando el contexto ambiental puede ayudar a comprender la disparidad de resultados entre diferentes estudios y aportar información relevante sobre la capacidad de las poblaciones para responder a diferentes fuentes de estrés (Forcada & Hoffman 2014).

Diversidad y estructura genética: marcadores neutros *vs.* funcionales

El tipo de marcadores a utilizar en los estudios de genética de poblaciones también ha sido objeto de importante controversia. Generalmente se considera que los marcadores microsatélites son evolutivamente neutros. Sin embargo, estos marcadores a menudo se encuentran localizados en regiones codificantes del genoma y, por lo tanto, pueden influir en diferentes procesos evolutivos (Li *et al.* 2004). De forma general se espera que los marcadores neutros detecten HFC bajo la hipótesis de efecto general o debido al efecto local si se encuentran ligados a loci funcionales (David 1998; Hansson *et al.* 2001; Hansson & Westerberg 2002; Balloux *et al.* 2004), mientras que solo los marcadores funcionales podrían detectar HFC debido a un efecto directo (Olano-Marin *et al.* 2011ab; Laine *et al.* 2012). Sin embargo, no existen muchos trabajos que hayan analizado las relaciones entre heterocigosidad y eficacia biológica empleando loci funcionales diferentes a los localizados en el complejo mayor de histocompatibilidad (Kupper *et al.* 2010; Olano-Marin *et al.* 2011ab; Laine *et al.* 2012). El empleo de marcadores con diferente funcionalidad puede ayudar a aclarar la importancia relativa del efecto del conjunto del genoma o el de unos pocos loci en la intensidad y tendencia de las correlaciones entre heterocigosidad y eficacia biológica (David 1998; Hansson & Westerberg 2002). Por otro lado, el empleo de ambos tipos de marcadores no ha sido una práctica muy extendida en los estudios sobre genética del paisaje (ej. Freedman *et al.* 2010), a pesar de que las diferencias de funcionalidad de las distintas regiones genómicas en las que se localizan pueden ayudar a entender mejor los procesos adaptativos o neutrales que modelan los patrones de diferenciación genética poblacional. Por estos motivos, el empleo tanto de marcadores neutrales como de marcadores localizados en regiones funcionales del genoma puede contribuir de manera notable a mejorar el entendimiento de los mecanismos responsables de las HFC y de los factores que determinan la estructuración genética de las poblaciones (Orsini *et al.* 2013).

Especie de estudio

El herrerillo común *Cyanistes caeruleus* es un pequeño (9-12 gramos) paseriforme insectívoro asociado generalmente a bosques caducifolios y mixtos (Cramp & Perrins 1993). Exhibe un plumaje vistoso compuesto por una amplia gama de colores. En la cabeza presenta un antifaz de color negro con mejillas blancas y un píleo azul que es considerado un importante rasgo ornamental relacionado con el grado de atractivo sexual (Andersson *et al.* 1998; Hunt *et al.* 1998). Las alas y la cola son en buena medida de color azul y tiene una coloración ventral amarilla con una marcada banda negra sobre el pecho y el dorso verde-pardusco. Esta especie se adapta con facilidad a criar en cajas nido en las que introducen diversos materiales vegetales y animales para construir sus nidos. El herrerillo común realiza una puesta que

oscila entre los 4 y los 13 huevos. Tras un periodo de incubación de 13 días, los huevos eclosionan y los pollos son alimentados por ambos padres. Los pollos abandonan el nido trascurridos 17-20 días tras la fecha de eclosión. Cuando termina la temporada de cría, los individuos pasan de tener un comportamiento territorial a gregario y forman grupos de residentes que se alimentan conjuntamente.

Las poblaciones mediterráneas de herrerillo común se encuentran en el extremo sur del rango de distribución de la especie (Cramp & Perrins 1993). En esta región los bosques caducifolios se encuentran altamente fragmentados tanto por la acción del hombre como de manera natural, quedando a menudo relegados a pequeños parches poco conectados localizados en umbrías o zonas elevadas. Las características propias del hábitat de la especie en ambientes mediterráneos, junto con su baja capacidad dispersiva y la fácil monitorización de sus poblaciones, generan un escenario ideal para estudiar i) las consecuencias que la estructura y heterogeneidad del paisaje tienen en la diferenciación genética de las poblaciones y ii) las consecuencias que la diversidad genética tiene en la eficacia biológica de los individuos.

Área de estudio

El área de estudio se encuentra situada en los Montes de Toledo y abarca una extensión de unos 1200 km² (Fig. 1). Presenta un clima mediterráneo continental caracterizado por una gran amplitud térmica y gran variabilidad interanual de las precipitaciones, lo cual se manifiesta en periodos de sequía alternados con años relativamente húmedos. El área de estudio se compone de tres zonas que albergan diferentes poblaciones. La primera de ellas se localiza en el Parque Nacional de Cabañeros (39°24´N, 3°35´W, Ciudad Real-Toledo) y fue monitorizada durante las primaveras de 2007 a 2008. La segunda se sitúa en Quintos de Mora (39º25´N 4º04´W, Toledo) y se trata de una finca gestionada por el Organismo Autónomo de Parques Nacionales (Ministerio de Agricultura, Alimentación y Medio Ambiente). En esta zona se realizó el seguimiento de las poblaciones desde el año 2007 al año 2014. La tercera zona de estudio se encuentra situada en el entorno de la localidad de San Pablo de los Montes (39º31´N 4º21´W, Toledo) y su seguimiento se desarrolló durante las primaveras de 2011 a 2013. Estas tres zonas no están situadas en bosques continuos sino que se encuentran separadas principalmente por zonas de cultivos, formando un paisaje altamente heterogéneo compuesto por un mosaico de parches con gran variedad de hábitats y usos del suelo. Las áreas más húmedas de umbría están mayormente dominadas por bosques caducifolios de roble melojo (*Quercus pyrenaica*) aunque también es posible encontrar arces (*Acer monspessulanum*) y fresnos (*Fraxinus agustifolia)* junto con otras especies de estrato arbustivo como el rusco (*Ruscus aculeatus*), el

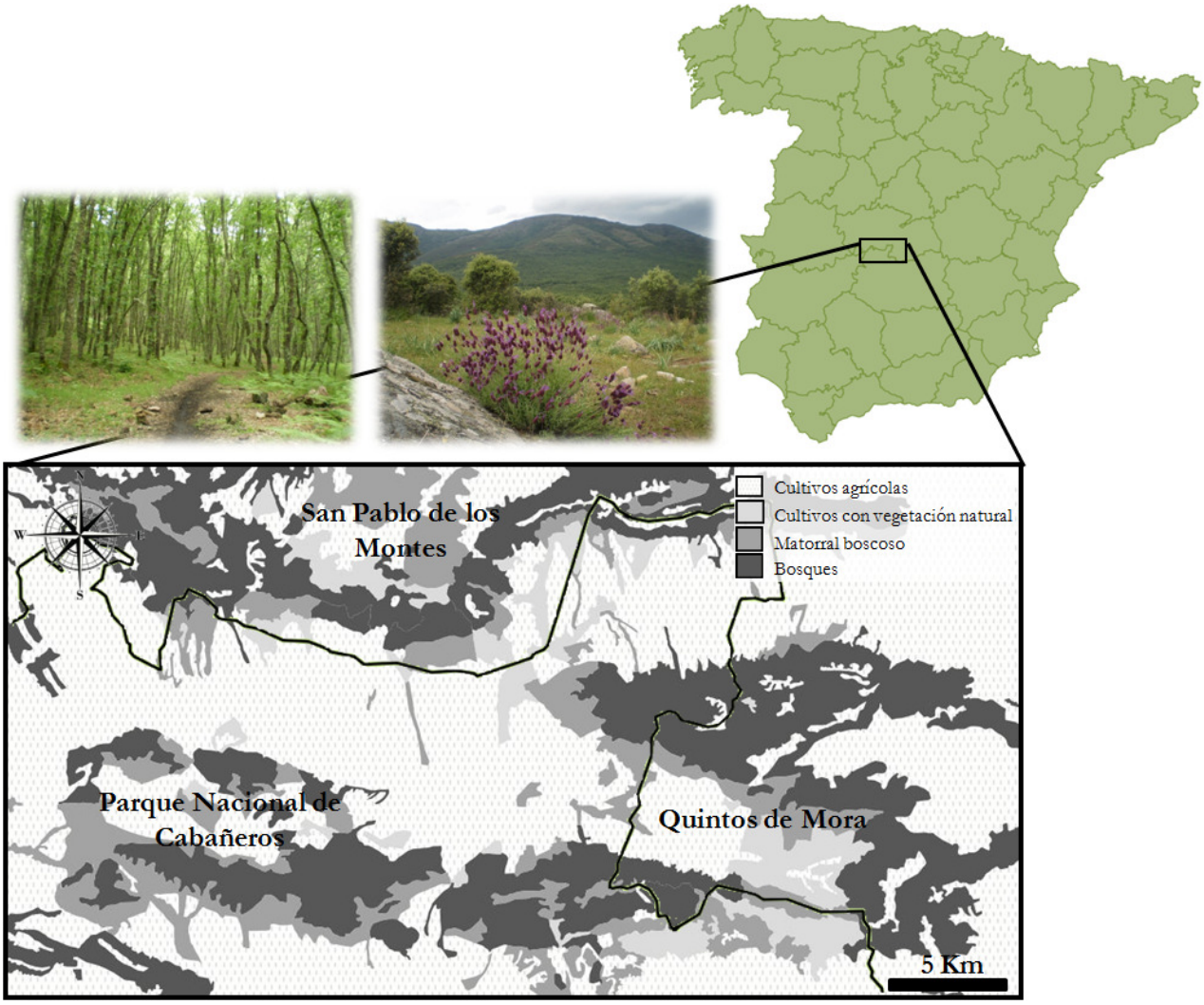


Figura 1 Localización de la zona de estudio y usos del suelo mayoritarios según Corine Land Cover 2006.

majuelo (*Crataegus monogyna*), el rosal silvestre (*Rubus sp.*) o la jara pringosa (*Cistus ladanifer*). La vegetación dominante en las llanuras y en las áreas de solana está compuesta por encinas (*Quercus ilex*), brezo (*Erica* spp.) o lavanda (*Lavandula angustifolia*). También se pueden encontrar plantaciones de pinar (*Pinus pinea*, *Pinus pinaster*) distribuidas a lo largo del paisaje.

Procedimiento general de campo

El seguimiento de las poblaciones de herrerillo común se realizó desde finales de Marzo hasta principios de Julio. Se tomaron datos de la fecha y tamaño de puesta, fecha de eclosión, tamaño de nidada y número de volantones. Los individuos adultos fueron capturados por medio de una trampa colocada en la caja nido cuando cebaban a sus pollos a la edad de 8 días. Se les midió el ala con una regla (± 0.5 mm), el tarso con un calibre (± 0.1 mm) y fueron pesados con una báscula (± 0.1 g). Además, tanto los individuos adultos como los pollos fueron anillados para su posterior identificación y se les extrajo una muestra de sangre (≤ 25 µl) mediante punción de la vena braquial.

Procedimiento general de análisis genéticos

Los individuos de herrerillo común fueron genotipados utilizando 26 marcadores microsatélites, 14 clasificados como neutrales y 12 como presumiblemente funcionales al estar localizados en regiones del genoma que son transcritas activamente a ARN. A partir de las muestras de sangre obtenidas en el campo se realizó la extracción y purificación de ADN genómico mediante kits comerciales y se procedió a la amplificación de los marcadores mediante reacciones en cadena de la polimerasa (PCR). Posteriormente, se realizó el genotipado de los individuos con el programa GENEMAPPER 3.7 (Applied Biosystems) y los análisis de desviación del equilibrio de Hardy-Weinberg y del posible ligamiento entre pares de marcadores usando GENEPOP versión web 4.0.10 (Garnier-Gere & Dillmann 1992; Raymond & Rousset 1995). Los índices de diversidad genética *HL* y H_O fueron calculados usando CERNICALIN (Aparicio *et al.* 2006) y la estructura y diferenciación genética de las poblaciones fue analizada con los programas STRUCTURE 2.3.4 y ARLEQUIN 3.1.

OBJETIVOS

Los objetivos fundamentales de esta tesis doctoral son, por un lado, contribuir a mejorar el conocimiento sobre los factores que determinan la estructuración genética de las poblaciones naturales en hábitats fragmentados y, por otro, utilizar nuevas aproximaciones para entender mejor la relación entre diversidad genética individual y distintos componentes de la eficacia biológica, teniendo en cuenta además el contexto ambiental en el que son analizadas. Para ello se han estudiado múltiples poblaciones mediterráneas de herrerillo común y combinado tanto datos de campo como moleculares basados en marcadores neutros y funcionales. Los objetivos específicos de esta tesis son los siguientes:

Capítulo I: Examinar por medio de datos moleculares y de captura-marcaje-recaptura las consecuencias genéticas de la dispersión del herrerillo común a diferentes escalas espaciales. En este capítulo se estudia si los patrones de dispersión difieren entre sexos y en qué medida son reflejados en la estructuración genética poblacional.

Capítulo II: Estudiar el papel que la distancia geográfica, la composición del paisaje, y las condiciones ambientales locales tienen en la diferenciación genética de las poblaciones y analizar en qué medida las inferencias obtenidas difieren entre marcadores neutros y funcionales.

Capítulo III: Identificar los linajes de malaria aviar que infectan al herrerillo común en nuestra zona de estudio y determinar los niveles de prevalencia en las distintas poblaciones analizadas.

Capítulo IV: Analizar si la probabilidad de ser infectado por parásitos de malaria aviar está relacionada con la diversidad genética individual y evaluar en qué medida esta asociación difiere entre poblaciones con

distintos niveles de prevalencia a estos parásitos o cuando es analizada utilizando diferentes tipos de marcadores genéticos (neutros *vs.* funcionales).

Capítulo V: Estudiar la relación entre la probabilidad de supervivencia interanual y la diversidad genética individual estimada mediante marcadores neutros y funcionales y analizar la influencia que las condiciones ambientales (precipitación y temperatura) tienen en la intensidad de selección de la heterocigosidad.

Capítulo VI: Analizar la relación entre heterocigosidad estimada mediante marcadores neutros y funcionales y la expresión de ornamentos sexuales secundarios en machos de herrerillo común.

REFERENCIAS

Acevedo-Whitehouse K, Spraker TR, Lyons E*, et al.* 2006. Contrasting effects of heterozygosity on survival and hookworm resistance in California sea lion pups. *Mol. Ecol.* 15: 1973-1982.

Andersson S, Ornborg J, Andersson M. 1998. Ultraviolet sexual dimorphism and assortative mating in blue tits. *Proc. R. Soc. B-Biol. Sci. 2*65: 445-450.

Annavi G, Newman C, Buesching CD*, et al.* 2014. Heterozygosity-fitness correlations in a wild mammal population: accounting for parental and environmental effects. *Ecol. Evol.* 4: 2594-2609.

Aparicio JM, Cordero PJ, Veiga JP. 2001. A test of the hypothesis of mate choice based on heterozygosity in the spotless starling. *Anim. Behav.* 62: 1001-1006.

Aparicio JM, Ortego J, Cordero PJ. 2006. What should we weigh to estimate heterozygosity, alleles or loci? *Mol. Ecol.* 15: 4659-4665.

Audo MC, Diehl WJ. 1995. Effect of quantity and quality of environmental-stress on multilocus heterozygosity growth relationships in *Eisenia fetid*a (Annelida, Oligochaeta). *Heredity* 75: 98-105.

Balloux F, Amos W, Coulson T. 2004. Does heterozygosity estimate inbreeding in real populations? *Mol. Ecol.* 13: 3021-3031.

Bolund E, Martin K, Kempenaers B, Forstmeier W. 2010. Inbreeding depression of sexually selected traits and attractiveness in the zebra finch. *Anim. Behav.* 79: 947-955.

Bradburd GS, Ralph PL, Coop GM. 2013. Disentangling the effects of geographic and ecological isolation on genetic differentiation. *Evolution* 67: 3258-3273.

Cassinello J, Gomendio M, Roldan ERS. 2001. Relationship between coefficient of inbreeding and parasite burden in endangered gazelles. *Conserv. Biol.* 15: 1171-1174.

Chapman JR, Nakagawa S, Coltman DW, Slate J, Sheldon BC. 2009. A quantitative

review of heterozygosity-fitness correlations in animal populations. *Mol. Ecol.* 18: 2746-2765.

Charlesworth D, Charlesworth B. 1987. Inbreeding depression and its evolutionary consequences. *Annu. Rev. Ecol. Evol. Syst.* 18: 237-268.

Coltman DW, Pilkington JG, Smith JA, Pemberton JM. 1999. Parasite-mediated selection against inbred Soay sheep in a free-living, island population. *Evolution* 53: 1259-1267.

Cramp S, Perrins CM (eds). 1993. *The birds of the Western Palearctic, vol 7*. Oxford: Oxford University Press.

Cristescu R, Cahill V, Sherwin WB, *et al.* 2009. Inbreeding and testicular abnormalities in a bottlenecked population of koalas (*Phascolarctos cinereus*). *Wildlife Res.* 36: 299-308.

Da Silva A, Luikart G, Yoccoz NG, Cohas A, Allaine D. 2006. Genetic diversity-fitness correlation revealed by microsatellite analyses in European alpine marmots (*Marmota marmota*). *Conserv. Genet.* 7:371-382.

David P. 1998. Heterozygosity-fitness correlations: new perspectives on old problems. *Heredity* 80: 531-537.

Davis MB, Shaw RG, Etterson JR. 2005. Evolutionary responses to changing climate. *Ecology* 86: 1704-14.

Fahrig L. 2003 Effects of habitat fragmentation on biodiversity. *Annu. Rev. Ecol. Evol. Syst.* 34: 487-515.

Falconer DS, Mackay TFC. 1996. *Introduction to quantitative genetics*, 3rd edn. Essex: Longman.

Felsenstein J. 1976. The theoretical population genetics of variable selection and migration. *Annu. Rev. Ecol. Evol. Syst.* 10:253-80.

Foley JA, DeFries R, Asner GP, Barford C, Bonan G, Carpenter SR, Chapin FS, Coe MT, Daily GC, Gibbs HK, *et al.* 2005. Global consequences of land use. *Science* 309: 570-574.

Forcada J, Hoffman JI. 2014. Climate change selects for heterozygosity in a declining fur seal population. *Nature* 511: 462-465.

Frankham R. 1996. Relationship of genetic variation to population size in wildlife. *Conserv. Biol.* 10: 1500-1508.

Freedman AH, Thomassen HA, Buermann, W, Smith TB. 2010. Genomic signals of diversification along eco-logical gradients in a tropical lizard. *Mol. Ecol.* 19: 3773-3788.

Gage MJ. 2006. Does reduced heterozygosity depress sperm quality in wild rabbits (*Oryctolagus cuniculus*)? Response to Slate and Pemberton. *Curr. Biol.* 16: R791-R792.

Garnier-Gere P, Dillmann C. 1992. A computer-program for testing pairwise

linkage disequilibria in subdivided populations. *J. Hered.* 83:239-239.

Griffith SC, Owens IPF, Thuman KA (2002) Extra pair paternity in birds: a review of interspecific variation and adaptive function. *Mol. Ecol.* 11: 2195-2212.

Hansson B, Bensch S, Hasselquist D, Akesson M. 2001. Microsatellite diversity predicts recruitment of sibling great reed warblers. *Proc. R. Soc. B-Biol. Sci.* 268: 1287-1291.

Hansson B, Westerberg L. 2002. On the correlation between heterozygosity and fitness in natural populations. *Mol. Ecol.* 11: 2467-2474.

Hoffmann AA, Hercus MJ. 2000. Environmental stress as an evolutionary force. *Bioscience* 50, 217-226.

Hunt S, Bennett ATD, Cuthill IC, Griffiths R. 1998. Blue tits are ultraviolet tits. *Proc. R. Soc. B-Biol. Sci.* 265: 451-455.

Keller LF, Waller DM. 2002. Inbreeding effects in wild populations. *Trends Ecol. Evol.* 17: 230-241.

Kempenaers B. 2007. Mate choice and genetic quality: A review of the heterozygosity theory. *Adv. Study Behav.* 37: 189-278.

Kimura M. 1955. Solution of a process of random genetic drift with a continuous model. *Proc. Natl. Acad. Sci. U.S.A.* 41:144-50.

Kuepper C, Kosztolanyi A, Augustin J*, et al.* 2010. Heterozygosity-fitness correlations of conserved microsatellite markers in Kentish plovers *Charadrius alexandrinus*. *Mol. Ecol.* 19: 5172-5185.

Laine VN, Herczeg G, Shikano T, Primmer CR. 2012. Heterozygosity-behaviour correlations in nine-spined stickleback (*Pungitius pungitius*) populations: contrasting effects at random and functional loci. *Mol. Ecol.* 21: 4872-4884.

Leclaire S, White J, Arnoux E*, et al.* 2011. Integument coloration signals reproductive success, heterozygosity, and antioxidant levels in chick-rearing black-legged kittiwakes. *Naturwissenschaften* 98: 773-782.

Lesbarreres D, Primmer CR, Laurila A, Merila J. 2005. Environmental and population dependency of genetic variability-fitness correlations in *Rana temporaria*. *Mol. Ecol.* 14: 311-323.

Li YC, Korol AB, Fahima T, Nevo E. 2004. Microsatellites within genes: Structure, function, and evolution. *Mol. Biol. Evol.* 21: 991-1007.

Marr AB, Arcese P, Hochachka WM, Reid JM, Keller LF. 2006. Interactive effects of environmental stress and inbreeding on reproductive traits in a wild bird population. *J. Anim. Ecol.* 75: 1406-1415.

Marshall RC, Buchanan KL, Catchpole CK. 2003. Sexual selection and individual genetic diversity in a songbird. *Proc. R. Soc. B-Biol. Sci.* 270: S248-S250.

McRae BH, Beier P. 2007. Circuit theory predicts gene flow in plant and animal populations. *Proc. Natl. Acad. Sci. U.S.A.* 104: 19885-19890.

Muller HJ. 1964. The relation of recombination to mutational advance. *Mutat. Res.* 1: 2-9.

Murphy MA, Dezzani R, Pilliod DS, Storfer A. 2010 Landscape genetics of high mountain frog metapopulations. *Mol. Ecol.* 19: 3634-3649.

Nosil P, Vines TH, Funk DJ. 2005. Perspective: Reproductive isolation caused by natural selection against immigrants from divergent habitats. *Evolution* 59: 705-719

Olano-Marin J, Mueller JC, Kempenaers B.2011a. Correlations between heterozygosity and reproductive success in the blue tit (*Cyanistes caeruleus*): an analysis of inbreeding and single locus effects. *Evolution* 65: 3175-3194.

Olano-Marin J, Mueller JC, Kempenaers B. 2011b. Heterozygosity and survival in blue tits (*Cyanistes caeruleus*): contrasting effects of presumably functional and neutral loci. *Mol. Ecol.* 20: 4028-4041.

Orsini L, Vanoverbeke J, Swillen I, Mergeay J, De Meester L. 2013. Drivers of population genetic differentiation in the wild: isolation by dispersal limitation, isolation by adaptation and isolation by colonization *Mol. Ecol.* 22: 5983-5999.

Ortego J, Calabuig G, Cordero PJ, Aparicio JM. 2007. Egg production and individual genetic diversity in lesser kestrels. *Mol. Ecol.* 16: 2383-2392.

Pemberton J. 2004. Measuring inbreeding depression in the wild: the old ways are the best. *Trends Ecol. Evol.* 19: 613-615.

Pierson JC, Allendorf FW, Saab V, Drapeau P, Schwartz MK. 2010. Do male and female black-backed woodpeckers respond differently to gaps in habitat? *Evol. Appl.* 3: 263-278.

Pflueger FJ, Balkenhol N. 2014. A plea for simultaneously considering matrix quality and local environmental conditions when analysing landscape impacts on effective dispersal. *Mol. Ecol.* 23: 2146-2156.

Porlier M, Garant D, Perret P, Charmantier A. 2012. Habitat-linked population genetic differentiation in the blue tit *Cyanistes caeruleus*. *J. Hered.* 103: 781-791.

Raymond M, Rousset F (1995) GENEPOP (Version-1.2) – Population-genetic software for exact tests and ecumenicism. *J. Hered.* 86: 248-249.

Reid JM. 2007. Secondary sexual ornamentation and non-additive genetic benefits of female mate choice. *Proc. R. Soc. B-Biol. Sci.* 274: 1395-1402.

Richter SC, Nunziata SO. 2014. Survival to metamorphosis is positively related to genetic variability in a critically endangered amphibian species. *Anim. Conserv.* 17: 265-274.

Scott TM, Koehn RK. 1990 The effect of environmental-stress on the relationship of heterozygosity to growth-rate in the coot clam *Mulinia lateralis* (Say). *J. Exp. Mar. Biol. Ecol.* 135: 109-116.

Sexton JP, Hangartner SB, Hoffmann AA. 2014. Genetic isolation by environment or distance: which pattern of gene flow is most common? *Evolution* 68:1-15.

Slatkin M.1993. Isolation by distance in equilibrium and noequilibrium populations. *Evolution* 47: 264-279.

Szulkin M, Bierne N, David P. 2010. Heterozygosity-fitness correlations: a time for reappraisal *Evolution* 64: 1202-1217.

Szulkin M, Garant D, McCleery RH, Sheldon BC. 2007. Inbreeding depression along a life-history continuum in the great tit. *J. Evol. Biol.* 20: 1531-1543.

Tiira K, Laurila A, Peuhkuri N, Piironen J, Aikio S, Ranta E, Primmer CR. 2003. Aggressiveness is associated with genetic diversity in landlocked salmon (*Salmo salar). Mol. Ecol.* 12: 2399-2407.

Tiira, K, Laurila A, Enberg K, Piironen J, Aikio S, Ranta E, Primmer CR. 2006. Do dominants have higher heterozygosity? Social status and genetic variation in brown trout, *Salmo trutta. Behav. Ecol. Sociobiol.* 59: 657-665.

Tsitrone A, Rousset F, David P. 2001. Heterosis, marker mutational processes and population inbreeding history. *Genetics* 159: 1845-1859.

Väli U, Einarsson A, Waits L, Ellegren H. 2008. To what extent do microsatellite markers reflect genome-wide genetic diversity in natural populations? *Mol. Ecol.* 17: 3808-3817.

van Oosterhout C, Trigg RE, Carvalho GR, *et al.* 2003. Inbreeding depression and genetic load of sexually selected traits: how the guppy lost its spots. *J. Evol. Biol.* 16: 273-281.

Voegeli B, Saladin V, Wegmann M, Richner H. 2012. Parasites as mediators of heterozygosity-fitness correlations in the great tit (*Parus major*). *J. Evol. Biol.* 25: 584-590.

Wang IJ. 2012. Environmental and topographic variables shape genetic structure and effective population sizes in the endangered Yosemite toad. *Divers. Distrib.* 18: 1033-1041.

Wang IJ, Bradburd G. 2014. Isolation by environment. *Mol. Ecol.* 23:5649-5662.

Willi Y, Van Buskirk J, Hoffmann AA. 2006. Limits to the adaptive potential of small populations. *Annu. Rev. Ecol. Evol. Syst.* 37: 433-458.

Wright S. 1945. Isolation by distance under diverse systems of mating. *Genetics* 30: 571-572.

1. Genetic structure reflects natal dispersal movements at different spatial scales in the blue tit, *Cyanistes caeruleus*

Ortego J, García-Navas V, Ferrer ES and Sanz JJ

Animal Behaviour 82: 131-137 (2011)

Genetic structure reflects natal dispersal movements at different spatial scales in the blue tit, *Cyanistes caeruleus*

Abstract

The study of the genetic consequences of dispersal is a central topic in evolutionary, conservation and behavioural research. However, few studies have simultaneously considered dispersal movements from marked individuals and contemporary patterns of gene flow. We analysed the link between dispersal behaviour and gene flow in four populations of blue tits with different degrees of connectivity. For this purpose, we monitored four breeding patches and used genotypic and capture-mark-recapture data to study the genetic consequences of dispersal at different spatial scales. Data on natal dispersal movements revealed that both males and females dispersed less than expected under a random pattern of settlement at the two large spatial scales considered: the whole study area and the two main localities. However, natal dispersal distance was lower than expected under random settlement within natal patches in males whereas an opposite pattern was found for females. Accordingly, microsatellite data revealed limited gene flow between the localities studied and an isolation-by-distance pattern of genetic structure that was particularly strong at the large spatial scale (i.e. considering geographically distant breeding patches). Finally, the strong male philopatry was reflected by a stronger genetic structure and a lower admixed ancestry in this sex. Overall, we found evidence that restricted dispersal and fragmentation may have both contributed to reduce interpopulation gene flow at different spatial scales in a forest species and that there is concordance between genetic studies and those based on capture-mark-recapture.

INTRODUCTION

Dispersal is a life-history trait that plays a fundamental role in population dynamics, influencing species distribution and the genetics and structure of populations (Clobert *et al.* 2001). Restricted dispersal can result in increased population fragmentation and genetic differentiation (Martínez-Cruz *et al.* 2007; Coulon *et al.* 2008; Tzika *et al.* 2008). This population subdivision is expected to reduce effective population sizes which can ultimately decrease genetic variability and the ability of populations to respond to selection and adapt to novel environmental conditions (Frankham 1996; Spielman *et al.* 2004; Frankham 2005; Willi *et al.* 2006). On the other hand, restricted dispersal and population fragmentation can also preserve genetic variation from the homogenizing effects of gene flow, favouring the maintenance of local adaptations and promoting intra-specific diversity or speciation (Smith *et al.* 1997; Smith *et al.* 2005; Milá *et al.* 2009). Thus, the study of the genetic consequences of dispersal has important theoretical and

applied implications which can be considered major topics in behavioural, evolutionary and conservation research (Saunders *et al.* 1991; Fahrig 2002).

The advent and application of DNA polymorphic markers has opened up the possibility that researchers can estimate realized gene-flow and infer how individuals move across the landscape (Manel *et al.* 2003). However, few studies have simultaneously considered dispersal movements from marked individuals and contemporary patterns of gene-flow (Riley *et al.* 2006; Boulet *et al.* 2007; Coulon *et al.* 2008; Coulon *et al.* 2010; Alcaide *et al.* 2009). Thus, more empirical information is required to reconcile the patterns of dispersal and genetic structure observed in spatially structured populations (i.e. meta-populations) (Ortego *et al.* 2008; Alcaide *et al.* 2009; Coulon *et al.* 2010). Simultaneous analyses of patterns of dispersal and genetic structure considering contrasting scenarios of human induced or natural population fragmentation and isolation at different spatial scales could help to resolve this problem (Boulet *et al.* 2007; Coulon *et al.* 2008; Coulon *et al.* 2010).

In this study, we analyze the link between dispersal behaviour and gene-flow in four populations of blue tits (*Cyanistes caeruleus*) with different degrees of connectivity. This forest species can be considered mostly sedentary and disperses over short distances which would make it particularly prone to population fragmentation (Matthysen *et al.* 2005; Foerster *et al.* 2006; Valcu & Kempenaers 2008; Table 1). Furthermore, the short generation time of this species (modal life span = 2 years) can favour the detection of genetic shifts (e.g. changes in allele frequency or genetic diversity) at very short spatiotemporal scales (Hailer *et al.* 2006; Ortego *et al.* 2007). In particular, the Mediterranean populations of the blue tit often show a high degree of isolation due to both natural and human induced habitat fragmentation of the deciduous forests optimal for this species (Blanco *et al.* 1997). For these reasons, the Mediterranean populations of blue tits offer an ideal scenario to study the genetic consequences of restricted dispersal and population fragmentation. In this study we monitored four populations (two localities with two patches each) and used detailed capture-mark-recapture information and genotypic data to analyse the genetic consequences of dispersal behaviour at different spatial scales. In particular, we tested the following predictions: 1) because of the small body size, sedentary behaviour and habitat fragmentation we expected (1.1) the study populations of blue tits would show very restricted natal dispersal movements and (1.2) dispersal distances would be particularly short in males, the less dispersive sex in most bird species (Greenwood 1980); 2) we also expected low gene flow and significant genetic differentiation between patches and localities with limited or no dispersal between them (Coulon *et al.* 2008); 3) if males are the less dispersive sex, we predict that this sex would show a more marked genetic structure than females; 4) finally, we

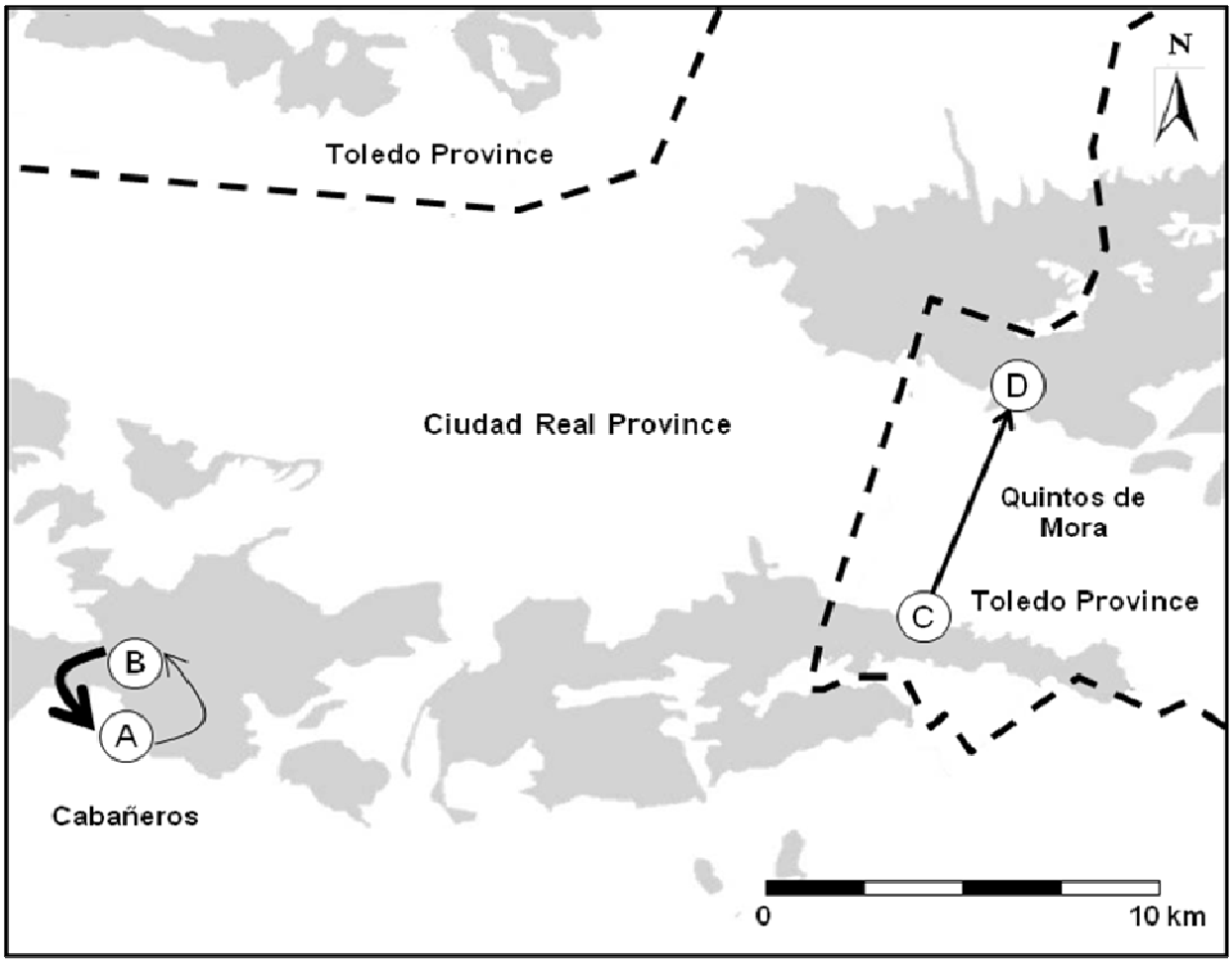


Figure 1 Map of the study area showing the spatial location of the studied blue tit populations at Cabañeros (A: Anchurones; B: Brezoso) and Quintos de Mora (C: Gil García; D: Valdeyernos). Shaded area represents woodlands. Arrows indicate observed dispersal movements and direction. Arrow thickness is proportional to the number of dispersed individuals.

expected that genetic similarity between individuals decreases with geographical distance (i.e. isolation by distance) because of migration-drift equilibrium (Hutchison & Templeton 1999).

MATERIALS AND METHODS

Study areas and population monitoring

We studied blue tits in two localities of central Spain, the Cabañeros National Park (Ciudad Real and Toledo provinces, 39°24´N, 3°35´W) and Quintos de Mora (Toledo province, 39º25´N 4º04´W) (Fig. 1). Both study localities comprise mainly oak-dominated woodlands with Mediterranean scrublands. In each of these two localities we monitored two breeding patches, El Brezoso and Anchurones in Cabañeros National Park and Valdeyernos and Gil García in Quintos de Mora (Fig. 1). Nest-boxes in Cabañeros (Anchurones: 150 nestboxes; El Brezoso: 100 nestboxes) and Quintos de Mora (Gil García: 100 nestboxes; Valdeyernos: 100 nestboxes)

were monitored from April to June during 2007-2009. Basic data on reproduction were obtained by frequent checks of nestboxes from nest building until fledging (Sanz & García-Navas 2009). Adult birds were captured when feeding 8-days-old chicks by means of a spring trap shutting the entrance hole as the bird entered the nest. Birds were sexed by the presence/absence of a brood patch (Sanz & García-Navas 2009). Blood samples (≤ 25 μl) for genetic analyses were obtained by brachial venipuncture and stored in 96 % ethanol. All adults and nestlings were individually marked with aluminium rings for further identification (Sanz & García-Navas 2009; García-Navas *et al.* 2009).

Natal dispersal movements

Natal dispersal distance was calculated as the Euclidean distance from the natal to the first-breeding nest-box (Greenwood & Harvey 1982). We analyzed differences between sexes in observed natal dispersal distances using a Generalized Linear Mixed Model (GLMM) implemented with the GLIMMIX macro of SAS (SAS Institute 2004). Dispersal distance was analyzed using a normal error structure and identity link function. Cohort, locality identity (Cabañeros, Quintos de Mora), breeding patch identity (Anchurones, Brezoso, Gil García or Valdeyernos) nested within locality and brood identity nested within breeding patch were included as random effects.

We also used Monte Carlo simulations to evaluate the possibility that observed natal dispersal patterns have occurred by chance and are merely constrained by the spatial distribution of breeding sites (Manly 1991; Serrano *et al.* 2008). For this purpose, we considered three different null models: 1) that individuals settle randomly in any nestbox located within the whole study area; 2) that individuals settle randomly in any nestbox located within their natal locality (i.e. Cabañeros or Quintos de Mora); 3) that

Table 1 Natal dispersal distances (m) recorded for different blue tit populations. *N*, sample size. *Sample size is reported for both sexes combined

Population	Sex	Mean/S.E.	Median	*N*	Reference
France	Males	463 / -	-	35 *	Parejo *et al.* 2007
France	Females	1356 / -	-	35 *	Parejo *et al.* 2007
Austria	Combined	328 / -	-	65	Foerster *et al.* 2006
Belgium	Males	- / -	256	70 *	Matthysen *et al.* 2005
Belgium	Females	- / -	936	70 *	Matthysen *et al.* 2005
Germany	Males	- / -	365	218 *	Winkel & Frantzen 1991
Germany	Females	- / -	700	218 *	Winkel & Frantzen 1991
Spain	Males	331 / 32	250	77	Present study
Spain	Females	649 / 112	442	89	Present study

individuals settle randomly in any nestbox located within their natal patch (i.e. Anchurones, Brezoso, Gil García or Valdeyernos). We performed three different simulations randomly assigning each individual to a nestbox and only constraining its settlement according with any of the three different null models considered above. Then we calculated the distance between the natal nestbox for each individual and the randomly assigned nestbox to generate the expected frequency distribution of dispersal distances (i.e. the null model; e.g. Serrano *et al.* 2008). Our study sites contain a high number of nest-boxes and many of them (~ 40-50 %) remain unoccupied every year (see Valcu & Kempenaers 2008). Thus, the three null models considered that natal dispersal decisions are not constrained by intra-specific competition or nest site availability (e.g. Serrano *et al.* 2008). Simulations of the three null models were repeated 1000 times to obtain the expected frequency distribution of median dispersal distances under the different scenarios. The expected distribution of median dispersal distances was compared with the observed median dispersal distance. Tests of significance were generated by counting the number of randomized cases that resulted in an equal or larger/smaller value than the observed median dispersal distance and dividing by the total number of randomizations (Manly 1991). Natal dispersal distances are larger in females than in males in the populations studied and so data were analysed separately for each sex (see below).

Microsatellite genotyping

We genotyped 212 breeding individuals (Anchurones: n = 33; Brezoso: n = 74; Gil García: n = 54; Valdeyernos: n = 51) across 14 polymorphic microsatellite markers (García-Navas *et al.* 2009). We used NucleoSpin Tissue Kits (Machery-Nagel) to extract and purify genomic DNA from the blood samples. Approximately 5 ng of template DNA was amplified in 10-μL reaction volumes containing 1X reaction buffer (67 mM Tris-HCL, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01 % Tween-20, EcoStart Reaction Buffer, Ecogen, Barcelona, Spain), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each dye-labelled primer (FAM, PET, VIC or NED) and 0.1 U of Taq DNA EcoStart Polymerase (Ecogen). All reactions were carried out on a Mastercycler EpgradientS (Eppendorf) thermal cycler. The PCR programme used was 9 min denaturing at 95 ºC followed by 35 cycles of 30 s at 94 ºC, 45 s at the annealing temperature (García-Navas *et al.* 2009) and 45 s at 72 ºC, ending with a 5 min final elongation stage at 72 ºC. Amplification products were electrophoresed using an ABI 310 Genetic Analyzer (Applied Biosystems) and genotypes were scored using GENEMAPPER 3.7 (Applied Biosystems). Microsatellite genotypes were tested for departure from Hardy-Weinberg equilibrium (HWE) at each locus using an exact test (Guo & Thompson 1992) based on 900 000 Markov chain iterations as implemented in the program ARLEQUIN 3.1 (Excoffier *et al.* 2005). We also used ARLEQUIN 3.1 to test for linkage

equilibrium within each pair of loci using a likelihood-ratio statistic, whose distribution was obtained by a permutation procedure (Excoffier *et al.* 2005).

Spatial genetic structure

We investigated genetic structure among the studied blue tit localities and patches. For this purpose we calculated pairwise fixation indexes (F_{ST}) between the different localities and patches and tested their significance with Fisher's exact tests after 10 000 permutations as implemented in ARLEQUIN 3.1 (Excoffier *et al.* 2005). We also analyzed the spatial genetic structure using an individual-based approach as implemented in the program STRUCTURE version 2.3.3 (Pritchard *et al.* 2000; Falush *et al.* 2003; Hubisz *et al.* 2009). This program is a Bayesian model-based clustering method which assigns individuals to populations based on their multilocus genotypes (Pritchard *et al.* 2000; Falush *et al.* 2003). For *K* population clusters, the program estimates the probability of the data [Pr(X|*K*)] and the probability of individual membership in each cluster using a Markov chain Monte Carlo (MCMC) method. We ran the program assuming correlated allele frequencies and admixture (Pritchard *et al.* 2000; Falush *et al.* 2003) and using "prior population information" option to take sampling locations into account (Hubisz *et al.* 2009). We conducted five independent runs for each value of *K* to estimate the true number of clusters with 10^6 MCMC cycles, following a burn-in period of 100 000 iterations. The simulated values of *K* ranged from 1-4. The number of populations best fitting the data set (*X*) was defined using log probabilities [Pr(X|*K*)] and ΔK, as described in (Evanno *et al.* 2005). Thus, the estimated number of subpopulations is taken to be the value of *K* at which Pr(X|*K*) plateaus. All analyses on spatial genetic structure were performed for all sampled individuals together and considering male and female genotypes separately. The deviation from a pure genotype (admixed ancestry) was calculated for each individual using the assignment scores provided by STRUCTURE analyses (e.g. Ortego & Bonal 2010). Then, we used a one-way ANOVA to analyze differences between sexes in the degree of admixed ancestry.

Isolation by distance

We explored the occurrence of an isolation-by-distance pattern of spatial genetic structure comparing pair-wise relatedness and Euclidean geographical distances between individuals. We used the software MARK (K. Ritland; www.genetics.forestry.ubc.ca/ritland/programs.html) to calculate pairwise relatedness values for all individuals using Queller & Goodnight's estimator. We also calculated the pairwise geographic distance between all individuals using an EXCEL spreadsheet. Finally, we used Mantel tests to analyze the association between distance matrixes using ZT software (Bonnet & Van de Peer 2002). The significance of Mantel test was assessed by 10 000 randomizations of the genetic

distance matrix. The test was one-tailed as only a positive correlation between geographical and genetic distances is expected (e.g. Gómez *et al.* 2007; Ortego *et al.* 2010). This approach was used to analyze all sampled individuals and only considering male-male and female-female comparisons in the whole study area and for Cabañeros and Quintos de Mora localities separately.

Ethical note

The study was done under licence from the Spanish institutional authorities (Environmental Agency of the Community of Castilla-La Mancha and the Ringing Office of the Ministry of Environment). These institutions provided permits for capturing and ringing blue tits and all these activities were performed following general ethical guidelines for animal welfare and nature conservation. All the breeding adults were captured with spring traps when the chicks were 8 days old (day of hatching = 0). Chicks were banded on day 13 after hatching. The blood-sampling procedure did not have any obvious adverse effects on the birds and our trapping and handling did not cause any harm, nest desertion or any other damage to the individuals or their nests.

RESULTS

Natal dispersal movements

We recaptured 166 individuals that were ringed as nestlings, 62 individuals at Cabañeros (Anchurones: 10 males, 10 females; El Brezoso: 21 males, 21 females) and 104 individuals at Quintos de Mora (Gil García: 27 males, 25 females; Valdeyernos: 19 males, 33 females). We did not record any movement between localities (Cabañeros-Quintos de Mora). However, we recorded some movements between close patches: from Gil García to Valdeyernos (2 females), from Anchurones to El Brezoso (1 male), from El Brezoso to Anchurones (2 males, 5 females) (Fig. 1). No individual moved from Valdeyernos to Gil García. After controlling for random effects, natal dispersal distances were significantly lower in males than in females (GLMM: $F_{1, 164} = 6.57$; $P = 0.011$) (Table 1).

Monte Carlo simulations revealed that both male and female median dispersal distances were lower than expected under random dispersal assuming that individuals can potentially settle in any nestbox located in the whole study area ($P < 0.001$; Fig. 2a). Similarly, median dispersal distances in both males and females were lower than expected under random dispersal assuming that individuals can potentially settle in any nestbox located within their natal locality (i.e. Cabañeros or Quintos de Mora) ($P < 0.001$; Fig. 2b). However, males dispersed less than expected under random dispersal within their natal patch (i.e. Anchurones, Brezoso, Gil García or Valdeyernos) ($P =$

0.049; Fig. 2c) but an opposite pattern was found for females (P = 0.001; Fig. 2c).

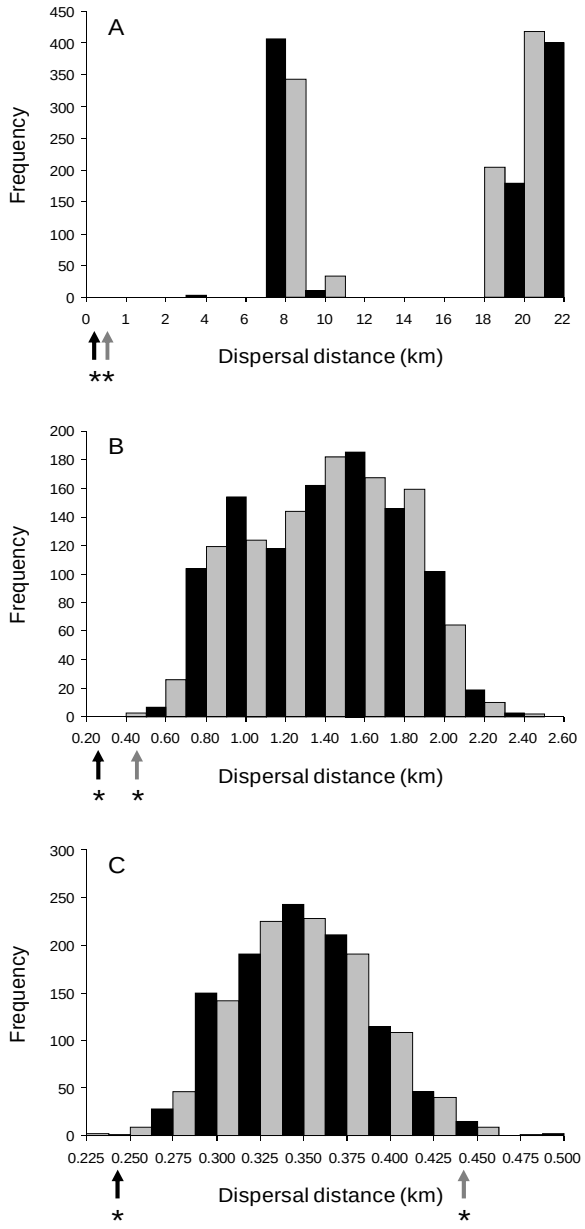


Figure 2 Frequency distribution of median dispersal distances expected for male (black bars) and female (grey bars) blue tits assuming different scenarios of random dispersal: (A) that individuals settle in any nestbox located in the whole study area; (B) that individuals settle in any nestbox located within their natal area (Cabañeros or Quintos de Mora); (C) that individuals settle in any nestbox located within their natal patch (Anchurones, Brezoso, Gil García or Valdeyernos). Arrows indicate the observed value for each sex (male: black arrow; female: grey arrow). *: $P < 0.05$.

Spatial genetic structure

On average, individuals were successfully typed at 13.4 ± 1.3 (S.E.) microsatellite loci. After applying sequential Bonferroni corrections to compensate for multiple statistical tests, we found that no locus deviated from HWE. There was no evidence of genotypic linkage disequilibrium at any pair of loci (all $P >$ 0.05). Pairwise F_{ST} values revealed a significant genetic differentiation between Cabañeros and Quintos de Mora localities (F_{ST} = 0.003; P = 0.006). This pattern was mainly driven by a marked genetic structure in males (F_{ST} = 0.011; $P < 0.001$) that was not significant for females (F_{ST} = 0.001; P = 0.201). Pooling male and female genotypes, we also found significant pair-wise F_{ST} values between different patches located in the two main study areas (Table 2). When we considered each sex separately, we found significant F_{ST} values between some patches located in different localities in males but never in females (Table 2).

STRUCTURE analyses considering all individuals revealed a maximum Pr(X|K) for K = 2 (Fig. 3a, 4a), indicating the presence of two genetic clusters in the study area. Genetic differentiation was particularly strong between El Brezoso and either of the two patches located in Quintos de Mora locality, although all the analyses revealed high levels of genetic admixture (Fig. 4a). Analyses considering only male genotypes also revealed a maximum Pr(X|K) for K = 2, with a similar pattern of genetic structure to that obtained in the global analyses

Table 2 Pairwise population F_{ST} values between sampling patches considering all individuals (below the diagonal) and male and female genotypes separately (above the diagonal, first male and then female values). Values in bold are statistically significant after sequential Bonferroni correction (α = 0.05).

	Anchurones	Brezoso	Gil García	Valdeyernos
Anchurones	-	0.001/0.005	0.007/0.007	0.004/0.007
Brezoso	0.005	-	**0.014**/0.003	**0.014**/-0.002
Gil García	**0.008**	**0.005**	-	0.002/0.002
Valdeyernos	0.006	0.004	0.004	-

including all individuals (Fig. 3b, 4b). However, analyses for females revealed a maximum Pr(X|*K*) for *K* = 1, indicating no genetic subdivision for this sex (Fig. 3c, 4c). All these patterns were consistent over different runs for the same *K* (Fig. 3). The deviation from pure genotypes was significantly lower in males, suggesting a higher admixed ancestry in females (one-way ANOVA: $F_{1, 210}$ = 1241.54; *P* < 0.001). Overall, these analyses show the presence of two genetic clusters for males but indicate a complete lack of genetic structure in females.

Isolation by distance

Isolation-by-distance analyses revealed a significant negative correlation between genetic relatedness and geographical distances considering all individuals (*r* = -0.0556; *P* < 0.001) or analyzing male (*r* = -0.112; *P* < 0.001) and female (*r* = -0.037; *P* = 0.002) genotypes separately. Considering only data from Cabañeros locality, we found no significant correlation between genetic and geographic distances considering all individuals (*r* = 0.024; *P* = 0.962) or analyzing male (*r* = 0.003; *P* = 0.543) and female (*r* = -0.029; *P* = 0.119) genotypes separately. In Quintos de Mora locality we found a marginally significant negative correlation between genetic relatedness and geographic distances (*r* = -0.018; *P* = 0.087) which was mainly driven by male genotypes (*r* = -0.068; *P* = 0.009). By contrast, we found no correlation between genetic relatedness and geographic distances when only female genotypes were considered (*r* = 0.024; *P* = 0.829).

DISCUSSION

We found evidence that restricted dispersal contributes to reducing interpopulation gene flow at different spatial scales. Our results revealed that males and females disperse shorter distances than expected from null models of random dispersal within both the whole study area and the studied localities (Quintos de Mora and Cabañeros). However, natal dispersal distance was lower than expected under random settlement within natal patches in

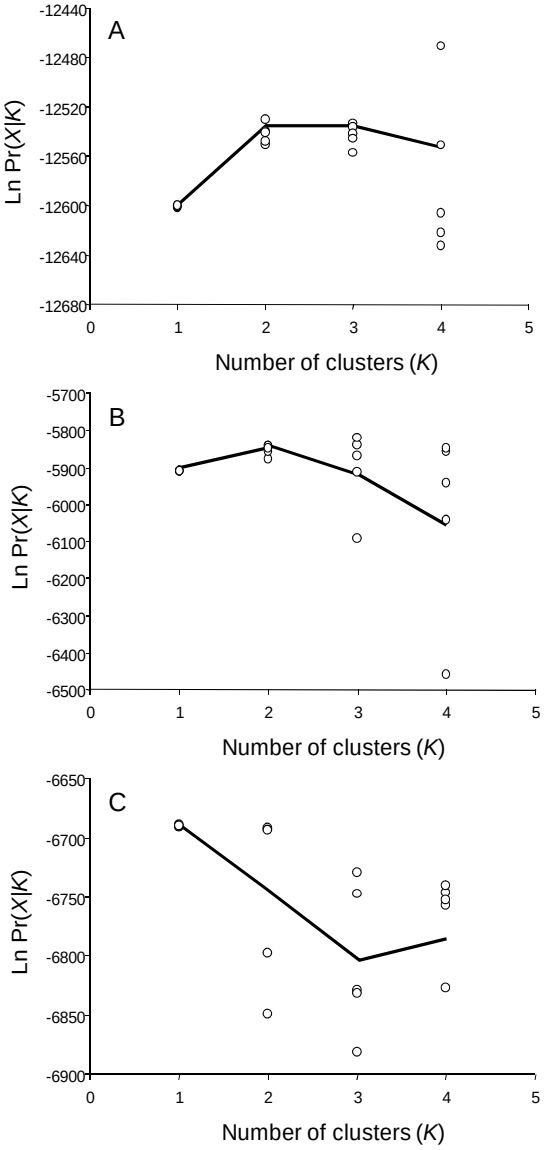


Figure 3 Results of Bayesian clustering analyses considering (A) all individuals and (B) male and (C) female genotypes separately. For each number of population clusters tested (*K*), Pr(X | *K*) is the probability of the data.

males, whereas an opposite pattern was found for females. These results suggest that behavioural/physical barriers to dispersal could differ between the sexes and the different spatial scales analysed (Pierson *et al.* 2010). In males, behavioural constraints are probably the main factor limiting movements within natal patches where suitable habitat shows a continuous distribution in the absence of any physical barrier to dispersal (Greenwood 1980; see below). By contrast, habitat fragmentation could reduce dispersal between habitat patches in females even if this sex is behaviourally predisposed to, or physiologically capable of, dispersing over large distances in continuous habitats (Harris & Reed 2002). As suggested for other forest passerines, females may be reluctant to enter inadequate habitats (agricultural land, scrublands, cleared forest or grassland) owing to increased predation risk and this could result in a fragmentation derived behavioural inhibition of gene flow (Harris & Reed 2002). Results on dispersal patterns suggest that habitat fragmentation would have a lower impact on male-mediated gene flow because male dispersal is limited even within natal patches showing a continuous distribution of suitable habitats (Pierson *et al.* 2010). In any case, it should be considered that the consequences of habitat fragmentation on male gene-flow are expected to be very dependent on the size of the patch under consideration and smaller patches than those studied here may result in decreased male dispersal rates.

Information on gene-flow inferred from genotypic data is mainly concordant with the observed dispersal patterns although such parallelism depends on the spatial scale analyzed (Coulon *et al.* 2008; Alcaide *et al.* 2009). Absence of dispersal between localities (Cabañeros, Quintos de Mora; Fig. 1) is reflected by highly significant F_{ST} values and global individual-based clustering analyses. However, although dispersal events were rarely

observed between patches within Quintos de Mora, there was a complete absence of genetic structure within this locality, whether considering F_{ST} values or individual-based clustering analyses (Fig. 4). We also found a globally significant correlation between genetic and geographical distances, indicating an isolation-by-distance pattern of genetic structure (Wright 1943; Slatkin 1993; Hutchison & Templeton 1999). The high admixture levels revealed by STRUCTURE analyses suggest that the global pattern of isolation by distance may be compatible with a migration-drift equilibrium scenario, that is, restricted dispersal from nearby populations may have resulted in a significant correlation between genetic and geographical distances (Hutchison & Templeton 1999; Coulon *et al.* 2008). Alternatively, the observed pattern could have been generated through serial colonization from geographically close populations and subsequent genetic drift as a consequence of persistent founder effects (Ramachandran *et al.* 2005; Mills *et al.* 2007; Ortego *et al.* 2010). Analyses of isolation-by-distance within the two localities showed a significant correlation between genetic and geographical distances only in Quintos de Mora. In this locality only 1.92% of dispersal events observed involved movements between patches, suggesting that restricted dispersal could have

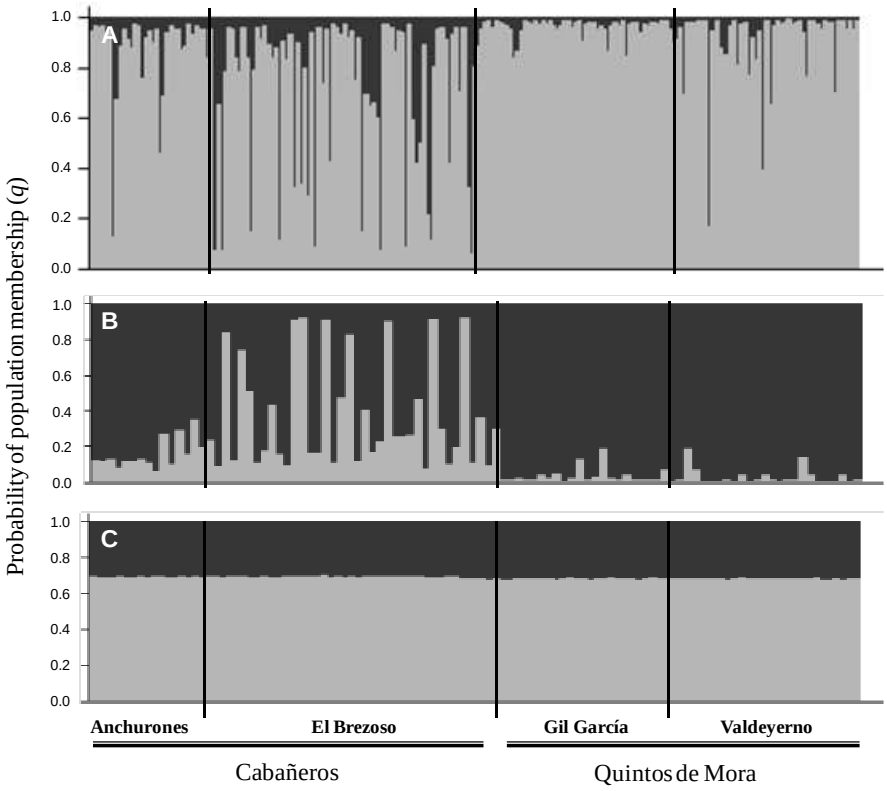


Figure 4 Results of genetic assignment based on Bayesian method implemented in the program STRUCTURE considering (A) all individuals and (B) male and (C) female genotypes separately. Each individual is represented by a thin vertical line, which is partitioned into two coloured segments that represent the individual's probability of belonging to the cluster with that colour.

.

resulted in the observed isolation-by-distance pattern of genetic structure (Hutchison & Templeton 1999; Coulon *et al.* 2008) which is not reflected by F_{ST} values or individual-based clustering analyses (Van de Casteele *et al.* 2003; Ortego *et al.* 2008). Thus, the blue tit population at this locality shows an 'isolation-by-distance' model rather than an 'island' model of genetic structure (Van de Casteele & Matthysen 2006). By contrast, the absence of correlation between genetic and geographical distances within Cabañeros locality could be the result of the homogenising effects of migration caused by the relatively frequent dispersal events (12.9 %) observed between the two patches studied within this locality (Hutchison & Templeton 1999).

Capture-mark-recapture results showed female-biased natal dispersal in the blue tit populations. As suggested for most bird species, the strong male philopatry is probably related to the greater role in territory and resource defence of this sex which may particularly benefit from being in a familiar locality and settling in natal areas (Greenwood 1980). This female-biased natal dispersal is partially reflected by the observed patterns of genetic structure and gene flow. Despite lower sample sizes in males, some pair-wise F_{ST} values were significant in males but none in females (Table 2). Similarly, individual-based clustering analyses also showed a higher admixed ancestry in females and a significant genetic subdivision between Quintos de Mora and Cabañeros in males that is completely absent in females (Fig. 3, 4). Finally, males showed a significant isolation by distance pattern of genetic structure within Quintos de Mora locality, which was not present when only female genotypes were considered. Thus, the genotypic results also showed that the observed male sex-biased dispersal is also reflected by stronger genetic structure in the most dispersive sex (Pierson *et al.* 2010).

Overall, this study shows that there is concordance between dispersal and the observed patterns of genetic structure, indicating that results from capture-mark-recapture studies can provide useful information on the contemporary levels of gene flow present in natural populations (Coulon *et al.* 2008; Pierson *et al.* 2010). Future landscape genetic approaches would futher contribute to identifying the barriers limiting gene flow in this and other organisms inhabiting highly fragmented landscapes (Smith *et al.* 1997, 2005; Cushman *et al.* 2006)

ACKNOWLEDGEMENTS

We wish to thank to Pedro J. Cordero (Instituto de Investigación en Recursos Cinegéticos, IREC) for allowing us to carry out the genetic analyses in his lab. Two anonymous referees provided useful discussion and valuable comments on an earlier draft of this manuscript. We thank board of the Cabañeros National Park and Centro Quintos de Mora for the facilities offered during the fieldwork. During this work VGN and JO were supported by

respective pre-doctoral FPI (MEC) and postdoctoral JAE-Doc (CSIC) fellowships. This study was funded by the grants: 69/2003 (Organismo Autónomo de Parques Nacionales-Ministerio de Medio Ambiente), GCL2007-61395 (Ministerio de Educación y Ciencia), and POIC10-0269-632 (Junta de Comunidades de Castilla-La Mancha-European Social Fund).

REFERENCES

Alcaide M, Serrano D, Tella JL, Negro JJ. 2009. Strong philopatry derived from capture-recapture records does not lead to fine-scale genetic differentiation in lesser kestrels. *J. Anim. Ecol.* 78: 468-475.

Blanco E, Casado M A, Costa-Tenorio M, Escribano R, García M, Génova M, Gómez A, Gómez F, Moreno JC, Morla C, Regato P, Saínz H. 1997. *Los bosques ibéricos. Una interpretación geobotánica.* Barcelona: Planeta.

Bonnet E, Van de Peer Y. 2002. ZT: a software tool for simple and partial Mantel tests. *J. Stat. Soft.* 10: 1-12.

Boulet M, Couturier S, Côté SD, Otto RD, Bernatchez L. 2007. Integrative use of spatial, genetic, and demographic analyses for investigating genetic connectivity between migratory, montane, and sedentary caribou herds. *Mol. Ecol.* 16: 4223-4240.

Clobert J, Danchin E, Dhondt AA, Nichols JD. 2001. *Dispersal.* Oxford: Oxford University Press.

Coulon A, Fitzpatrick JW, Bowman R, Stith B M, Makarewich C A, Stenzler L M, Lovette IJ. 2008. Congruent population structure inferred from dispersal behaviour and intensive genetic surveys of the threatened Florida scrub-jay (*Aphelocoma coerulescens*). *Mol. Ecol.* 17: 1685-1701.

Coulon A, Fitzpatrick JW, Bowman R, Lovette IJ. 2010. Effects of habitat fragmentation on effective dispersal of Florida scrub-jays. *Conserv. Biol.* 24: 1080-1088.

Cushman SA, McKelvey KS, Hayden J, Schwartz MK. 2006. Gene flow in complex landscapes: Testing multiple hypotheses with causal modeling. *Am. Nat.* 168: 486-499.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol. Ecol.* 14: 2611-2620.

Excoffier L, Laval G, Schneider S. 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evol. Bioinf.* 1: 47-50.

Falush D, Stephens M, Pritchard JK. 2003. Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. *Genetics* 164: 1567-1587.

Foerster K, Valcu M, Johnsen A, Kempenaers B. 2006. A spatial genetic structure and effects of relatedness on mate choice in a wild bird population. *Mol. Ecol.* 15: 4555-4567.

Frankham R. 1996. Relationship of genetic variation to population size in wildlife. *Conserv. Biol.* 10: 1500-1508.

Frankham R. 2005. Genetics and extinction. *Biol. Conserv.* 126: 131-140.

García-Navas, V, Ortego J, Sanz, JJ. 2009. Heterozygosity-based assortative mating in blue tits (*Cyanistes caeruleus*): implications for the evolution of mate choice. *Proc. R. Soc. B-Biol. Sci.* 276: 2931-2940.

Gómez A, Montero-Pau J, Lunt DH, Serra M, Campillo S. 2007. Persistent genetic signatures of colonization in *Brachionus manjavacas* rotifers in the Iberian Peninsula. *Mol. Ecol.* 16: 3228-3240.

Greenwood PJ. 1980. Mating systems, philopatry and dispersal in birds and mammals. *Anim. Behav.* 28: 1140-1162.

Greenwood, PJ, Harvey, PH. 1982. The natal and breeding dispersal of birds. *Annu. Rev. Ecol. Evol. Syst.* 13: 1-21.

Guo SW, Thompson EA. 1992. A monte-carlo method for combined segregation and linkage analysis. *Am. J. Hum. Genet.* 51: 1111-1126.

Hailer F, Helander B, Folkestad AO, Ganusevich SA, Garstad S, Hauff P, Koren C, Nygard T, Volke V, Vilà C, Ellegren H. 2006. Bottlenecked but long-lived: high genetic diversity retained in white-tailed eagles upon recovery from population decline. *Biol. Lett.* 2: 316-319.

Harris RJ , Reed JM. 2002. Behavioral barriers to non-migratory movements of birds. *Ann. Zool. Fenn.* 39: 275-290.

Hubisz MJ, Falush D, Stephens M, Pritchard, JK. 2009. Inferring weak population structure with the assistance of sample group information. *Mol. Ecol. Resour.* 9: 1322-1332.

Hutchison DW, Templeton AR. 1999. Correlation of pairwise genetic and geographic distance measures: Inferring the relative influences of gene flow and drift on the distribution of genetic variability. *Evolution* 53: 1898-1914.

Manel S, Schwartz MK, Luikart G, Taberlet P. 2003. Landscape genetics: combining landscape ecology and population genetics. *Trends Ecol. Evol.* 18: 189-197.

Manly BFJ. 1991. *Randomization and Monte Carlo methods in biology*. New York: Chapman & Hall.

Martínez-Cruz B, Godoy JA, Negro JJ. 2007. Population fragmentation leads to spatial and temporal genetic structure in the endangered Spanish imperial eagle. *Mol. Ecol.* 16: 477-486.

Matthysen E, Van de Casteele T, Adriaensen F. 2005. Do sibling tits (*Parus major*, *P. caeruleus*) disperse over similar distances and in similar directions? *Oecologia* 143: 301-307.

Milá B, Wayne RK, Fitze P, Smith TB. 2009. Divergence with gene flow and fine-scale phylogeographical structure in the wedge-billed woodcreeper, *Glyphorynchus spirurus*, a Neotropical rainforest bird. *Mol. Ecol.* 18: 2979-2995.

Mills S, Lunt DH , Gómez A. 2007. Global isolation by distance despite strong regional phylogeography in a small metazoan. *BMC Evol. Biol.* 7: 10.

Ortego J, Bonal R. 2010. Natural hybridisation between kermes (*Quercus coccifera* L.) and holm oaks (*Q. ilex* L.) revealed by microsatellite markers. *Plant Biol.* 12: 234-238.

Ortego J, Aparicio JM, Calabuig G, Cordero PJ. 2007. Increase of heterozygosity in a growing population of lesser kestrels. *Biol. Lett.* 3: 585-588.

Ortego J, Calabuig G, Aparicio JM, Cordero PJ. 2008. Genetic consequences of natal dispersal in the colonial lesser kestrel. *Mol. Ecol.* 17: 2051-2059.

Ortego J, Aguirre MP, Cordero PJ. 2010. Population genetics of *Mioscirtus wagneri*, a grasshopper showing a highly fragmented distribution. *Mol. Ecol.* 19: 472-483.

Parejo D, White J, Clobert J, Dreiss A, Danchin E. 2007. Blue tits use fledgling quantity and quality as public information in breeding site choice. *Ecology* 88: 2373-2382.

Pierson JC, Allendorf FW, Saab V, Drapeau P, Schwartz MK. 2010. Do male and female black-backed woodpeckers respond differently to gaps in habitat? *Evol. Appl.* 3: 263-278.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155: 945-959.

Queller DC, Goodnight KF. 1989. Estimating relatedness using genetic-markers. *Evolution* 43: 258-275.

Ramachandran S, Deshpande O, Roseman CC, Rosenberg NA, Feldman MW, Cavalli-Sforza LL. 2005. Support from the relationship of genetic and geographic distance in human populations for a serial founder effect originating in Africa. *Proc. Nat. Acad. Sci. U.S.A.* 102: 15942-15947.

Riley SPD, Pollinger JP, Sauvajot RM, York EC, Bromley C, Fuller TK, Wayne RK. 2006. A southern California freeway is a physical and social barrier to gene flow in carnivores. *Mol. Ecol.* 15: 1733-1741.

Sanz JJ, García-Navas V. 2009. Eggshell pigmentation pattern in relation to breeding performance of blue tits *Cyanistes caeruleus*. *J. Anim. Ecol.* 78: 31-41.

SAS Institute. 2004. *SAS/STAT 9.1 user's guide*. Cary, NC: SAS Institute Inc.

Serrano D, Carrete M, Tella JL. 2008. Describing dispersal under habitat constraints: A randomization approach in lesser kestrels. *Basic Appl. Ecol.* 9: 771-778.

Slatkin M. 1993. Isolation by distance in equilibrium and nonequilibrium populations. *Evolution* 47: 264-279.

Smith TB, Wayne RK, Girman DJ, Bruford MW. 1997. A role for ecotones in generating rainforest biodiversity. *Science* 276: 1855-1857.

Smith TB, Calsbeek R, Wayne RK, Holder, KH, Pires D, Bardeleben C. 2005. Testing alternative mechanisms of evolutionary divergence in an African rain forest passerine bird. *J. Evol. Biol.* 18: 257-268.

Spielman D, Brook BW, Frankham R. 2004. Most species are not driven to extinction before genetic factors impact them. *Proc. Nat. Acad. Sci. U.S.A.* 101: 15261-15264.

Tzika AC, Rosa SFP, Fabiani A, Snell HL, Snell HM, Marquez C, Tapia W, Rassmann K, Gentile G, Milinkovitch MC. 2008. Population genetics of Galapagos land iguana (genus *Conolophus*) remnant populations. *Mol. Ecol.* 17: 4943-4952.

Valcu M, Kempenaers B. 2008. Causes and consequences of breeding dispersal and divorce in a blue tit, *Cyanistes caeruleus*, population. *Anim. Behav.* 75: 1949-1963.

Van de Casteele T, Galbusera P, Schenck T, Matthysen E. 2003. Seasonal and lifetime reproductive consequences of inbreeding in the great tit *Parus major*. *Behav. Ecol.* 14: 165-174.

Van de Casteele T, Matthysen E. 2006. Natal dispersal and parental escorting predict relatedness between mates in a passerine bird. *Mol. Ecol.* 15: 2557-2565.

Willi Y, Van Buskirk J, Hoffmann AA. 2006. Limits to the adaptive potential of small populations. *Annu. Rev. Ecol. Evol. Syst.* 37: 433-458.

Winkel W, Frantzen M. 1991. Population-dynamics of the blue tit (*Parus caeruleus*) - Long-term investigations in the Braunschweig region. *J. Ornithol.* 132: 81-96.

Wright S. 1943. Isolation by distance. *Genetics* 28: 139-156.

2. The influence of landscape configuration and environment on population genetic structure in a sedentary passerine: insights from putatively functional and neutral loci

Ferrer ES, García-Navas V, Bueno-Enciso J, Barrientos R, Serrano-Davies E, Cáliz-Campal C, Sanz JJ and Ortego J

Under review

The influence of landscape configuration and environment on population genetic structure in a sedentary passerine: insights from putatively functional and neutral loci

Abstract

The study of the factors structuring genetic variation can help to infer the neutral and adaptive processes shaping the demographic and evolutionary trajectories of natural populations. Here, we analyse the role of isolation-by distance (IBD), isolation-by-resistance (IBR, defined by landscape composition), and isolation by environment (IBE, estimated as habitat and elevation dissimilarity) in structuring genetic variation of 25 populations of blue tit (*Cyanistes caeruleus*). We typed 1385 individuals at two subsets of loci, classified as putatively neutral (14 loci) or functional (12 loci) by considering whether the genomic region where they are located is being actively transcribed to RNA. Population genetic differentiation was mostly detected using the panel of neutral markers. Landscape genetic analyses showed a pattern of IBD for all loci and the panel of neutral markers, but genetic differentiation estimated at functional loci was only explained by IBR models considering high resistance for natural vegetation and low resistance for agricultural lands. Finally, the absence for IBE suggests a lack of divergent selection pressures associated with differences in habitat and elevation. Overall, our study shows that markers located in different regions of the genome can yield contrasting inferences on landscape-level patterns of realized gene flow in natural populations.

INTRODUCTION

The study of the factors structuring genetic variation can help to infer the neutral and adaptive processes shaping the demographic and evolutionary trajectories of natural populations (Nosil *et al.* 2005; Freedman *et al.* 2010). Genetic differentiation between populations has been often found to increase with geographical distance due to migration-genetic drift equilibrium in organisms with restricted dispersal (i.e., isolation-by-distance, IBD; Wright 1945; Slatkin 1993; Hutchison & Templeton 1999).

However, landscape configuration can also have profound effects on gene flow and explain patterns of genetic differentiation among populations better than Euclidean geographical distances. This has led to the emerging concept of isolation-by-resistance (IBR) (McRae 2006; McRae & Beier 2007). IBR is defined as the correlation between genetic differentiation and resistance distances that summarize information on the probability an individual disperses from one population to the other across all potential paths weighted by the "friction" or "resistance" to movement offered by discrete (e.g., roads, rivers; Coulon *et al.*

2006; Quéméré *et al.* 2010) or continuous landscape elements (e.g., habitat suitability; He *et al.* 2013; Seymour *et al.* 2013; Ortego *et al.* 2015). The growing interest to understand the influence of landscape composition on the genetic structure of populations has led to the development of the field of landscape genetics, which has successfully integrated molecular tools, geographical information systems and different analytical techniques to identify the specific landscape features favouring or impeding gene flow between populations (Manel *et al.* 2003; Storfer *et al.* 2007; Storfer *et al.* 2010). Landscape genetics has been proven a valuable approach to predict the responses of organisms to habitat alteration and infer unexpected migration pathways for species with cryptic life-history traits or for which dispersal movements are difficult to track due to different technical or logistic reasons (e.g., Wang *et al.* 2009; Jha & Kremen 2013).

Geographical distances, landscape composition, and the dispersal potential of organisms are not the only factors behind the emergence of population genetic structure (Wang *et al.* 2013; Wang & Bradburg 2014). Dispersal is a complex process and immigrant individuals arriving to new habitat patches also have to cope with new environmental conditions, achieve a territory, and mate and reproduce in an unfamiliar place to which they may not be well adapted (Wheelwright & Mauck 1998; Hansson *et al.* 2004; Nosil *et al.* 2005; Postma & van Noordwijk 2005; Garant *et al.* 2007). As a result, natural and sexual selection against immigrants is expected to reduce rates of realized dispersal and gene flow among populations experiencing contrasting environmental or socioecological conditions (Shafer & Wolf 2013; Sexton *et al.* 2014; Wang & Bradburg 2014). Accordingly, different studies have found remarkable genetic differentiation between nearby populations established in different habitat types (Andrew *et al.* 2012; Porlier *et al.* 2012a), altitudinal ranges (Murphy *et al.* 2010) or environmental conditions (Wang *et al.* 2013). In the case of animals, dispersers may also prefer to move through and settle in areas similar to their natal sites, i.e., the previous experience shapes later preference, which can also result in disrupted gene flow among populations experiencing different environmental conditions (Davis & Stamps 2004; Stamps *et al.* 2009; Wang & Bradburg 2014). Thus, isolation-by-environment (IBE) can shape patterns of genetic differentiation irrespective of the spatial location of populations due to the interaction between landscape heterogeneity, natural/sexual selection and/or biased dispersal (Wang & Bradburg 2014). However, IBD, IBR and IBE are not mutually exclusive processes so that pathways constraining individual movement across the landscape and different selective and behavioural processes can all contribute to shape the patterns of gene flow in natural populations (Wang *et al.* 2013; Shafer & Wolf 2013; Wang & Bradburg 2014). In this sense, incorporating ecological theory into landscape genetic studies can help to get a more comprehensive view on the neutral

and micro-evolutionary processes shaping the distribution of genetic variability at small spatiotemporal scales (Garroway *et al.* 2013; Coster *et al.* 2015; Peterman *et al.* 2015).

In this study, we analyse the relative role of geography, landscape composition, and environment in structuring genetic variation in the blue tit (*Cyanistes caeruleus*), a small passerine widespread in forested habitats of the western Palaearctic (Illera *et al.* 2011). Previous studies on blue tits have shown the presence of fine-spatial genetic structure (Foerster *et al.* 2003; Ortego *et al.* 2011; Olano-Marin *et al.* 2010) and reduced gene flow among geographically close populations due to local adaptation processes (Porlier *et al.* 2012a, b; García-Navas *et al.* 2014). Taking advantage of extensive genotypic data (1385 individuals typed at 26 microsatellite markers), we explore the landscape-level processes shaping gene flow among 25 Mediterranean populations of blue tits experiencing different environmental conditions and degree of geographical isolation, Mediterranean environments provide a fascinating framework to examine this issue because they are generally characterized by a fine-grained mosaic and an extreme spatial heterogeneity resulting from natural (e.g., fire) and human disturbances (agriculture, forestry, grazing) (Blondel 2006; Blondel & Aronson 1999). We used circuit theory to generate different isolation-by-resistance scenarios (McRae 2006; McRae & Beier 2007) and test their importance in shaping contemporary patterns of gene flow relative to geographical distance and environmental dissimilarity (Wang 2013; Wang *et al.* 2013). Further, we used two subsets of loci, classified as putatively neutral or functional by considering whether the genomic region where they are located is being actively transcribed to RNA (*sensu* Olano-Marin *et al.* 2011a, b; Ferrer *et al.* 2014). The combination of both sets of markers will help to understand whether the factors (e.g., neutral vs. adaptive processes) shaping observed patterns of genetic differentiation generate distinct signatures across genomic regions with different functions and architecture. In spite of this question has recently received considerable attention in the context of landscape genetics (Shafer & Wolf 2013; Wang & Bradburd 2014), it has been rarely tested (see Freedman *et al.* 2010 for an exception). In particular, we (i) hypothesize that geography (isolation-by-distance, IBD), landscape composition (isolation-by-resistance, IBR) and heterogeneous ecological conditions (isolation-by-environment, IBE) contribute to shape patterns of genetic differentiation in the studied populations. Given that our functional markers are located in multiple genomic regions and likely subject to different selective pressures on different traits (Olano-Marin *et al.* 2011a), we expect that they (*ii*) show more subtle patterns of genetic structure than neutral markers and (*iii*) are less impacted by the different processes contributing to shape contemporary patterns of gene flow in the studied populations.

MATERIALS AND METHODS

Study system

During 2008-2013, we studied 25 blue tit populations located in fragmented woodlands scattered within an area of 150 km2 in Montes de Toledo, central Spain (Fig. 1). Seventeen populations were located in San Pablo de los Montes (Toledo province, 39º31´N 4º21´W), six in Quintos de Mora (Toledo province, 39º25´N 4º04´W), and two in Cabañeros National Park (Ciudad Real and Toledo provinces, 39°24´N, 3°35´W) (Table 1; see Fig. 1). These three areas are mainly separated by farmlands dominated by extensive cereal crops and olive groves (Fig. 1). Accordingly, the study area exhibits considerable habitat heterogeneity resulting in a mosaic-like pattern, a typical landscape configuration of the Mediterranean region (Blondel & Aronson 1999). Within forested areas we can discern three habitat types: 1) deciduous forests of Pyrenean oak (*Quercus pyrenaica*), which predominate in the most humid environments (shaded slopes and gullies); 2) evergreen forests of holm oak (*Q. rotundifolia*) widely distributed in the sunny slopes and plains, and 3) coniferous plantations (*Pinus pinea* and *P. pinaster*) derived from afforestation policies carried out in the 60s. All studied blue tit populations are established in woodlots supplied with nestboxes, of which 21 are located in deciduous Pyrenean oak forests, three in evergreen holm oak woodlands, and one in a pine plantation (see Table 1 for more details). Some forest patches show a high degree of isolation as they are separated by scrublands, croplands and grazing land for livestock. Therefore, forested areas in this region do not constitute a continuous habitat; rather these can be defined as small woodland remnants into a matrix of kaleidoscopic quality (García-Canseco 1997; Tornero 2003). Orographically, the study area (Montes de Toledo) constitutes one of the main mountain systems in the Iberian Peninsula. The most important ranges of this system are located in north-western Ciudad Real Province and south-western Toledo Province, where they form a denuded high land with low-medium elevations (max. 1447 m). The studied populations are located at different altitudes (ranging from 700 to 1200 m), depending on whether they are sited in flood-plains or in mountain ranges (see Table 1), which could lead to local adaptation processes and genetic isolation (e.g., Milá *et al.* 2009; Thomassen *et al.* 2010).

Sample collection

Adult blue tits were captured when feeding 8-day-old chicks by means of spring traps. Birds were individually marked with aluminium rings for further identification and bled by puncturing the brachial vein. Blood samples were stored on FTA cards (Whatman Bioscience, Florham Park, NJ, USA) or in Eppendorf tubes with 96% ethanol until needed for genetic analyses.

Table 1 Attributes of the 25 blue tit populations analysed in the present study. The following information is given: location of the populations in the main study localities (SPM: San Pablo de los Montes; QM: Quintos de Mora; CNP: Cabañeros National Park), population codes, geographical coordinates, number of individuals analysed in each population (*n*), elevation, and habitat type (*Qr*: holm oak forest; *Qp*: Pyrenean oak forest; *Pp*: pinewood).

Area	Population	Code	Latitude	Longitude	*n*	Elevation (m)	Habitat
SPM	NVI	1	39.564	-4.363	16	836	*Qr*
SPM	NAH	2	39.556	-4.362	10	856	*Qp*
SPM	MAR	3	39.534	-4.377	159	953	*Qp*
SPM	MAJ	4	39.538	-4.368	58	1014	*Qp*
SPM	BAN	5	39.529	-4.354	13	1075	*Qp*
SPM	CAS	6	39.528	-4.344	45	1104	*Qp*
SPM	MOR	7	39.528	-4.325	94	1220	*Qp*
SPM	ERM	8	39.532	-4.327	9	1068	*Qp*
SPM	FRI	9	39.531	-4.318	10	1100	*Qp*
SPM	ROB	10	39.509	-4.359	18	866	*Qp*
SPM	FCA	11	39.496	-4.355	10	820	*Qp*
SPM	PLA	12	39.521	-4.280	15	1057	*Qp*
SPM	FCE	13	39.519	-4.277	11	1052	*Qp*
SPM	CAN	14	39.518	-4.257	11	1022	*Qp*
SPM	ARR	15	39.512	-4.288	10	962	*Qp*
SPM	AVE	16	39.512	-4.277	52	1068	*Qp*
SPM	FUB	17	39.523	-4.257	93	1033	*Qp*
QM	VAL	18	39.442	-4.094	211	908	*Qp*
QM	POS	19	39.422	4.092	11	842	*Qr*
QM	NAV	20	39.391	-4.116	75	746	*Qp*
QM	CAZ	21	39.385	-4.107	76	768	*Pp*
QM	GIL	22	39.379	-4.124	237	826	*Qp*
QM	FCO	23	39.363	-4.080	32	919	*Qp*
CNP	BRE	24	39.357	-4.351	78	764	*Qp*
CNP	ANC	25	39.342	-4.366	33	711	*Qr*

Microsatellite genotyping and basic genetic statistics

A total of 1385 individuals were genotyped across 26 polymorphic microsatellite markers (see Supporting Information Table S1). These markers were classified as presumably functional or neutral as described by Olano-Marin *et al.* (2011a, b) (see also Olano-Marin *et al.* 2010; Ferrer *et al.* 2014). We used NucleoSpin Blood Kits (Macherey-Nagel, Duren, Germany) to extract and purify genomic DNA from the blood samples. Approximately 5 ng of template DNA was amplified in 10-μL reaction volumes containing 1X reaction buffer (67 mM Tris-HCL, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01 % Tween-20, EcoStart Reaction Buffer, Ecogen, Barcelona, Spain), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each dye-labelled primer (FAM, PET, VIC or NED) and 0.1 U of Taq DNA EcoStart Polymerase (Ecogen). The

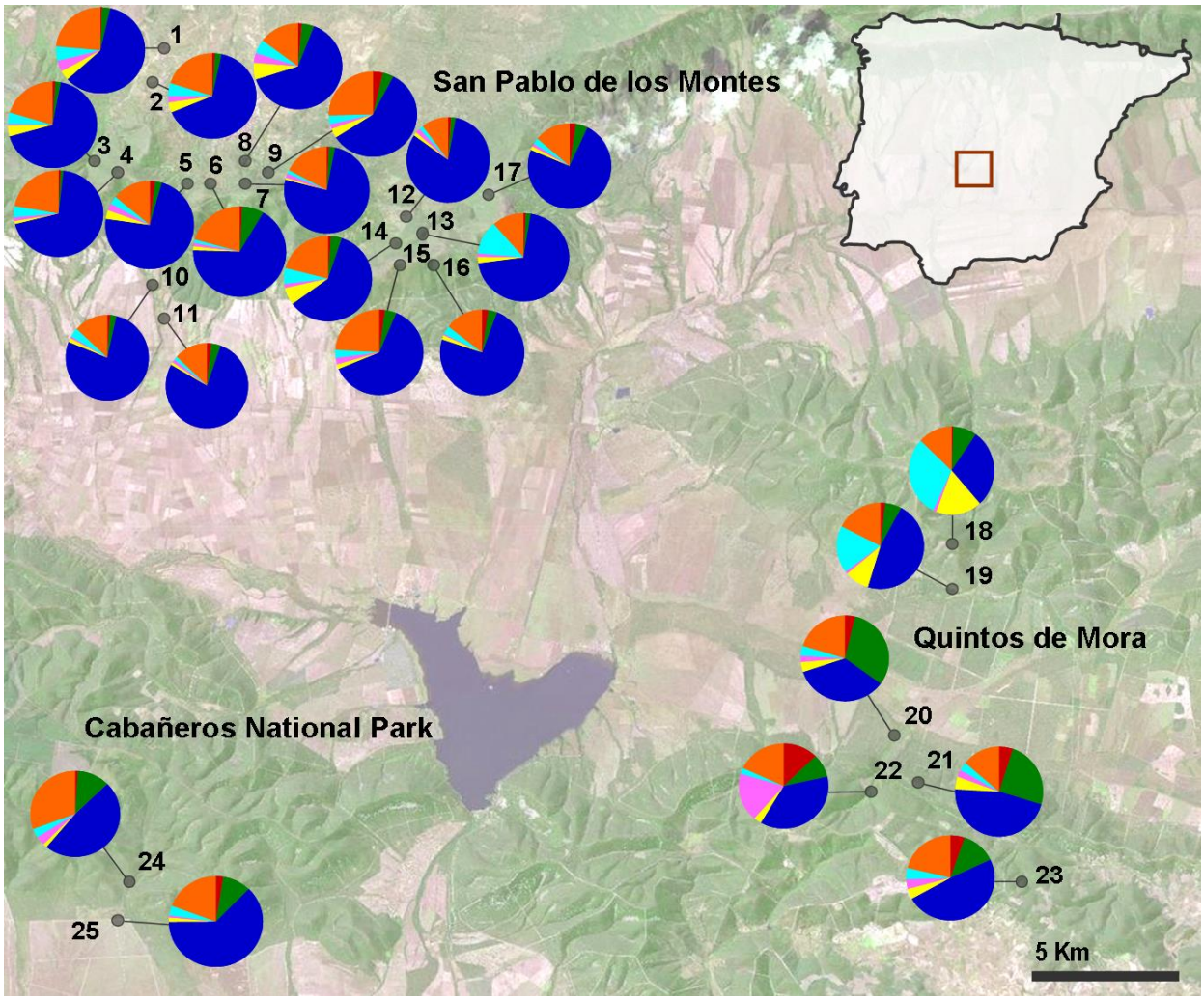


Figure 1 Map of the study area showing the location of the studied blue tit populations and their genetic assignment based on the Bayesian method implemented in the program STRUCTURE considering seven genetic clusters and all typed markers (functional and neutral loci). The admixture proportions generated by STRUCTURE were represented using pie charts, with each colour indicating a different genotypic cluster. Population codes are indicated in Table 1. The inset map shows the location of the study area in the Iberian Peninsula.

PCR programme used was 9 min denaturing at 95 ºC followed by 40 cycles of 30 s at 94 ºC, 45 s at the annealing temperature (Table S1) and 45 s at 72 ºC, ending with a 10 min final elongation stage at 72 ºC. Amplification products were electrophoresed using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and genotypes were scored using GENEMAPPER 3.7 (Applied Biosystems).

We tested for linkage disequilibrium (LD) between pair of loci and deviations from Hardy-Weinberg equilibrium (HWE) using GENEPOP web version 4.2 (Raymond & Rousset 1995). Probabilities of significance were assessed applying a Markov chain method (Guo & Thompson 1992) using 200 batches, 1000 iterations per batch and 1000 dememorization steps as implemented in GENEPOP. The LD correlation coefficient (*r*LD) between alleles at different loci was used to estimate the magnitude of LD. *r*LD was computed with

the program LINKDOS (http://genepop.curtin.edu.au/linkdos.html ; Garnier-Gere & Dillmann 1992). Sequential Bonferroni corrections were applied to account for multiple comparisons (Rice 1989).

Spatial genetic structure

We investigated the genetic differentiation between the studied blue tit populations calculating pairwise F_{ST} values and testing their significance with Fisher's exact tests after 10 000 permutations as implemented in ARLEQUIN 3.1 (Excofier *et al.* 2005). Pairwise F_{ST} values were calculated for all markers and separately for the subsets of functional and neutral loci. We also analysed the spatial genetic structure using the Bayesian clustering method implemented in STRUCTURE 2.3.4, which assigns individuals to populations based on their multilocus genotypes (Pritchard *et al.* 2000; Falush *et al.* 2003). We ran STRUCTURE assuming correlated allele frequencies and admixture (Pritchard *et al.* 2000; Falush *et al.* 2003) and using the "prior population information" option to take sampling locations into account (Hubisz *et al.* 2009). First, we conducted five independent runs for each value of K = 1-25 with a burn-in of 100000 MCMC steps followed by 100000 iterations. This allowed us to determine basic parameters and the most likely number of genetic clusters (K) and avoid very time-consuming runs for large values of K. Given that the most likely number of genetic clusters inferred in these preliminary analyses was always largely below K = 10 (see Results), we performed five additional runs for each value of K = 1-10 using the same settings. The most likely value of K was determined using log probabilities [Pr(X|K)] (Pritchard *et al.* 2000) and the ΔK method (Evanno *et al.* 2005). We used STRUCTURE HARVESTER (Earl & vonHoldt 2012) to compile and visualize the results from STRUCTURE, CLUMPP to align multiple runs for the same value of K (Jakobsson & Rosenberg 2007) and DISTRUCT (Rosenberg 2004) to visualize the resulting Q matrix. Analyses in STRUCTURE were also performed for all makers together and considering functional and neutral markers separately.

Landscape genetic analyses

We examined the effects of geographical distance (isolation-by-distance, IBD), resistance distances defined by landscape configuration (isolation-by-resistance, IBR) and environmental dissimilarity (isolation-by-environment, IBE) on the genetic differentiation of populations. To test the effect of IBD, we calculated a matrix of Euclidean geographical distances between all pairs of populations. We applied circuit theory to model gene flow across the highly heterogeneous studied landscape and determine the impact of different isolation by resistance (IBR) scenarios on observed patterns of genetic differentiation (McRae 2006; e.g., McRae & Beier 2007; McRae *et al.* 2008). We used ARCMAP 10.0 (ESRI,

Redlands, CA, USA) and the Corine-land cover map (1: 100 000) from the Spanish National Centre of Geographic Information (CNIG; https://www.cnig.es) to create raster layers with a spatial resolution standardized to a 50-m grid cell size. We reclassified land uses into three landscape element classes that *a priori* are likely to determine the distribution and dispersal patterns in forest passerines: 1) woodlands; 2) non-forested natural or semi-natural habitats, mostly constituted by scrublands and meadows; 3) non-natural habitats, mostly including open agricultural lands and, in a much lesser extent, reservoirs, villages and other developed areas (e.g., Desrochers & Hannon 1997; Bélisle *et al.* 2001; Matthysen *et al.* 2001; Pavlova *et al.* 2012). We generated different IBR scenarios assigning a range of resistance values to each of the three habitat classes (Table 2). This allowed us to identify the resistance values for each habitat class that best fit our data on genetic differentiation (e.g., Andrew *et al.* 2012; Seymour *et al.* 2013). We used CIRCUITSCAPE 3.5.8 to calculate resistance distance matrices between all pairs of populations considering an eight-neighbour cell connection scheme (McRae 2006). We also created a null model with all cells having an equal resistance (value = 1), which is expected to yield similar results than Euclidean geographical distance but has been suggested to be more appropriate to compare with models of IBR generated with CIRCUITSCAPE (Lee-Yaw *et al.* 2009; Munshi-South 2012; Jha & Kremen 2013). Finally, we tested two isolation-by-environment (IBE) scenarios based on differences in elevation and habitat type between the studied populations, which have been previously found to be involved in processes of local adaptation and influence patterns of genetic differentiation in a range of organisms (reviewed in Shafer & Wolf 2013; Sexton *et al.* 2014). We used a digital elevation model (DEM) from the Spanish National Centre of Geographic Information to calculate a matrix of differences in elevation between each pair of studied populations (Table 1). We generated a pairwise matrix of habitat differences considering whether the populations were located in deciduous forests, evergreen sclerophyllous forests, or coniferous forests (Table 1). All comparisons within the same habitat type were assigned a value of 0, whereas deciduous *vs.* evergreen forest, coniferous *vs.* evergreen forest, and coniferous *vs.* deciduous forest comparisons were assigned a value of 1 (e.g., Andrew *et al.* 2012).

The relationships between genetic differentiation (F_{ST}) and matrices of geographical distance (IBD), landscape resistance (IBR), and environmental dissimilarity (IBE) were examined using multiple matrix regressions with randomization (MMRR) using the MMRR function script (Wang 2013) implemented in R 3.0.2. These analyses were performed for pairwise F_{ST} values calculated for all makers and considering functional and neutral markers separately.

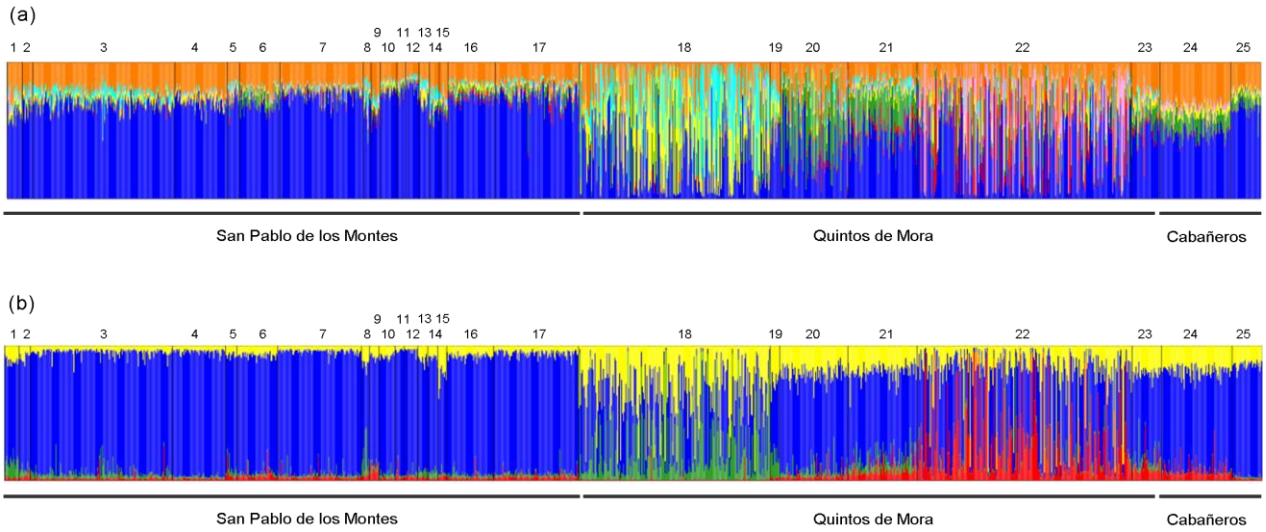


Figure 2 Results of genetic assignments of blue tits (n = 1385 individuals) based on the Bayesian method implemented in the program STRUCTURE considering (a) the seven genetic clusters inferred for all typed markers (functional and neutral loci) and (b) the four genetic clusters inferred for the subset of neutral loci. Each individual is represented by a thin vertical line, which is partitioned into coloured segments that represent the individual's probability of belonging to the cluster with that colour. Individuals are grouped according to their populations of origin using vertical black lines. Population codes are indicated in Table 1.

RESULTS

Microsatellite data

Genetic diversity was higher for the panel of neutral markers in comparison with the subset of functional loci, but these differences were only statistically significant in terms of allelic richness (one-way ANOVAs, H_E: $F_{1,24}$ = 2.60, P = 0.119; H_O: $F_{1,24}$ = 2.08, P = 0.161; allelic richness: $F_{1,24}$ = 4.90, P = 0.036; Table S1, Supporting Information). We observed significant departures from HWE in 18 of 650 population-locus combinations after correcting for multiple comparisons. Such deviations involved loci *Pca2* (FCO and MAR populations), *Pca3* (NAV population), *Pca8* (GIL and NAV populations), *Pdoμ5* (FCA, FUB, and VAL populations), *Pocc6* (GIL population), *CcaTgu11* (CAS, GIL, MAR and VAL populations) and *CcaTgu15* (CAZ, FCO, GIL, MAR, and VAL populations). We only found evidence of significant LD between some pair of loci located within the same chromosome for GIL (*Mcyμ4-Tg053*, *Pca3-Pdoμ5*, *Pca8-Tgu07, Pca9-Pocc1*, *PK12-TG05-053*, *PK12-CcaTgu15*, *Pocc6-Tgu07*), VAL (*Mcyμ4-Tg053*, *Mcyμ4-Tgu14*, *PK12-CcaTgu15*, *Tgu15-Tgu14*), BRE (*Pca3-Pdoμ5*), and MAR (*PK12-Mcyμ4*). Given that LD and departures from HWE were not consistent across loci and populations, all markers were used in subsequent analyses.

Spatial genetic structure

After sequential Bonferroni corrections, we found 10 significant pairwise F_{ST} values for analyses combining genotypic data from

Table 2 Multiple Matrix Regressions with Randomization (MMRR) for genetic differentiation (F_{ST}; estimated for all markers and the subsets of neutral and functional loci) in relation with 25 isolation-by-resistance (IBR) scenarios considering different resistance values (1 = lowest resistance; 100 = highest resistance) for the three land cover types considered in this study: forests, non-forested natural vegetation habitats, and lands mostly devoted to agriculture. Values in bold indicate the most significant model for each group of loci.

				All loci			Neutral loci			Functional loci		
Model	Forest	Non-forested vegetation	Agricultural lands	R^2	β	*P*	R^2	β	*P*	R^2	β	*P*
Null Model	1	1	1	**0.047**	**0.329**	**0.031**	**0.044**	**0.318**	**0.036**	0.008	0.141	0.408
Model 1	1	1	50	0.003	0.054	0.681	0.048	0.218	0.168	0.007	-0.088	0.457
Model 2	1	1	100	0.002	0.048	0.694	0.045	0.210	0.172	0.008	-0.088	0.460
Model 3	1	50	1	0.000	-0.022	0.9	0.005	-0.075	0.660	0.003	0.060	0.756
Model 4	1	50	50	0.000	-0.023	0.883	0.002	-0.054	0.739	0.001	0.043	0.819
Model 5	1	50	100	0.000	-0.024	0.86	0.002	-0.046	0.617	0.001	0.036	0.852
Model 6	1	100	1	0.001	-0.032	0.834	0.006	-0.078	0.636	0.002	0.049	0.806
Model 7	1	100	50	0.001	-0.034	0.823	0.004	-0.066	0.674	0.001	0.039	0.851
Model 8	1	100	100	0.008	0.081	0.836	0.003	-0.062	0.711	0.001	0.032	0.877
Model 9	50	1	1	0.006	0.085	0.601	0.036	0.204	0.228	0.009	-0.101	0.618
Model 10	50	1	50	0.007	0.09	0.597	0.051	0.242	0.161	0.011	-0.117	0.525
Model 11	50	1	100	0.007	0.091	0.544	0.054	0.249	0.123	0.012	-0.118	0.510
Model 12	50	50	1	0.044	0.342	0.089	0.000	-0.046	0.831	0.064	0.412	0.053
Model 13	50	50	100	0.034	0.264	0.064	0.042	0.294	0.054	0.003	0.089	0.570
Model 14	50	100	1	0.035	0.285	0.185	0.000	0.002	0.990	0.063	0.385	0.061
Model 15	50	100	50	0.033	0.265	0.132	0.007	0.126	0.480	0.029	0.252	0.129
Model 16	50	100	100	0.027	0.232	0.14	0.010	0.144	0.348	0.019	0.195	0.239
Model 17	100	1	1	0.005	0.078	0.64	0.035	0.197	0.260	0.009	-0.105	0.597
Model 18	100	1	50	0.006	0.082	0.632	0.046	0.229	0.161	0.012	-0.118	0.521
Model 19	100	1	100	0.006	0.083	0.61	0.050	0.237	0.158	0.012	-0.121	0.526
Model 20	100	50	1	0.037	0.263	0.151	0.040	0.275	0.167	0.001	0.055	0.802
Model 21	100	50	50	0.03	0.228	0.18	0.054	0.307	0.084	0.000	0.008	0.969
Model 22	100	50	100	0.026	0.208	0.234	0.055	0.303	0.072	0.000	-0.007	0.973
Model 23	100	100	1	0.043	0.335	0.097	0.001	-0.063	0.465	**0.066**	**0.416**	**0.048**
Model 24	100	100	50	0.053	0.372	0.027	0.020	0.228	0.180	0.025	0.258	0.131

neutral and functional markers (Table S2, Supporting Information. When the two, subsets of markers were analysed separately, we found 17 and 5 significant pairwise F_{ST} values for the panels of neutral and functional loci, respectively (Table S3, Supporting Information). Pairwise F_{ST} values estimated for neutral and functional loci were not significantly correlated (Mantel test: r = -0.018, P = 0.396). STRUCTURE analyses considering all typed loci and the statistic Δ*K* indicated that the most likely number of genetic clusters was K = 7 (Fig. 2a and Fig. S1 and S2, Supporting Information). STRUCTURE analyses run for the subset of neutral markers showed an optimal value of K = 4 according to the statistic Δ*K* (Fig. 2b and Fig. S1 and S3, Supporting Information). However, when the panel of functional markers was analysed separately the value of Pr(X|*K*) reached a maximum at K = 1 (Fig. S1, Supporting Information). Overall, STRUCTURE analyses for all loci and the

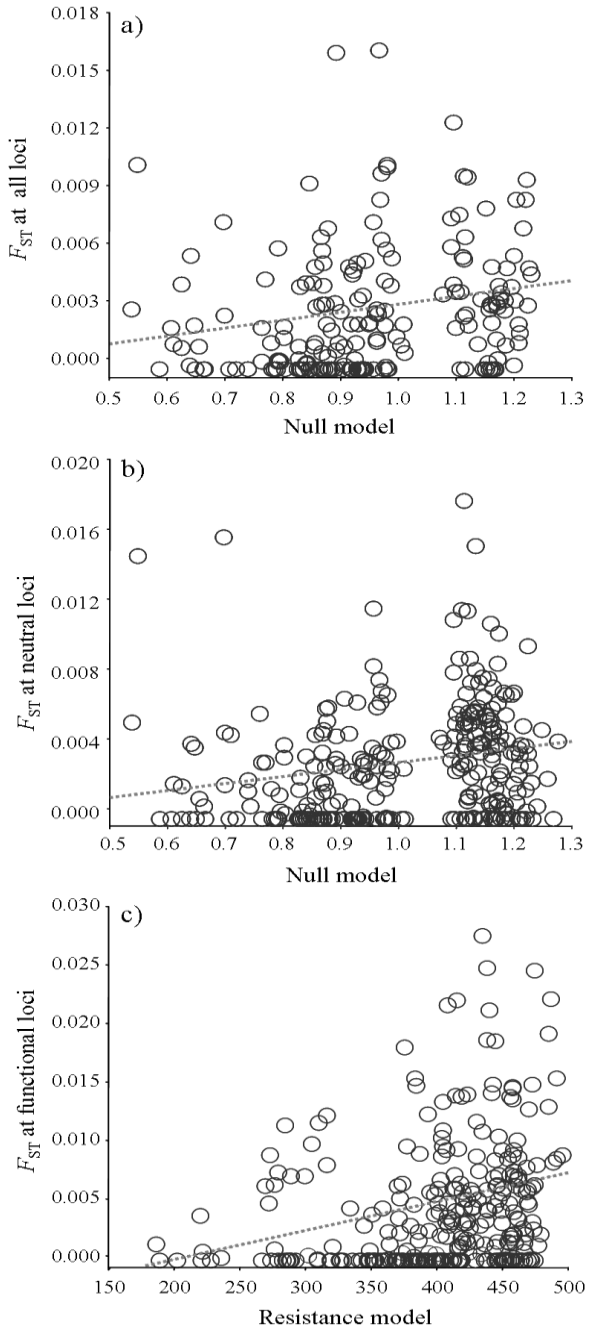


Figure 3 Relationship between genetic differentiation (F_{ST}) and the best explanatory model of resistance obtained using CIRCUITSCAPE for (a) all markers (null model, i.e., resistance = 1 for all habitat classes), (b) neutral markers (null model) and (c) functional markers (resistance = 500 for forest and non-forested natural vegetation and resistance = 1 for lands mostly devoted to agriculture).

panel of neutral markers revealed high levels of genetic admixture and subtle levels of genetic differentiation among populations from the three main study areas. Both STRUCTURE analyses and pairwise F_{ST} values also showed that VAL and GIL tended to show the highest levels of genetic differentiation both between them and with all the other studied populations (Fig. 1-2).

We performed additional analyses to ascertain whether the contrasting patterns of genetic structure inferred with the subsets of neutral and functional loci were due to the higher genetic diversity of neutral loci and/or the different number of typed markers in each group (14 neutral markers vs. 12 functional markers; Table S1, Supporting Information). For this purpose, we removed the two most diverse loci (*Pca3* and *Pca8*; Table S1, Supporting Information) from the panel of neutral markers and re-calculated F_{ST} values and re-ran STRUCTURE analyses. After the removal of loci *Pca3* and *Pca8*, genetic diversity did not significantly differ between the subsets of neutral and functional loci (one-way ANOVAs, H_E: $F_{1, 22} = 1.34$, $P = 0.258$; H_O: F1, 22 = 1.08, $P = 0.309$; allelic richness: $F_{1, 22} = 2.84$, $P = 0.105$). For this subset of 12 neutral loci, we found 19 significant pairwise F_{ST} values and STRUCTURE analyses showed that $\Pr(X|K)$ increased up to $K = 6$ and the statistic ΔK indicated that the most likely number of genetic clusters was $K = 2$ (Fig. S4 and S5, Supporting Information).

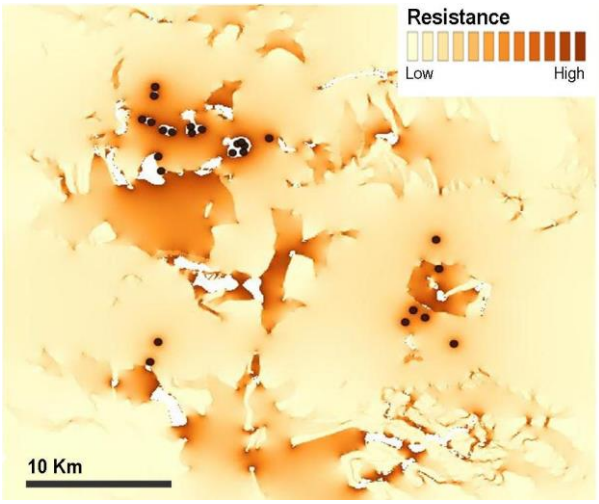


Figure 4 Landscape resistance model for which we obtained the highest correlation with genetic differentiation (F_{ST}) for functional markers (resistance = 500 for forest and non-forested natural vegetation and resistance = 1 for lands mostly devoted to agriculture). Lighter colours represent low resistance and darker colours represent high resistance

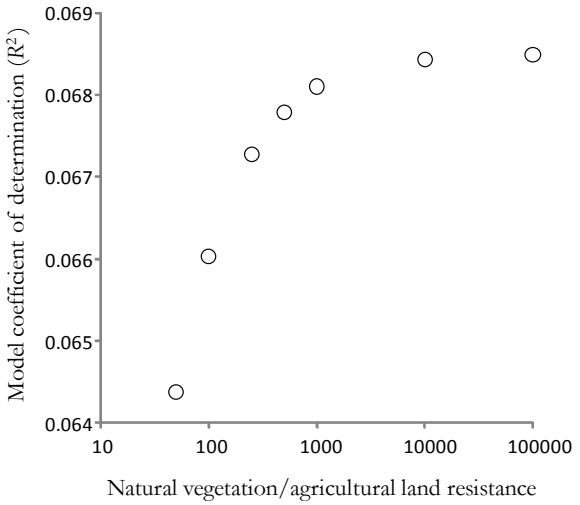


Figure 5 Coefficient of determination (R^2) for models analysing genetic differentiation (F_{ST}) estimated at functional markers in relation with isolation-by-resistance (IBR) distance matrices calculated with CIRCUITSCAPE considering different resistance ratios for natural vegetation (forests and non-forested natural habitats) and lands mostly devoted to agriculture. All models considered the minimum resistance (resistance = 1) for agricultural land and increasing resistance values (from 10 to 100000) for forests and non-forested natural habitats. Resistance ratios are log-transformed for illustrative purposes.

Landscape genetic analyses

Genetic differentiation (F_{ST}) estimated at all loci and the panel of neutral markers was associated with geographical distances (all loci: t = 3.55, P = 0.037; neutral loci: t = 3.94, P = 0.032) but not with differences in habitat type (all loci: t = 0.75, P = 0.733; neutral loci: t = 2.55, P = 0.309) or elevation (all loci: t =1.11, P = 0.464; neutral loci: t =1.32, P = 0.397). Genetic differentiation estimated at the subset of functional markers was not associated with geographical distance (t =0.06, P = 0.575) or environmental dissimilarity (habitat: t =-0.05, P = 0.978; elevation: t = 0.13, P = 0.926). Different models of isolation by resistance (IBR) explained genetic differentiation estimated at all markers and the subsets of neutral and functional loci (Table 2). The null model (i.e., all cells were assigned the same value), which is similar to Euclidean geographical distance (r = 0.96, P = 0.001), explained genetic differentiation for all loci and neutral loci (Table 2; Fig. 3a-b). However, genetic differentiation estimated at functional loci was better explained by IBR models considering high resistance values for woodlands and non-forested natural vegetation and the minimum resistance value (i.e., resistance = 1) for lands mostly devoted to agriculture (Table 2; Fig. 3c and Fig. 4). The null model considering equal resistance to all habitat classes did not explain genetic differentiation estimated at functional loci (Table 2). We generated further IBR models progressively increasing resistance for woodlands and non-forested natural

vegetation habitat classes (10, 25, 50, 100, 250, 500, 1000, 10000, and 100000) to evaluate if this contributes to increase the goodness of fit of the model (e.g., Andrew *et al.* 2012). Model goodness of fit increased with the "forested and non-forested habitats"/ "agricultural land" resistance ratio, but the strength of the relationship between genetic differentiation and IBR stabilized beyond a ratio 500:1(Fig. 5; Table S4, Supporting Information).

DISCUSSION

We found that neutral and functional loci yielded contrasting patterns of genetic structure for the studied blue tit populations. Population genetic differentiation was subtle and mostly detected using the panel of neutral markers. Landscape genetic analyses showed a pattern of IBD for all loci and the panel of neutral markers, but genetic differentiation estimated at the subset of functional loci was better explained by IBR models considering high resistance values for natural vegetation and low resistance to managed areas mostly devoted to agriculture. Finally, we did not find any support for IBE in our study system, suggesting a lack of micro-evolutionary changes and divergent selection pressures associated with differences in the two environmental components analysed.

Population genetic structure

STRUCTURE analyses and pairwise F_{ST} values indicated the presence of a subtle pattern of genetic structure across all the studied populations (Fig. 1). All individuals and populations showed a high degree of genetic admixture for any value of K considered and most significant pairwise F_{ST} values involved comparisons between populations from different areas (Fig. 1). The only exception was the significant genetic structure observed within Quintos de Mora, which was mostly due to the high genetic differentiation existing between VAL and GIL and some comparisons between these and other populations (Fig. 1). A previous study showed that divergent natural selection has resulted in phenotypic divergence between these two populations, which may have reduced realized gene flow despite they are located less than 7 km apart and sporadic exchange of individuals has been recorded (García-Navas *et al.* 2014).

When data were analysed separately for the subsets of neutral and functional loci, STRUCTURE analyses revealed that the most optimal clustering solution for functional markers was the presence of a single genetic cluster, whereas analyses focused on neutral markers showed an optimal value of $K = 4$. These differences were not due to the different number of markers employed or their degree of polymorphism, suggesting that patterns of genetic structure observed for the two subsets of loci may be consequence of their location in genomic regions submitted to different evolutionary pressures. Analyses

of neutrality based on the F_{ST} outliers tests implemented in Lositan (Beaumont & Nichols 1996; Antao *et al.* 2008) and Bayescan (Foll & Gagiotti 2008) indicated that no locus deviated significantly from neutral expectations (data not shown). Thus, the functional loci do not seem to be under strong selection despite they are located inside or flanking coding gene sequences that are being actively expressed to RNA. Loci under directional or disruptive selection are expected to show more marked genetic structure than neutral loci if incipient local adaptive processes have not yet reduced genome-wide gene flow, but our results point to the opposite direction (Shafer & Wolf 2013; see Zhao *et al.* 2013; Oetjen *et al.* 2007). One possibility to explain the observed pattern is that most functional loci are under stabilizing selection, which is more difficult to detect by outlier tests particularly when populations are weakly differentiated (Beaumont & Balding 2004). Thus, the presence of functional constraints and the effects of purifying selection may explain the lower levels of genetic differentiation and polymorphism in the subset of functional loci (Oetjen *et al.* 2007; Olano-Marin *et al.* 2011a; Agostini *et al.* 2013).

Isolation-by-resistance

Pairwise genetic differentiation at neutral and functional loci were not significantly correlated, suggesting that both kind of loci may capture different aspects of the same demographic phenomena (Freedman *et al.* 2010; Zhao *et al.* 2013). Accordingly, landscape genetic analyses based on circuit theory showed that the null model considering equal resistance for all habitat classes (equivalent to Euclidean geographical distance) was the one that best explained genetic differentiation estimated at all loci and the panel of neutral markers. However, contrary to our expectations, inter-population gene flow estimated for the subset of functional loci was not significantly associated with geographical distance; the best-fitting model was the one considering the lowest resistance (i.e., resistance = 1) for open croplands and a high resistance (resistance > 100) for natural vegetation (woodlands, scrublands and meadows). Model goodness of fit reached a plateau when considering resistance values for natural vegetation ~500 times higher than those assigned to open agricultural lands (Fig. 5). Studies comparing genetic structure in fragmented and continuous landscapes have often shown lower dispersal and gene-flow between patches separated by a matrix of unsuitable habitat (Broquet *et al.* 2006; Martínez-Cruz *et al.* 2007; Coulon *et al.* 2008; Dutta *et al.* 2013). However, habitat fragmentation can also induce the opposite process if individuals are forced to disperse longer distances across unfavourable habitats to find a suitable breeding site, a fact that may explain the obtained results for the subset of functional loci (Wiens 2001; Veit *et al.* 2005; Mylecraine *et al.* 2008; Harrison *et al.* 2013). Finally, it should be kept in mind that we found subtle genetic differentiation, which indicates widespread

gene flow and a limited impact of landscape composition in contemporary patterns of genetic structure at the spatial scale here considered.

Isolation-by-environment

Our analyses based on habitat and elevation dissimilarity did not reveal the presence of IBE in our study system, a pattern that may be explained by different reasons. Elevation has been found to be an important driver of genetic and phenotypic divergence in different bird species (e.g., Milá *et al.* 2009; Thomassen *et al.* 2010), but we did not find any signal of isolation-by-altitude. Although populations located at different elevations show significant differences in the timing of breeding in our study area (*unpubl. data*), our results suggest that such differences in phenology may represent a purely plastic response to temporal differences in prey availability associated with local environmental conditions (García-Navas & Sanz 2011) and that these populations do not exhibit different reaction norms, which are only possible under reduced gene flow. Regarding the second environmental factor, i.e. habitat type, a previous study carried out on Corsican populations of blue tit (*C. caeruleus ogliastrae*) revealed the existence of habitat-linked genetic differentiation (Porlier *et al.* 2012a). This suggests that local adaptation might reduce gene flow among populations from different habitat types (deciduous *vs.* evergreen oak forests) and/or that divergent selection (in response to habitat-specific selection regimes) is strong relative to gene flow (Porlier *et al.* 2012a). Thereby, the observed differences in morphological and reproductive traits among Corsican blue tit populations inhabiting deciduous and evergreen forests can be explained as consequence of asymmetrical gene flow resulting from source-sink dynamics between these two habitat types (Dias *et al.* 1996; Blondel 2007; Blondel *et al.* 2006). In our study populations, habitat type (deciduous *vs.* evergreen) does not seem to impose important selective pressures as indicated by ecological and behavioural studies in which both forest types were compared (García-Navas & Sanz 2012; García-Navas *et al.* 2012). Such studies do not support the existence of local maladaption to the less suitable habitat (evergreen forests) in contrast with that reported in Corsican blue tits (cf. Dias & Blondel 1996). At this point, we must acknowledge that the low number of localities sampled in evergreen forests may have reduced our statistical power to detect such effects. However, no single significant pairwise F_{ST} comparison involved populations from different habitats, which suggests that the lack of isolation-by-habitat may be genuine or that considerable gene flow prevents local adaptation to persist over time.

Despite our number of markers and sample sizes are both way far larger than those employed in most previous studies exploring the role of environment in structuring genetic variation, it should be also considered that IBE generally only explains ~5% of total genetic differentiation (Shafer & Wolf 2013), which could have reduced the ability of our loci to

detect incipient or ephemeral adaptation processes that may be taking place in some of our study populations (Shafer & Wolf 2013; e.g., García-Navas *et al.* 2014). Finally, it should be also taken into account that we cannot totally reject the hypothesis of IBE given that other factors not analysed in this study (e.g., food supply: García-Navas & Sanz 2011; calcium availability: García-Navas *et al.* 2011; disease risk: Garroway *el al.* 2013) could potentially lead to micro evolutionary changes and cryptic processes of local adaptation (e.g., Garroway *el al.* 2013; McDevitt *et al.* 2013).

Conclusions

Overall, our study shows that markers located in different regions of the genome can yield contrasting inferences about the patterns of population genetic structure. It is particularly remarkable the low genetic differentiation observed for the subset of functional loci in comparison the panel of neutral loci. The subtle pattern of genetic structure and the lack of IBE indicate widespread gene flow in our study system, which may be explained by long-distance dispersal movements through unsuitable habitat matrix as suggested by the best explanatory IBR model for functional markers. As far as we know this study is one of the first to incorporate local factors (i.e., conditions at each sampling locality) in conjunction with landscape factors describing matrix quality to examine how the environment influences realized dispersal and gene flow (Pflüger & Balkenhol 2014). Future studies performed at larger spatial scales, considering other potential drivers of local adaptation, and based on genome-wide data obtained using high throughput sequencing technology will help to get a better understanding on the neutral and adaptive processes determining effective dispersal and the resulting patterns of genetic structure in natural populations (Andrews & Luikart 2014; e.g., Garroway *el al.* 2013; Orsini *et al.* 2013; Geraldes *et al.* 2014).

ACKNOWLEDGEMENTS

We thank the board of the Cabañeros National Park, Centro Quintos de Mora and the Council of San Pablo de los Montes for the facilities offered during the fieldwork. We are indebted to Pedro J. Cordero for allowing us to carry out the genetic analyses in his lab and Víctor Noguerales for technical support. This study was funded by grants 69/2003 (Organismo Autónomo de Parques Nacionales-Ministerio de Medio Ambiente), GCL2007-61395 (Ministerio de Educación y Ciencia), CGL2010-21933-C02-01 (Ministerio de Ciencia e Innovación) and POIC10-0269-7632 (Junta de Comunidades de Castilla-La Mancha-European Social Fund). ESF and JBE are supported by a PhD scholarship from Junta de Comunidades de Castilla-La Mancha-European Social Fund, VGN is supported by a Forschungskredit of the University of Zurich (FK-14-103), RB is supported by a Juan de la Cierva fellowship (Ministerio de Ciencia e Innovación), ESD is supported by

a PhD scholarship from Ministerio de Ciencia e Innovación-European Social Fund (FPI Program) and JO is supported by a research contract funded by Severo Ochoa Program (SEV-2012-0262).

REFERENCES

Agostini C, Papetti C, Patarnello T, Mark FC, Zane L, Marino IAM. 2013. Putative selected markers in the *Chionodraco* genus detected by interspecific outlier tests. *Polar Biol.* 36:1509-1518.

Andrew RL, Ostevik KL, Ebert DP, Rieseberg LH. 2012. Adaptation with gene flow across the landscape in a dune sunflower. *Mol. Ecol.* 21:2078-2091.

Antao T, Lopes A, Lopes RJ, Beja-Pereira A, Luikart G. 2008. LOSITAN: A workbench to detect molecular adaptation based on a *F*(st)-outlier method. *BMC Bioinf.* 9: 323.

Beaumont MA, Balding DJ. 2004. Identifying adaptive genetic divergence among populations from genome scans. *Mol. Ecol.* 13:969-980.

Beaumont MA, Nichols RA. 1996. Evaluating loci for use in the genetic analysis of population structure. *Proc. R. Soc. B-Biol. Sci.* 263:1619–1626

Bélisle M, Desrochers A, Fortin MJ. 2001. Influence of forest cover on the movements of forest birds: A homing experiment. *Ecology* 82:1893-1904.

Blondel J. 2006. The "design" of Mediterranean landscapes: a millennial history of humans and ecological systems during the Historic Period. *Human Ecol.* 34:713-729.

Blondel J. 2007. Coping with habitat heterogeneity: the story of Mediterranean blue tits. *J. Ornithol.* 148:3-15.

Blondel J, Aronson J. 1999. *Biology and Wildlife of the Mediterranean Region*. Oxford: Oxford University Press.

Blondel J, Thomas DW, Charmantier A, Perret P, Bourgault P, Lambrechts MM. 2006. A thirty year study of phenotypic and genetic variation of blue tits in Mediterranean habitat mosaics. *BioScience* 56:661-673

Broquet T, Ray N, Petit E, Fryxell JM, Burel F. 2006. Genetic isolation by distance and landscape connectivity in the American marten (*Martes americana*). *Landscape Ecol.* 21:877-889.

Coster SS, Babbitt KJ, Cooper A, Kovach AI. 2015. Limited influence of local and landscape factors on finescale gene flow in two pond-breeding amphibians. *Mol. Ecol.* 24:742-758.

Coulon A, Fitzpatrick JW, Bowman R, Stith BM, Makarewich CA, Stenzler LM, Lovette IJ. 2008. Congruent population structure inferred from dispersal behaviour and intensive genetic surveys of the threatened Florida scrub-jay (*Aphelocoma coerulescens*). *Mol. Ecol.* 17:1685-1701.

Coulon A, Guillot G, Cosson JF, Angibault JMA, Aulagnier S, Cargnelutti B, Galan M, Hewison AJM. 2006. Genetic structure is influenced by landscape features: empirical evidence from a roe deer population. *Mol. Ecol.* 15:1669-1679.

Davis JM, Stamps JA. 2004. The effect of natal experience on habitat preferences. *Trends Ecol. Evol.* 19:411-416

Desrochers A, Hannon SJ. 1997. Gap crossing decisions by forest songbirds during the post-fledging period. *Conserv. Biol.* 11:1204-1210.

Dias PC, Blondel J. 1996. Local specialization and maladaptation in the Mediterranean blue tit (*Parus caeruleus*). *Oecologia* 107:79-86.

Dias P, Verheyen G, Raymond M. 1996. Source-sink populations in Mediterranean blue tits: evidence using single-locus minisatellite probes. *J. Evol. Biol.* 9:965–978.

Dutta T, Sharma S, Maldonado JE, Wood TC, Panwar HS, Seidensticker J. 2013. Fine-scale population genetic structure in a wide-ranging carnivore, the leopard (*Panthera pardus fusca*) in central India. *Divers. Distrib.* 19:760-771.

Earl DA, vonHoldt BM. 2012. Structure Harvester: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.* 4:359-361.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol. Ecol.* 14:2611-2620.

Excoffier L, Laval G, Schneider S. 2005. Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evol. Bioinf.* 1:47-50.

Falush D, Stephens M, Pritchard JK. 2003. Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. *Genetics* 164:1567-1587.

Ferrer ES, García-Navas V, Sanz JJ, Ortego J. 2014. Individual genetic diversity and probability of infection by avian malaria parasites in blue tits (*Cyanistes caeruleus*). *J. Evol. Biol.* 27:2468-2482.

Foerster K, Delhey K, Johnsen A, Lifjeld JT, Kempenaers B. 2003. Females increase offspring heterozygosity and fitness through extra-pair matings. *Nature* 425:714-717.

Foll M, Gaggiotti OE. 2008. A genome scan method to identify selected loci appropriate for both dominant and codominant markers: A Bayesian perspective. *Genetics* 180:977-993

Freedman AH, Thomassen HA, Buermann, W, Smith TB. (2010) Genomic signals of diversification along eco-logical gradients in a tropical lizard. *Mol. Ecol.* 19:3773-3788.

Garant D, Forde SE, Hendry AP. 2007. The multifarious effects of dispersal and gene flow on contemporary adaptation. *Funct. Ecol.* 21:434-443.

García-Navas V, Sanz JJ. 2011. The importance of a main dish: Nestling diet and foraging behaviour in Mediterranean blue tits in relation to prey phenology. *Oecologia* 165:639-649.

García-Navas V, Sanz JJ, Merino S, Martínez-de la Puente J, Lobato E, del Cerro S, Rivero J, Ruiz de Castañeda R, Moreno J. 2011. Experimental evidence for the role of calcium in eggshell pigmentation pattern and breeding performance in blue tits *Cyanistes caeruleus*. *J. Ornithol.* 152:1-82.

García-Navas V, Sanz JJ. 2012. Environmental and within-nest factors influencing nestling feeding patterns in Mediterranean blue tits (*Cyanistes caeruleus*). *Condor* 114:612-620.

García-Navas V, Ferrer ES, Sanz JJ. 2012. Plumage yellowness predicts foraging ability in the blue tit *Cyanistes caeruleus*. *Biol. J. Linn. Soc.* 106:418-429.

García-Navas V, Ferrer ES, Sanz JJ, Ortego J. 2014. The role of immigration and local adaptation on fine-scale genotypic and phenotypic population divergence in

a less mobile passerine. *J. Evol Biol.* 27:1590-1603.

García-Canseco V. 2000. *Parque Nacional de Cabañeros*. Ciudad Real: Ecohabitat.

Garnier-Gere P, Dillmann C. 1992. A computer-program for testing pairwise linkage disequilibria in subdivided populations. *J. Hered.* 83:239-239.

Garroway CJ, Radersma R, Sepil I, Santure AW, De Cauwer I, Slate J, Sheldon BC. 2013. Fine-scale genetic structure in a wild bird population: The role of limited dispersal and environmentally based selection as causal factors. *Evolution* 67:3488-3500.

Gascon C, Malcolm JR, Patton JL, da Silva MNF, Bogart JP, Lougheed SC, Peres CA, Neckel S, Boag PT. 2000. Riverine barriers and the geographic distribution of Amazonian species. *Proc. Natl. Acad. Sci. U.S.A.* 97:13672-13677.

Geraldes A, Farzaneh N, Grassa CJ, McKown AD, Guy RD, Mansfield SD, Douglas CJ, Cronk QCB. 2014. Landscape genomics of *Populus trichocarpa*: the role of hybridization, limited gene flow, and natural selection in shaping patterns of population structure. *Evolution* 68:3260-3280.

Guo SW, Thompson EA. 1992. A monte-carlo method for combined segregation and linkage analysis. *Am. J. Hum. Genet.* 51:1111-1126.

Hansson B, Bensch S, Hasselquist D. 2004. Lifetime fitness of short- and long-distance dispersing great reed warblers. *Evolution* 58:2546-2557.

Harrisson KA, Pavlova A, Amos JN, Takeuchi N, Lill A, Radford JQ, Sunnucks P. 2013. Disrupted fine-scale population processes in fragmented landscapes despite large-scale genetic connectivity for a widespread and common cooperative breeder: the superb fairy-wren (*Malurus cyaneus*). *J. Anim. Ecol.* 82:322-333.

He QX, Edwards DL, Knowles LL. 2013. Integrative testing of how environments from the past to the present shape genetic structure across landscapes. *Evolution* 67:3386-3402.

Hubisz MJ, Falush D, Stephens M, Pritchard JK. 2009. Inferring weak population structure with the assistance of sample group information. *Mol. Ecol. Resour.* 9:1322-1332.

Hutchison DW, Templeton AR. 1999. Correlation of pairwise genetic and geographic distance measures: Inferring the relative influences of gene flow and drift on the distribution of genetic variability. *Evolution* 53:1898-1914.

Illera JC, Koivula K, Broggi J, Paeckert M, Martens J, Kvist L. 2011. A multi-gene approach reveals a complex evolutionary history in the *Cyanistes* species group. *Mol. Ecol.* 20:4123-4139.

Jakobsson M, Rosenberg NA. 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* 23:1801-1806.

Jha S, Kremen C. 2013. Urban land use limits regional bumble bee gene flow. *Mol. Ecol.* 22:2483-2495.

Lee-Yaw JA, Davidson A, McRae BH, Green DM. 2009. Do landscape processes predict phylogeographic patterns in the wood frog? *Mol. Ecol.* 18:1863-1874.

Manel S, Schwartz MK, Luikart G, Taberlet P. 2003. Landscape genetics: combining landscape ecology and population genetics. *Trends Ecol. Evol.* 18:89-197.

Martínez-Cruz B, Godoy JA, Negro JJ. 2007. Population fragmentation leads to spatial and temporal genetic structure in the endangered Spanish imperial eagle. *Mol. Ecol.* 16:477-486.

Matthysen E, Adriaensen F, Dhondt AA. 2001. Local recruitment of great and blue tits (*Parus major*, *P. caeruleus*) in relation to study plot size and degree of isolation. *Ecography* 24:33-42.

McDevitt AD, Oliver MK, Piertney SB, Szafrańska PA, Konarzewski M, Zub K. 2013. Individual variation in dispersal associated with phenotype influences fine-scale genetic structure in weasels. *Conserv. Genet.* 14:499-509.

McRae BH. 2006. Isolation by resistance. *Evolution* 60:1551-1561.

McRae BH, Beier P. 2007. Circuit theory predicts gene flow in plant and animal populations. *Proc. Natl. Acad. Sci. U.S.A.* 104:19885-19890.

McRae BH, Dickson BG, Keitt TH, Shah VB. 2008. Using circuit theory to model connectivity in ecology, evolution and conservation. *Ecology* 89:2712-2724.

Milá, B, Wayne RK, Fitze P, Smith TB. 2009. Divergence with gene flow and fine-sclae phylogeographical structure in the wedge-billed woodcreeper, *Glyphorynchus spirurus*, a Neotropical rainforest bird. *Mol. Ecol.* 18:2979-2995.

Munshi-South J. 2012. Urban landscape genetics: canopy cover predicts gene flow between white-footed mouse (*Peromyscus leucopus*) populations in New York City. *Mol. Ecol.* 21:1360-1378.

Murphy MA, Dezzani R, Pilliod DS, Storfer A. 2010. Landscape genetics of high mountain frog metapopulations. *Mol. Ecol.* 19:3634-3649.

Mylecraine, KA, Bulgin NL, Gibbs HL, Vickery PD, Perkins DW. 2008. Limited genetic structure and evidence for dispersal among populations of the endangered Florida grasshopper sparrow, *Ammodramus savannarum floridanus*. *Conserv. Genet.* 9:1633-1638.

Nosil P, Vines TH, Funk DJ. 2005. Perspective: Reproductive isolation caused by natural selection against immigrants from divergent habitats. *Evolution* 59:705-719.

Oetjen K, Reusch TBH. 2007. Genome scans detect consistent divergent selection among subtidal *vs.* intertidal populations of the marine angiosperm *Zostera marina*. *Mol. Ecol.* 16:5156-5167.

Olano-Marin J, Dawson DA, Girg A, Hansson B, Ljungqvist M, Kempenaers B, Mueller JC. 2010. A genome-wide set of 106 microsatellite markers for the blue tit (*Cyanistes caeruleus*). *Mol. Ecol. Resour.* 10:516-532.

Olano-Marin J, Mueller JC, Kempenaers B. 2011a. Heterozygosity and survival in blue tits (*Cyanistes caeruleus*): contrasting effects of presumably functional and neutral loci. *Mol. Ecol.* 20:4028-4041.

Olano-Marin, J, Mueller JC, Kempenaers B. 2011b. Correlations between heterozygosity and reproductive success in the blue tit (*Cyanistes caeruleus*): an analysis of inbreeding and single locus effects. *Evolution* 65:3175-3194.

Orsini L, Vanoverbeke J, Swillen I, Mergeay J, De Meester L. 2013. Drivers of population genetic differentiation in the wild: isolation by dispersal limitation, isolation by adaptation and isolation by colonization. *Mol. Ecol.* 22:5983-5999.

Ortego J, García-Navas V, Ferrer ES, Sanz JJ. 2011. Genetic structure reflects natal dispersal movements at different spatial

scales in the blue tit, *Cyanistes caeruleus*. *Anim. Behav*. 82:131-137.

Ortego J, Gugger PF, Sork VL. 2015. Climatically stable landscapes predict patterns of genetic structure and admixture in the Californian canyon live oak. *J. Biogeogr*. 42:328-338.

Pavlova A, Amos JN, Goretskaia MI, Beme IR, Buchanan KL, Takeuchi N, Radford JQ, Sunnucks P. 2012. Genes and song: genetic and social connections in fragmented habitat in a woodland bird with limited dispersal. *Ecology* 93:1717-1727.

Peterman WE, Anderson TL, Ousterhout BH, Drake DL, Semlitsch RD, Eggert LS. 2015. Differential dispersal shapes population structure and patterns of genetic differentiation in two sympatric pond breeding salamanders. *Conserv. Genet*. 16:59-69.

Pflüger FJ, Balkenhol N. 2014. A plea for simultaneously considering matrix quality and local environmental conditions when analysing landscape impacts on effective dispersal. *Mol. Ecol*. 23:2146-2156.

Porlier M, Garant D, Perret P, Charmantier A. 2012a. Habitat-linked population genetic differentiation in the blue tit *Cyanistes caeruleus*. *J. Hered*. 103:781-791.

Porlier M, Charmantier A, Bourgault P, Perret P, Blondel J, Garant D. 2012b. Variation in phenotypic plasticity and selection patterns in blue tit breeding time: between- and within-population comparisons. *J. Anim. Ecol*. 81:1041-1051.

Postma E, van Noordwijk AJ. 2005. Gene flow maintains a large genetic difference in clutch size at a small spatial scale. *Nature* 443:65-68.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155:945-959.

Quéméré E, Crouau-Roy B, Rabarivola C, Jr Louis EE, Chikhi L. 2010. Landscape genetics of an endangered lemur (*Propithecus tattersalli*) within its entire fragmented range. *Mol. Ecol*. 19:1606-1621.

Raymond M, Rousset F. 1995. Genepop (Version-1.2) – Population-genetics software for exact tests and ecumenicism. *J. Hered*. 86:248-249.

Rice WR. 1989. Analyzing tables of statistical tests. *Evolution* 43:223-225.

Rosenberg NA. 2004. Distruct: a program for the graphical display of population structure. *Mol. Ecol. Notes* 4:137-138.

Sexton JP, Hangartner SB, Hoffmann AA. 2014. Genetic isolation by environment or distance: which pattern of gene flow is most common? *Evolution* 68:1-15.

Seymour M, Rasanen K, Holderegger R, Kristjansson BK. 2013. Connectivity in a pond system influences migration and genetic structure in threespine stickleback. *Ecol. Evol*. 3:492-502.

Shafer ABA, Wolf JBW. 2013. Widespread evidence for incipient ecological speciation: a meta-analysis of isolation by-ecology. *Ecol. Lett*. 16:940–950.

Slatkin M. 1993. Isolation by distance in equilibrium and non-equilibrium populations. *Evolution* 47:264-279.

Stamps JA, Krishnan VV, Willits NH. 2009. How different types of natal experience affect habitat preference. *Am. Nat*. 174:623-630

Storfer A, Murphy MA, Evans JS, Goldberg CS, Robinson S, Spear SF, Dezzani R, Delmelle E, Vierling L, Waits LP. 2007.

Putting the 'landscape' in landscape genetics. *Heredity* 98:128-142.

Storfer A, Murphy MA, Spear SF, Holderegger R, Waits LP. 2010. Landscape genetics: where are we now? *Mol. Ecol.* 19:3496-3514.

Thomassen HA, Cheviron ZA, Freedman AH, Harrigan RJ, Wayne RK, Smith TB. 2010. Spatial modelling and landscape-level approaches for visualizing intra-specific variation. *Mol. Ecol.* 19:3532-3548.

Tornero J. 2003. *Los Quintos de Mora*. Madrid: OAPN-Ministerio de Medio Ambiente.

Veit ML, Robertson RJ, Hamel PB, Friesen VL. 2005. Population genetic structure and dispersal across a fragmented landscape in cerulean warblers (*Dendroica cerulea*). *Conserv. Genet*. 6:159-174.

Wang IJ. 2013. Examining the full effects of landscape heterogeneity on spatial genetic variation: a multiple matrix regression approach for quantifying geographic and ecological isolation. *Evolution* 67:3403-3411.

Wang IJ, Bradburd G. 2014. Isolation by environment. *Mol. Ecol.* 23:5649-5662.

Wang IJ, Glor RE, Losos JB. 2013. Quantifying the roles of ecology and geography in spatial genetic divergence. *Ecol. Lett.* 16: 175-182.

Wang IJ, Savage WK, Shaffer HB. 2009. Landscape genetics and least-cost path analysis reveal unexpected dispersal routes in the California tiger salamander (*Ambystoma californiense*). *Mol. Ecol.* 18:1365-1374.

Wiens JA. 2001 The landscape context of dispersal. Pages 96–99 in J. Clobert, E. Danchin, A. A. Dhondt, J. D. Nichols, editors. *Dispersal.* New York, NY: Oxford University Press.

Wheelwright NT, Mauck RA. 1998. Philopatry, natal dispersal, and inbreeding avoidance in an island population of savannah sparrows. *Ecology* 79:755-767.

Wright S. 1945. Isolation by distance under diverse systems of mating. *Genetics* 30:571-572.

Zhao Y, Vrieling K, Liao H, Xiao M, Zhu Y, Rong J, Zhang W, Wang Y, Yang J, Chen J, Song Z. 2013. Are habitat fragmentation, local adaptation and isolation-by-distance driving population divergence in wild rice *Oryza rufipogon*? *Mol. Ecol.* 22:5531-5547.

SUPPORTING INFORMATION

Table S1 Panel of 26 microsatellite loci used to genotype blue tits. The following information is given: chromosome location in the zebra finch genome, category (neutral or functional), number of alleles (*K*), expected heterozygosity (H_E), observed heterozygosity (H_O), annealing temperature (T) and original reference or GenBank accession number for each locus.

Locus	Chromosome	Category	*K*	H_E	H_O	T (ºC)	Reference
PK11	Unassigned	Neutral	7	0.77	0.80	52	GenBank Acc. no.: AF041465
Pca3	4	Neutral	18	0.90	0.98	55	Dawson *et al.* 2000
Pca9	7	Neutral	11	0.71	0.70	62	Dawson *et al.* 2000
Pocc1	7	Neutral	11	0.86	0.93	55	Bensch *et al.* 1997
Pocc6	2	Neutral	16	0.85	0.73	62	Bensch *et al.* 1997
Pat-MP2-43	2	Neutral	7	0.48	0.45	59	Otter *et al.* 1998
PK12	5	Neutral	14	0.79	0.85	62	GenBank Acc. no.: AF041466
Ase18	3	Neutral	13	0.85	0.88	60	Richardson *et al.* 2000
Pdoμ5	4	Neutral	17	0.87	0.86	46	Griffith *et al.* 1999
Pca7	1	Neutral	13	0.88	0.88	60	Dawson *et al.* 2000
Pca4	8	Neutral	9	0.74	0.69	60	Dawson *et al.* 2000
Pca2	Unassigned	Neutral	9	0.76	0.73	60	Dawson *et al.* 2000
Mcyμ4	5	Neutral	13	0.83	0.84	50	Double *et al.* 1997
Pca8	2	Neutral	26	0.94	0.88	53	Dawson *et al.* 2000
CcaTgu28	23_random	Functional	8	0.64	0.67	60	Olano-Marin *et al.* 2010
PiJ14	7	Functional	12	0.88	0.89	60	Olano-Marin *et al.* 2010
TG05-053	5	Functional	8	0.75	0.74	55	Dawson *et al.* 2009
TG05-046	5	Functional	3	0.50	0.34	55	Dawson *et al.* 2009
CcaTgu15	5	Functional	4	0.66	0.82	60	Olano-Marin *et al.* 2010
Tg13-017	13	Functional	14	0.87	0.84	60	Dawson *et al.* 2010
CcaTgu19	10	Functional	21	0.92	0.96	60	Olano-Marin *et al.* 2010
CcaTgu11	3	Functional	6	0.74	0.71	60	Olano-Marin *et al.* 2010
CcaTgu8	2	Functional	3	0.58	0.52	63	Olano-Marin *et al.* 2010
Tgu07	6	Functional	6	0.68	0.76	55	Slate *et al.* 2007
CcaTgu14	5	Functional	13	0.78	0.73	55	Olano-Marin *et al.* 2010
CcaTgu7	2	Functional	5	0.72	0.59	55	Olano-Marin *et al.* 2010

Table S2 Pairwise F_{ST}-values estimated at all typed loci (functional and neutral) for the studied blue tit populations. Values in bold are statistically significant ($P < 0.05$) after sequential Bonferroni correction. Population codes are described in Table 1.

	ANC	BRE	CAZ	FCO	GIL	NAV	POS	VAL	MAR	AVE	ARR	CAN	FCE	PLA	ROB	FCA	ERM	FRI	FUB	MOR	CAS	MAJ	NVI	BAÑ	NAH
ANC	-																								
BRE	0.002	-																							
CAZ	0.004	0.004	-																						
FCO	0.004	0.003	0.000	-																					
GIL	0.005	0.003	0.002	0.003	-																				
NAV	0.005	**0.006**	0.003	0.004	**0.004**	-																			
POS	0.003	0.011	0.002	0.000	0.006	0.003	-																		
VAL	0.007	**0.007**	0.004	0.004	**0.005**	**0.005**	0.002	-																	
MAR	0.005	**0.007**	0.002	0.001	**0.004**	0.002	0.001	**0.004**	-																
AVE	0.006	0.007	0.004	0.003	0.005	0.006	0.008	**0.008**	0.003	-															
ARR	0.000	0.000	0.003	0.000	0.000	0.003	0.006	0.004	0.003	0.000	-														
CAN	0.002	0.001	0.001	0.004	0.003	0.002	0.000	0.002	0.003	0.006	0.001	-													
FCE	0.000	0.007	0.004	0.001	0.004	0.007	0.000	0.006	0.002	0.002	0.003	0.001	-												
PLA	0.009	0.013	0.005	0.004	0.009	0.010	0.010	0.013	0.007	0.002	0.008	0.011	0.003	-											
ROB	0.003	0.007	0.004	0.000	0.003	0.002	0.000	0.003	0.000	0.000	0.000	0.002	0.006	0.004	-										
FCA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-									
ERM	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.003	0.000	0.000	-								
FRI	0.001	0.000	0.002	0.001	0.000	0.000	0.008	0.001	0.001	0.005	0.000	0.000	0.004	0.004	0.005	0.003	0.000	-							
FUB	0.005	0.006	0.003	0.001	0.003	0.004	0.004	0.004	0.002	0.000	0.000	0.002	0.005	0.001	0.000	0.000	0.000	0.000	-						
MOR	0.003	0.004	0.002	0.000	0.002	0.003	0.003	**0.004**	0.001	0.002	0.001	0.001	0.001	0.006	0.000	0.000	0.000	0.000	0.001	-					
CAS	0.003	0.005	0.001	0.003	0.004	0.004	0.004	0.005	0.000	0.002	0.003	0.000	0.005	0.005	0.000	0.000	0.000	0.000	0.003	0.000	-				
MAJ	0.005	0.006	0.004	0.003	0.004	0.006	0.000	0.005	0.001	0.002	0.002	0.002	0.001	0.008	0.000	0.000	0.000	0.004	0.002	0.000	0.002	-			
NVI	0.007	0.009	0.001	0.006	0.005	0.005	0.003	0.002	0.001	0.006	0.000	0.005	0.004	0.011	0.000	0.000	0.000	0.001	0.001	0.001	0.000	0.004	-		
BAÑ	0.000	0.005	0.002	0.002	0.001	0.004	0.003	0.003	0.000	0.000	0.000	0.005	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.003	-	
NAH	0.014	0.017	0.009	0.005	0.010	0.009	0.007	0.009	0.006	0.011	0.009	0.017	0.006	0.010	0.000	0.000	0.007	0.016	0.001	0.007	0.010	0.000	0.000	0.000	-

Table S3 Pairwise F_{ST}-values estimated at neutral (below the diagonal) and functional (above the diagonal) for the studied blue tit populations. Values in bold are statistically significant ($P < 0.05$) after sequential Bonferroni correction. Population codes are described in Table 1.

	ANC	BRE	CAZ	FCO	GIL	NAV	POS	VAL	MAR	AVE	ARR	CAN	FCE	PLA	ROB	FCA	ERM	FRI	FUB	MOR	CAS	MAJ	NVI	BAÑ	NAH
ANC	-	0.000	0.002	0.000	0.001	0.002	0.001	0.007	0.003	0.003	0.000	0.000	0.009	0.001	0.000	0.000	0.000	0.000	0.001	0.000	0.002	0.001	0.007	0.005	0.025
BRE	0.006	-	0.004	0.002	0.000	0.003	0.009	0.007	**0.007**	0.009	0.000	0.000	0.009	0.014	0.010	0.000	0.000	0.000	0.008	0.001	0.006	0.006	0.013	0.010	0.032
CAZ	0.005	0.004	-	0.000	0.004	0.004	0.000	0.005	0.002	0.006	0.004	0.000	0.009	0.004	0.009	0.000	0.000	0.002	0.005	0.001	0.002	0.004	0.002	0.009	0.025
FCO	0.007	0.004	0.000	-	0.002	0.002	0.000	0.003	0.000	0.000	0.000	0.000	0.002	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.003	0.001	0.009	0.006	0.016
GIL	**0.008**	**0.006**	0.001	0.003	-	0.003	0.004	**0.006**	**0.005**	0.007	0.000	0.000	0.007	0.009	0.006	0.000	0.000	0.000	0.002	0.001	0.006	0.003	0.009	0.006	0.022
NAV	0.007	**0.009**	0.002	0.006	**0.005**	-	0.001	0.004	0.002	0.008	0.000	0.001	0.011	0.007	0.004	0.000	0.000	0.000	0.001	0.000	0.005	0.004	0.008	0.007	0.015
POS	0.005	0.011	0.006	0.003	0.007	0.005	-	0.000	0.000	0.006	0.006	0.001	0.007	0.000	0.000	0.000	0.000	0.011	0.000	0.000	0.000	0.000	0.000	0.007	0.010
VAL	0.007	**0.008**	0.003	0.004	**0.004**	**0.007**	0.003	-	**0.005**	**0.014**	0.006	0.000	0.015	0.015	0.007	0.000	0.003	0.000	0.003	0.003	0.008	0.006	0.008	0.013	0.019
MAR	0.006	**0.006**	0.002	0.002	**0.004**	0.003	0.004	**0.004**	-	0.004	0.000	0.001	0.009	0.006	0.002	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.001	0.005	0.015
AVE	0.008	0.006	0.002	0.005	0.004	0.004	0.009	0.003	0.002	-	0.000	0.008	0.007	0.000	0.000	0.000	0.004	0.011	0.000	0.003	0.004	0.000	0.019	0.001	0.021
ARR	0.006	0.000	0.002	0.004	0.001	0.007	0.005	0.003	0.006	0.000	-	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.001	0.011	0.000	0.012
CAN	0.006	0.003	0.004	0.009	0.005	0.003	0.000	0.005	0.004	0.004	0.001	-	0.000	0.005	0.010	0.000	0.000	0.000	0.000	0.000	0.004	0.003	0.006	0.014	0.028
FCE	0.000	0.003	0.000	0.000	0.001	0.004	0.000	0.000	0.000	0.000	0.005	0.002	-	0.000	0.015	0.000	0.005	0.010	0.007	0.006	0.014	0.008	0.006	0.004	0.014
PLA	0.016	0.012	0.005	0.007	0.009	0.012	0.018	0.011	0.007	0.004	0.016	0.015	0.006	-	0.005	0.000	0.000	0.006	0.001	0.009	0.006	0.002	0.015	0.005	0.019
ROB	0.006	0.004	0.001	0.000	0.001	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.003	-	0.000	0.006	0.018	0.000	0.004	0.002	0.000	0.006	0.001	0.006
FCA	0.005	0.005	0.001	0.000	0.002	0.000	0.000	0.006	0.000	0.003	0.003	0.000	0.000	0.003	0.000	-	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000
ERM	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.006	0.000	0.000	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.014
FRI	0.004	0.000	0.002	0.001	0.001	0.006	0.006	0.003	0.003	0.001	0.000	0.000	0.001	0.004	0.000	0.003	0.000	-	0.000	0.000	0.000	0.003	0.003	0.012	0.033
FUB	0.008	0.005	0.001	0.004	0.003	**0.006**	0.008	**0.004**	0.003	0.001	0.000	0.004	0.003	0.002	0.000	0.002	0.000	0.000	-	0.003	0.005	0.000	0.006	0.001	0.005
MOR	0.006	0.006	0.002	0.003	**0.004**	**0.006**	0.005	**0.004**	0.002	0.002	0.003	0.005	0.000	0.004	0.000	0.000	0.000	0.001	0.000	-	0.000	0.000	0.002	0.003	0.022
CAS	0.004	0.005	0.000	0.004	0.003	0.003	0.011	0.003	0.000	0.001	0.003	0.000	0.000	0.005	0.000	0.000	0.002	0.000	0.001	0.002	-	0.001	0.000	0.012	0.022
MAJ	0.008	0.006	0.004	0.005	0.004	**0.007**	0.002	0.005	0.002	0.003	0.003	0.002	0.000	0.012	0.000	0.000	0.000	0.005	0.004	0.001	0.003	-	0.007	0.001	0.009
NVI	0.007	0.005	0.001	0.004	0.002	0.003	0.010	0.000	0.002	0.000	0.000	0.004	0.003	0.007	0.000	0.009	0.000	0.000	0.000	0.001	0.002	0.002	-	0.014	0.012
BAÑ	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-	0.002
NAH	0.006	0.005	0.000	0.000	0.000	0.004	0.005	0.001	0.000	0.003	0.007	0.008	0.000	0.004	0.000	0.000	0.002	0.005	0.000	0.000	0.000	0.000	0.000	0.000	-

Table S4 Multiple Matrix Regressions with Randomization (MMRR) for genetic differentiation (F_{ST}) estimated for the subset of functional loci in relation with 9 isolation-by-resistance (IBR) scenarios considering the minimum resistance value (resistance = 1) to lands mostly devoted to agriculture and high resistance values (ranging from 10 to 100000) for forests and non-forested natural vegetation habitats.

Forest	Non-forested vegetation	Agricultural lands	R^2	β	*P*
10	10	1	0.055	0.383	0.059
25	25	1	0.062	0.404	0.067
50	50	1	0.064	0.412	0.053
100	100	1	0.066	0.416	0.055
250	250	1	0.067	0.419	0.047
500	500	1	0.068	0.421	0.046
1000	1000	1	0.068	0.422	0.057
10000	10000	1	0.068	0.423	0.045
100000	100000	1	0.068	0.423	0.053

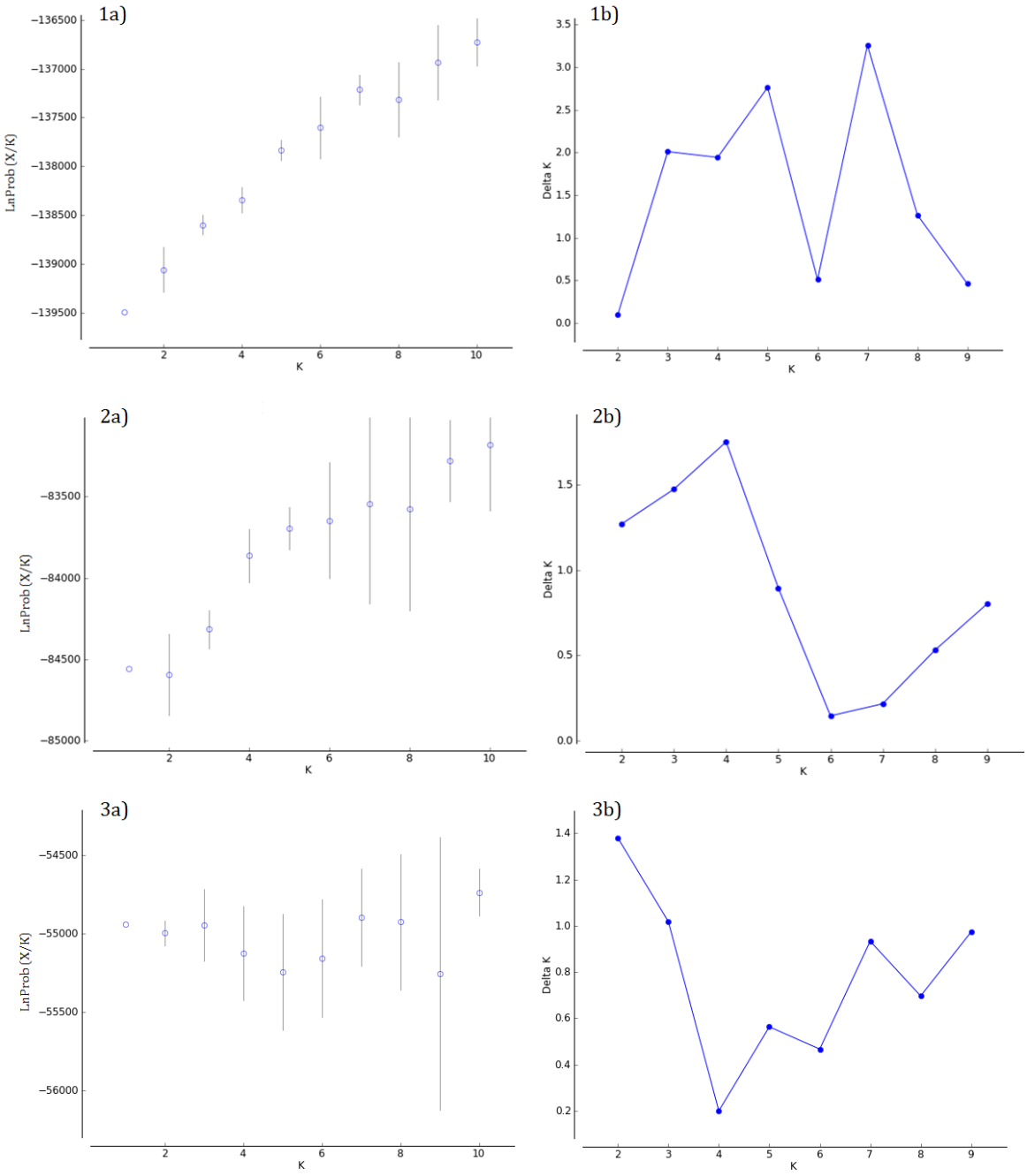


Figure S1 Results of Bayesian clustering analyses in STRUCTURE. Plots show (a) the mean (± SD) log probability of the data (ln Pr(X | *K*)) over 10 runs for each value of *K* from 1 to 10 (1a: all markers; 2a: neutral markers; 3a: functional markers) and (b) the Δ*K* statistic (1b: all markers; 2b: neutral markers; 3b: functional markers).

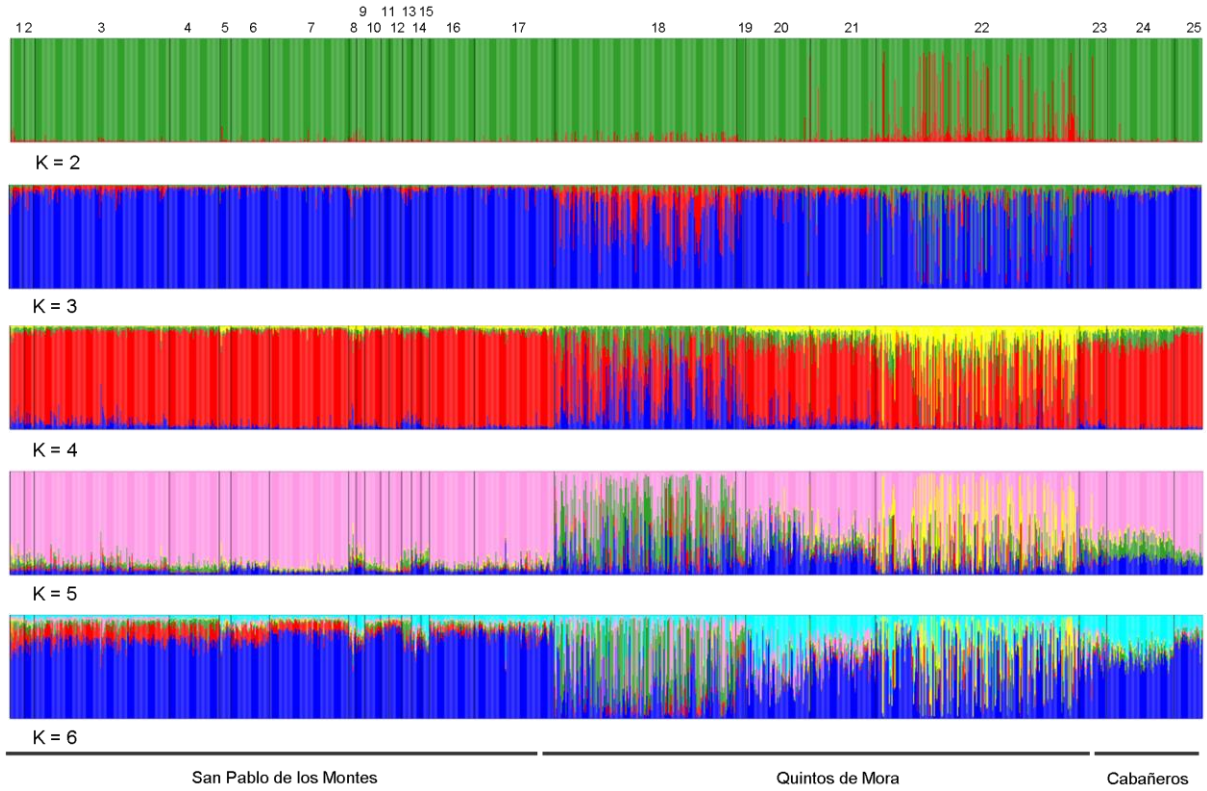


Figure S2 Results of genetic assignments of blue tits (n = 1385 individuals) based on the Bayesian method implemented in the program STRUCTURE considering all the markers (n = 26) and K = 2-6. Each individual is represented by a thin vertical line, which is partitioned into coloured segments that represent the individual's probability of belonging to the cluster with that colour. Individuals are grouped according to their populations of origin using vertical black lines. Population codes are indicated in Table 1.

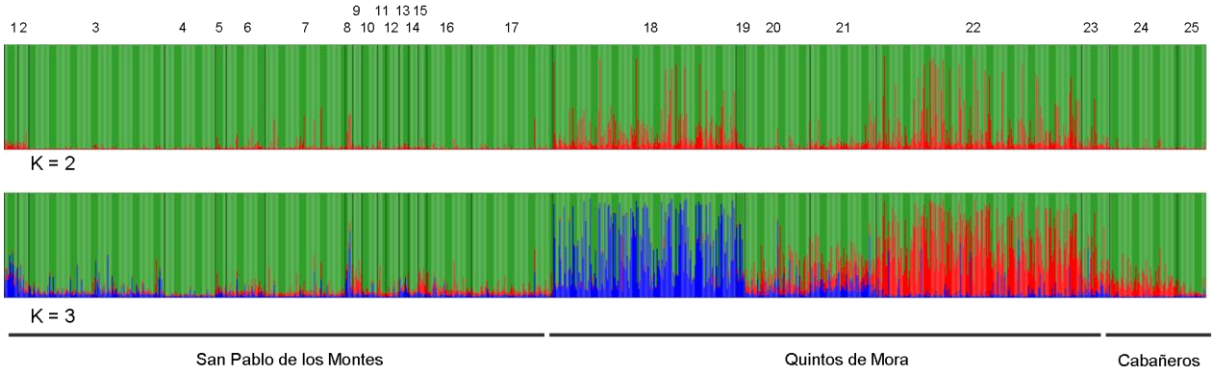


Figure S3 Results of genetic assignments of blue tits (n = 1385 individuals) based on the Bayesian method implemented in the program STRUCTURE considering all the subset of neutral markers (n = 14) and K = 2-3. Each individual is represented by a thin vertical line, which is partitioned into coloured segments that represent the individual's probability of belonging to the cluster with that colour. Individuals are grouped according to their populations of origin using vertical black lines. Population codes are indicated in Table 1.

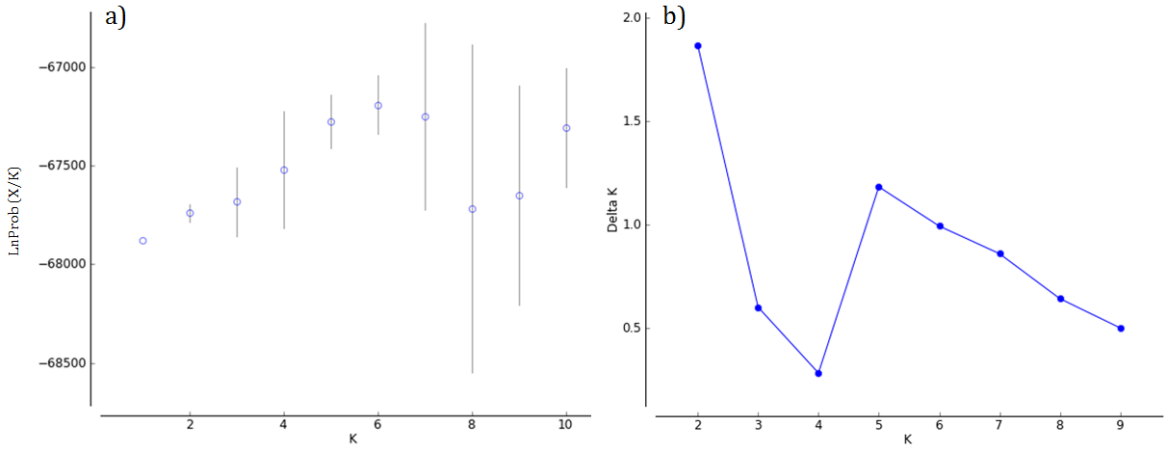


Figure S4 Results of Bayesian clustering analyses in STRUCTURE for the subset of neutral markers excluding the two most polymorphic markers (*Pca3* and *Pca8*). Plots show (a) the mean (± SD) log probability of the data (ln Pr(X | *K*)) over 10 runs for each value of *K* from 1 to 10 (b) the Δ*K* statistic.

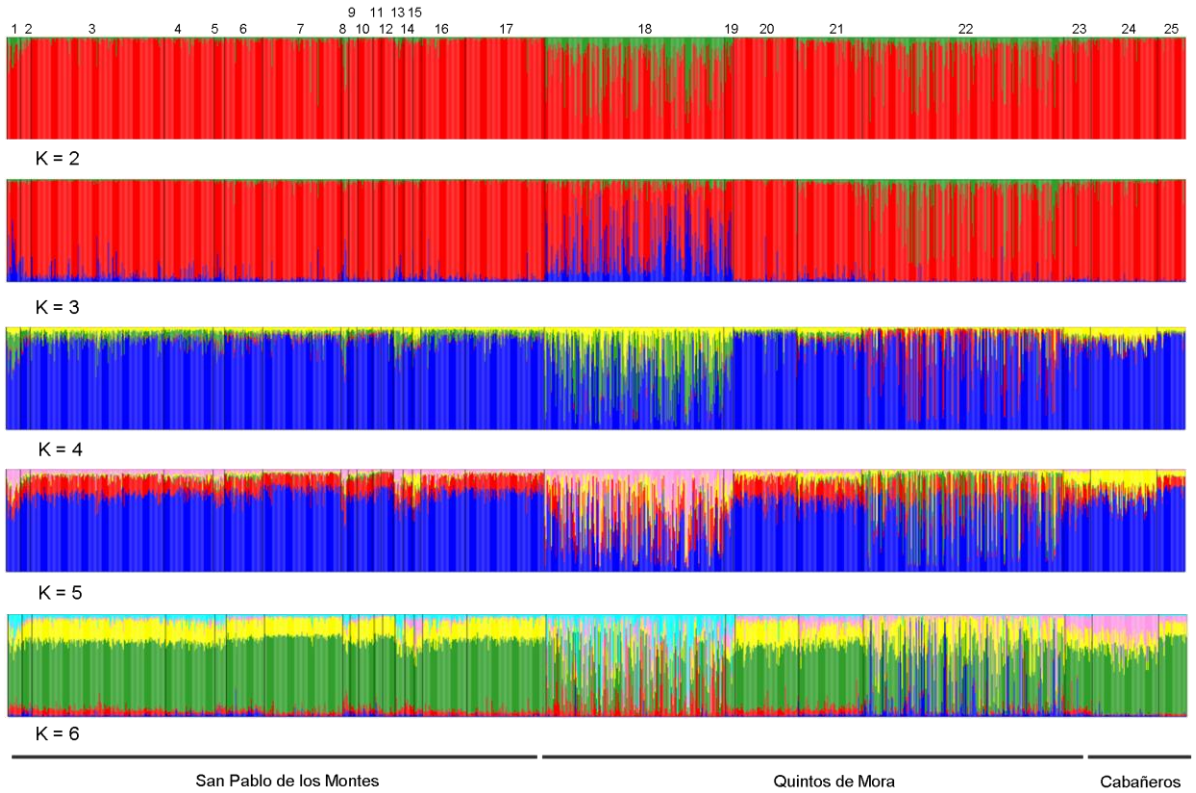


Figure S5 Results of genetic assignments of blue tits (*n* = 1385 individuals) based on the Bayesian method implemented in the program STRUCTURE considering the subset of neutral markers excluding the two most polymorphic markers (*Pca3* and *Pca8*) and *K* = 2-6. Each individual is represented by a thin vertical line, which is partitioned into coloured segments that represent the individual's probability of belonging to the cluster with that colour. Individuals are grouped according to their populations of origin using vertical black lines. Population codes are indicated in Table 1.

SUPPLEMENTARY REFERENCES

Bensch S, Price T, Kohn J. 1997. Isolation and characterization of microsatellite loci in a *Phylloscopus* warbler. *Mol. Ecol.* 6: 91-92.

Dawson DA, Chittock J, Jehle R, Whitlock A, Nogueira D, Pellatt J, Birkhead T, Burke T. 2005. Identification of 13 polymorphic microsatellite loci in the zebra finch, *Taeniopygia guttata* (Passeridae, Aves). *Mol. Ecol. Notes* 5: 298-301.

Dawson DA, Horsburgh GJ, Küpper C, Stewart IRK, Ball AD, Durrant KL, Hansson B, Bacon I, Bird S, Klein A, Krupa AP, Lee JW, Martín-Gálvez D, Simeoni M, Smith G, Spurgin LG, Burke T. 2010. New methods to identify conserved microsatellite loci and develop primer sets of high cross-species utility - as demonstrated for birds. *Mol. Ecol. Resour.* 10: 475-494.

Double MC, Dawson D, Burke T, Cockburn A. 1997. Finding the fathers in the least faithful bird: A microsatellite-based genotyping system for the superb fairy-wren *Malurus cyaneus*. *Mol. Ecol.* 6: 691-693.

Griffith SC, Stewart IRK, Dawson DA, Owens IPF, Burke T. 1999. Contrasting levels of extra-pair paternity in mainland and island populations of the house sparrow (*Passer domesticus*): is there an "island effect"? *Biol. J. Linn. Soc.* 68: 303-316.

Olano-Marin J, Dawson DA, Girg A, Hansson B, Ljungqvist M, Kempenaers B, Mueller JC. 2010. A genome-wide set of 106 microsatellite markers for the blue tit (*Cyanistes caeruleus*). *Mol. Ecol. Resour.* 10: 516-532.

Otter K, Ratcliffe L, Michaud D, Boag PT. 1998. Do female black-capped chickadees prefer high-ranking males as extra-pair partners? *Behav. Ecol. Sociobiol.* 43: 25-36.

Richardson DS, Jury FL, Dawson DA, Salgueiro P, Komdeur J, Burke T. 2000. Fifty Seychelles warbler (*Acrocephalus sechellensis*) microsatellite loci polymorphic in Sylviidae species and their cross-species amplification in other passerine birds. *Mol. Ecol.* 9: 2226-2231.

Slate J, Hale M, Birkhead TR. 2007. Simple sequence repeats in zebra finch (*Taeniopygia guttata*) expressed sequence tags: a new resource for evolutionary genetic studies of passerines. *BMC Genom.* 8: 52.

3. Molecular characterization of avian malaria parasites in three Mediterranean blue tit (*Cyanistes caeruleus*) populations

Ferrer ES, García-Navas V, Sanz JJ and Ortego J

Parasitology Research, 111: 2137-2142 (2012)

Molecular characterization of avian malaria parasites in three Mediterranean blue tit (*Cyanistes caeruleus*) populations

Abstract

We genetically analyzed malaria parasites (Protozoa) in three Mediterranean blue tit (*Cyanistes caeruleus*) populations from central Spain. A total of 853 breeding individuals were screened for parasites of the genera *Plasmodium* and *Haemoproteus* using a very efficient polymerase chain reaction (PCR) approach that amplifies a partial segment of the mitochondrial cytochrome *b* gene of these parasites. We have found six lineages of *Plasmodium* (SGS1, GRW11, COLL1, DELURB4, GRW04, and BLUTI10) parasitizing the studied populations but we did not detect any infection by *Haemoproteus*. One of the detected lineages (BLUTI10) has not been previously described in any bird species and this is the first study recording lineages DELURB4 and GRW04 in blue tits. SGS1 (belonging to the morphospecies *Plasmodium relictum*) was the most frequent lineage (overall prevalence 24 %), whereas the other lineages showed a much lower prevalence (<4 %). Only a small proportion (12.2 %) of positive amplifications of the most common lineage (SGS1) was detected in blood smears using light microscopy and infection intensities were very low (mean ± SE, 2.0 ± 1.4 parasites/2000 erythrocytes).We have also found strong inter-population variability in prevalence patterns (12-41% for lineage SGS1), suggesting important differences in parasite transmission rates among the geographically close studied localities.

INTRODUCION

Avian malaria is a mosquito-borne disease caused by parasites in the genus *Plasmodium* and *Haemoproteus* (*sensu* Pérez-Tris *et al.* 2005). These parasites have been widely used as study system to understand the dynamics of infectious diseases (e.g. Wood *et al.* 2007; Knowles *et al.* 2011; Lachish *et al.* 2011a) and their consequences in wild populations (e.g. Ortego *et al.* 2008; Martínez-de la Puente *et al.* 2010; Lachish *et al.* 2011b; Shurulinkow *et al.* 2012). The application of molecular methods to the study of these parasites has opened up the possibility of detecting genetically different lineages, further increasing the interest for this host-parasite system in recent years (Bensch *et al.* 2000; Pérez-Tris *et al.* 2005). For instance, molecular tools have revealed that the number of avian malaria species is much higher than previously thought and that host switching is extensive (e.g. Bensch *et al.* 2000; Ortego *et al.* 2007; Illera *et al.* 2008; Valkiunas *et al.* 2009).

The blue tit (*Cyanistes caeruleus*) is a model species that has been extensively studied in the fields of ecology, behaviour and evolution. Genetic characterization of avian malaria in this species has revealed the presence of several lineages of the genus

Plasmodium and *Haemoproteus* (Bensch *et al.* 2009; Szöllösi *et al.* 2011; Fig. 1). Some studies have also found that the two most widespread lineages (the *Plasmodium* SGS1 and the *Haemoproteus* PARUS1) have negative fitness consequences in this species (Martínez-de la Puente *et al.* 2010; Lachish *et al.* 2011a). The factors determining malaria prevalence and infection dynamics at a local spatial scale have been also recently studied, suggesting complex patterns that depend on both habitat and host characteristics (Shurulinkow & Chakarov 2006; Wood *et al.* 2007; Knowles *et al.* 2011; Lachish *et al.* 2011a). A recent study analyzing nine blue tit populations across Europe has reported strong differences among populations in parasite lineage composition, indicating that their transmission success is site specific (Szöllösi *et al.* 2011).

In spite of the ecological and genetic distinctiveness of blue tit populations from southern Europe (Blondel *et al.* 2006; Illera *et al.* 2011; García-Navas & Sanz 2011), avian malaria parasites have been only genetically characterized in a single population located at these latitudes and no information is available for any genuine Mediterranean population (Szöllösi *et al.* 2011). In the present study we characterize avian malaria in three Mediterranean blue tit populations from Montes de Toledo (central Spain), an area located close to the southern edge of the species distribution range (Illera *et al.* 2011). In the Mediterranean region, habitat fragmentation due to both natural and anthropogenic processes has resulted in mosaic landscapes (Blondel & Aronson 1999). In Montes de Toledo, prevailing climatic conditions have favoured the predominance of vegetation with low water requirements, while the deciduous oak woodlands preferred by blue tits are restricted to the most humid environments such as valleys and riverbanks (Blanco *et al.* 1997). This has probably contributed to increase population fragmentation and reduce dispersal and gene flow in comparison with other European blue tit populations (Ortego *et al.* 2011). For this reason, we predict that spatial heterogeneity in malaria transmission rates results in strong differences in parasite prevalence among the studied populations, a pattern which can be compared with that reported for populations studied across a much larger geographical scale (Szöllösi *et al.* 2011).

MATERIALS AND METHODS

The study was conducted in three localities of central Spain, Quintos de Mora (Toledo province, 39º25´N 4º04´W; 2008-2011 breeding seasons), San Pablo de los Montes (Toledo province, 39º31´N 4º21´W; 2011 breeding season), and Cabañeros National Park (Ciudad Real province, 39°24´N, 3°35´W; 2008 breeding season) (Fig. 1). The study area comprises deciduous forests dominated by Pyrenean oak *Quercus pyrenaica* and Mediterranean scrublands. Adult birds were captured when feeding 8 days old chicks by means of a spring trap shutting the entrance hole as the bird entered the nest. All birds were individually marked

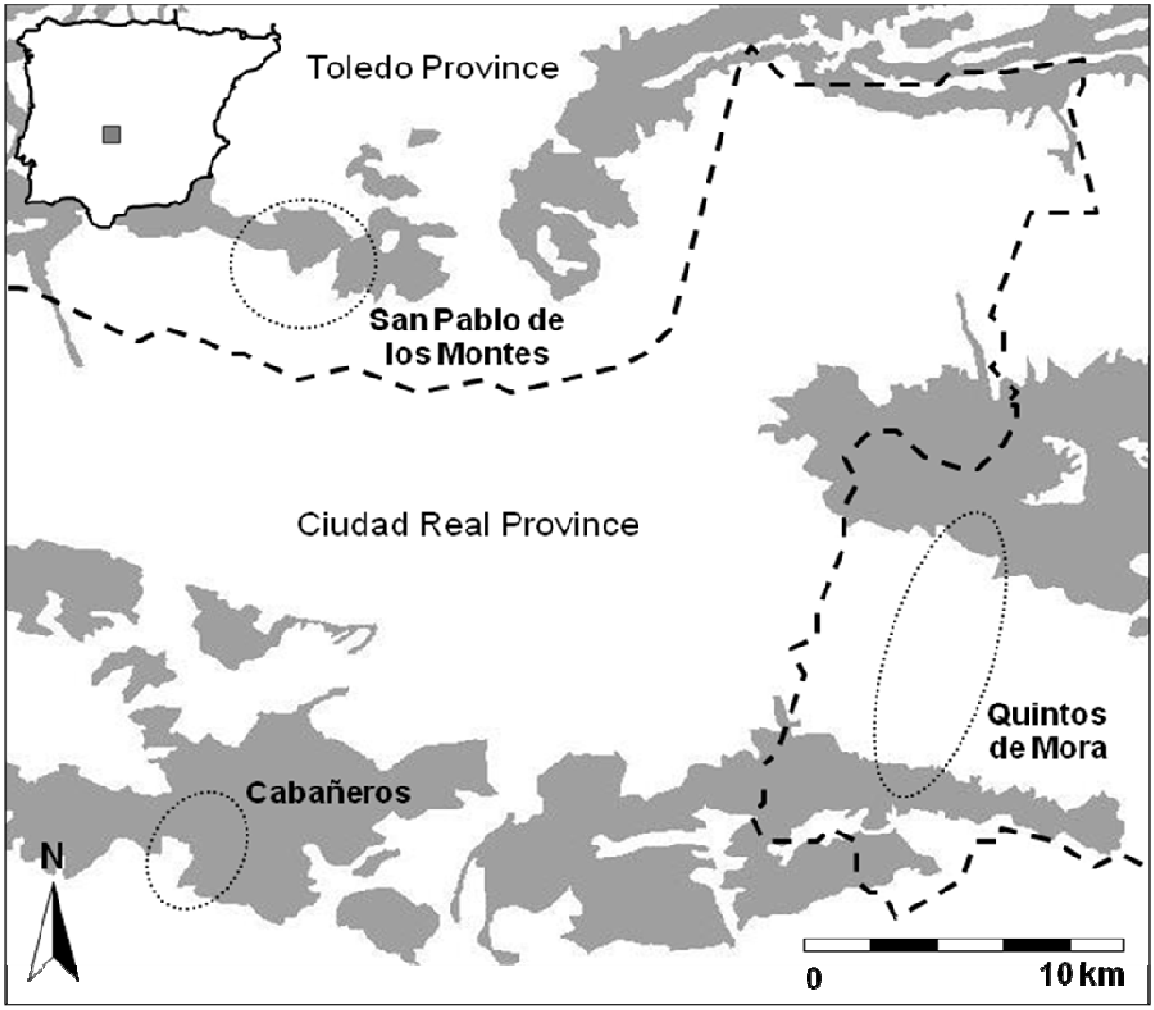


Figure 1 Map of the study area showing main woodlands (shaded areas) and the spatial location of the studied blue tit populations (dotted areas).

with aluminium rings for further identification. Blood samples (≤ 25 µl) for genetic analyses were obtained by brachial venipuncture and stored in ethanol 96 %.

We used NucleoSpin Tissue Kits (Macherey-Nagel) to extract and purify genomic DNA from the blood samples. A total of 853 adult individuals were screened for malaria infection using a highly efficient nested PCR that amplifies 480 bp of the cytochrome b of both *Plasmodium* and *Haemoproteous* parasites (Waldenström *et al.* 2004). We used negative controls (i.e. samples with ddH_2O instead of genomic DNA as template) and positive controls (i.e. DNA from individuals with known malarial infections) to ascertain that the outcome of each PCR run was not affected by contamination (Waldenström *et al.* 2004). Further, negative infections were confirmed by repeated PCR. Positive or negative infections (i.e. birds having or not gametocytes or merozoites in their blood stream) were scored separating PCR products on 2 % agarose gels. PCR

products from positive samples were purified using NucleoSpin Extract II (Macherey-Nagel) kits and sequenced on an ABI 310 Genetic Analyser (Applied Biosystems).

Sequences were edited and aligned using the program SEQUENCHER 5.0 (GeneCodes Corporation). A sequence divergence of at least one nucleotide was used to define lineages (Waldeström *et al.* 2004). Chromatograms were examined for conspicuous overlapping peaks indicative of co-infection (e.g. Wood *et al.* 2007). The obtained sequences were compared to the MalAvi database (Bensch *et al.* 2009) and by NCBI Blast to those other published sequences available from GenBank. Then, a phylogenetic tree was constructed in the program MEGA 3.1 using a neighbour-joining method with a Kimura two-parameter distance matrix (Kumar *et al.* 2001). Node support was tested using 1000 bootstrap replications. For comparison, we also included in this phylogenetic analysis all the avian malaria lineages (*Plasmodium* and *Haemoproteus*) previously isolated from blue tits according to the MalAvi database (Bensch *et al.* 2009) and other recent published studies (Szöllösi *et al.* 2011; Lachish *et al.* 2011a). The tree was rooted using a sequence of *Leucocytozoon* sp. also isolated in blue tits (lineage Parus4; GenBank accession number: AY393795).

For a subset of the blue tits captured in 2011 breeding season (San Pablo de los Montes: 280 individuals; Quintos de Mora: 37 individuals), we compared the estimates of prevalence based on the PCR approach with ocular examinations of blood smears. For this purpose, we smeared a drop of blood on an individually marked microscope slide. Blood smears were rapidly air dried, fixed with absolute ethanol and later stained in the laboratory with Giemsa's solution (1:10) for 45 min. At least 10000 erythrocytes per slide were examined at 1000 X magnification under oil immersion to determine presence of blood parasites. In positive smears, we estimated infection intensity as number of parasites/2 000 erythrocytes (Ortego & Espada 2007). Determination of prevalence and intensity of infection was carried out by the same person (E.S. Ferrer), who had no information about the origin of the samples except ring number of analyzed birds.

RESULTS AND DISCUSSION

We have identified six lineages of avian malaria infecting blue tits in the studied populations (Fig. 2). Two of these lineages (SGS1 and GRW11) belong to the morphospecies *Plasmodium relictum* (Lachish *et al.* 2011a) and have been previously found parasitizing several other blue tit populations (Fig. 2) and more than 50 bird species from 16 families worldwide distributed (MalAvi database). COLL1 is a less frequent lineage that has been recently found parasitizing another blue tit population from Austria (Szöllösi *et al.* 2011). Lineages DELURB4 and GRW04 have not been previously recorded parasitizing blue tits but they have been

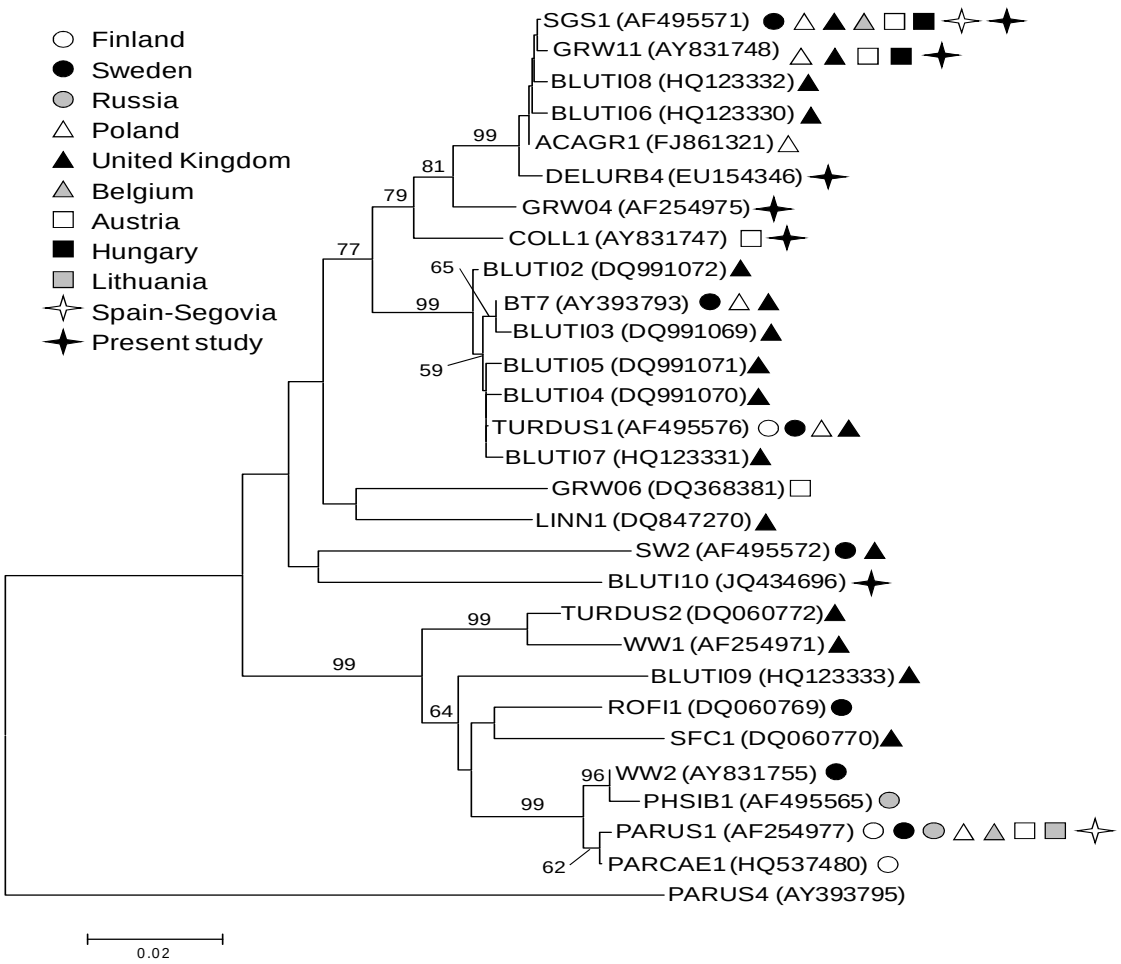


Figure 2 Neighbour joining tree (Kimura two-parameter distance) of avian malaria lineages infecting blue tits based on partial sequences of the cytochrome *b* gene. Occurrence of these lineages in blue tit populations from different European countries is indicated. We used a sequence from *Leucocytozoon* sp. isolated from a blue tit (lineage PARUS4) as an outgroup to root the tree. Bootstrap values are based on 1000 replicates and are shown when larger than 50. GenBank accession numbers of each isolate are shown in parentheses.

detected in several other passerines (MalAvi database). Finally, lineage BLUTI10 has not been hitherto isolated from any bird species. The sequence for this lineage has been deposited in the GenBank International Nucleotide Sequence Database with accession number JQ434696. Based on the occurrence of conspicuous double peaks in electropherograms, we detected three individuals carrying mixed infections that always involved the lineages SGS1 and COLL1 (Table 1). It is noticeable the absence of infections by *Haemoproteus*, despite lineage PARUS1 within this genus reaches 100% prevalence in a close Iberian population (Szöllösi *et al.* 2011). The blue tit is a sedentary species and shows very low dispersal rates in the study area (Ortego *et al.* 2011), indicating that all the detected lineages must be locally transmitted.

Sequence divergence between DELURB4 and SGS1-GRW11 is very low (<0.4 %, corresponding to 2bp

Table 1 Prevalence (%) of the different malaria lineages (the percentage of infected individuals in relation to all screened individuals in a population) detected in the three study populations. Absolute number of infected individuals with each lineage is shown in parentheses. [a]Chromatograms showing conspicuous overlapping peaks indicating co-infection with the lineages SGS1 and COLL1.

	Quintos de Mora					Cabañeros	San Pablo de los Montes
Year	2008 (*n* = 121)	2009 (*n* = 149)	2010 (*n* = 99)	2011 (*n* = 93)	Years combined	2008 (*n* = 111)	2011 (*n* = 280)
SGS1	19.8 (24)	16.1 (24)	8.1 (8)	20.4 (19)	16.2 (75)	11.7 (13)	41.4 (116)
GRW11	1.7 (2)	0.0 (0)	0.0 (0)	1.1 (1)	0.6 (3)	2.7 (3)	3.9 (11)
COLL1	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.4 (1)
DELURB4	0.8 (1)	0.0 (0)	0.0 (0)	2.2 (2)	0.6 (3)	0.0 (0)	2.1 (6)
GRW04	0.8 (1)	0.0 (0)	0.0 (0)	0.0 (0)	0.2 (1)	0.0 (0)	0.0 (0)
BLUTI10	0.8 (1)	0.0 (0)	0.0 (0)	0.0 (0)	0.2 (1)	0.9 (1)	0.4 (1)
SGS1+COLL1 [a]	0.8 (1)	0.0 (0)	0.0 (0)	0.0 (0)	0.2 (1)	0.9 (1)	0.4 (1)

synonymous substitutions), suggesting that DELURB4 may also belong to *P. relictum* (Bensch *et al.* 2004). Phylogenetic analyses placed GRW04 and COLL1 in the main clade including *P. relictum* (Fig. 2) (see also Zehtindjiev *et al.* 2008). These lineages have sequence divergences with SGS1-GRW11 (2-3%) similar to those previously reported among reproductively isolated avian malaria parasites, suggesting that they could be independent evolutionary units (Bensch *et al.* 2004). A previous study has also reported that lineages SGS1 and GRW11 occur at much lower parasitemia than GRW04 within the same host species, indicating its ecological distinctiveness (Zehtindjiev *et al.* 2008). Finally, phylogenetic analyses placed lineage BLUTI10 in a different clade (Fig. 2). Lineage BLUTI10 probably belongs to a different species according to the sequence divergences (6-8 %) with the other lineages detected in the studied populations (Bensch *et al.* 2004).

As found in previous studies, the ability of microscope examinations to detect haemosporidian infections was very low compared with the nested PCR approach (Waldenström *et al.* 2004; Ortego *et al.* 2007). No positive amplification for the lineages COLL1 and GRW04 was detected in blood smears using light microscopy (Table 1). Only 12.2 % of positive amplifications of lineage SGS1 were also positive by microscopic examination of blood smears and infection intensities were very low (mean ± S.E. = 2.0 ± 1.4 parasites/2000 erythrocytes; *n* = 16). Two individuals infected with the lineages GRW11 (16.7 %; mean ± S.E. = 2.3 ± 1.1 parasites/2000 erythrocytes) and

DELURB4 (33 %; mean ± S.E. = 1.0 ± 0.0 parasites/2000 erythrocytes) also showed infections detectable by traditional microscopic methods. The single individual infected with lineage BLUETI10 that was also examined at blood smears had detectable infections by microscopic examination (5.0 parasites / 2000 erythrocytes).

Prevalence strongly differed among the detected lineages (Table 1). SGS1 was the most frequent lineage, whereas other lineages showed a much lower prevalence (<4 %) (Table 1). Considering that lineages GRW11-DELURB4 probably belong to the same species that SGS1, the lower frequency of the former could just reflect genetic bottlenecks or other demographic phenomena (Vardo & Schall 2008). The fact that the other recovered lineages (COLL1, BLUTI10, and GRW04) have been only detected in a few individuals suggests that they may represent sporadic infections by malaria lineages that mainly depend on other hosts species (e.g. Szöllösi *et al.* 2011). Another possibility is that a high virulence of these lineages causes high mortality rates among infected individuals during the acute phase, thus, reducing the observed prevalence as a result of only live birds are sampled (Ortego *et al.* 2008). Probability of parasitism combining all the studied lineages strongly differed among study populations (General Linear Model, GLM: $F_{1, 850} = 13.38$; $P < 0.001$) and years ($F_{1, 850} = 3.86$; $P = 0.009$). A similar pattern was found only considering individuals infected with the most frequent lineage SGS1 (area: $F_{1, 850} = 11.12$; $P < 0.001$; year: $F_{2, 850} = 2.55$; $P = 0.055$). Post-hoc Tukey tests showed that probability of parasitism was significantly different between San Pablo de Los Montes and the other studied localities both considering all the lineages combined and only SGS1 (all *P*-values < 0.001). However, we found no significant differences between Cabañeros and Quintos de Mora (*P*-values > 0.5). This inter-population variability could be due to different landscape features and /or climate among the studied localities that may strongly influence vector abundance (Surulinkow & Chakarov 2006; Ortego *et al.* 2007; Shurulinkow & Ilieva 2009). These differences may be also maintained by the low dispersal and high isolation of the studied blue tit populations (Ortego *et al.* 2011; see also Illera *et al.* 2008). Given that Cabañeros and San Pablo de los Montes were only sampled during one breeding season (2008 and 2011, respectively), we repeated the analyses only comparing these populations with Quintos de Mora within the same study year. We found significant differences between Quintos de Mora and San Pablo de los Montes (all lineages: $F_{1, 371} = 15.17$; $P < 0.001$; lineage SGS1: $F_{1, 371} = 18.91$; $P < 0.001$), but not between Quintos de Mora and Cabañeros (all lineages: $F_{1, 230} = 2.93$; $P = 0.088$; lineage SGS1: $F_{1, 230} = 2.99$; $P = 0.085$). We also found inter-annual differences in probability of parasitism within Quintos de Mora (all lineages: $F_{1, 459} = 4.77$; $P = 0.003$; lineage SGS1: $F_{1, 459} = 3.20$; $P = 0.023$). Post-hoc Tukey tests showed that this pattern was driven by the lower probability of

parasitism in 2010 in comparison with 2008 and 2011 breeding seasons (Table 1). Inter-annual variation of avian malaria prevalence has been observed in other studies and may be consequence of temporal fluctuation of vector abundance or due to the different origin of immigrant individuals in different years (see Surulinkow and Ilieva 2009 and references therein).

Overall, we have detected six lineages of avian malaria infecting the studied blue tit populations. All these lineages showed very low levels of parasitemia that cannot be efficiently detected using traditional microscopic examination of blood smears. We have also found strong spatial variability in infection patterns among geographically close localities, indicating that these fragmented populations constitute an ideal study system to analyze the factors determining the patterns of avian malaria transmission at the landscape scale.

ACKNOWLEDGEMENTS

We wish to thank Pedro J. Cordero for allowing us to carry out the genetic analyses in his laboratory and Javier Bueno and Rafael Barrientos for their help during sample collection at San Pablo de los Montes locality. We thank the board of the Cabañeros National Park, Centro Quintos de Mora and the Council of San Pablo de Los Montes for the facilities offered during the fieldwork. This study was funded by grants 69/2003 (Organismo Autónomo de Parques Nacionales-Ministerio de Medio Ambiente), GCL2007-61395 (Ministerio de Educación y Ciencia), CGL2010-21933-C02-01 (Ministerio de Ciencia e Innovación) and POIC10-0269-7632 (Junta de Comunidades de Castilla-La Mancha-European Social Fund). ESF was supported by a pre-doctoral fellowship from Junta de Comunidades de Castilla-La Mancha-European Social Fund and VGN enjoyed a FPI pre-doctoral fellowship from Ministerio de Ciencia e Innovación-European Social Fund. J.O was supported by a Juan de la Cierva contract (Ministerio de Ciencia e Innovación).

REFERENCES

Bensch S, Stjernman M, Hasselquist D, Östman Ö, Hansson B, Westerdahl H, Torres-Pinheiro R. 2000. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proc. R. Soc. B-Biol. Sci.* 267: 1583-1589.

Bensch S, Pérez-Tris J, Waldenström J, Hellgren O. 2004. Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: Multiple cases of cryptic speciation? *Evolution* 58: 1617-1621.

Bensch S, Hellgren O, Pérez-Tris J. 2009. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial

cytochrome *b* lineages. *Mol. Ecol. Res.* 9: 1353-1358.

Blanco E, Casado MA, Costa M *et al.* 1997. *Los Bosques Ibéricos. Una Interpretación Geobotánica*. Madrid: Planeta.

Blondel J, Aronson J. 1999. *Biology and Wildlife of the Mediterranean Region*. Oxford: Oxford University Press.

Blondel J, Thomas DW, Charmantier A, Perret P, Bourgault P, Lambrechts MM. 2006. A thirty-year study of phenotypic and genetic variation of blue tits in Mediterranean habitat mosaics. *Bioscienc*e 56: 661-673.

García-Navas V, Sanz JJ. 2011. The importance of a main dish: nestling diet and foraging behaviour in Mediterranean blue tits in relation to prey phenology. *Oecologia* 165: 639-649.

Illera JC, Emerson BC, Richardson DS. 2008. Genetic characterization, distribution and prevalence of avian pox and avian malaria in the Berthelot's pipit (Anthus berthelotii) in Macaronesia. *Parasitol. Res.* 103: 1435-1443

Illera JC, Koivula K, Broggi J, Packert M, Martens J, Kvist L. 2011. A multi-gene approach reveals a complex evolutionary history in the *Cyanistes* species group. *Mol. Ecol.* 20: 4123-4139.

Knowles SCL, Wood MJ, Alves R, Wilkin TA, Bensch S, Sheldon BC. 2011. Molecular epidemiology of malaria prevalence and parasitaemia in a wild bird population. *Mol. Ecol.* 20: 1062-1076.

Kumar S, Tamura K, Jakobsen IB, Nei M. 2001. *Mega3: Molecular Evolutionary Genetics Analysis Software*. Tempe, AZ: Arizona State University.

Lachish S, Knowles SCL, Alves R, Wood MJ, Sheldon BC. 2011a. Fitness effects of endemic malaria infections in a wild bird population: the importance of ecological structure. *J. Anim. Ecol.* 80: 1196-1206.

Lachish S, Knowles SCL, Alves R, Wood MJ, Sheldon BC. 2011b. Infection dynamics of endemic malaria in a wild bird population: parasite species-dependent drivers of spatial and temporal variation in transmission rates. *J. Anim. Ecol.* 80: 1207-1216.

Martínez-de la Puente J, Merino S, Tomás G *et al.* 2010. The blood parasite *Haemoproteus* reduces survival in a wild bird: a medication experiment. *Biol. Lett.* 6: 663-665.

Ortego J, Calabuig G, Cordero PJ, Aparicio JM. 2007. Genetic characterization of avian malaria (Protozoa) in the endangered lesser kestrel, *Falco naumanni*. *Parasitol. Res.* 101: 1153-1156.

Ortego J, Cordero PJ, Aparicio JM, Calabuig G. 2008. Consequences of chronic infections with three different avian malaria lineages on reproductive performance of lesser kestrels (*Falco naumanni*). *J. Ornithol.* 149: 337-343.

Ortego J, Espada F. 2007. Ecological factors influencing disease risk in eagle owls *Bubo bubo*. *Ibis* 149: 386-395

Ortego J, García-Navas V, Ferrer ES, Sanz JJ. 2011. Genetic structure reflects natal dispersal movements at different spatial scales in the blue tit, *Cyanistes caeruleus*. *Anim. Behav*. 82: 131-137.

Pérez-Tris J, Hasselquist D, Hellgren O, Krizanauskiene A, Waldenström J, Bensch S. 2005. What are malaria parasites? *Trends Parasitol.* 21: 209-211.

Shurulinkov P, Chakarov N, Daskalova G (2012) Blood parasites, body condition, and wing length in two subspecies of yellow wagtail (Motacilla flava) during migration. *Parasitol. Res*. 110: 2043-2051

Shurulinkow P, Chakarov N. 2006. Prevalence of blood parasites in different local populations of reed warbler (Acrocephalus scirpaceus) and great reed warbler (Acrocephalus arundinaceus). *Parasitol. Res*. 99: 588-592

Shurulinkow P, Ilieva M. 2009. Spatial and temporal differences in the blood parasite fauna of passerine birds during the spring migration in Bulgaria. *Parasitol. Res*. 104: 1453-1458

Szöllösi E, Cichon M, Eens M *et al*. 2011. Determinants of distribution and prevalence of avian malaria in blue tit populations across Europe: separating host and parasite effects. *J. Evol. Biol.* 24: 2014-2024.

Valkiunas G, Iezhova TA, Loiseau C, Smith TB, Sehgal RNM. 2009. New malaria parasites of the subgenus Novyella in African rainforest birds, with remarks on their high prevalence, classification and diagnostics. *Parasitol. Res*. 104: 1061-1077

Vardo AM, Schall JJ. 2007. Clonal diversity of a lizard malaria parasite, *Plasmodium mexicanum*, in its vertebrate host, the western fence lizard: role of variation in transmission intensity over time and space. *Mol. Ecol.* 16: 2712-2720.

Waldenström J, Bensch S, Hasselquist D, Ostman O. 2004. A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. *J. Parasitol.* 90: 191-194.

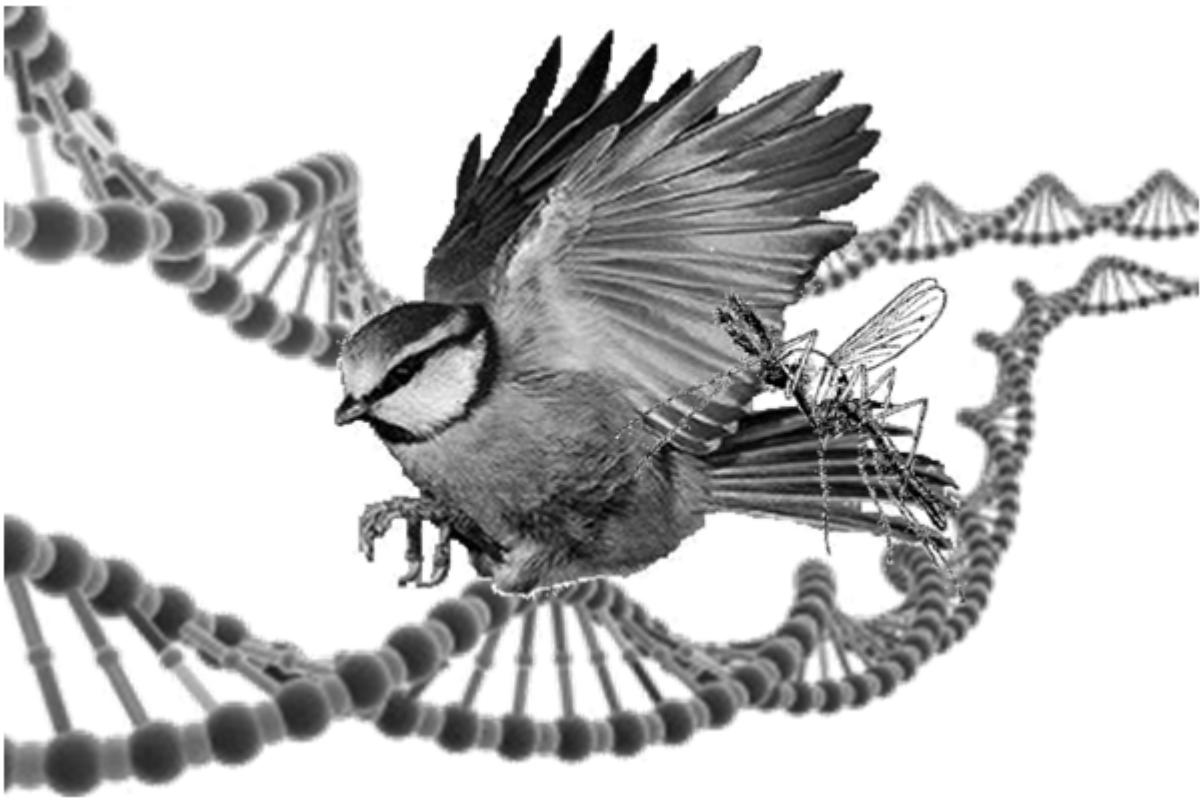
Wood MJ, Cosgrove CL, Wilkin TA, Knowles SCL, Day KP, Sheldon BC. 2007. Within-population variation in prevalence and lineage distribution of avian malaria in blue tits, *Cyanistes caeruleus*. *Mol. Ecol.* 16: 3263-3273.

Zehtindjiev P, Ilieva M, Westerdahl H, Hansson B, Valkiunas G, Bensch S. 2008. Dynamics of parasitemia of malaria parasites in a naturally and experimentally infected migratory songbird, the great reed warbler *Acrocephalus arundinaceus*. *Exp. Parasitol.* 119: 99-110.

4. Individual genetic diversity and probability of infection by avian malaria parasites in blue tits (*Cyanistes caeruleus*)

Ferrer ES, García-Navas V, Sanz JJ and Ortego J

Journal of Evolutionary Biology, 27: 2468-2482 (2014)



Individual genetic diversity and probability of infection by avian malaria parasites in blue tits (*Cyanistes caeruleus*)

Abstract

Understanding the importance of host genetic diversity for coping with parasites and infectious diseases is a long-standing goal in evolutionary biology. Here, we study the association between probability of infection by avian malaria (*Plasmodium relictum*) and individual genetic diversity in three blue tit (*Cyanistes caeruleus*) populations that strongly differ in prevalence of this parasite. For this purpose, we screened avian malaria infections and genotyped 789 blue tits across 26 microsatellite markers. We used two different arrays of markers: 14 loci classified as neutral and 12 loci classified as putatively functional. We found a significant relationship between probability of infection and host genetic diversity estimated at the subset of neutral markers that was not explained by strong local effects and did not differ among the studied populations. This relationship was not linear, and probability of infection increased up to values of homozygosity by locus (*HL)* around 0.15, reached a plateau at values of *HL* from 0.15 to 0.40 and finally declined among a small proportion of highly homozygous individuals (*HL* > 0.4). We did not find evidence for significant identity disequilibrium, which may have resulted from a low variance of inbreeding in the study populations and/or the small power of our set of markers to detect it. A combination of subtle positive and negative local effects and/or a saturation threshold in the association between probability of infection and host genetic diversity in combination with increased resistance to parasites in highly homozygous individuals may explain the observed negative quadratic relationship. Overall, our study highlights that parasites play an important role in shaping host genetic variation and suggests that the use of large sets of neutral markers may be more appropriate for the study of heterozygosity-fitness correlations.

INTRODUCTION

Genetic diversity is essential for individuals and populations to cope with novel and changing environmental conditions (Coltman *et al.* 1999; Willi *et al.* 2006). A plethora of studies have reported that reduced genetic diversity decreases fitness (David 1998; Coltman & Slate, 2003; Chapman *et al.* 2009; Szulkin *et al.* 2010) and increases extinction risk of natural populations (Saccheri *et al.* 1998; Spielman *et al.* 2004). For this reason, understanding the consequences of genetic diversity on different components of fitness is a central question in evolutionary and conservation biology (Keller & Waller 2002; Chapman *et al.* 2009; Szulkin *et al.* 2010). An important negative consequence of reduced levels of individual genetic diversity is a lower resistance to parasites (e.g. Coltman *et al.* 1999; Hawley *et al.* 2005; Acevedo-

Whitehouse *et al.* 2006; Luikart *et al.* 2008; Rijks *et al.* 2008), a phenomenon that can also lead to higher disease susceptibility at the whole population level if severe demographic bottlenecks have depleted genetic variability (O'Brien *et al.* 1988; e.g. Meagher *et al.* 1999; Whiteman *et al.* 2006; Pearman & Garner 2009; Whitehorn *et al.* 2011). Different genetic mechanisms can explain the association between individual genetic diversity and increased susceptibility to parasites and diseases (Keller & Waller 2002). Genetically more diverse individuals are expected to have a higher probability of carrying adaptive alleles implicated in immune response and recognition of pathogens that can confer resistance to a wider array of parasite species or genotypes (Keller & Waller 2002; Puurtinen *et al.* 2004; Reid *et al.* 2007; Fossoy *et al.* 2009). In turn, high levels of individual genetic diversity can also reduce the susceptibility to diseases through overdominance at genes not directly involved in immune response but involved in the capacity to clear and survive an infection (Coltman *et al.* 1999). For instance, high enzyme polymorphism at genes affecting physiological processes such as oxygen consumption, cellular homeostasis, and metabolic efficiency are likely to ultimately influence vulnerability to pathogens (Mitton & Grant 1984; Ferguson & Drahushchak 1990; Kristensen *et al.* 2002; Pedersen *et al.* 2005; Luong *et al.* 2007). Finally, individuals with low genetic diversity have a higher chance of expressing recessive deleterious alleles, which would decrease their capacity to resist infectious diseases and survive (Coltman *et al.* 1999; Keller & Waller, 2002). Thus, low genetic diversity can increase susceptibility to pathogens through its influence on different immunological, physiological and biochemical mechanisms (Coltman *et al.* 1999; Acevedo-Whitehouse *et al.* 2003).

The relationship between parasitism and host genetic diversity can also potentially vary across different genomic regions not directly involved in immune defence (Szulkin & David 2011). Microsatellite loci, the most common markers used in HFCs studies, have been generally assumed to be evolutionary neutral, but there is growing evidence on their implication in different biological processes (Li *et al.* 2004). Differences in the functionality of the markers employed to estimate heterozygosity can help to elucidate the relative importance of genome-wide/inbreeding effects ("general effect hypothesis") or effects at a single or few loci ("local and direct effect hypothesis") on observed heterozygosity-fitness correlations (HFC) (David 1998; Hansson & Westerberg 2002). A few recent empirical studies have shown that microsatellite markers located in functional and non-functional regions of the genome can behave differentially in terms of signal intensity and patterns of HFC (see Da Silva *et al.* 2009; Küpper *et al.* 2010; Olano-Marin *et al.* 2011a, b; Szulkin & David 2011; Laine *et al.* 2012). However, both classes of loci have not been yet employed to study the association between host genetic diversity and resistance to infectious diseases, an approach that could contribute to get a

better understanding on the underlying mechanisms behind parasite-host genotype interactions (Szulkin & David 2011).

The strength of the relationship between fitness and individual genetic diversity or inbreeding has been often found to increase under stressful and harsh environmental conditions (reviewed in Armbruster & Reed 2005; Fox & Reed 2011). Previous studies have shown that the stress imposed by parasites can increase the strength of HFCs (e.g. Voegeli *et al.* 2012; Coltman *et al.* 1999) and exacerbate the negative effects of inbreeding (e.g. Carr *et al.* 2003). However, no study has yet analyzed differences in heterozygosity-dependent parasite resistance across populations experiencing contrasting patterns of parasite pressure. If the strength of the association between parasitism and host genetic diversity increases during disease outbreaks or in populations experiencing high parasitism rates, this could buffer the expected loss of genetic diversity due to parasite-mediated demographic declines and ultimately increase the chance of population viability (e.g. Coltman *et al.* 1999; see also Forcada & Hoffman 2012). Thus, studies comparing populations with differences in parasite pressure can provide insight into the population's responses to infectious diseases and help to further understand the demographic and genetic consequences of parasites and its role as mediators of HFC in wild populations (Coltman *et al.* 1999; Voegeli *et al.* 2012).

Here, we study the association between individual genetic diversity and probability of avian malaria infections in three Mediterranean blue tit (*Cyanistes caeruleus*) populations that strongly differ in prevalence of these parasites (Ferrer *et al.* 2012). The negative consequences of avian malaria on different components of fitness have been reported in different species (e.g. van Riper *et al.* 1986; Merino *et al.* 2000; Sol *et al.* 2003; Ortego *et al.* 2008; Atkinson & Samuel 2010; Lachish *et al.* 2011). We have previously found six different lineages of avian malaria parasitizing our studied blue tit populations, but the lineage SGS1 (*Plasmodium relictum*) is involved in most infections (Ferrer *et al.* 2012). Previous studies have reported that both chronic and acute infections by *P. relictum* have negative fitness consequences in blue tits (Knowles *et al.* 2010; Lachish *et al.* 2011), including effects on mortality and survival probability in both this and other bird species (Atkinson *et al.* 2000; Atkinson & Samuel 2010; Lachish *et al.* 2011). Thus, this parasite probably exerts important evolutionary pressures that may shape host genetic composition and diversity at both specific loci involved in immune defence and at other parts of the genome related to physiological condition or health status (Ortego *et al.* 2007a; Reid *et al.* 2007; Fossoy *et al.* 2009). Mediterranean blue tits often have to cope with the extreme heterogeneity of fragmented forest habitats (Blondel & Aronson 1999), show a sedentary behaviour and tend to disperse across very short distances (Blondel *et al.* 2006; Ortego *et al.* 2011a), factors that may increase inbreeding rates and favour the detection of heterozygosity-fitness

correlations (HFC) (Balloux *et al.* 2004; Szulkin *et al.* 2010). Accordingly, previous studies have found HFCs in blue tits for individual quality, female fecundity, reproductive performance and survival (Foerster *et al.* 2003; García-Navas *et al.* 2009; Olano-Marin *et al.* 2011a, b). Thus, Mediterranean blue tits constitute an ideal study system to explore the association between individual genetic diversity and probability of infection by avian malaria.

In this study we have genotyped 789 blue tits across 26 polymorphic microsatellite markers to estimate individual genetic diversity and investigate its association with the probability of infection by avian malaria parasites screened using a highly efficient polymerase chain reaction (Waldenström *et al.* 2004). We used two different arrays of markers, classified as potentially neutral (14 loci) or functional (12 loci) by considering whether the genomic region where the markers are located is transcribed to RNA (*sensu* Olano-Marin *et al.* 2011a, b; see also Da Silva *et al.* 2009; Küpper *et al.* 2010; Laine *et al.* 2012). With this information, we tested the following specific hypotheses: We predicted that (i) more heterozygous individuals have a lower probability of infection by avian malaria parasites and (ii) the strength of such relationship increases in populations with a higher prevalence of the studied parasite; (iii) we analyzed whether the association between heterozygosity and parasitism reflects a genome-wide effect (general effect hypothesis; Weir & Cockerham 1973; David 1998) or is explained by single locus effects due to their functional nature (direct effect hypothesis; David 1998; Hansson & Westerberg 2002) or strong linkage disequilibrium with other functional loci (local effect hypothesis; David 1998; Hansson *et al.* 2001; Hansson & Westerberg 2002); and (iv) finally, to further understand the underlying mechanism behind the correlation between heterozygosity and probability of infection, we analyzed whether this association is better explained by functional loci, neutral loci or a combination of both. Neutral markers can cause this association either by general effects or local effects if they happen to be linked to functional loci. Direct effects and strong local effects, however, are only likely to be caused by functional markers (Olano-Marin *et al.* 2011a, b; Laine *et al.* 2012).

MATERIALS AND METHODS

Study species and sampling

The study was conducted in three blue tit populations located in Montes de Toledo (central Spain): Quintos de Mora (Toledo province, 39º25´N 4º04´W; 2008-2011 breeding seasons), San Pablo de los Montes (Toledo province, 39º31´N 4º21´W; 2011 breeding season), and Cabañeros (Ciudad Real and Toledo provinces, 39°24´N, 3°35´W; 2008 breeding season) (see Table 1 for more details). Adult birds (Quintos de Mora: 228 females and 175 males; San Pablo de los Montes: 160 females and 119 males; Cabañeros: 54 females and 53 males) were captured when feeding 8 days old

chicks by means of a spring-trap shutting the entrance hole as the bird entered the nest. Individuals were weighed using a pocket balance (accuracy ± 0.1g) and their wing length measured to the nearest 0.1 mm using a stopped ruler. Birds were sexed by the presence/absence of a brood patch and aged according to Svensson (1992) as juveniles (yearlings) or experienced breeders (second-year and older birds). All birds were individually marked with aluminium rings for further identification. Blood samples (≤ 25 µl) for genetic analyses were obtained by brachial venipuncture and stored in ethanol 96 %.

Microsatellite markers

We genotyped blue tits across 27 polymorphic microsatellite markers (Table 2). These markers were classified as presumably functional or neutral as described by Olano-Marin *et al.* (2011a, b) (see also Olano-Marin *et al.* 2010). We used NucleoSpin Blood Kits (Macherey-Nagel) to extract and purify genomic DNA from the blood samples. Approximately 5 ng of template DNA was amplified in 10-µL reaction volumes containing 1X reaction buffer (67 mM Tris-HCL, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01 % Tween-20, EcoStart Reaction Buffer, Ecogen, Barcelona, Spain), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 µM of each dye-labelled primer (FAM, PET, VIC or NED) and 0.1 U of *Taq* DNA EcoStart Polymerase (Ecogen). The PCR programm used was 9 min denaturing at 95 ºC followed by 40 cycles of 30 s at 94 ºC, 45 s at the annealing temperature (Table 2) and 45 s at 72 ºC, ending with a 10 min final elongation stage at 72 ºC. Amplification products were electrophoresed using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and genotypes were scored using

Table 1 Geographical location of the studied populations and subpopulations of blue tits (*Cyanistes caeruleus*) in Montes de Toledo, sample size (*n*), genetic diversity (mean ± S.E.) estimated as homozygosity by locus (*HL*) at all loci (HL_{Total}) and neutral ($HL_{Neutral}$) and functional loci ($HL_{Functional}$) separately, and prevalence of avian malaria (lineage SGS1).

Population/Subpopulation	Latitude	Longitude	*n*	HL_{Total}	$HL_{Neutral}$	$HL_{Functional}$	Prevalence
Quintos de Mora			403	0.218±0.004	0.188±0.005	0.258±0.007	17.12 %
Gil García	39.37976	-4.12593	215	0.223±0.006	0.186±0.007	0.274±0.009	13.95 %
Valdeyernos	39.44008	-4.09091	156	0.214±0.007	0.194±0.009	0.240±0.010	18.59 %
Fuente del Común	39.36316	-4.07830	32	0.201±0.012	0.175±0.016	0.235±0.025	31.25 %
San Pablo de los Montes			279	0.210±0.005	0.187±0.006	0.242±0.007	41.94 %
Marchés	39.54413	-4.37361	101	0.220±0.009	0.188±0.010	0.268±0.014	42.57 %
Morra	39.52895	-4.32851	80	0.207±0.008	0.194±0.011	0.221±0.014	38.75 %
Fuente Cerecera	39.51296	-4.27795	40	0.203±0.009	0.189±0.014	0.221±0.014	50.00 %
Fuenlabrada	39.52414	-4.25709	46	0.198±0.010	0.168±0.013	0.240±0.018	34.78 %
Robledillo	39.50862	-4.35865	12	0.219±0.026	0.202±0.028	0.243±0.030	58.33 %
Cabañeros			107	0.219±0.007	0.203±0.010	0.241±0.011	13.08 %
Anchurones	39.34241	-4.36783	33	0.207±0.014	0.182±0.018	0.240±0.022	21.21 %
Brezoso	39.36307	-4.35798	74	0.225±0.008	0.213±0.012	0.241±0.013	9.46 %

GENEMAPPER 3.7 (Applied Biosystems). Linkage disequilibrium (LD) between loci and deviations from Hardy-Weinberg equilibrium (HWE) were tested with GENEPOP web version 4.0.10 (Raymond & Rousset 1995). Probabilities of significance were computed applying a Markov chain method (Guo & Thompson 1992) using 200 batches and 1000 iterations per batch as implemented in GENEPOP. The LD correlation coefficient (*r*LD) between alleles at different loci was used to estimate the magnitude of LD. *r*LD was computed with the program LinkDos (http://genepop.curtin.edu.au/linkdos.html ; Garnier-Gere & Dillmann, 1992). Sequential Bonferroni corrections were applied to account for multiple comparisons (Rice 1989).

Genetic structure

We analyzed patterns of spatial genetic structure using the Bayesian Markov chain Monte Carlo clustering analysis implemented in the program STRUCTURE 2.3.3 (Pritchard *et al.* 2000; Falush *et al.* 2003; Hubisz *et al.* 2009). STRUCTURE assigns individuals to populations based on their multilocus genotypes (Pritchard *et al.* 2000; Falush *et al.* 2003). We ran STRUCTURE assuming correlated allele frequencies and admixture and conducted ten independent runs for each value of K = 1-10 to estimate the "true" number of clusters with 200000 MCMC cycles, following a burn-in step of 100000 iterations. The number of populations best fitting the data set was defined using both log probabilities [Pr(X|*K*)] (Pritchard *et al.* 2000) and the ΔK method (Evanno *et al.* 2005), as implemented in STRUCTURE HARVESTER (Earl & vonHoldt 2012).

Heterozygosity, identity disequilibrium, and inbreeding

We used two metrics to estimate individual genetic diversity: (i) uncorrected heterozygosity (H_O), calculated as the proportion of loci at which an individual is heterozygous; (ii) homozygosity by locus (*HL*), a microsatellite derived measure that improves heterozygosity estimates in open populations by weighting the contribution of each locus to the homozygosity value depending on their allelic variability (Aparicio *et al.* 2006). H_O and *HL* were calculated using CERNICALIN, an Excel spreadsheet available on request.

Identity disequilibrium (ID), defined as the correlation in heterozygosity and/or homozygosity across loci within individuals (Weir & Cockerham 1973), arises when there is enough variation in individual inbreeding coefficients within the population (Ballux *et al.* 2008; Szulkin *et al.* 2010). We used two methods to analyze the presence of identity disequilibrium and test whether heterozygosity measured at our set of microsatellite loci was representative of

Table 2 Microsatellite loci used to genotype blue tits: chromosome location in the zebra finch (*Taeniopygia guttata*) genome, category (neutral or functional), number of alleles (*K*), expected heterozygosity (H_E), observed heterozygosity (H_O) (based on 789 individuals) and annealing temperature (T) for each locus.

Locus	Chromosome	Category	*K*	H_E	H_O	T (ºC)	Reference
Pca7	1	Neutral	17	0.90	0.88	60	Dawson *et al.* 2000
Pocc6	2	Neutral	25	0.86	0.82	62	Bensch *et al.* 1997
Pat-MP2-43	2	Neutral	12	0.55	0.54	59	Otter *et al.* 1998
Pca8	2	Neutral	70	0.96	0.93	53	Dawson *et al.* 2000
Ase18	3	Neutral	20	0.86	0.83	60	Richardson *et al.* 2000
Pca3	4	Neutral	31	0.89	0.87	55	Dawson *et al.* 2000
Pdoμ5	4	Neutral	42	0.91	0.81	46	Griffith *et al.* 1999
PK12	5	Neutral	25	0.86	0.86	62	GenBank Acc. no.: AF041466
Mcyμ4	5	Neutral	18	0.84	0.82	50	Double *et al.* 1997
Pca9	7	Neutral	16	0.81	0.80	62	Dawson *et al.* 2000
Pocc1	7	Neutral	16	0.85	0.84	55	Bensch *et al.* 1997
Pca4	8	Neutral	19	0.75	0.73	60	Dawson *et al.* 2000
PK11	Unassigned	Neutral	13	0.81	0.81	52	GenBank Acc. no.: AF041465
Pca2	Unassigned	Neutral	15	0.76	0.67	60	Dawson *et al.* 2000
CcaTgu8	2	Functional	8	0.61	0.58	63	Olano-Marin *et al.* 2010
CcaTgu7	2	Functional	8	0.75	0.76	55	Olano-Marin *et al.* 2010
CcaTgu11	3	Functional	7	0.73	0.71	60	Olano-Marin *et al.* 2010
TG05-053	5	Functional	9	0.77	0.77	55	Dawson *et al.* 2009
TG05-046	5	Functional	4	0.49	0.52	55	Dawson *et al.* 2009
CcaTgu15	5	Functional	9	0.65	0.64	60	Olano-Marin *et al.* 2010
CcaTgu14	5	Functional	22	0.83	0.78	55	Olano-Marin *et al.* 2010
Tgu07	6	Functional	7	0.67	0.69	55	Slate *et al.* 2007
PiJ14	7	Functional	18	0.88	0.87	60	Olano-Marin *et al.* 2010
CcaTgu19	10	Functional	51	0.93	0.91	60	Olano-Marin *et al.* 2010
Tg13-017	13	Functional	21	0.86	0.84	60	Dawson *et al.* 2010
CcaTgu25 *	18	Functional	26	0.85	0.63	63	Olano-Marin *et al.* 2010
CcaTgu28	23_random	Functional	13	0.73	0.72	60	Olano-Marin *et al.* 2010

*This locus deviated from Hardy–Weinberg equilibrium across all populations/years and was discarded for further analyses.

genome-wide inbreeding: (*i*) we calculated heterozygosity-heterozygosity correlations (HHC) following Balloux *et al.* (2004). If our microsatellites markers carry information about genome-wide levels of heterozygosity, then comparing two random subsets of such markers should yield a positive, significant correlation (Balloux *et al.* 2004). The correlation is obtained by repeatedly and randomly dividing the genotyped loci in half, and calculating an estimate of individual multilocus heterozygosity for both sets of loci. The mean correlation between these sets will then yield the HHC (Balloux *et al.* 2004). We used the r package "rhh" to run 1000 randomizations of the markers and estimate the average HHC coefficient (*r*) and the 95% confidence intervals (Alho *et al.* 2010); (*ii*) we calculated the parameter g^2 described by David *et al.* (2007), a central measure of identity disequilibrium that quantifies the excess of multiple heterozygotes at the studied loci relative to

the expectation under random association (i.e., covariance in heterozygosity) (see Szulkin *et al.* 2010). We used the software RMES to calculate g^2 and test whether this parameter differs significantly from zero (David *et al.* 2007).

Pedigree data are only available for Quintos de Mora population because the two other studied populations have been only monitored during one (San Pablo de los Montes; 201 breeding season) or a few breeding seasons (Cabañeros; 2006-2008 breeding seasons). Individual inbreeding coefficients (*f*) were estimated from the reconstructed pedigree using PEDIGREE VIEWER (http://www-personal.une.edu.au/~bkinghor/pedigree.htm). Only one recruit to the study populations was highly inbred, and we compared its level of multilocus heterozygosity with those values obtained for other individuals also captured within Quintos de Mora during the same breeding season (e.g. Ortego *et al.* 2011b).

Screening for avian malaria infections

Each individual was screened for avian malaria infection using a highly efficient nested PCR protocol that amplifies a 524-bp fragment (including primers) of the mitochondrial cytochrome *b* gene of both *Plasmodium* and *Haemoproteous* parasites (Waldenström *et al.* 2004). This method consists in two rounds, an initial 20 cycles of PCR using the primers HAEMNF and HAEMNR2 and a final 35 cycles of PCR using the internally nested primers HAEMF and HAEMR2 (Waldenström *et al.* 2004). For the second PCR, 1 μL of the PCR product from the initial PCR was used as template. All PCRs were performed in 25 μL volumes, and we routinely used positive (i.e. DNA from individuals with known malarial infections) and negative controls (i.e. samples with ddH_2O instead of genomic DNA as template) to ascertain that the outcome of each PCR run was not affected by contamination (Waldenström *et al.* 2004). The PCR programme, thermal profile, and reagent proportions were as described by Waldenström *et al.* (2004), with the exception of using a 9 min denaturing at 95 ºC rather than 3 min at 94 ºC because we used a hot-start polymerase (EcoStart, Ecogen). Reagents were the same used for microsatellite amplifications. Amplification of the 26 microsatellite loci above described was used as a control for DNA quality. These amplifications were successful in all cases. Further, negative infections were confirmed by repeated PCR. Positive or negative second round PCR products (i.e. birds having or not gametocytes or merozoites in their blood stream) were scored by electrophoresis on 2% agarose gels stained with ethidium bromide and determining the presence/absence of a band of the expected size under UV light. PCR products from positive samples were purified using NucleoSpin Extract II (Macherey-Nagel) kits and bidirectionally sequenced on an ABI 310 Genetic Analyser (Applied Biosystems). Sequences were edited and aligned using the program

SEQUENCHER 5.0 (GeneCodes Corporation, Ann Arbor, MI, USA).

Statistical analyses: general effects

We analyzed the relationship between multilocus heterozygosity and probability of infection by avian malaria parasites using Generalized Linear Mixed Models (GLMMs) with a binomial error structure and a logit link function. We constructed two separate GLMMs, fitting *HL* or H_O as explanatory variables together with non-genetic terms (fixed factors: sex and age; covariates: body mass and wing length) that could potentially influence avian malaria infections. Additionally, we ran GLMMs including as predictors heterozygosity (*HL* or H_O) calculated with the subset of neutral ($HL_{Neutros}$ or $H_{O\ Neutros}$) and functional markers ($HL_{Functional}$ or $H_{O\ Functional}$). Heterozygosity measured with neutral and functional markers are not correlated (see results section) and, for this reason, both variables were included together into the same models. We included breeding pair, year and population and subpopulation identity as random effects in all the models. Initially, each GLMM was constructed with all explanatory terms fitted, including first-order interactions and quadratic effects to account for potential nonlinear relationships. Final models were selected following a backward procedure, by progressively eliminating non-significant variables. The significance of the remaining variables was tested again until no additional variable reached significance. The result is the minimal most adequate model for explaining the variability in the response variable, where only the significant explanatory variables are retained. All random effects were retained in the final models. GLMMs were run using the package lme4 in R 3.0.0 (R Development Core Team, 2012).

Statistical analyses: local effects

For those cases where models included an estimate of multilocus heterozygosity, we followed the approach described in Szulkin *et al.* (2010) to test whether single locus heterozygosity (SLH) effects explain more variance than multilocus heterozygosity (MLH) effects. In brief, we used an *F*-test ratio test to compare the model including MLH (i.e. the model described in the previous section) and a similar model in which we replaced MLH with "normalized" SLH at all markers incorporated as binary variables (Szulkin *et al.* 2010; e.g. Olano-Marin *et al.* 2010a,b; Ruiz-López *et al.* 2012). Only individuals with complete genotypes for all loci were included in these analyses (e.g. Ruiz-López *et al.* 2012).

We also explored the effects of SLH by fitting one GLMM per locus, that is 26 models in total. We calculated the effect size for each locus as the partial correlation coefficient obtained from their respective models (Nakagawa & Culhill 2007). We used a binomial sign test to analyze whether positive and negative effects occurred equally and χ^2 tests to investigate whether

the number of positive/negative or significant/non-significant effects differed between the subsets of neutral and functional loci. Finally, we used a general linear model (GLM) analysis to test whether the absolute effect size of SLH was associated with marker diversity (estimated as H_E, H_O and allelic richness; Table 2) or differed between the groups of neutral and functional loci (e.g. Olano-Marin *et al.* 2010a; Ruiz-López *et al.* 2012).

RESULTS

Microsatellite data

Observed heterozygosity at each locus ranged from 0.51 to 0.93, with 4-70 alleles per locus (Table 2). After applying sequential Bonferroni corrections to compensate for multiple statistical tests, loci *Pdoμ5*, *CcaTgu8* and *CcaTgu15* showed significant deviations from HWE in the Cabañeros population. Locus *CcaTgu1*5 also showed deviations in Quintos de Mora population during a single study year. Locus *CcaTgu25* showed deviations across all study years and populations and it was discarded for further analyses. After sequential Bonferroni corrections, significant LD was detected for loci *TG05-053*/*CcaTgu15* in Cabañeros population and loci *Ase18*/*CcaTgu14* in San Pablo population. For the two loci not assigned to any chromosome (*Pca2* and *PK11*; Table 2), we only found significant LD between *PK11* and *Pdoμ5* in Quintos de Mora population for one of the study years. As the LD correlation coefficient (*r*LD) between these pairs of loci was very small (range: 0.062-0.074) and we found no consistent LD across study years/populations, none of these markers was discarded (e.g. Olano-Marin *et al.* 2011).

Genetic structure

STRUCTURE analyses indicated an optimal value of $K = 2$ according with the method of Evanno *et al.* (2005) (Fig. S1), but most individuals and all populations showed a considerable degree of genetic admixture (Fig. S2). The genetic assignment of individuals did not show any clear geographical pattern of genetic structure (Fig. S2), suggesting that the inferred clusters may be due to the presence of individuals with different genetic backgrounds (e.g. originated from genetically differentiated populations; see also Olano-Marin *et al.* 2010a for a similar pattern).

Heterozygosity, identity disequilibrium, and inbreeding

HL and H_O were highly correlated considering all loci or the subsets of neutral and functional markers ($r > 0.95$ and $P < 0.001$ in all cases). Subsequent statistical analyses and figures are only presented for *HL* because this measure is a better estimator of genome-wide heterozygosity and inbreeding in open populations

subjected to immigration and admixture (Aparicio *et al.* 2006). H_O data provided analogous results and are available from the authors. *HL* values for each population and subpopulation are indicated in Table 1. *HL* estimated at the subset of functional markers was not correlated with *HL* at the subset of neutral markers (r = 0.02, P = 0.567). Average (±S.E.) *HL* did not differ among populations (all markers: $F_{2, 786}$ = 0.98, P = 0.375; neutral markers: $F_{2, 786}$ = 0.98, P = 0.375; functional markers: $F_{2, 786}$ = 0.98, P = 0.375). *HL* did not differ among the different subpopulations for all markers or the subset of neutral markers (all Ps > 0.1). *HL* estimated at the subset of functional markers did not differ among subpopulations within Cabañeros or San Pablo de los Montes. However, *HL* estimated at the subset of functional markers differed among subpopulations within Quintos de Mora ($F_{2, 402}$ = 3.29, P = 0.038) and post hoc Tukey tests showed that these differences were due to a lower *HL* in Valdeyernos than in Gil Garcia (P = 0.048) (Table 1).

HHC were non-significant (i.e. 95% quantiles crossed zero) when all the studied individuals were considered (all markers: r = 0.016, 95% CI = -0.036-0.064; neutral markers: r = -0.017, 95% CI = -0.064-0.038; functional markers: r = 0.001, 95% CI = -0.033-0.043). We also analyzed HHC within each of the three studied populations. HHC were not significant in Quintos de Mora (all markers: r = 0.017, 95% CI = -0.054-0.088; neutral markers: r = 0.005, 95% CI = -0.072-0.086; functional markers: r = 0.010, 95% CI = -0.056-0.069), San Pablo de los Montes (all markers: r = 0.003, 95% CI = -0.072-0.090; neutral markers: r = -0.061, 95% CI = -0.132-0.016; functional markers: r = 0.004, 95% CI = -0.064-0.076) or Cabañeros (all markers: r = 0.033, 95% CI = -0.092-0.171; neutral markers: r = 0.014, 95% CI = -0.104-0.152; functional markers: r = -0.025, 95% CI = -0.159-0.103). The parameter g^2 did not differ significantly from zero when all the individuals were considered (all markers: g^2 = -0.003, P = 0.682; neutral markers: g^2 = -0.0004, P = 0.645; functional markers: g^2 = 0.006, P = 0.329) or when individuals from Quintos de Mora (all markers: g^2 = 0.002, P = 0.372; neutral markers: g^2 = 0.0020, P = 0.100; functional markers: g^2 = 0.010, P = 0.266), Cabañeros (all markers: g^2 = -0.011, P = 0.724; neutral markers: g^2 = -1.1617, P = 0.465; functional markers: g^2 = 0.009, P = 0.575) or San Pablo de los Montes (all markers: g^2 = -0.008, P = 0.748; neutral markers: g^2 = -0.0036, P = 0.988; functional markers: g^2 = 0.013, P = 0.438) were analyzed separately.

Only 144 out of 619 breeding individuals captured at Quintos de Mora population over the six study years were recruits with both parents known (23.3 %). The other individuals were founders, immigrants, or recruits whose parents could not be captured or identified (i.e. individuals with f assumed to be zero). Of the 144 breeding individuals with known ancestry (at least both parents known) only one (0.7%) resulted from a consanguineous mating involving full siblings (f = 0.25).

Table 3 GLMMs (binomial error and logit link function; n = 789 individuals) for probability of infection by avian malaria (lineage SGS1) in relation to non-genetic terms (covariates: body mass, wing length; fixed factors: sex, age) and homozygosity by locus (*HL*) estimated for (a) all loci (HL_{Total}) or the (b) subsets of neutral ($HL_{Neutral}$) and functional ($HL_{Functional}$) loci.

	Estimate ± S.E.	Test	*P*
a) All loci			
Rejected terms			
HL_{Total}		Z = 0.356	0.722
HL_{Total}^2		Z = 0.126	0.899
Sex		Z = 1.403	0.161
Age		Z = -0.238	0.812
Body mass		Z = -1.422	0.155
Wing length		Z = 0.590	0.555
b) Subsets of neutral and functional loci			
Explanatory terms			
Intercept	-2.467 ± 0.578		
$HL_{Neutral}$	10.856 ± 3.242	Z = 3.349	<0.001
$HL_{Neutral}^2$	-23.095 ± 7.507	Z = -3.076	0.002
Rejected terms			
$HL_{Functional}$		Z = -1.073	0.283
$HL_{Functional}^2$		Z = -1.084	0.278
Sex		Z = 1.297	0.195
Age		Z = -0.163	0.871
Body mass		Z = -1.243	0.214
Wing length		Z = 0.622	0.534

This individual had a significantly lower heterozygosity (all markers: HL = 0.291; neutral markers: HL = 0.218; functional markers: HL = 0.387) than the other individuals (n = 114, mean ± S.D, all markers: HL = 0.220±0.008, t = -8.85, P < 0.001; neutral markers: HL = 0.190±0.010, t = -2.84, P = 0.005; functional markers: HL = 0.259 ± 0.011, t = -11.20, P < 0.001) also captured during the same breeding season within Quintos de Mora population.

We have also found two other crosses between related individuals (a mother-son pair, f = 0.25; a grandfather-granddaughter pair, f = 0.125), but none of their offspring recruited in the population.

Avian malaria infections

Prevalence of avian malaria (lineage SGS1) at each population and subpopulation is indicated in Table 1. Probability of infection differed among populations (Wald = 59.64, P < 0.001) and post hoc analyses showed that these differences were due to a higher probability of infection in San Pablo de los Montes than in Quintos de Mora (Wald = 4.64, P < 0.001) and Cabañeros (Wald = 48.64, P < 0.001) (see also Ferrer *et al.* 2012). Probability of infection did not differ among subpopulations within San Pablo de los Montes (Wald = 3.64, P = 0.456) or Cabañeros (Wald = 2.64, P = 0.104). However, we found that probability of infection slightly differed among subpopulations within Quintos de Mora (Wald = 5.98, P = 0.050). A post hoc analysis showed that these differences were due to a higher probability of infection in Fuente del Común than in Gil García (Wald = 5.77, P = 0.016) (Table 1).

Association between heterozygosity and parasitism: general effects

We found no association between probability of infection by the lineage SGS1 of avian malaria and heterozygosity

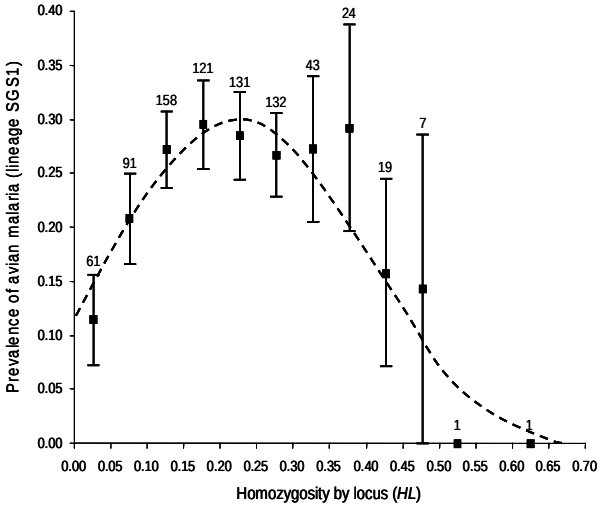


Figure 1 Mean ± 1 S.E. prevalence of avian malaria in relation to homozygosity by locus (*HL*) for the subset of "neutral" loci. Dashed line shows predicted values from the quadratic model. Numbers above bars indicate sample size for each category.

estimated at all typed loci (Table 3a) or the subset of functional markers (Table 3b). However, we found a highly significant quadratic relationship between probability of infection and individual heterozygosity estimated at neutral loci (Table 3b). Probability of infection increases with homozygosity up to values of *HL* around 0.15, reaches a plateau at values of *HL* from 0.15 to 0.40 and finally declines in highly homozygous individuals (*HL* > 0.4) (Fig. 1). The other studied variables (sex, age, body mass, and wing length) were not significantly associated with probability of infection in any model (Table 3). Other quadratic terms and interactions between independent variables were not significant ($P > 0.1$ in all cases).

Genetic diversity did not significantly differ between the subsets of neutral and functional loci, but tended to be higher in the former (H_E: $F_{1, 24} = 3.86$, $P = 0.061$; H_O: $F_{1, 24} = 2.66$, $P = 0.116$; allelic richness: $F_{1, 24} = 2.83$, $P = 0.105$) (Table 2). We performed an additional analysis to ascertain whether the differences in the HFC obtained for MLH estimated at the subsets of neutral and functional markers were due to their differences in genetic diversity and/or the different number of typed markers in each group (14 neutral markers *vs.* 12 functional markers; Table 2). For this purpose, we removed the two most diverse loci (*Pca8* and *Pdoμ5*; Table 2) from the calculation of MLH at neutral markers and ran another GLMM using this new variable. After the removal of these two loci, genetic diversity did not significantly differ between the subsets of neutral and functional loci (H_E: $F_{1, 22} = 2.30$, $P = 0.144$; H_O: $F_{1, 22} = 1.67$, $P = 0.209$; allelic richness: $F_{1, 22} = 1.06$, $P = 0.313$) and we still found a significant quadratic relationship between probability of infection and *HL* estimated at the subset of neutral loci (linear term of *HL*: $Z = 2.454$, $P = 0.014$; quadratic term of *HL*: $Z = -2.041$, $P = 0.041$).

We also analyzed the data for each population separately to see whether the relationship between probability of infection and host genetic diversity differs among the studied populations (Table 1). Although the degree of spatial genetic structure within the whole study area is low, these analyses also help to avoid the possibility that the association between probability of avian malaria infections and individual genetic diversity has merely resulted from population stratification

(Slate *et al.* 2004; Slate & Pemberton 2006). In Quintos de Mora and San Pablo de los Montes, probability of infection increased (linear term of *HL*: Quintos de Mora: Z = 2.794, P = 0.005; San Pablo de los Montes: Z = 2.046, P = 0.040) and then declined (quadratic term of *HL*: Quintos de Mora: Z = -2.340, P = 0.019; San Pablo de los Montes: Z = -1.944, P = 0.051) with *HL* estimated at the subset of neutral loci. A similar pattern was found in Cabañeros, but the association was not significant (linear term of *HL*: Z = 0.750, P = 0.453; quadratic term of *HL*: Z = -1.152, P = 0.249). Probability of infection was not associated with the linear or quadratic term of heterozygosity estimated at all loci or the subset of functional markers in any population (all Ps > 0.06). We also re-analyzed the data from Quintos de Mora excluding the subpopulation with higher prevalence to avian malaria (Fuente del Común; Table 1) and we still found that probability of infection increased (linear term of *HL*: Z = 3.410, P < 0.001) and then declined (quadratic term of *HL*: Z = -2.963, P = 0.003) with *HL* estimated at the subset of neutral loci. Finally, we re-analysed the complete data set but including population or subpopulation as fixed factors (rather than as random effects) and their respective interactions with *HL* and its quadratic term. No interaction was significant (all Ps > 0.3), indicating that the relationship between probability of infection and host genetic diversity did not differ among the studied populations/subpopulations.

Association between heterozygosity and parasitism: local effects

The model including SLH at all neutral loci did not improve the variance explained by the model including the significant effect of MLH at this subset of loci ($F_{12, 657}$ = 1.23, P = 0.258). No single locus effect was significant after sequential Bonferroni correction (Table 4; Fig. 2). The probability of obtaining positive or negative effects, irrespective of the statistical significance, was not different from 0.5 and the direction and significance of SLH effects did not differ between the subsets of neutral and functional markers (all Ps > 0.5). Absolute effect size of SLH was not correlated with marker genetic diversity (H_E: $F_{1, 24}$ = 0.07, P = 0.79; H_O: $F_{1, 24}$ = 0.24, P = 0.631; allelic richness: $F_{1, 24}$ = 0.10, P = 0.76) and did not differ between the subsets of neutral and functional loci ($F_{1, 24}$ = 2.18, P = 0.153).

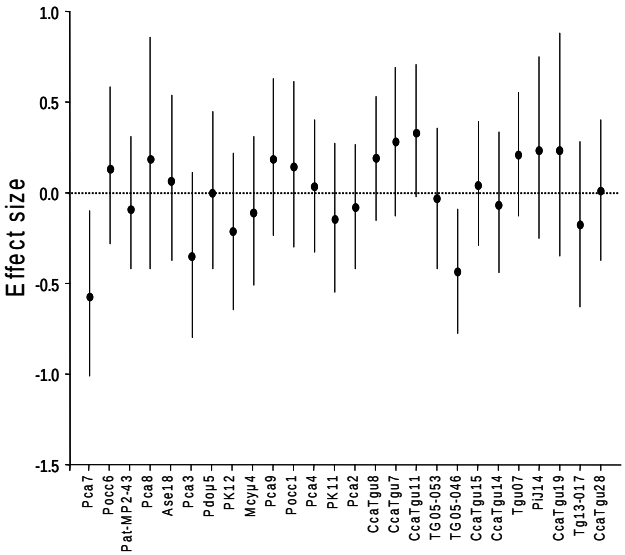


Figure 2 Effect sizes and 95% confidence intervals of single-locus heterozygosity (SLH) for probability of infection by avian malaria.

Table 4 Test for the effects of single locus heterozygosity (SLH) on probability of infection by avian malaria (lineage SGS1). Table shows effect sizes, 95% confidence intervals and *P*-values. No test was significant after sequential Bonferroni correction.

Locus	Category	Effect size	95% Ci (Lower/Upper)	Test	*P*
Pca7	Neutral	-0.576	-1.026/-0.112	Z = -2.477	0.013
Pocc6	Neutral	0.127	-0.287/0.561	Z = 0.588	0.556
Pat-MP2-43	Neutral	-0.095	-0.419/0.299	Z = -0.577	0.564
Pca8	Neutral	0.182	-0.432/0.860	Z = 0.555	0.579
Ase18	Neutral	0.061	-0.366/0.510	Z = 0.275	0.783
Pca3	Neutral	-0.358	-0.804/0.106	Z = -1.544	0.122
Pdoμ5	Neutral	-0.009	-0.433/0.434	Z = -0.041	0.967
PK12	Neutral	-0.217	-0.651/0.235	Z = -0.963	0.335
Mcyμ4	Neutral	-0.114	-0.516/0.304	Z = -0.544	0.587
Pca9	Neutral	0.180	-0.223/0.603	Z = 0.858	0.391
Pocc1	Neutral	0.136	-0.304/0.601	Z = 0.589	0.556
Pca4	Neutral	0.028	-0.330/0.398	Z = 0.154	0.878
PK11	Neutral	-0.149	-0.545/0.262	Z = -0.724	0.469
Pca2	Neutral	-0.085	-0.423/0.259	Z = -0.489	0.625
CcaTgu8	Functional	0.184	-0.168/0.542	Z = 1.019	0.308
CcaTgu7	Functional	0.279	-0.120/0.695	Z = 1.342	0.179
CcaTgu11	Functional	0.325	-0.040/0.703	Z = 1.718	0.086
TG05-053	Functional	-0.038	-0.417/0.353	Z = -0.195	0.845
TG05-046	Functional	-0.438	-0.775/-0.104	Z = -2.563	0.010
CcaTgu15	Functional	0.038	-0.299/0.381	Z = 0.222	0.825
CcaTgu14	Functional	-0.070	-0.453/0.325	Z = -0.356	0.722
Tgu07	Functional	0.204	-0.148/0.565	Z = 1.119	0.263
PiJ14	Functional	0.229	-0.258/0.752	Z = 0.892	0.373
CcaTgu19	Functional	0.228	-0.362/0.880	Z = 0.726	0.468
Tg13-017	Functional	-0.180	-0.615/0.274	Z = -0.794	0.427
CcaTgu28	Functional	0.004	-0.360/0.377	Z = 0.020	0.984

DISCUSSION

We found a significant relationship between probability of infection by lineage SGS1 of avian malaria and individual genetic diversity across the three studied blue tit populations. However, contrary to our expectations, the relationship was not linear and individuals with high and low genetic diversity showed a lower probability of being infected than individuals with an intermediate level of heterozygosity. Further, this association was significant only for MLH estimated at the panel of neutral markers, and we did not find any relationship between parasitism and host

genetic diversity estimated at the subset of functional markers. Finally, the association between probability of infection and host genetic diversity did not change across populations with remarkably different levels of malaria prevalence, indicating that contrasting patterns of parasite pressure do not change the shape of the observed relationship in our study system.

Non-linear association between heterozygosity and parasitism

Non-linear relationships between host genetic diversity and parasitism have been also found in previous studies estimating genetic variability at microsatellite markers (Ortego *et al.* 2007a; Blanchet *et al.* 2009; Ruiz-López *et al.* 2012) and genes involved in immune response (e.g. Wegner *et al.* 2003a, b; Westerdahl *et al.* 2005). We found that the positive effects of individual genetic diversity in terms of reduced probability of infection follow a saturation curve, quickly reaching a plateau after which lower levels of heterozygosity do not result in increased risk of infection. This may indicate that the benefits of increased genetic diversity are particularly patent among highly heterozygous individuals and readily stabilize due to a similarly low capacity to clean or avoid infections below a certain threshold of genetic diversity (*HL*~0.15). Only a few (<0.4%) highly homozygous individuals (*HL* > 0.4) are responsible of the negative quadratic pattern. Different factors may explain the unexpected lower probability of infection among these highly homozygous individuals.

A possible explanation for the observed negative quadratic pattern between parasitism and host genetic diversity could be related to the existence of a bias in mortality, in such a way that highly homozygous individuals infected by avian malaria do not survive acute infections and only those that have never been exposed to the parasite (i.e. uninfected) are among the sampled individuals (Ortego *et al.* 2007b; Westerdahl *et al.* 2005; Westerdahl 2007). If this is the case, we would expect (i) that the frequency of infected individual among highly homozygous individuals is lower than in highly heterozygous individuals and (ii) that a higher mortality rate among highly

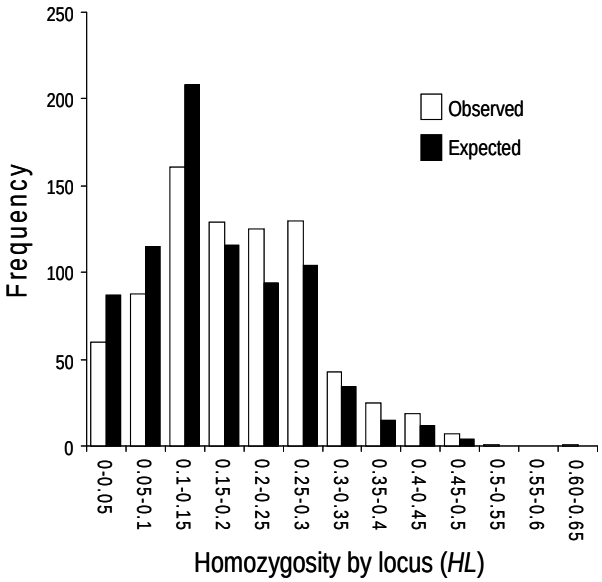


Figure 3 Observed (open bars) and expected (black bars) distribution of homozygosity by locus (*HL*) for the subset of neutral loci. The expected distribution of *HL* was obtained via simulations of 789 multilocus genotypes (i.e. a figure equal to our total number of sampled individuals) based on observed allele frequencies within each subpopulation and assuming random mating.

homozygous individuals reduces its frequency in the population in comparison with that expected in the absence of positive heterozygosity-based selection. However, we found that the frequency of infected individuals did not significantly differ between highly heterozygous (*HL* < 0.15) and highly homozygous (*HL* > 0.40) individuals (2 × 2 contingency table; χ^2 = 0.451, *P* = 0.502). To see whether the frequency of highly homozygous individuals in the population is lower than expected in absence of heterozygosity-based selection, we simulated multilocus genotypes for the subset of neutral loci considering the allele frequencies observed in each subpopulation. We found that the observed frequency of highly homozygous individuals tended to be higher than expected in absence of positive heterozygosity-based selection (2 × 2 contingency table; χ^2 = 2.829, *P* = 0.093) (Fig. 3). These analyses indicate that a bias in mortality proposed in previous studies to explain a negative quadratic association between probability of infection and individual genetic diversity is not likely to be the reason behind such pattern in our study system (Westerdahl *et al.* 2005; Westerdahl 2007). Thus, an alternative explanation for the lower probability of infection among highly homozygous individuals is that it reflects increased resistance to parasites among this small proportion of presumably highly inbreed individuals due to the purging of deleterious recessive alleles (Barsett & Charlesworth 1991; Dudash *et al.* 1997), which may have uncoupled the expected relationship between individual genetic diversity and resistance to parasites (Wiehn *et al.* 2002; Haag *et al.* 2003).

Blanchet *et al.* (2009) and Ruiz-López *et al.* (2012) also reported that hosts with intermediate levels of heterozygosity had higher ectoparasite load than highly homozygous or heterozygous individuals. These studies did not find evidence for strong local effects, but suggested that a combination of both positive and negative small local HFCs (irrespective of their statistical significance) may be behind the observed quadratic relationship at the multilocus level and interpreted that the global pattern could be caused by parasite-mediated disruptive selection on genetic diversity (see also Neff 2004; Küpper *et al.* 2010; Olano-Marin *et al.* 2010a, b; Mueller *et al.* 2011). Similarly, we did not find any significant single locus effect, but the proportion of positive and negative local effects was similar (Fig. 2), and this may also explain the negative quadratic relationship between host genetic diversity and parasitism observed in our studied blue tit populations.

General *vs.* local effects

There is no consensus about whether heterozygosity measured across a set of markers can reflect genome-wide heterozygosity and therefore inbreeding levels (Balloux *et al.* 2004; Forstmeier *et al.* 2012). We found no evidence that local effects are the underlying mechanism behind the observed association between

probability of infection by avian malaria parasites and individual genetic diversity. The model including SLH at all neutral loci did not improve the variance explained by the model including MLH at this subset of loci, and no single locus effect was significant (Table 4; Fig. 2). However, we found that HHC (Balloux *et al.* 2004) and parameter g^2 (David *et al.* 2007; Szulkin *et al.* 2010) did not differ significantly from zero, suggesting that heterozygosity measured at our set of microsatellite loci may not be representative of inbreeding. It should be considered that a non-significant correlation in heterozygosity among markers (HHC or g^2) cannot be regarded as disproving general effects, as the studied traits can capture the effect of potentially many more loci than the number of typed markers (Szulkin *et al.* 2010; e.g. Forcada & Hoffman 2014). Accordingly, a recent simulation-based study has demonstrated that failure to detect ID cannot be taken as strong evidence that inbreeding depression is not behind observed HFC and showed that many studies are likely to detect HFC caused by inbreeding depression, but fail to detect ID (Kardos *et al.* 2014). This study also suggested that ID is only likely to be detected when variance of inbreeding is high or many loci are used (>100 microsatellites), a situation that is not likely to the case in most studies on natural populations. Our study is well above the typical number of 10-14 microsatellite loci employed in most studies on HFC, but way too far from the number of loci necessary to detect ID in the presence of a moderate to low variance of inbreeding. The very limited dispersal capacity of blue tits has probably resulted in the patterns of fine spatial-scale genetic structure and matings between relatives reported in different populations of this species (García-Navas *et al.* 2009; Olano-Marin *et al.* 2010a; Ortego *et al.* 2010a). This may increase the variance in genome-wide genetic diversity and increase the chance of detecting HFC (Balloux *et al.* 2004, Slate *et al.* 2004). Pedigree data were only available for a small proportion of our sampled individuals (<20%), and for most of them (>80%), we exclusively had information on the identity of their parents, which is likely to have resulted in we have failed to identify most inbreed individuals. Despite these limitations of our pedigree data, we have identified two cases of mating between close relatives (García-Navas *et al.* 2009) and the only inbreed recruit had significantly lower heterozygosity than other supposedly outbreed individuals, which suggests that our set of markers may be reflecting genome-wide inbreeding. Thus, inbreeding rates may be enough for HFCs arise in our and other studied blue tit populations (Foerster *et al.* 2003; García-Navas *et al.* 2009; Olano-Marin *et al.* 2010a, b), but they are also likely to be below the threshold necessary to detect ID using our set of typed markers (Kardos *et al.* 2014; see also Forcada & Hoffman 2014 for a recent empirical study).

Neutral *vs.* functional markers

We have found an association between probability of avian malaria infections and

genetic diversity at neutral loci, but such association was not significant when only considering the subset of functional loci or a combination of both groups of loci. In our case, these differences are not attributable to differences in genetic diversity between our panels of neutral and functional markers (see also Olano-Marin *et al.* 2010a; Szulkin & David 2011). One possibility to explain this discrepancy is that heterozygosity (or homozygosity) at functional markers may be selected by many processes related with their different specific functions (or those from their closely linked genes), which may reduce their ability to reflect genome-wide heterozygosity (see also Mueller *et al.* 2011; Szulkin & David 2011). The capacity to survive and clear an infection is likely to be associated with many physiological traits whose heterozygosity may be better reflected by neutral markers. Accordingly, a previous study on blue tits also found that a similar set of neutral loci provides more power to detect HFC and identity disequilibrium than functional loci, indicating that the former may capture better the effects of inbreeding (Olano-Marin *et al.* 2010a). HFCs reported at functional loci (or loci located in functional genomic regions) have been generally interpreted as evidence of local or direct effects (David 1998; Hansson *et al.* 2001; Hansson & Westerberg 2002) and, thus, the ability of these markers to detect HFCs may depend on whether their specific functions are related to the particular studied traits (e.g. Da Silva *et al.* 2009; Küpper *et al.* 2010; Olano-Marin *et al.* 2010b; Laine *et al.* 2012).

Conclusions

This study shows that host genetic diversity is associated with probability of infection by avian malaria parasites. The fact that we did not find any evidence for local effects and that the association between probability of infection and individual genetic diversity was exclusively explained by heterozygosity at putatively neutral loci suggests that the observed effects may be mediated by inbreeding and/or of genome-wide genetic diversity. Given that heterozygosity is heritable in our study populations, avian malaria could be an important factor shaping host genetic diversity in this system (García-Navas *et al.* 2009). Different studies have demonstrated that genetic variation in genes involved in parasite defence is not necessarily linked to levels of variation in the rest of the genome and they can strongly differ in their responses to demographic changes and parasite-mediated selection pressures (Westerdahl *et al.* 2005; Oliver *et al.* 2009; Oliver & Piertney 2012). Thus, our study and others previously finding an association between parasitism and heterozygosity estimated at putatively neutral markers suggest that genetic diversity at loci not directly involved in immune defence is also relevant for the resistance to infectious diseases (Acevedo-Whitehouse *et al.* 2006; Ortego *et al.* 2007a). The fact that the strength and the shape of the relationship between probability of infection and heterozygosity did not differ among populations strongly differing in malaria prevalence suggests that host genetic diversity is likely to similarly

respond to infectious diseases under different scenarios of parasite pressure. Overall, our study highlights that parasites play a major role in shaping genetic variation in wild populations and suggests that the use of larger sets of neutral markers not associated with functional genomic regions may be more appropriate to the study of HFCs, particularly if functional loci related to the specific studied traits have not been identified. The application of new available high-throughput sequencing technology will help to extraordinarily increase the number of employed loci and improve the estimates of genome-wide heterozygosity in future HFC studies (e.g. Hoffman *et al.* 2014).

ACKNOWLEDGEMENTS

We wish to thank Pedro J. Cordero for allowing us to carry out the genetic analyses in his laboratory and Javier Bueno and Rafael Barrientos for their help during sample collection at San Pablo de los Montes. Two anonymous referees provided useful discussion and valuable comments on an earlier draft of this manuscript. We thank the board of the Cabañeros National Park, Centro Quintos de Mora and the Council of San Pablo de Los Montes for the facilities offered during the fieldwork. This study was funded by grants 69/2003 (Organismo Autónomo de Parques Nacionales-Ministerio de Medio Ambiente), GCL2007-61395 (Ministerio de Educación y Ciencia), CGL2010-21933-C02-01 (Ministerio de Ciencia e Innovación) and POIC10-0269-7632 (Junta de Comunidades de Castilla-La Mancha-European Social Fund). ESF was supported by a pre-doctoral scholarship from Junta de Comunidades de Castilla-La Mancha-European Social Fund and VGN enjoyed a FPI pre-doctoral scholarship from Ministerio de Ciencia e Innovación-European Social Fund. J.O was supported by "Juan de la Cierva" and "Severo Ochoa-EBD" post-doctoral fellowships (Ministerio de Ciencia e Innovación).

REFERENCES

Acevedo-Whitehouse K, Gulland F, Greig D, Amos W. 2003. Disease susceptibility in California sea lions. *Nature* 422: 35.

Acevedo-Whitehouse K, Spraker TR, Lyons E, Melin SR, Gulland F, Delong RL *et al.* 2006. Contrasting effects of heterozygosity on survival and hookworm resistance in California sea lion pups. *Mol. Ecol.* 15: 1973-1982.

Alho JS, Välimäki K, Merilä J. 2010. Rhh: an R extension for estimating multilocus heterozygosity and heterozygosity-heterozygosity correlation. *Mol. Ecol. Resour.* 10: 720-722.

Aparicio JM, Ortego J, Cordero PJ. 2006. What should we weigh to estimate heterozygosity, alleles or loci? *Mol. Ecol.* 15: 4659-4665.

Armbruster P, Reed DH. 2005. Inbreeding depression in benign and stressful environments. *Heredity* 95: 235-242.

Atkinson CT, Dusek RJ, Woods KL, Iko WM. 2000. Pathogenicity of avian malaria in experimentally-infected Hawaii amakihi. *J. Wildl. Dis.* 36: 197-204.

Atkinson CT, Samuel MD. 2010. Avian malaria *Plasmodium relictum* in native Hawaiian forest birds: epizootiology and demographic impacts on 'apapane *Himatione sanguinea*. *J. Avian Biol.* 41: 357-366.

Balloux F, Amos W, Coulson T. 2004. Does heterozygosity estimate inbreeding in real populations? *Mol. Ecol.* 13: 3021-3031.

Barrets CH, Charleswort D. 1991. Effects of a change in the level of inbreeding on the genetic load. *Nature* 352: 522-524.

Bensch S, Price T, Kohn J. 1997. Isolation and characterization of microsatellite loci in a *Phylloscopus* warbler. *Mol. Ecol.* 6: 91-92.

Blanchet S, Rey O, Berthier P, Lek S, Loot G. 2009. Evidence of parasite-mediated disruptive selection on genetic diversity in a wild fish population. *Mol. Ecol.* 18: 1112-1123.

Blondel J, Aronson J. 1999. *Biology and Wildlife of the Mediterranean Region*. Oxford: Oxford University Press.

Blondel J, Thomas DW, Charmantier A, Perret P, Bourgault P, Lambrechts MM. 2006. A thirty-year study of phenotypic and genetic variation of blue tits in Mediterranean habitat mosaics. *Bioscience* 56: 661-673.

Carr DE, Murphy JF, Eubanks MD. 2003. The susceptibility and response of inbred and outbred *Mimulus guttatus* to infection by *Cucumber mosaic virus*. *Evol. Ecol.* 17: 85-103.

Chapman JR, Nakagawa S, Coltman DW, Slate J, Sheldon BC. 2009. A quantitative review of heterozygosity-fitness correlations in animal populations. *Mol. Ecol.* 18: 2746-2765.

Coltman DW, Pilkington JG, Smith JA, Pemberton JM. 1999. Parasite-mediated selection against inbred Soay sheep in a free-living, island population. *Evolution* 53: 1259-1267.

Coltman DW, Slate J. 2003. Microsatellite measures of inbreeding: A meta-analysis. *Evolution* 57: 971-983.

Da Silva A, Gaillard JM, Yoccoz NG, Hewison AJM, Galan M, Coulson T, *et al.* 2009. Heterozygosity-fitness correlations revealed by neutral and candidate gene markers in roe deer from a long-term study. *Evolution* 63: 403-417.

David P. 1998. Heterozygosity-fitness correlations: new perspectives on old problems. *Heredity* 80: 531-537.

David P, Pujol B, Viard F, Castella V, Goudet J. 2007. Reliable selfing rate estimates from imperfect population genetic data. *Mol. Ecol.* 16: 2474-2487.

Dawson DA, Hanotte O, Greig C, Stewart IRK, Burke T. 2000. Polymorphic microsatellites in the blue tit *Parus caeruleus* and their cross-species utility in 20 songbird families. *Mol. Ecol.* 9: 1941-1944.

Dawson DA, Horsburgh GJ, Kupper C, Stewart IRK, Ball AD, Durrant KL. *et al.* 2010. New methods to identify conserved microsatellite loci and develop primer sets of high cross-species utility - as demonstrated for birds. *Mol. Ecol. Resour.* 10: 475-494.

Double MC, Dawson D, Burke T, Cockburn A. 1997. Finding the fathers in the least faithful bird: A microsatellite-based genotyping system for the superb fairy-wren *Malurus cyaneus*. *Mol. Ecol.* 6: 691-693.

Dudash MR, Carr DE, Fenster CB. 1997. Five generations of enforced selfing and outcrossing in *Mimulus guttatus*: Inbreeding depression variation at the population and family level. *Evolution* 51: 54-65.

Earl DA, vonHoldt BM. 2012. Structure Harvester: a website and program for visualizing Structure output and implementing the Evanno method. *Conserv. Genet. Resour.* 4: 359-361.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software Structure: a simulation study. *Mol. Ecol.* 14: 2611-2620.

Falush D, Stephens M, Pritchard JK. 2003. Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. *Genetics* 164, 1567-1587.

Ferguson MM, Drahushchak LR. 1990. Disease resistance and enzyme heterozygosity in rainbow-trout. *Heredity* 64: 413-417.

Ferrer ES, Garcia-Navas V, Sanz JJ, Ortego J. 2012. Molecular characterization of avian malaria parasites in three Mediterranean blue tit (*Cyanistes caeruleus*) populations. *Parasitol. Res.* 111: 2137-2142.

Foerster K, Delhey K, Johnsen A, Lifjeld JT, Kempenaers B. 2003. Females increase offspring heterozygosity and fitness through extra-pair matings. *Nature* 425: 714-717.

Forcada J, Hoffman JI. 2014. Climate change selects for heterozygosity in a declining fur seal population. *Nature* 511: 462-465.

Forstmeier W, Schielzeth H, Mueller JC, Ellegren H, Kempenaers B. 2012. Heterozygosity-fitness correlations in zebra finches: microsatellite markers can be better than their reputation. *Mol. Ecol.* 21: 3237-3249.

Fossoy F, Johnsen A, Lifjeld JT. 2009. Cell-mediated immunity and multi-locus heterozygosity in bluethroat nestlings. *J. Evol. Biol.* 22: 1954-1960.

Fox CW, Reed DH. 2011. Inbreeding depression increases with environmental stress: An experimental study and meta-analysis. *Evolution* 65: 246-258.

García-Navas V, Ortego J, Sanz J J. 2009. Heterozygosity-based assortative mating in blue tits (*Cyanistes caeruleus*): implications for the evolution of mate choice. *Proc. R. Soc. B-Biol. Sci.* 276: 2931-2940.

Garnier-Gere P, Dillmann C. 1992. A computer-program for testing pairwise linkage disequilibria in subdivided populations. *J. Hered.* 83: 239-239.

Griffith SC, Stewart IRK, Dawson DA, Owens IPF, Burke T. 1999. Contrasting levels of extra-pair paternity in mainland and island populations of the house sparrow (*Passer domesticus*): is there an 'island effect'? *Biol. J. Linn. Soc.* 68: 303-316.

Guo SW, Thompson EA. 1992. A monte-carlo method for combined segregation and linkage analysis. *Am. J. Hum. Genet.* 51: 1111-1126.

Haag CR, Sakwinska O, Ebert D. 2003. Test of synergistic interaction between infection and inbreeding in *Daphnia magna*. *Evolution* 57: 777-783.

Hansson B, Bensch S, Hasselquist D, Akesson M. 2001. Microsatellite diversity predicts recruitment of sibling great reed warblers. *Proc. R. Soc. B-Biol. Sci.* 268: 1287-1291.

Hansson B, Westerberg L. 2002. On the correlation between heterozygosity and fitness in natural populations. *Mol. Ecol.* 11: 2467-2474.

Hawley DM, Sydenstricker KV, Kollias GV, Dhondt AA. 2005. Genetic diversity predicts pathogen resistance and cell-mediated immunocompetence in house finches. *Biol. Lett.* 1: 326-329.

Hoffman JI, Simpson F, David P, Rijks JM, Kuiken T, Thorne MAS, Lacy RC, Dasmahapatra KK. 2014. High-throughput sequencing reveals inbreeding depression in a natural population. *Proc. Natl. Acad. Sci. U.S.A.* 111: 3775-3780.

Hubisz MJ, Falush D, Stephens M, Pritchard JK. 2009. Inferring weak population structure with the assistance of sample group information. *Mol. Ecol. Resour.* 9: 1322-1332.

Kardos M, Allendorf FW , Luikart G. 2014. Evaluating the role of inbreeding depression in heterozygosity-fitness correlations: how useful are tests for identity disequilibrium? *Mol. Ecol. Resour.*, 14: 519-530.

Keller LF, Waller DM. 2002. Inbreeding effects in wild populations. *Trends Ecol. Evol.* 17: 230-241.

Knowles SCL, Palinauskas V, Sheldon BC. 2010. Chronic malaria infections increase family inequalities and reduce parental fitness: experimental evidence from a wild bird population. *J. Evol. Biol.* 23: 557-569.

Kristensen TN, Dahlgaard J, Loeschcke V. 2002. Inbreeding affects Hsp70 expression in two species of *Drosophila* even at benign temperatures. *Evol. Ecol. Res.* 4: 1209-1216.

Kupper C, Kosztolanyi A, Augustin J, Dawson DA, Burke T, Szekely T. 2010. Heterozygosity-fitness correlations of conserved microsatellite markers in Kentish plovers *Charadrius alexandrinus*. *Mol. Ecol.* 19: 5172-5185.

Lachish S, Knowles SCL, Alves R, Wood MJ, Sheldon BC. 2011. Fitness effects of endemic malaria infections in a wild bird population: the importance of ecological structure. *J. Anim. Ecol.* 80: 1196-1206.

Laine VN, Herczeg G, Shikano T, Primmer CR. 2012. Heterozygosity-behaviour correlations in nine-spined stickleback (*Pungitius pungitius*) populations: contrasting effects at random and functional loci. *Mol. Ecol.* 21: 4872-4884.

Li YC, Korol AB, Fahima T, Nevo E. 2004. Microsatellites within genes: Structure, function, and evolution. *Mol. Biol. Evol.* 21: 991-1007.

Luikart G, Pilgrim K, Visty J, Ezenwa VO, Schwartz MK. 2008. Candidate gene microsatellite variation is associated with parasitism in wild bighorn sheep. *Biol. Lett.* 4: 228-231.

Luong LT, Heath BD, Polak M. 2007. Host inbreeding increases susceptibility to ectoparasitism. *J. Evol. Biol.* 20: 79-86.

Meagher S. 1999. Genetic diversity and *Capillaria hepatica* (Nematoda) prevalence in Michigan deer mouse populations. *Evolution* 53: 1318-1324.

Merino S, Moreno J, Sanz JJ, Arriero E. 2000. Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). *Proc. R. Soc. B-Biol. Sci.* 267: 2507-2510.

Mitton JB, Grant MC. 1984. Associations among protein heterozygosity, growth-rate, and developmental homeostasis. *Annu. Rev. Ecol. Evol. Syst.* 15: 479-499.

Mueller JC, Hermisson J, Olano-Marin J, Hansson B, Kempenaers B. 2011. Linking genetic mechanisms of heterozygosity-fitness correlations to footprints of selection at single loci. *Evol. Ecol.* 25: 1-11.

Nakagawa S, Cuthill IC. 2007. Effect size, confidence interval and statistical significance: a practical guide for biologists. *Biol. Rev.* 82: 591-605.

Neff BD. 2004. Stabilizing selection on genomic divergence in a wild fish population. *Proc. Natl. Acad. Sci. U. S. A.* 101: 2381-2385.

Obrien SJ, Evermann JF. 1988. Interactive influence of infectious disease and genetic diversity in natural populations. *Trends Ecol. Evol.* 3: 254-259.

Olano-Marin J, Dawson DA, Girg A, Hansson B, Ljungqvist M, Kempenaers B. *et al.* 2010. A genome-wide set of 106 microsatellite markers for the blue tit (*Cyanistes caeruleus*). *Mol. Ecol. Resour.* 10: 516-532.

Olano-Marin J, Mueller JC, Kempenaers B. 2011a. Correlations between heterozygosity and reproductive success in the blue tit (*Cyanistes caeruleus*): an analysis of inbreeding and single locus effects. *Evolution* 65: 3175-3194.

Olano-Marin J, Mueller JC, Kempenaers B. 2011b. Heterozygosity and survival in blue tits (*Cyanistes caeruleus*): contrasting effects of presumably functional and neutral loci. *Mol. Ecol.* 20: 4028-4041.

Oliver MK, Lambin X, Cornulier T, Piertney SB. 2009. Spatiotemporal variation in the strength and mode of selection acting on major histocompatibility complex diversity in water vole (*Arvicola terrestris*) metapopulations. *Mol. Ecol.* 18: 80-92.

Oliver MK, Piertney SB. 2012. Selection maintains MHC diversity through a natural population bottleneck. *Mol. Biol. Evol.* 29: 1713-1720.

Ortego J, Aparicio JM, Calabuig G, Cordero PJ. 2007a. Risk of ectoparasitism and genetic diversity in a wild lesser kestrel population. *Mol. Ecol.* 16: 3712-3720.

Ortego J, Cordero PJ, Aparicio JM, Calabuig G. 2007b. No relationship between individual genetic diversity and prevalence of avian malaria in a migratory kestrel. *Mol. Ecol.* 16: 4858-4866.

Ortego J, Cordero PJ, Aparicio JM, Calabuig G. 2008. Consequences of chronic infections with three different avian malaria lineages on reproductive performance of lesser kestrels (*Falco naumanni*). *J. Ornithol.* 149: 337-343.

Ortego J, García-Navas V, Ferrer ES, Sanz JJ. 2011a. Genetic structure reflects natal dispersal movements at different spatial scales in the blue tit, *Cyanistes caeruleus*. *Anim. Behav.* 82: 131-137.

Ortego J, Yannic G, Shafer ABA, Mainguy J, Festa-Bianchet M, Coltman DW. *et al.* 2011b. Temporal dynamics of genetic variability in a mountain goat (*Oreamnos americanus*) population. *Mol. Ecol.* 20: 1601-1611.

Otter K, Ratcliffe L, Michaud D, Boag PT. 1998. Do female black-capped chickadees prefer high-ranking males as extra-pair partners? *Behav. Ecol. Sociobiol.* 43: 25-36.

Pearman PB, Garner TWJ. 2005. Susceptibility of Italian agile frog populations to an emerging strain of Ranavirus parallels population genetic diversity. *Ecol. Lett.* 8: 401-408.

Pedersen KS, Kristensen TN, Loeschcke V. 2005. Effects of inbreeding and rate of inbreeding in *Drosophila melanogaster* - Hsp70 expression and fitness. *J. Evol. Biol.* 18: 756-762.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics*, 155: 945-959.

Puurtinen M, Hytonen M, Knott KE, Taskinen J, Nissinen K, Kaitala V. 2004. The effects of mating system and genetic variability on susceptibility to trematode parasites in a freshwater snail, *Lymnaea stagnalis*. *Evolution* 58: 2747-2753.

R Development Core Team 2012. R: A Language and Environment for Statistical Computing. Version 3.0.2. R Foundation for Statistical Computing, Vienna, Austria. http://www.r-project.org.

Raymond M, Rousset F. 1995. GENEPOP (Version-1.2) - Population-genetics software for exact tests and ecumenicism. *J. Hered.* 86: 248-249.

Reid JM, Arcese P, Keller LF, Elliott KH, Sampson L, Hasselquist D. 2007. Inbreeding effects on immune response in free-living song sparrows (*Melospiza melodia*). *Proc. R. Soc. B-Biol. Sci.* 274: 697-706.

Rice WR. 1989. Analyzing tables of statistical tests. *Evolution* 43: 223-225.

Richardson DS, Jury FL, Dawson DA, Salgueiro P, Komdeur J, Burke T. 2000. Fifty Seychelles warbler (*Acrocephalus sechellensis*) microsatellite loci polymorphic in Sylviidae species and their cross-species amplification in other passerine birds. *Mol. Ecol.* 9: 2226-2231.

Rijks JM, Hoffman JI, Kuiken T, Osterhaus ADME, Amos W. 2008. Heterozygosity and lungworm burden in harbour seals (*Phoca vitulina*). *Heredity* 100: 587-593.

Ruiz-Lopez MJ, Monello RJ, Gompper ME, Eggert LS. 2012. The effect and relative importance of neutral genetic diversity for predicting parasitism varies across parasite taxa. *PLoS One* 7: e45404.

Saccheri I, Kuussaari M, Kankare M, Vikman P, Fortelius W, Hanski I. 1998. Inbreeding and extinction in a butterfly metapopulation. *Nature* 392: 491-494.

Slate J, David P, Dodds KG, Veenvliet BA, Glass BC, Broad TE. *et al.* 2004. Understanding the relationship between the inbreeding coefficient and multilocus heterozygosity: theoretical expectations and empirical data. *Heredity* 93: 255-265.

Slate J, Hale MC, Birkhead TR. 2007. Simple sequence repeats in zebra finch (*Taeniopygia guttata*) expressed sequence tags: a new resource for evolutionary genetic studies of passerines. *BMC Genomics* 8: 52.

Slate J, Pemberton J. 2006. Does reduced heterozygosity depress sperm quality in

wild rabbits (*Oryctolagus cuniculus*)? *Curr. Biol.* 16: R790-R791.

Sol D, Jovani R, Torres J. 2003. Parasite mediated mortality and host immune response explain age-related differences in blood parasitism in birds. *Oecologia* 135: 542-547.

Spielman D, Brook BW, Frankham R. 2004. Most species are not driven to extinction before genetic factors impact them. *Proc. Natl. Acad. Sci. U.S.A.* 101: 15261-15264.

Svensson L. 1992. *Identification guide to European Passerines*. Stockholm: Lars Svensson.

Szulkin M, Bierne N, David P. 2010. Heterozygosity-fitness correlations: a time for reappraisal. *Evolution* 64: 1202-1217.

Szulkin M, David P. 2011. Negative heterozygosity-fitness correlations observed with microsatellites located in functional areas of the genome. *Mol. Ecol.* 20: 3949-3952.

Van Riper C, Vanriper SG, Goff ML, Laird M. 1986. The epizootiology and ecological significance of malaria in hawaiian land birds. *Ecol. Monogr.* 56: 327-344.

Voegeli B, Saladin V, Wegmann M, Richner H. 2012. Parasites as mediators of heterozygosity-fitness correlations in the great tit (*Parus major*). *J. Evol. Biol.* 25: 584-590.

Waldenström J, Bensch S, Hasselquist D, Ostman O. 2004. A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. *J. Parasitol.* 90: 191-194.

Wegner KM, Kalbe M, Kurtz J, Reusch TBH, Milinski M. 2003a. Parasite selection for immunogenetic optimality. *Science* 301: 1343-1343.

Wegner KM, Reusch TBH, Kalbe M. 2003b. Multiple parasites are driving major histocompatibility complex polymorphism in the wild. *J. Evol. Biol.* 16: 224-232.

Weir BS, Cockerham CC. 1973. Mixed self and random mating at 2 loci. *Gen. Res.* 21: 247-262.

Westerdahl H. 2007. Passerine MHC: genetic variation and disease resistance in the wild. *J. Ornithol.* 148: S469-S477.

Westerdahl H, Waldenström J, Hansson B, Hasselquist D, von Schantz T, Bensch S. 2005. Associations between malaria and MHC genes in a migratory songbird. *Proc. R. Soc. B-Biol. Sci.* 272: 1511-1518.

Whitehorn PR, Tinsley MC, Brown MJF, Darvill, B, Goulson, D. 2011. Genetic diversity, parasite prevalence and immunity in wild bumblebees. *Proc. R. Soc. B-Biol. Sci.* 278: 1195-1202.

Whiteman NK, Matson KD, Bollmer JL, Parker PG. 2006. Disease ecology in the Galapagos hawk (*Buteo galapagoensis*): host

genetic diversity, parasite load and natural antibodies. *Proc. R. Soc. B-Biol. Sci*. 273: 797-804.

Wiehn J, Kopp K, Rezzonico S, Karttunen S, Jokela J. 2002. Family-level covariation between parasite resistance and mating system in a hermaphroditic freshwater snail. *Evolution* 56: 1454-1461.

Willi Y, Van Buskirk J, Hoffmann AA. 2006. Limits to the adaptive potential of small populations. *Annu. Rev. Ecol. Evol. Syst.* 37: 433-458.

SUPPORTING INFORMATION

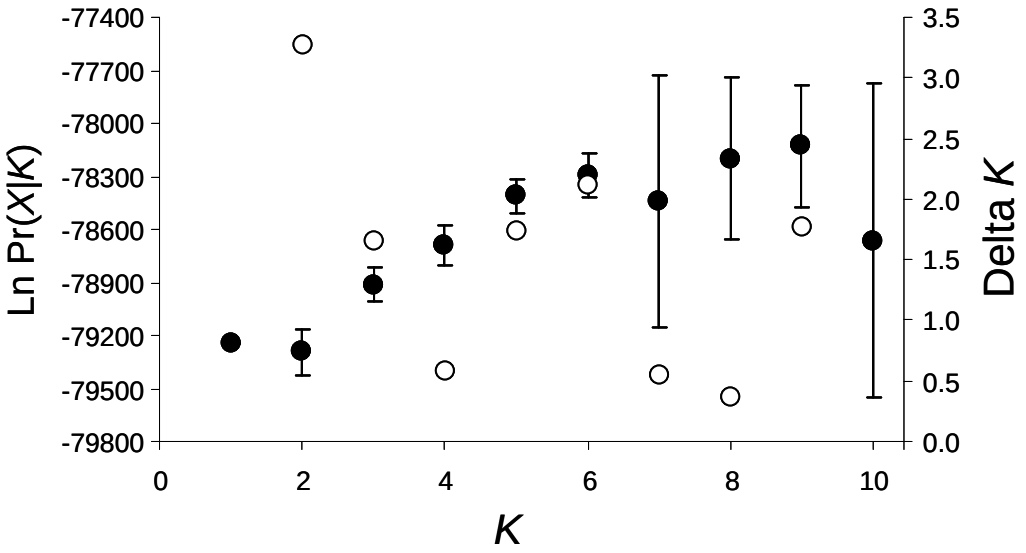


Figure S1 Results of Bayesian clustering analyses in STRUCTURE. Plots show the mean (± S.D.) log probability of the data (ln Pr(X | *K*)) over 10 runs (left axis, black dots and error bars) for each value of *K*. The magnitude of Δ*K* as a function of *K* indicates the most likely number of genetic clusters (*K* = 3) in the STRUCTURE analyses (right axis, open dots).

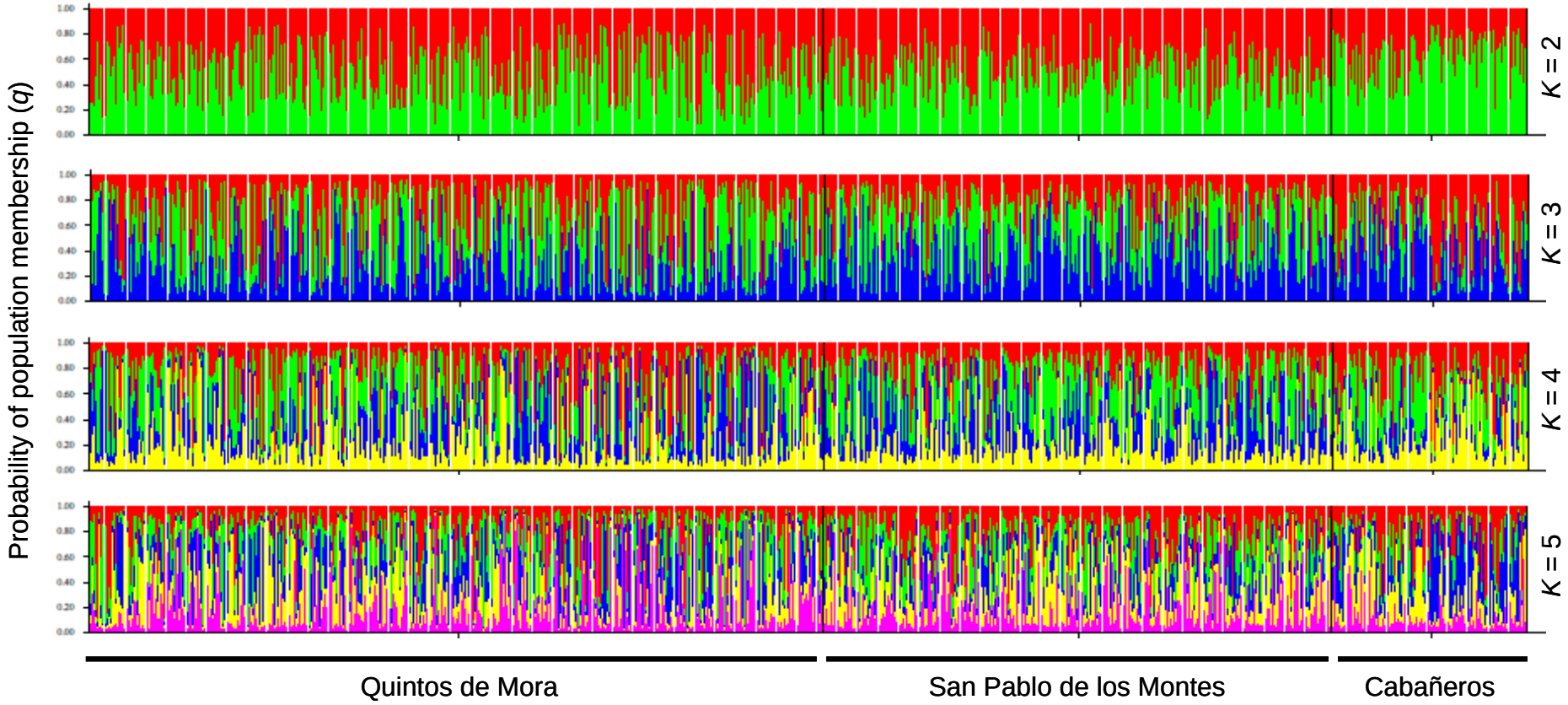
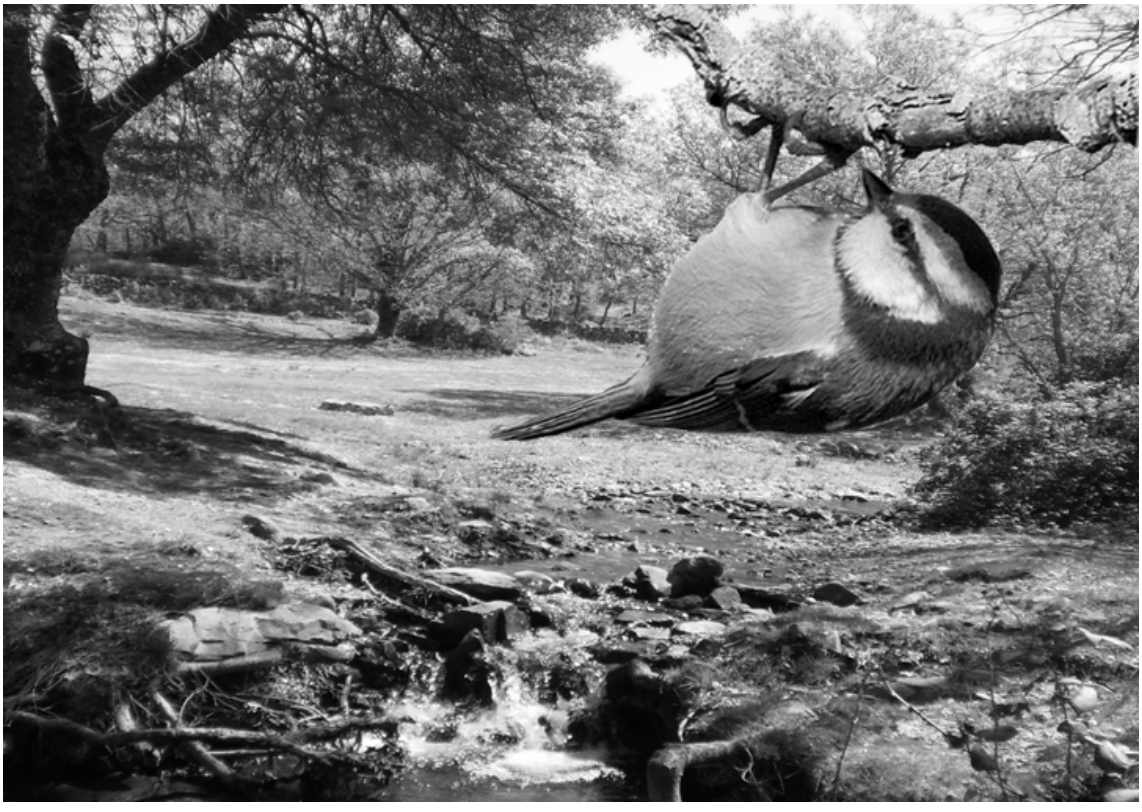


Figure S2 Results of genetic assignments based on the Bayesian method implemented in the program STRUCTURE considering genetic clusters from $K = 2$ to $K = 5$. Each individual is represented by a thin vertical line, which is partitioned into coloured segments that represent the individual's probability of belonging to the cluster with that colour.

5. The strength of selection on heterozygosity at both functional and neutral genomic regions increases with environmental harshness in blue tits

Ferrer ES, García-Navas V, Sanz JJ, Ortego J

Under review



The strength of selection on heterozygosity at both functional and neutral genomic regions increases with environmental harshness in blue tits

Abstract

The extent of inbreeding depression and the magnitude of heterozygosity-fitness correlations (HFC) have been suggested to depend on the environmental context in which they are assayed, but little evidence is available for wild populations. In the present study, we combine extensive molecular and capture-mark-recapture data from a blue tit (*Cyanistes caeruleus*) population to (*i*) analyze the relationship between heterozygosity and probability of inter-annual survival and (*ii*) test whether environmental stress imposed by physiologically suboptimal temperatures and rainfall influence the magnitude of HFC and the strength of selection on heterozygosity each year. To address these questions we used two different arrays of markers: 14 loci classified as neutral and 12 loci classified as putatively functional. We found significant relationships between heterozygosity and adult survival probability that were most likely explained by variation in genome-wide heterozygosity. The strength of selection for heterozygosity differed among years and was positively associated with annual accumulated precipitation. Annual mean heterozygosity increased over time, suggesting a micro-evolutionary response to selection over the course of the study period. Finally, neutral and functional loci showed similar trends, but the former had stronger effect sizes and seemed to reflect better genome-wide heterozygosity. Overall, our results show that HFC can be context-dependent, emphasizing the need for considering the role of environmental heterogeneity as a key factor when exploring the consequences of individual genetic diversity on fitness in natural populations.

INTRODUCTION

Inbreeding and reduced levels of genetic diversity have been found to negatively impact different components of fitness, including reproductive performance Seddon *et al.* 2004; Heber & Briskie 2010; Ortego *et al.* 2010), immune response (Hawley *et al.* 2005; Rantala & Roff 2007), resistance to parasites (Reid *et al.* 2007; Charpentier *et al.* 2008) and survival (Acevedo-Whitehouse *et al.* 2006; Richner & Nunziata 2014). The positive association between individual genetic diversity and fitness-related traits arise when homozygotes are less fit than heterozygotes due to either heterozygote advantage (i.e., overdominance) or recessivity of deleterious or partly deleterious alleles (Charlesworth & Charlesworth 1987; Keller & Waller 2002). The study of this relationship in wild populations has traditionally been problematic due to the difficulty of obtaining well-resolved pedigrees to estimate individual coancestry, and an alternative approach consisting in

measuring genetic diversity at a set of loci has become popular (Pemberton 2004; reviewed in Chapman *et al.* 2009). Consequently, in most studies individual genetic diversity is assessed using microsatellite markers, which are only expected to reflect genome-wide heterozygosity if different processes, fundamentally inbreeding, genetic drift, genetic admixture and bottlenecks, contribute to the generation of identity disequilibrium (ID) Balloux *et al.* 2004; Szulkin *et al.* 2010. Although ID is considered to be the fundamental cause of heterozygosity-fitness correlations (HFC) ("general effect hypothesis"; David 1998), it has been suggested that HFC may also result from functional overdominance at the scored loci *per se* ("direct effect hypothesis"; David 1998; e.g. Li *et al.* 2004) or as consequence of some markers being linked to genes under selection ("local effect hypothesis"; Hansson & Westerberg 2002; Slate *et al.* 2004; García-Navas *et al.* 2014). Despite a considerable number of studies have analyzed the association between different components of fitness and marker-based estimates of heterozygosity, the relative importance of the above described hypotheses to explain observed HFC is still controversial and matter of ongoing debate (Chapman *et al.* 2009; Szulkin *et al.* 2010; Miller & Coltman 2014).

The extent of inbreeding depression and the magnitude of HFC have been suggested to depend on the specific environmental context in which they are assayed (Miller 1994; Armbruster & Reed 2005; Marr *et al.* 2006; Fox & Reed 2011). Different studies have found that inbreeding depression and HFC only arise or manifest more strongly when resources are limited (food supply: Lesbarèrres *et al.* 2005; water shortage: Vranckx *et al.* 2014) or under stressful conditions imposed by different biotic (parasites: Coltman *et al.* 1999; Voegeli *et al.* 2012; Carr *et al.* 2003; Haag *et al.* 2003; competitors: Cheptou *et al.* 2000) and abiotic factors (thermal stress: Scott & Koehn 1990; adverse weather conditions: Marr *et al.* 2006; Forcada & Hoffman 2014). Thus, the environmental context (i.e., the "stressfulness" of the environment) under which HFC are analyzed can have profound effects on the inferred consequences of reduced levels of genetic diversity for a given population. In this sense, the high spatiotemporal variability in the conditions experienced by individuals (e.g., weather, parasitism pressure, competition, etc.) could explain the apparently contradictory results found in different HFC studies on wild populations (Coltman & Slate 2003; Armbruster & Reed 2005; Chapman *et al.* 2009; Fox & Reed 2011). The study of genetic diversity-environment interactions also has important implications for understanding the potential of populations to cope with different sources of stress and predict their future demographic and evolutionary dynamics in response to climate change and habitat fragmentation (Reed *et al.* 2002, 2007; Liao & Reed 2009; Forcada & Hoffman 2014). However, studies on HFC performed in natural

populations have rarely considered the impact of environmental heterogeneity and evidence on this respect generally comes from experimental or laboratory studies (but see Coltman *et al.* 1999; Marr *et al.* 2006; Annavi *et al.* 2014; Forcada & Hoffman 2014).

In the present study we combine extensive molecular and capture-mark-recapture data to analyze the relationship between heterozygosity and probability of inter-annual survival in a short-lived passerine with limited dispersal, the blue tit (*Cyanistes caeruleus*). Previous studies carried out on this species have found significant heterozygosity-fitness correlations considering different life-history traits (reproductive performance García-Navas *et al.* 2009; Foerster *et al.* 2003; Olano-Marin *et al.* 2011a; survival probability: Olano-Marin *et al.* 2011b; parasite resistance: Ferrer *et al.* 2014). Consanguineous matings have been reported in these populations, which may increase variance in inbreeding and the chance of detecting HFC due to extensive ID across the genome (Balloux *et al.* 2004; Szulkin *et al.* 2010). In addition, we examine the relationship between environmental variability and the strength of selection on heterozygosity. As other small passerines, the blue tit has a high metabolic rate and a limited capacity to deposit long-term body reserves (McNab 2002), factors that are likely to increase its susceptibility to environmental stress imposed by food shortage or severe weather conditions (Marr *et al.* 2006). For these reasons, our study system is particularly amenable to study HFC and their interactions with environment.

Specifically, we monitored inter-annual survival over six consecutive years (2008-2013) and genotyped all adult blue tits across 26 microsatellite markers to estimate individual genetic diversity. Further, we used two different arrays of markers, classified as putatively neutral (14 loci) or functional (12 loci) by considering whether the genomic region where they are located is transcribed to RNA (*sensu* Olano-Marin *et al.* 2011ab; see also Da Silva *et al.* 2009; Küpper *et al.* 2010; Laine *et al.* 2012), which allowed us to test potential differences in HFC and genetic-environment interactions between these subsets of loci. First, we analyzed (*i*) the relationship between probability of inter-annual survival and individual genetic diversity and tested whether such associations (*ii*) varied between functional and neutral loci and (*iii*) were better explained by a genome-wide effect ("general effect hypothesis") or strong linkage disequilibrium between the employed markers and genes under selection ("local effect hypothesis"). Second, (*iv*) we studied whether environmental stress imposed by physiologically suboptimal temperatures and rainfall influence the magnitude of HFC and the intensity of selection on heterozygosity (e.g., Keller *et al.* 2002; Szulkin & Sheldon 2007; Forcada & Hoffman 2014). Finally, (*v*) we analyzed the change in heterozygosity over time to test for a microevolutionary response to

selection (e.g., Bensch *et al.* 2006; Kaeuffer *et al.* 2007; Forcada & Hoffman 2014).

MATERIALS AND METHODS

Study system and general field procedures

During seven consecutive years (2008-2014) we have monitored a blue tit population established in two nearby plots (Gil García: 39º22´N 4º07´W; Valdeyernos: 39º26´N 4º05´W) located in Quintos de Mora (Montes de Toledo, central Spain). Both study plots consist of a Pyrenean oak (*Quercus pyrenaica*) forest (~22 ha) containing 100 nestboxes (see García-Navas & Sanz 2011 for more details). The climate is meso-mediterranean with mean temperatures ranging from 24-26°C in July to 4-6°C in January and 300-600 mm of rainfall concentrated in spring and autumn. Regular frosts take place at night during the winter, but temperatures very rarely fall below -5ºC and snowfalls are anecdotal (Tornero 2003).

Adult blue tits were captured while feeding 8-days old nestlings by means of spring traps, weighed using an electronic portable balance (accuracy ± 0.1 g) and their tarsus and wing length measured with a digital calliper (to the nearest 0.01 mm) and a stopped ruler (to the nearest 0.1 mm), respectively. Upon capture, we determined sex and age (juveniles or ≥2 yr breeders) based on plumage characteristics according to Svensson (1992). We knew the exact age of around 73% of individuals that were ringed as fledglings or juveniles (i.e., nearly three quarters of birds that we tracked from their born or their first reproduction). For all other individuals, we considered that they were captured in their second year if they presented adult plumage (e.g., Ortego *et al.* 2007). All birds were banded with metal rings (if they were not already) and bled (20-30 µl) by puncturing the brachial vein. Blood samples were stored on FTA cards (Whatman Bioscience, Florham Park, NJ, USA) or in Eppendorf tubes with 96% ethanol until needed for genetic analyses. All morphometric measurements were taken by the same observer (VGN).

Survival estimates

In this study, we focus on the probability of inter-annual survival to analyze i) if it is associated with individual genetic diversity and ii) whether the intensity of selection on heterozygosity each year is related with the environmental conditions experienced by the individuals until the next breeding season (see below). Inter-annual survival was assessed through intensive capture-mark-recapture of breeding individuals across all the study years (capture effort = 82.72%) under the assumption that individuals not returning to the study area and not breeding in the subsequent year had died (survival = local survival). Individuals recaptured the following breeding season were coded as "1", whereas those that were not recovered were coded as "0" (e.g., Olano-Marin *et al.* 2011b).

Microsatellite genotyping

We genotyped a total of 388 blue tits using a panel of 26 polymorphic microsatellite markers (see electronic supplementary material, Table S1). These markers were classified as presumably functional or neutral as described by Olano-Marin *et al.* (2011a,b). We extracted DNA using NucleoSpin Blood Kits (Macherey-Nagel, Duren, Germany) and amplified it in 10-μL reaction volumes containing 1 μL of purified DNA (~5 ng), 1X reaction buffer (67 mM Tris-HCL, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01 % Tween-20, EcoStart Reaction Buffer, Ecogen, Barcelona, Spain), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each dye-labelled primer (FAM, PET, VIC or NED) and 0.1 U of Taq DNA EcoStart Polymerase (Ecogen). The PCR programme used was 9 min denaturing at 95 ºC followed by 40 cycles of 30 s at 94 ºC, 45 s at the annealing temperature (Table S1) and 45 s at 72 ºC, ending with a 10 min final elongation step at 72 ºC. Amplification products were electrophoresed using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and genotypes were scored using GENEMAPPER 3.7 (Applied Biosystems).

Basic genetic statistics

Linkage disequilibrium (LD) between loci and deviations from Hardy-Weinberg equilibrium (HWE) were tested with the software GENEPOP on the Web (http://genepop.curtin.edu.au/; Raymond & Rousset, 1995). Probabilities of significance were computed applying a Markov chain method (Guo & Thompson 1992) using 200 batches and 1000 iterations per batch as implemented in GENEPOP. Correlation coefficient (*r*LD) between alleles at different loci was used to estimate the magnitude of LD. *r*LD was computed using the program LINKDOS (http://genepop.curtin.edu.au/linkdos.html ; Garnier-Gere & Dillmann 1992). We applied sequential Bonferroni corrections to account for multiple comparisons (Rice 1989).

Heterozygosity estimates and identity disequilibrium

We used homozygosity by locus (*HL*) to estimate individual genetic diversity, an index that improves heterozygosity estimates in open populations by weighting the contribution of each locus to the homozygosity value depending on their allelic variability (Aparicio *et al.* 2006). We calculated *HL* for all typed markers (HL_{Total}) and separately for the subsets of neutral ($HL_{Neutral}$) and functional loci ($HL_{Functional}$). *HL* values were calculated using CERNICALIN, an Excel spreadsheet available on request. We used the inverse of *HL* (i.e., 1-*HL*) as an estimate of individual heterozygosity in subsequent analyses.

Identity disequilibrium, defined as the correlation in heterozygosity and/or homozygosity across loci, is considered to be the fundamental cause of HFC (Szulkin *et al.* 2010). We analysed the presence of identity disequilibrium to determine

whether heterozygosity measured at our set of microsatellite loci is representative of genome-wide inbreeding. For this purpose, we calculated the parameter g^2, a central measure of identity disequilibrium that quantifies the excess of double heterozygotes at two loci relative to the expectation under random association (David *et al.* 2007). This estimate is constant for any pair of loci considered and only depends on the mean and variance of inbreeding in the population (David *et al.* 2007; Szulkin *et al.* 2010). We used the software RMES to calculate g^2 and test whether this parameter differed significantly from zero (David *et al.* 2007).

Heterozygosity-fitness correlations: multi-locus effects

We used an information-theoretic model selection approach to analyse the association between probability of inter-annual survival and individual heterozygosity (Burnham & Anderson 1998). For each year, we constructed two separated generalized linear mixed models (GLMMs, binomial error and logit link function), one including as predictor variable individual heterozygosity (i.e., 1-*HL*) calculated for all loci (HL_{Total}) and another including as predictor variables heterozygosity estimated for the subsets of neutral ($HL_{Neutral}$) and functional ($HL_{Functional}$) markers (e.g., Ferrer *et al.* 2014). Study plot and sex were included as fixed factors in all the models. Moreover, we included body condition (estimated as the residuals of a multiple regression of body mass on wing and tarsus length) and the age of individuals as covariates and mating pair identity as random effect in all the models. We ranked the resulting models following a model-selection approach on the basis of the Akaike's information criterion corrected for small sample size (AICc; Burnham & Anderson 1998). AICc values for each model were rescaled (ΔAICc) calculating the difference between the AICc value of each model and the minimum AICc obtained among all competing models (i.e. the best model has ΔAICc = 0). Models with ΔAICc ≤ 2 were considered equivalent (Burnham & Anderson 1998). In cases where model selection as a function of AICc did not give a single model, we performed an averaging of equivalent models (i.e. models with ΔAICc ≤ 2; Burnham & Anderson 2002). We calculated the mean of the predictor estimators, their unconditional standard errors (USE) and confidence intervals (CI), and the relative importance of each variable in the final averaged model (Σ ωi, the sum of Akaike weights of models with ΔAICc ≤ 2 in which the variable was included). Parameter estimates were considered significant when their 95% CI did not span zero [64]. Model selection and averaging was performed using the R package lme4 with binomial error and AICcmodavg (R Core Team 2012).

Heterozygosity-fitness correlations: single-locus effects

First, we examined whether multilocus heterozygosity (*MLH*) explained more variance than single-locus heterozygosity (*SLH*) following the approach described in Szulkin *et al.* (2010). We performed *F*-test ratio tests to compare models including *MLH* with those in which we replaced *MLH* with "normalized" *SLH* at all markers for each year (Szulkin *et al.* 2010). Second, we analysed the effect of single locus heterozygosity (*SLH*) by fitting one GLMM per locus and year. Effect size was calculated for each locus as the partial correlation coefficient obtained from its respective model (Nakagawa & Culhill 2007). Finally, we used a general linear model (GLM) to analyse whether absolute effect sizes of single locus heterozygosities were associated with marker variability (allelic richness and observed and expected heterozygosity, included as covariates) and differed between neutral and putatively functional loci (marker category was included as a fixed factor) (e.g., Olano-Marin *et al.* 2011ab; Ruiz-López *et al.* 2012; Ferrer *et al.* 2014).

Environmental data

Meteorological data used to determine the local environmental conditions experienced by individuals from the end of reproduction (July 1st) until the beginning of the next breeding season (March 31st) were obtained from the meteorological station located in Quintos de Mora (39° 25'N, 4° 04'W). We calculated three parameters as potential indicators of environmental harshness and that may influence the survival prospects and/or the individual physiological state in our study species. First, we calculated the number of days with temperature ≥ 35ºC or ≤ 15ºC. These temperatures represent the upper and lower critical limits of the thermal neutral zone (TNZ; i.e., the temperature tolerance range of an endotherm organism) for blue tits and other small passerines beyond of which the energy expenditure and oxygen consumption increases (Dawson & Carey 1976; Haftorn & Reinertsen 1985; Reinertsen & Haftorn 1986; Gavrilow & Dolnik 1985; Thomas *et al.* 2001). Second, we calculated the number of days with temperatures below 0 ºC (freezing degree-days, FDD), because frosts are likely to increase considerably energy expenditure for thermoregulation, particularly in Mediterranean small passerines adapted to mild climates (Broggi *et al.* 2007). Third, we calculated the accumulated precipitation (in millimeters, mm). Rainfall can dramatically increase energy demands for thermoregulation and energy expenditure for flight (through reduced maneuverability as feathers become water-logged) (Stalmaster & Gessaman 1984; Wilson *et al.* 2004). In addition, rainfall can reduce food availability and birds' capacity to acquire this resource, particularly in insectivorous species because arthropods are less active during adverse weather (e.g. Avery & Krebs 1984; Arlettaz *et al.* 2010), which can lead to a reduction in body condition and increase the likelihood of starvation and mortality

(Franklin *et al.* 2000; McDonald *et al.* 2004; Cowley & Siriwardena 2005; Öberg *et al.* 2015).

Selection analysis

The strength of the association between heterozygosity and fitness is expected to depend on factors such as the intensity of selection and the level of LD in the population (Armbruster & Reed 2005; Fox & Reed 2011). For each year, we estimated the directional selection differentials (S) (Falconer & Mackay 1996; Kingsolver *et al.* 2001; Wheelwright *et al.* 2014) for heterozygosity in relation with probability of inter-annual survival, a direct measure of fitness. Selection differentials were calculated for each year as the covariance of individual heterozygosity and standardized survival (i.e., S = cov[ω,heterozygosity]). Selection differentials were estimated for all markers (S_{Total}) and the subsets of neutral (S_{Neutral}) and functional ($S_{\text{Functional}}$) loci separately. We then analyzed the association between S and the three cues indicative of environmental harshness described in the previous section (see *Environmental data*) using linear regressions. Thereby, we tested whether environmental severity intensifies the strength of selection on heterozygosity. Finally, we analyzed the change in annual mean heterozygosity values over time using linear regressions. By this way, we tested for a microevolutionary response to selection during the study period (e.g., Charmantier *et al.* 2004; Forcada & Hoffman 2014).

RESULTS

Basic genetic statistics

Observed heterozygosity at each locus ranged from 0.48 to 0.94, with 3-60 alleles per locus (see electronic supplementary material, Table S1). Neutral loci tended to have higher allelic richness (A_R) and observed (H_O) and expected heterozygosity (H_E) than functional loci, but these differences were not statistically significant (one-way ANOVAs, A_R: $F_{1, 24} = 3.60$, $P = 0.069$; H_O: $F_{1, 24} = 2.77$, $P = 0.108$; H_E: $F_{1, 24} = 3.90$, $P = 0.059$). No loci consistently deviated from HWE or exhibited LD across all years and therefore all markers were used in subsequent analyses (see also Ferrer *et al.* 2014).

Identity disequilibrium

The g^2 estimator of identity disequilibrium calculated for all markers was positive in all study years and differed significantly from zero in 2008, 2009 and 2012 (see electronic supplementary material, Table S2). When the subset of neutral loci were analysed separately, g^2 values were also always positive and differed significantly for zero in 2009 and 2010 and were marginally significant in 2011 and 2012 (Table S2). For the subset of functional markers, g^2 value was only positive in 2011, 2012 and 2013 and only differed significantly from zero in 2012 (Table S2).

Table 1 GLMMs (binomial error and logit link function) for probability of inter-annual survival in relation with heterozygosity estimated at all loci (HL_{Total}) and different non-genetic terms (locality, age, body condition and sex) for the six study years (2008-2013). We performed model averaging of the best ranked equivalent models (ΔAICc ≤ 2) to obtain parameter estimates and unconditional standard errors (U.S.E.) (see Table S2 in electronic supplementary material). Variables are sorted according with their relative importance based on the sum of Akaike weights (Σ ωi) of those models with ΔAICc ≤ 2 in which the variable was present. Bold type indicates significant variables, i.e. variables for which their unconditional 95 % confidence interval (CI) did not cross zero.

	Estimate ± U.S.E.	Σ ωi	Lower 95% CI	Upper 95% CI
(a) 2008				
Sex	1.57 ± 0.62	0.49	**0.35**	**2.79**
Age	0.30 ± 0.54	0.17	-1.36	0.76
Body condition	-0.35 ± 0.49	0.09	-1.35	0.62
(b) 2009				
Locality	0.99 ± 0.41	0.48	**0.18**	**1.79**
Body condition	1.04 ± 0.43	0.48	**0.19**	**1.88**
HL_{Total}	3.04 ± 2.13	0.24	-1.13	7.22
(c) 2010				
Body condition	-0.71 ± 0.46	0.52	-1.61	0.19
Locality	0.46 ± 0.53	0.10	-0.58	1.5
Age	-0.18 ± 0.29	0.08	-0.76	0.39
Sex	0.26 ± 0.45	0.08	-0.62	1.14
HL_{Total}	1.38 ± 2.92	0.07	-4.33	7.01
(d) 2011				
HL_{Total}	-5.09 ± 2.31	0.50	**-9.62**	**-0.56**
Locality	0.72 ± 0.51	0.29	-0.28	1.72
Body condition	-0.41 ± 0.45	0.14	-1.28	0.47
Sex	-0.21 ± 0.32	0.10	-0.83	0.41
(e) 2012				
HL_{Total}	8.62 ± 4.29	0.62	**0.21**	**17.03**
Locality	1.64 ± 0.8	0.62	**0.07**	**3.20**
Age	-0.53 ± 0.36	0.53	-1.24	0.18
Body condition	-0.67 ± 0.74	0.20	-2.13	0.78
Sex	0.81 ± 0.73	0.20	-0.63	2.24
(f) 2013				
Body condition	0.45 ± 0.52	0.34	-0.56	1.47
Age	3.41 ± 0.00	0.25	-0.31	0.58

Heterozygosity-fitness correlations: multi-locus effects

Probability of survival was significantly associated with individual heterozygosity estimated at all loci in 2011 and 2012 (Table 1 and Table S3) and with heterozygosity estimated at the subset of neutral markers in 2011 (Tables 2 and Table S4). Yet, heterozygosity estimated at the subset of

Table 2 GLMMs (binomial error and logit link function) for probability of inter-annual survival in relation with heterozygosity estimated at the subset of neutral ($HL_{Neutral}$) and functional ($HL_{Functional}$) loci and different non-genetic terms (locality, age, body condition and sex) for the six study years (2008-2013). We performed model averaging of the best ranked equivalent models ($\Delta AICc \leq 2$) to obtain parameter estimates and unconditional standard errors (U.S.E.) (see Table S3 in electronic supplementary material). Variables are sorted according with their relative importance based on the sum of Akaike weights ($\Sigma\ \omega i$) of those models with $\Delta AICc \leq 2$ in which the variable was present. Bold type indicates significant variables, i.e. variables for which their unconditional 95 % confidence interval (CI) did not cross zero.

	Estimate ± U.S.E.	Σ ωi	Lower 95% CI	Upper 95% CI
(a) 2008				
Sex	1.58 ± 0.63	0.47	**0.35**	**2.79**
Body condition	-0.33 ± 0.50	0.15	-1.31	0.65
$HL_{Functional}$	-2.30 ± 2.40	0.12	-7.00	2.41
$HL_{Neutral}$	2.01 ± 2.94	0.06	-3.76	7.78
Age	-0.30 ± 0.54	0.05	-1.36	0.76
(b) 2009				
Locality	0.98 ± 0.41	0.55	**0.18**	**1.78**
Body condition	1.05 ± 0.43	0.55	**0.20**	**1.90**
$HL_{Neutral}$	2.61 ± 1.74	0.24	-0.81	6.03
$HL_{Functional}$	0.95 ± 1.39	0.07	-1.78	3.68
(c) 2010				
Body condition	-0.71 ± 0.46	0.39	-1.61	0.19
Locality	0.46 ± 0.53	0.07	-0.58	1.5
Age	-0.18 ± 0.29	0.06	-0.76	0.39
$HL_{Functional}$	1.14 ± 1.94	0.06	-2.66	4.94
Sex	0.26 ± 0.45	0.06	-0.62	1.14
(d) 2011				
$HL_{Neutral}$	-4.97 ± 2.34	0.45	**-9.56**	**-0.38**
$HL_{Functional}$	-1.05 ± 1.63	0.45	-4.25	2.14
Locality	0.97 ± 0.51	0.36	-0.03	1.98
Age	0.17 ± 0.23	0.07	-0.29	0.62
Body condition	-0.24 ± 0.01	0.06	**-0.25**	**-0.23**
Sex	-0.23 ± 0.46	0.06	-1.13	0.67
(e) 2012				
Locality	1.82 ± 0.85	0.67	**0.14**	**3.49**
$HL_{Functional}$	3.05 ± 2.41	0.67	-1.66	7.77
Age	-0.58 ± 0.38	0.59	-1.32	0.15
$HL_{Neutral}$	5.13 ± 3.28	0.41	-1.30	11.57
Sex	1.10 ± 0.77	0.29	-0.40	2.60
Body condition	-0.73 ± 0.79	0.22	-2.27	0.82
(f) 2013				
$HL_{Functional}$	5.98 ± 21.39	0.27	-35.94	47.91
$HL_{Neutral}$	60.64 ± 47.89	0.17	-33.22	154.51
Body condition	0.48 ± 0.52	0.17	-0.55	1.51
Sex	2.78 ± 6.96	0.06	-10.86	16.43

functional loci had no significant effect on probability of survival in any year (Table 2 and Table S4). The direction of the association differed between years; probability of survival decreased with individual heterozygosity in 2011, and the opposite pattern was found the next year (Tables 1-2). Regarding non-genetic terms, we found that the probability of survival was positively associated with body condition in 2009 and negatively in 2011, males were more likely to survive than females in 2008, and the likelihood of inter-annual survival was higher in Gil García than in Valdeyernos in 2009 and 2012 (Tables 1-2). The interaction between heterozygosity and sex or locality had no significant effect in any year (i.e., CIs crossed zero in all cases), indicating that the association between probability of survival and individual genetic diversity did not differ between males and females and the two studied localities.

Heterozygosity-fitness correlations: single-locus effects

We did not find significant differences in the variance explained by the models including multilocus heterozygosity (*MLH*) compared to the models including single locus heterozygosity (*SLH*) in any year or considering any subset of loci (all $P_S > 0.1$). Furthermore, no single locus effect was significant after sequential Bonferroni correction (see electronic supplementary material, Table S5). Absolute effect size of SLH was not correlated with marker genetic diversity (estimated as H_O, H_E or A_R; Table S1) and did not differ between the subsets of neutral and functional loci in any year (all $P_S > 0.05$).

Selection

Selection differentials (*S*) estimated at all loci were positive in all years except for 2008 and 2011, for neutral loci were positive in all years except 2011 and for

Table 3. Linear regressions between the three studied parameters associated with environmental harshness (TNZ days: number of days with temperature ≥35 ºC or ≤ 15ºC; FDD: freezing-degree days, calculated as the number of days with temperature < 0 ºC; accumulated precipitation) and selection differentials (*S*) for heterozygosity estimated at all markers (HL_{Total}) and the subsets of neutral ($HL_{Neutral}$) and functional loci ($HL_{Functional}$) for each study year. Table shows *r* values and *P*-values are indicated in parentheses.

	TNZ days	FDD	Accumulated precipitation
HL_{Total}	0.294 (*P* = 0.572)	0.050 (*P* = 0.926)	0.911 (*P* = 0.011)
$HL_{Neutral}$	0.321 (*P* = 0.535)	0.043 (*P* = 0.935)	0.862 (*P* = 0.027)
$HL_{Functional}$	0.232 (*P* = 0.658)	0.104 (*P* = 0.845)	0.946 (*P* = 0.004)

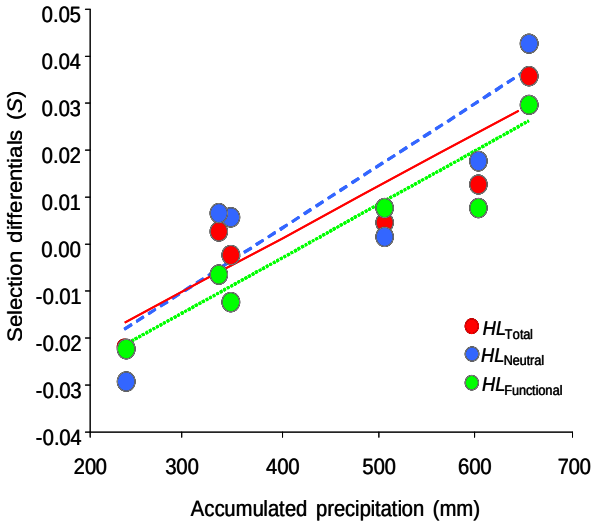


Figure 1 Relationship between annual accumulated precipitation and selection differentials (*S*) for heterozygosity estimated at all markers (HL_{Total}) and the subsets of neutral ($HL_{Neutral}$) and functional loci ($HL_{Functional}$). Regression lines are shown.

functional loci were negative in 2008, 2011 and 2013 (Table S2). *S* estimated at neutral and functional loci were significantly correlated ($r = 0.897$, $P = 0.015$), indicating that selection on heterozygosity acts in a similar way in both sets of markers. When we analyzed the association between *S* and the variables used as proxies for environmental harshness, we found a positive association with annual accumulative precipitation for all the subsets of markers (Table 3; Fig. 1). This indicates that selection on heterozygosity was higher in years with harder climatic conditions, i.e., wetter years. However, *S* was not associated with the number of days that reached temperatures below 0 ºC or beyond the limits of the thermal neutral zone (Table 3).

Annual mean heterozygosity estimated at neutral and functional loci were significantly correlated ($r = 0.844$, $P = 0.034$). Annual mean heterozygosity values estimated at all loci and the subset of neutral loci increased marginally and significantly, respectively, over the course of the study period (HL_{Total}: $r = 0.799$, $P = 0.056$; $HL_{Neutral}$: $r = 0.831$, $P = 0.040$; $HL_{Functional}$: $r = 0.566$, $P = 0.240$; Fig. 2), suggesting the existence of directional selection on heterozygosity.

DISCUSSION

We found significant relationships between individual genetic diversity estimated using different subsets of markers and adult survival probability in our study system. However, the direction and magnitude of HFC differed among years and they were only significantly explained by multi-locus heterozygosity calculated at all markers or the panel of neutral markers, but not by genetic diversity estimated at the subset of functional loci. We also found a highly significant positive relationship between the strength of selection for heterozygosity and annual accumulated precipitation, which indicates that inter-annual differences in HFC may be explained by the different environmental conditions to which individuals are exposed each year (Armbruster & Reed 2005; Fox & Reed 2011). Hence, our results reinforce the expectation of stronger selection under

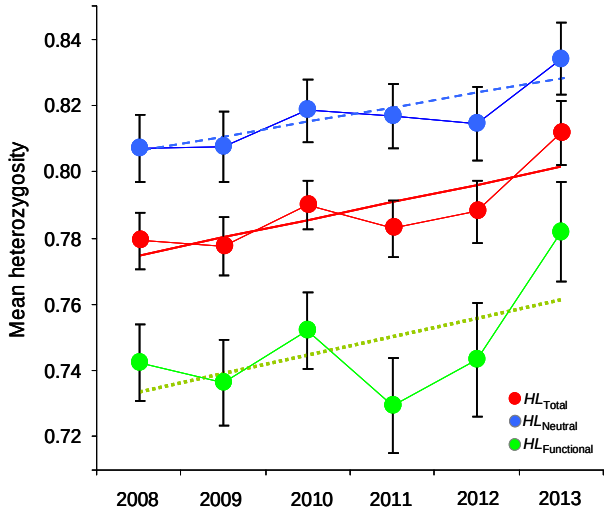


Figure 2 Temporal change in mean (± SE) heterozygosity estimated at all markers (HL_{Total}) and the subsets of neutral ($HL_{Neutral}$) and functional loci ($HL_{Functional}$) over the study period. Trend lines are shown.

adverse environmental conditions in wild populations (Endler 1986). We also found that heterozygosity increased over time, suggesting a micro-evolutionary response to selection over the course of the study period (Bensch *et al.* 2006; Kaeuffer *et al.* 2007; Forcada & Hoffman 2014).

Heterozygosity and fitness

We found different effects of multilocus heterozygosity on probability of survival depending on the year of study. In 2012, we detected a positive significant relationship between individual genetic diversity and probability of survival, but an opposite pattern was found in the previous year. Individuals from 2012 breeding season had to cope with a severe winter (high rainfall and incidence of FDD above the average; Table 2) which may have resulted in stronger natural selection against homozygous individuals (Keller *et al.* 1994; Armbruster & Reed 2005; Fox & Reed 2011). On the contrary, we found a counterintuitive negative association between heterozygosity and probability of survival in birds that reproduced in the previous year. Individuals that bred in 2011 experienced more benign environmental conditions during the following winter (low rainfall; Table S2), which may have resulted in a superior advantage of more inbred individuals due to local maladaptation of outbreed individuals (i.e., outbreeding depression; Marshall & Spalton 2000; Marr *et al.* 2002). Outbreed individuals resulted from immigrant-immigrant and local-immigrant crosses may be less fit than local (purebred) individuals if the later are better adapted to their native habitats (Coltman 2005; e.g., Postma & van Noordwijk 2005; García-Navas *et al.* 2014), a pattern that may only arise when benign environmental conditions relax the strength of selection on heterozygosity. This result is in agreement with that reported by Keller *et al.* (2006) in song sparrows (*Melospiza melodia*) as they found that inbred males produced fewer offspring in cooler years, but their productivity was similar in comparison with their outbred contemporaries after warm springs. Further, we have previously found that immigrant females, which represent the bulk of gene flow in blue tits, tend to be more heterozygous than residents in our study populations (García-Navas *et al.* 2014). More heterozygous immigrants may exhibit higher fecundity and viability than local individuals under stressful conditions but be in competitive disadvantage if more

benign environmental circumstances make resident individuals superior due to their higher familiarity and/or adaptation to local conditions (Dias & Blondel 1996; Hansson *et al.* 2004). Thus, our results are in accordance with previous studies finding that the effects of heterozygosity on fitness are complex and positive and negative correlations can coexist due to the particular environmental context prevailing in different years within the same population (Lesbarrères *et al.* 2005; Chapman *et al.* 2010; Annavi *et al.* 2014).

Our analyses aimed to disentangle the underlying mechanism behind the observed HFC suggest that they are most likely explained by a general effect. Single-locus heterozygosity models did not improve the variance explained by multilocus heterozygosity models, indicating that genome-wide heterozygosity is probably behind HFC in our study population (Szulkin *et al.* 2010). We neither found significant HFC when estimating heterozygosity from the subset of functional loci, which is the group of markers most likely to show local or direct effects due to their location inside or flanking coding gene sequences that are being actively expressed to RNA (David *et al.* 1998; Li *et al.* 2004; Olano-Marin *et al.* 2011ab). Further, we did not find heterozygosity at any particular marker, either neutral or functional, to be associated with fitness in any year. Thus, genome-wide inbreeding seems to be the most plausible explanation for the observed HFC, a hypothesis that is also partially supported by the fact that we found evidence for ID (i.e., positive and significant g^2 values) in different study years (David *et al.* 2007; Szulkin *et al.* 2010; e.g. García-Navas *et al.* 2014). In particular, all g^2 values were positive and many of them significant or marginally significant for all loci and the subset of neutral markers, whereas most g^2 values were not significant and often negative for the subset of functional loci. These results are similar to those found in a previous study on blue tits comparing both kind of markers and support the idea that neutral loci capture better the effects of genome-wide inbreeding than functional markers (Olano-Marin *et al.* 2011a). Certain characteristics present in both populations (population substructure, high phylopatry and occurrence of incestuous matings) may have contributed to generate ID, increasing the power of markers to reflect inbreeding and thus favouring the detection of significant HFC (García-Navas *et al.* 2009; Olano-Marin *et al.* 2011ab; Ferrer *et al.* 2014).

Selection on heterozygosity and environment

Several studies have found that the association between genetic diversity (or inbreeding) and different components of fitness become stronger under stressful conditions (Ambruster & Reed 2005; Fox & Reed 2011), but most evidence on this respect comes from experimental approaches and only a few studies have analyzed such interactions in wild

populations (Rowe & Beebee 2005; Marr *et al.* 2006; Szulkin & Sheldon 2007; Forcada & Hoffman 2014). Here, we quantified the relationship between heterozygosity and fitness along an environmental continuum, comprising harsh (wet-cool) and benign (dry-warm) years. Our results support that inter-annual variability in levels of stress induced by environmental conditions can affect the strength of selection on heterozygosity and the magnitude of HFC (Keller *et al.* 2002). Among the studied environmental parameters, only accumulated rainfall predicted the strength of selection on heterozygosity (Fig. 1). Severe rainfall events can lead to an increase in energy expenditure for flight and thermoregulation and a reduction in foraging efficiency, which can jeopardize substantially the fitness and survival prospects of songbirds (Avery & Krebs 1984; Stalmaster & Gessaman 1984; Wilson *et al.* 2004). Accordingly, different studies have reported negative consequences of rainfall in terms of reproductive performance (Radford *et al.* 2001; McDonald *et al.* 2004; Arlettaz *et al.* 2010; Öberg *et al.* 2015) and juvenile and adult survival (Franklin *et al.* 2000; McDonald *et al.* 2004; Cowley & Siriwardena 2005; Öberg *et al.* 2015), which highlights the important role of this meteorological variable as a selective agent in different life stages. The lack of association between temperature-related parameters and the strength of selection on heterozygosity may be explained by the climate prevailing in the study area, which is characterized by mild winters with little or no snowfall. In the Mediterranean region, winter conditions are less severe in comparison with temperate areas at higher latitudes and thus low temperatures are not likely to constitute an important stressor for birds in this region. In the same vein, the observed low inter-annual variability in the number of days with extreme temperatures (i.e., beyond the thermal neutrality zone; see Table S2) could explain that the strength of selection on heterozygosity with respect to such variable has been maintained stable over the study period, irrespective of its relevance or not as a selective pressure. On the contrary, despite in the Mediterranean region the seasonal pattern of precipitation is predictable, annual rainfall can vary markedly from year to year (Blondel & Aronson 1999). Accordingly, we found strong variability in accumulated precipitation within our small temporal series, which included dry and wet years (range: 258-656 mm; Table S2). This fact may have increased variance in strength of selection among years with respect to this environmental variable. Thus, temporal and spatial variability in selective pressures may be an important factor explaining the direction and magnitude of the relationship between individual genetic diversity and fitness, which suggests the need to investigate HFC over a wide range of environmental conditions (Armbruster & Reed 2005).

We found selection differentials and gradients to be correlated for the subsets of functional and neutral markers, indicating that heterozygosity across different regions

of the genome are submitted to similar evolutionary pressures with respect to genetic diversity. Despite HFC were predominately detected using all markers or only the panel of neutral loci, the association between probability of survival and heterozygosity estimated at both subset of markers showed similar trends in most years (see Table 2). Thus, differences in significance between both subsets of loci may be related with the lower power of functional markers to reflect genome-wide inbreeding due to non-directional selective pressures (e.g., stabilizing selection) acting on functional regions contribute to slightly reduce ID with the rest of the genome (Olano-Marin *et al.* 2011a).

Consistent selection on a heritable trait should lead to an evolutionary response. Here, we documented positive selection on heterozygosity in most years. This fact together with the moderate level of narrow-sense heritability (Mitton *et al.* 1993) reported for heterozygosity in our studied blue tit populations (h^2 = 0.3; García-Navas *et al.* 2009) can explain the increase in heterozygosity observed over the course of the study period (Fig. 2). Our results are thus in agreement with previous studies carried out in bottlenecked populations where selection against homozygotes is suggested as a possible mechanism for the maintenance or increase of heterozygosity over time (Bensch *et al.* 2006; Kaeuffer *et al.* 2007; Forcada & Hoffman 2014).

Overall, our study shows the importance of considering the environmental context to get a better understanding on the consequences of individual genetic diversity on fitness. Studies based on larger temporal series (e.g., Szulkin & Seldon 2007; Forcada & Hoffman 2014) and considering genome-wide data (e.g., Forstmeier *et al.* 2012; Hoffman *et al.* 2014) will enable us to address the interaction between heterozygosity, fitness and environment in a more comprehensive and accurate way. These new avenues of research will help to understand whether the strength of HFC in natural populations is influenced by stochastic, cyclic or directional environmental changes (Forcada & Hoffman 2014).

ACKNOWLEDGEMENTS

We thank the board of Centro Quintos de Mora (Organismo Autónomo Parques Nacionales) for the facilities offered during the fieldwork. We are indebted to Pedro J. Cordero for allowing us to carry out the genetic analyses in his lab. We thank Conchi Cáliz for her advice during genotyping and Erik Postma for suggestions and comments on the selection analyses. This study was funded by Ministerio de Educación y Ciencia (GCL2007-61395), Ministerio de Ciencia e Innovación (CGL2010-21933-C02-01) and Junta de Comunidades de Castilla-La Mancha-European Social Fund (POIC10-0269-7632). ESF is supported by a pre-doctoral scholarship from Junta de Comunidades de Castilla-La Mancha-European Social Fund, VGN is supported by a Forschungskredit of the University of Zurich (FK-14-103) and JO is supported by

a research contract funded by Severo Ochoa Program (SEV-2012-0262).

REFERENCES

Acevedo-Whitehouse K, Spraker TR, Lyons E, Melin SR, Gulland F, Delong RL, Amos W. 2006. Contrasting effects of heterozygosity on survival and hookworm resistance in California sea lion pups. *Mol. Ecol.* 15: 1973-1982.

Annavi G, Newman C, Buesching CD, Macdonald DW, Burke T, Dugdale HL. 2014. Heterozygosity-fitness correlations in a wild mammal population: accounting for parental and environmental effects. *Ecol. Evol.* 4: 2594-2609.

Aparicio JM, Ortego J, Cordero PJ. 2006. What should we weigh to estimate heterozygosity, alleles or loci? *Mol. Ecol.* 15: 4659-4665.

Armbruster P, Reed DH. 2005. Inbreeding depression in benign and stressful environments. *Heredity* 95: 235-242.

Arlettaz R, Schaad M, Reichlin TS, Schaub M. 2010. Impact of weather and climate variation on hoopoe reproductive ecology and population growth. *J. Ornithol.* 151: 889-899.

Avery MI, Krebs JR. 1984. Temperature and foraging success of great tits *Parus major* hunting for spiders. *Ibis* 126: 33-38.

Balloux F, Amos W, Coulson T. 2004. Does heterozygosity estimate inbreeding in real populations? *Mol. Ecol.* 13: 3021-3031.

Bensch S, Andren H, Hansson B, Pedersen HC, Sand H, Sejberg D, Wabakken P, Akesson M, Liberg O. 2006. Selection for heterozygosity gives hope to a wild population of inbred wolves. *PLoS One* 1: e72.

Blondel J, Aronson J. 1999. *Biology and wildlife of the Mediterranean region*. Oxford: Oxford University Press.

Broggi J, Hohtola E, Koivula K, Orell M, Thomson RL, Nilsson JA. 2007. Sources of variation in winter basal metabolic rate in the great tit. *Funct. Ecol.* 21: 528-533.

Burnham KP, Anderson DR. 1998. *Model selection and inference: a practical information-theoretic approach*. New York, NY: Springer-Verlag.

Burnham KP, Anderson DR. 2002. *Model selection and multi-model inference: a practical information-theoretic approach*. New York, NY: Springer-Verlag.

Carr DE, Murphy JF, Eubanks MD. 2003. The susceptibility and response of inbred and outbred *Mimulus guttatus* to infection by *Cucumber mosaic virus*. *Evol. Ecol.* 17: 85-103.

Chapman JR, Nakagawa S, Coltman DW, Slate J, Sheldon BC. 2009. A quantitative review of heterozygosity-fitness correlations in animal populations. *Mol. Ecol.* 18: 2746-2765.

Charlesworth D, Charlesworth B. 1987. Inbreeding depression and its evolutionary consequences. *Annu. Rev. Ecol. Evol. Syst.* 18: 237-268.

Charmantier A, Kruuk LEB, Blondel J, Lambrechts MM. 2004. Testing for microevolution in body size in three blue tit populations. *J. Evol. Biol.* 17: 732-743.

Charpentier MJE, Williams CV, Drea CM. 2008. Inbreeding depression in ring-tailed lemurs (*Lemur catta*): genetic diversity predicts parasitism, immunocompetence, and survivorship. *Conserv. Genet.* 9: 1605-1615.

Cheptou PO, Imbert E, Lepart J, Escarre J. 2000. Effects of competition on lifetime estimates of inbreeding depression in the outcrossing plant *Crepis sancta* (Asteraceae). *J. Evol. Biol.* 13: 522-531.

Coltman DW. 2005. Evolutionary genetics-differentiation by dispersal. *Nature* 433: 23-24.

Coltman DW, Pilkington JG, Smith JA, Pemberton JM. 1999. Parasite-mediated selection against inbred Soay sheep in a free-living, island population. *Evolution* 53: 1259-1267.

Coltman DW, Slate J. 2003 Microsatellite measures of inbreeding: A meta-analysis. *Evolution* 57: 971-983.

Cowley E, Siriwardena GM. 2005. Long-term variation in survival rates of sand martins *Riparia riparia*: dependence on breeding and wintering ground weather, age and sex, and their population consequences. *Bird Study* 52: 237-251.

Da Silva A, Gaillard J-M, Yoccoz NG, Hewison AJM, Galan M, Coulson T, Allaine D, Vial L, Delorme D, Van Laere G *et al.* 2009. Heterozygosity-fitness correlations revealed by neutral and candidate gene markers in roe deer from a long-term study. *Evolution* 63: 403-417.

David P. 1998. Heterozygosity-fitness correlations: new perspectives on old problems. *Heredity* 80: 531-537.

David P, Pujol B, Viard F, Castella V, Goudet J. 2007. Reliable selfing rate estimates from imperfect population genetic data. *Mol. Ecol.* 16: 2474-2487.

Dawson WR, Carey C. 1976. Seasonal acclimatization to temperature in *Cardueline finches*. 1. Insulative and metabolic adjustments. *J. Comp. Physiol.* 112: 317-333.

Dias PC, Blondel J. 1996. Local specialization and maladaptation in the Mediterranean blue tit (*Parus caeruleus*). *Oecologia* 107: 79-86.

Endler JA. 1986. *Natural selection in the wild.* Princeton, NJ: Princeton University Press.

Falconer DS, Mackay TFC. 1996. *Introduction to Quantitative Genetics,* 4th edition. Harlow: Longman.

Ferrer ES, García-Navas V, Sanz JJ, Ortego J. 2014. Individual genetic diversity and probability of infection by avian malaria

parasites in blue tits (*Cyanistes caeruleus*). *J. Evol. Biol.* 27: 2468-2482.

Foerster K, Delhey K, Johnsen A, Lifjeld JT, Kempenaers B. 2003. Females increase offspring heterozygosity and fitness through extra-pair matings. *Nature* 425: 714-717.

Forcada J, Hoffman JI. 2014. Climate change selects for heterozygosity in a declining fur seal population. *Nature* 511: 462-465.

Forstmeier W, Schielzeth H, Mueller JC, Ellegren H, Kempenaers B. 2012. Heterozygosity-fitness correlations in zebra finches: microsatellite markers can be better than their reputation. *Mol. Ecol.* 21: 3237-3249.

Fox CW, Reed DH. 2011. Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. *Evolution* 65: 246-258.

Franklin AB, Anderson DR, Gutierrez RJ, Burnham KP. 2000. Climate, habitat quality, and fitness in northern spotted owl populations in northwestern California. *Ecol. Monogr.* 70: 539-590.

García-Navas V, Cáliz-Campal C, Ferrer ES, Sanz JJ, Ortego J. 2014. Heterozygosity at a single locus explains a large proportion of variation in two fitness-related traits in great tits: a general or a local effect? *J. Evol. Biol.* 27: 2807-2819.

García-Navas V, Ferrer ES, Sanz JJ, Ortego J. 2014. The role of immigration and local adaptation on fine-scale genotypic and phenotypic population divergence in a less mobile passerine. *J. Evol. Biol.* 27: 1590-1603.

García-Navas V, Ortego J, Sanz JJ. 2009. Heterozygosity-based assortative mating in blue tits (*Cyanistes caeruleus*): implications for the evolution of mate choice. *Proc. R. Soc. B-Biol. Sci.* 276: 2931-2940.

García-Navas V, Sanz JJ. 2011. The importance of a main dish: nestling diet and foraging behaviour in Mediterranean blue tits in relation to prey phenology. *Oecologia* 165: 639-649.

Garniergere P, Dillmann C. 1992 A computer-program for testing pairwise linkage disequilibria in subdivided populations. *J. Hered.* 83: 239-239.

Gavrilow VM, Dolnik VR. 1985. Basal metabolic rate, thermorregulation and existence energy in birds, world data. *Acta Int. Ornithol. Congr.* 18: 421-466.

Guo SW, Thompson EA. 1992. A monte-carlo method for combined segregation and linkage analysis. *Am. J. Hum. Genet.* 51: 1111-1126.

Haag CR, Sakwinska O, Ebert D. 2003. Test of synergistic interaction between infection and inbreeding in *Daphnia magna*. *Evolution* 57: 777-783.

Haftorn S, Reinertsen RE. 1985. The effect of temperature and clutch size on the energetic cost of incubation in a free-

living blue tit (*Parus caeruleus*). *Auk* 102: 470-478.

Hansson B, Bensch S, Hasselquist D. 2004. Lifetime fitness of short- and long-distance dispersing great reed warblers. *Evolution* 58: 2546-2557.

Hansson B, Westerberg L. 2002. On the correlation between heterozygosity and fitness in natural populations. *Mol. Ecol.* 11: 2467-2474.

Hawley DM, Sydenstricker KV, Kollias GV, Dhondt AA. 2005. Genetic diversity predicts pathogen resistance and cell-mediated immunocompetence in house finches. *Biol. Lett.* 1: 326-329.

Heber S, Briskie JV. 2010. Population bottlenecks and increased hatching failure in endangered birds. *Conserv. Biol.* 24: 1674-1678.

Hoffman JI, Simpson F, David P, Rijks JM, Kuiken T, Thorne MAS, Lacy RC, Dasmahapatra KK. 2014. High-throughput sequencing reveals inbreeding depression in a natural population. *Proc. Natl. Acad. Sci. U.S.A.* 111: 3775-3780.

Husby A, Visser ME, Kruuk LEB. 2011. Speeding up microevolution: the effects of increasing temperature on selection and genetic variance in a wild bird population. *PLoS Biol.* 9: e1000585.

Kaeuffer R, Coltman DW, Chapuis J-L, Pontier D , Reale D. 2007. Unexpected heterozygosity in an island mouflon population founded by a single pair of individuals. *Proc. R. Soc. B-Biol. Sci.* 274: 527-533.

Keller LF, Arcese P, Smith JNM, Hochachka WM, Stearns SC. 1994. Selection against inbred song sparrow during a natural-population bottleneck. *Nature* 372: 356-357.

Keller LF, Grant PR, Grant BR , Petren K. 2002. Environmental conditions affect the magnitude of inbreeding depression in survival of Darwin's finches. *Evolution* 56: 1229-1239.

Keller LF, Marr AB, Reid JM. 2006. The genetic consequences of small population size: inbreeding and loss of genetic variation Pp. 113–137 *in* Smith JNM, Keller LF, Marr AB, Arcese P (eds). *Conservation and Biology of Small Populations: the Song Sparrows of Mandarte Island.* New York, NY: Oxford University Press.

Keller LF, Waller DM. 2002. Inbreeding effects in wild populations. *Trends Ecol. Evol.* 17: 230-241.

Kingsolver JG, Hoekstra HE, Hoekstra JM, Berrigan D, Vignieri SN, Hill CE, Hoang A, Gibert P, Beerli P. 2001. The strength of phenotypic selection in natural populations. *Am. Nat.* 157: 245-261.

Kuepper C, Kosztolanyi A, Augustin J, Dawson DA, Burke T, Szekely T. 2010. Heterozygosity-fitness correlations of conserved microsatellite markers in Kentish plovers *Charadrius alexandrinus*. *Mol. Ecol.* 19: 5172-5185.

Laine VN, Herczeg G, Shikano T, Primmer CR. 2012. Heterozygosity-behaviour correlations in nine-spined stickleback (*Pungitius pungitius*) populations: contrasting effects at random and functional loci. *Mol. Ecol.* 21: 4872-4884.

Lesbarreres D, Primmer CR, Laurila A, Merila J. 2005. Environmental and population dependency of genetic variability-fitness correlations in *Rana temporaria*. *Mol. Ecol.* 14: 311-323.

Li YC, Korol AB, Fahima T, Nevo E. 2004. Microsatellites within genes: Structure, function, and evolution. *Mol. Biol. Evol.* 21: 991-1007.

Liao W, Reed DH. 2009. Inbreeding-environment interactions increase extinction risk. *Anim. Conserv.* 12: 54-61.

Marshall TC, Spalton JA. 2000. Simultaneous inbreeding and outbreeding depression in reintroduced Arabian oryx. *Anim. Conserv.* 3: 241-248.

Marr AB, Arcese P, Hochachka WM, Reid JM, Keller LF. 2006. Interactive effects of environmental stress and inbreeding on reproductive traits in a wild bird population. *J. Anim. Ecol.* 75: 1406-1415.

Marr AB, Keller LF, Arcese P. 2002. Heterosis and outbreeding depression in descendants of natural immigrants to an inbred population of song sparrows (*Melospiza melodia*). *Evolution* 56: 131-142.

Matsumura S, Arlinghaus R, Dieckmann U. 2012. Standardizing selection strengths to study selection in the wild: a critical comparison and suggestions for the future. *Bioscience* 62: 1039-1054.

McDonald PG, Olsen PD, Cockburn A. 2004. Weather dictates reproductive success and survival in the Australian brown falcon *Falco berigora*. *J. Anim. Ecol.* 73: 683-692.

McNab BK. 2002. *The physiological ecology of vertebrates: a view from energetics*. Ithaca, NY: Cornell University Press.

Miller PS. 1994. Is inbreeding depression more severe in a stressful environment. *Zoo Biol.* 13: 195-208.

Miller JM, Coltman DW. 2014. Assessment of identity disequilibrium and its relation to empirical heterozygosity fitness correlations: a meta-analysis. *Mol. Ecol.* 23: 1899-1909.

Mitton JB, Schuster WSF, Cothran EG, Defries JC. 1993. Correlation between the individual heterozygosity of parents and their offspring. *Heredity* 71: 59-63.

Nakagawa S, Cuthill IC. 2007. Effect size, confidence interval and statistical significance: a practical guide for biologists. *Biol. Rev.* 82: 591-605.

Oberg M, Arlt D, Part T, Laugen AT, Eggers S, Low M. 2015. Rainfall during parental care reduces reproductive and survival components of fitness in a passerine bird. *Ecol. Evol.* 5: 345-356.

Olano-Marin J, Mueller JC, Kempenaers B. 2011a. Correlations between heterozygosity and reproductive success

in the blue tit (*Cyanistes caeruleus*): an analysis of inbreeding and single locus effects. *Evolution* 65: 3175-3194.

Olano-Marin J, Mueller JC, Kempenaers B. 2011b. Heterozygosity and survival in blue tits (*Cyanistes caeruleus*): contrasting effects of presumably functional and neutral loci. *Mol. Ecol.* 20: 4028-4041.

Ortego J, Calabuig G, Bonal R, Muñoz A, Aparicio JM, Cordero PJ. 2009. Temporal variation of heterozygosity-based assortative mating and related benefits in a lesser kestrel population. *J. Evol. Biol.* 22: 2488-2495.

Ortego J, Calabuig G, Cordero PJ, Aparicio JM. 2007. Egg production and individual genetic diversity in lesser kestrels. *Mol. Ecol.* 16: 2383-2392.

Pemberton J. 2004. Measuring inbreeding depression in the wild: the old ways are the best. *Trends Ecol. Evol.* 19: 613-615.

Postma E, van Noordwijk AJ. 2005. Gene flow maintains a large genetic difference in clutch size at a small spatial scale. *Nature* 433: 65-68.

Radford AN, McCleery RH, Woodburn RJW, Morecroft MD. 2001. Activity patterns of parent great tits *Parus major* feeding their young during rainfall. *Bird Study* 48: 214-220

Rantala MJ, Roff DA. 2007. Inbreeding and extreme outbreeding cause sex differences in immune defence and life history traits in *Epirrita autumnata*. *Heredity* 98: 329-336.

Raymond M, Rousset F. 1995. Genepop (version-1.2)-population-genetics software for exact tests and ecumenicism. *J. Hered.* 86: 248-249.

Reed DH, Briscoe DA, Frankham R. 2002. Inbreeding and extinction: The effect of environmental stress and lineage. *Conserv. Genet.* 3: 301-307.

Reed DH, Nicholas AC, Stratton GE. 2007. Genetic quality of individuals impacts population dynamics. *Anim. Conserv.* 10: 275-283.

Reid JM. 2007. Secondary sexual ornamentation and non-additive genetic benefits of female mate choice. *Proc. R. Soc. B-Biol. Sci.* 274: 1395-1402.

Reinertsen RE, Haftorn S. 1986. Different metabolic strategies of northern birds for nocturnal survival. *J. Comp. Physiol. B.* 156: 655-663.

Rice WR. 1989. Analyzing tables of statistical tests. *Evolution* 43: 223-225.

Richter SC, Nunziata SO. 2014. Survival to metamorphosis is positively related to genetic variability in a critically endangered amphibian species. *Anim. Conserv.* 17: 265-274.

Rowe G, Beebee TJC. 2005. Intraspecific competition disadvantages inbred natterjack toad (*Bufo calamita*) genotypes over outbred ones in a shared pond environment. *J. Anim. Ecol.* 74: 71-76.

Ruiz-López MJ, Monello RJ, Gompper ME, Eggert LS. 2012. The effect and relative

importance of neutral genetic diversity for predicting parasitism varies across parasite taxa. *PLoS One* 7: e45404

Scott TM, Koehn RK. 1990. The effect of environmental-stress on the relationship of heterozygosity to growth-rate in the coot clam *Mulinia lateralis* (Say). *J. Exp. Mar. Biol. Ecol.* 135: 109-116.

Seddon N, Amos W, Mulder RA, Tobias JA. 2004. Male heterozygosity predicts territory size, song structure and reproductive success in a cooperatively breeding bird *Proc. R. Soc. B-Biol. Sci.* 271: 1823-1829.

Slate J, David P, Dodds KG, Veenvliet BA, Glass BC, Broad TE, McEwan JC. 2004. Understanding the relationship between the inbreeding coefficient and multilocus heterozygosity: theoretical expectations and empirical data. *Heredity* 93: 255-265.

Stalmaster MV, Gessaman JA. 1984. Ecological energetic and foraging behaviour of overwintering bald eagles. *Ecol. Monogr.* 54: 407-428.

Svensson L. 1992. *Identification guide to European Passerines*. Stockholm: Lars Svensson.

Szulkin M, Bierne N, David P. 2010. Heterozygosity-fitness correlations: a time for reappraisal *Evolution* 64: 1202-1217.

Szulkin M, Sheldon BC. 2007. The environmental dependence of inbreeding depression in a wild bird population. *PLoS One* 2: e1027.

Thomas DW, Blondel J, Perret P. 2001. Physiological ecology of Mediterranean blue tits (*Parus caeruleus*) 1. A test for inter-population differences in resting metabolic rate and thermal conductance as a response to hot climates. *Zoology* 104: 33-40.

Tornero J. 2003. *Los Quintos de Mora*. Madrid: Organismo Autónomo Parques Nacionales.

Voegeli B, Saladin V, Wegmann M, Richner H. 2012. Parasites as mediators of heterozygosity-fitness correlations in the great tit (*Parus major*). *J. Evol. Biol.* 25: 584-590.

Vranckx G, Jacquemyn H, Mergeay J, Cox K, Janssens P, Gielen BAS, Muys B, Honnay O. 2014. The effect of drought stress on heterozygosity-fitness correlations in pedunculate oak (*Quercus robur*). *Ann. Bot.* 113: 1057-1069.

Wheelwright NT, Keller LF, Postma E. 2014. The effect of trait type and strength of selection on heritability and evolvability in an island bird population. *Evolution* 68: 3325-3336.

Wilson GR, Cooper SJ, Gessaman JA. 2004. The effects of temperature and artificial rain on the metabolism of American kestrels (*Falco sparverius*). *Comp. Biochem. Phys. A.* 139: 389-394.

SUPPORTING INFORMATION

Table S1 Panel of 26 microsatellite loci used to genotype blue tits. The following information is given for each locus: chromosome location in the zebra finch genome, category (neutral or functional), number of alleles (*K*), expected heterozygosity (H_E), observed heterozygosity (H_O), annealing temperature (T) and original reference or GenBank accession number.

Locus	Chromosome	Category	*K*	H_E	H_O	T (ºC)	Reference
PK11	1	Neutral	12	0.80	0.80	52	Dawson *et al.* 2000
Pca3	2	Neutral	25	0.87	0.85	55	Bensch *et al.* 1997
Pca9	2	Neutral	15	0.80	0.81	62	Otter *et al.* 1998
Pocc1	2	Neutral	13	0.85	0.87	55	Dawson *et al.* 2000
Pocc6	3	Neutral	23	0.87	0.83	62	Richardson *et al.* 2000
Pat-MP2-43	4	Neutral	11	0.56	0.52	59	Dawson *et al.* 2000
PK12	4	Neutral	23	0.87	0.89	62	Griffith *et al.* 1999
Ase18	5	Neutral	21	0.87	0.83	60	GenBank Acc. no.:
Pdoμ5	5	Neutral	33	0.91	0.84	46	Double *et al.* 1997
Pca7	7	Neutral	17	0.89	0.84	60	Dawson *et al.* 2000
Pca4	7	Neutral	15	0.75	0.77	60	Bensch *et al.* 1997
Pca2	8	Neutral	11	0.78	0.67	60	Dawson *et al.* 2000
Mcyμ4	Unassigned	Neutral	16	0.85	0.82	50	GenBank Acc. no.:
Pca8	Unassigned	Neutral	60	0.96	0.94	53	Dawson *et al.* 2000
CcaTgu28	2	Functional	11	0.73	0.74	60	Olano-Marin *et al.* 2010
PiJ14	2	Functional	17	0.88	0.89	60	Olano-Marin *et al.* 2010
TG05-053	3	Functional	7	0.76	0.77	55	Olano-Marin *et al.* 2010
TG05-046	5	Functional	3	0.50	0.48	55	Dawson *et al.* 2009
CcaTgu15	5	Functional	7	0.66	0.64	60	Dawson *et al.* 2009
Tg13-017	5	Functional	18	0.86	0.84	60	Olano-Marin *et al.* 2010
CcaTgu19	5	Functional	35	0.93	0.92	60	Olano-Marin *et al.* 2010
CcaTgu11	6	Functional	11	0.77	0.67	60	Slate *et al.* 2007
CcaTgu8	7	Functional	8	0.61	0.61	63	Olano-Marin *et al.* 2010
Tgu07	10	Functional	7	0.66	0.68	55	Olano-Marin *et al.* 2010
CcaTgu14	13	Functional	22	0.83	0.78	55	Dawson *et al.* 2010
CcaTgu7	23_random	Functional	6	0.75	0.75	55	Olano-Marin *et al.* 2010

Table S2 Estimates of environmental harshness (TNZ days: number of days with temperature ≥35 ºC or ≤ 15ºC; FDD: freezing-degree days, calculated as the number of days with temperature < 0 ºC; Precip.: accumulated precipitation in mm) used in this study (calculated from July 1st to March 31st), selection differentials (*S*) for heterozygosity and g^2 values calculated for all markers and the subsets of neutral and functional loci. *P*-values for g^2 are indicated in parentheses and those statistically significant are in bold.

Year	Environmental harshness			Selection differentials (*S*)			Identity disequilibrium (g^2)		
	TNZ	FDD	Precip.	S_{Total}	$S_{Neutral}$	$S_{Functional}$	g^2_{Total}	$g^2_{Neutral}$	$g^2_{Functional}$
2008	239	15	351.8	-0.002	0.006	-0.012	0.003 (*P* = **0.028**)	0.002 (*P* = 0.177)	-0.001 (*P* = 0.585)
2009	267	32	600.9	0.013	0.018	0.008	0.006 (*P* = **0.001**)	0.080 (*P* < **0.001**)	-0.006 (*P* =0.884)
2010	237	22	505.3	0.005	0.002	0.008	0.001 (*P* = 0.151)	0.078 (*P* < **0.001**)	-0.002 (*P* = 0.733)
2011	234	46	238.2	-0.022	-0.029	-0.022	0.002 (*P* = 0.100)	0.003 (*P* = 0.087)	0.004 (*P* = 0.239)
2012	237	44	656.0	0.036	0.043	0.030	0.006 (*P* = **0.003**)	0.004 (*P* = 0.077)	0.017 (*P* = **0.010**)
2013	238	34	339.2	0.003	0.007	-0.006	0.001 (*P* = 0.285)	0.001 (*P* = 0.423)	0.002 (*P* = 0.302)

Table S3 Model selection to assess the association between probability of inter-annual survival and genetic [A: heterozygosity estimated at all loci, HL_{Total}] and non-genetic terms [B: locality; C: age; D: body condition; E: sex] for the six study years (2008-2013). Mating pair identity was included as random effect in all the models. Only best ranked equivalent models with $\Delta AICc \leq 2$ are shown.

Model no.	Model	*K*	AIC_c	ΔAIC_c	ωi
(a) 2008					
1	E	3	146.42	0.00	0.23
2	C+E	4	147.08	0.06	0.17
3	D+E	4	148.27	1.85	0.09
(b) 2009					
1	B+D	4	154.95	0.00	0.24
2	A+B+D	5	155.01	0.05	0.24
(c) 2010					
1	D	3	162.92	0.00	0.19
2	B+D	4	164.29	1.36	0.10
3	C+D	4	164.67	1.74	0.08
4	E+D	4	164.72	1.80	0.08
5	A+D	4	164.84	1.92	0.07
(d) 2011					
1	A+B	4	144.15	0.00	0.13
2	A	3	144.16	0.01	0.13
3	A+D	4	145.22	1.06	0.08
4	B	3	145.31	1.15	0.07
5	A+B+D	5	145.81	1.66	0.06
6	A+E	4	146.11	1.96	0.05
7	A+B+E	5	146.12	1.97	0.05
(e) 2012					
1	A+B+C	5	85.95	0.00	0.21
2	A+B+C+D	6	86.96	1.00	0.12
3	A+B+C+E	6	86.98	1.02	0.12
4	A+B	4	87.51	1.55	0.09
5	A+B+C+D+E	7	87.96	2.00	0.08
(f) 2013					
1	D	5	79.00	0.00	0.25
2	C+D	3	80.91	1.91	0.09

Table S4 Model selection to assess the association between probability of inter-annual survival and genetic [A: heterozygosity estimated at the subset of neutral loci, $HL_{Neutral}$; B: heterozygosity estimated at the subset of functional loci, $HL_{Functional}$] and non-genetic terms [C: locality; D: age; E: body condition; F: sex] for the six study years (2008-2013). Mating pair identity was included as random effect in all the models. Only best ranked equivalent models with ΔAICc ≤ 2 are shown.

Model no.	Model	*K*	AIC_c	ΔAIC_c	ωi
(a) 2008					
1	F	3	146.42	0.00	0.14
2	E+F	4	147.08	0.66	0.10
3	B+F	4	147.61	1.19	0.07
4	A+F	4	148.07	1.65	0.06
5	B+E+F	5	148.27	1.85	0.05
6	D+F	4	148.27	1.85	0.05
(b) 2009					
1	A+C+E	5	154.95	0.00	0.24
2	C+E	4	155.01	0.05	0.24
3	B+C+E	5	156.66	1.86	0.07
(c) 2010					
1	E	3	162.92	0.00	0.14
2	E+C	4	164.29	1.36	0.07
3	E+D	4	164.67	1.74	0.06
4	E+B	4	164.71	1.79	0.06
5	E+F	4	164.72	1.80	0.06
(d) 2011					
1	A+B+C	5	137.59	0.00	0.17
2	A+B	4	138.89	1.30	0.09
3	A+B+C+D	6	139.34	1.75	0.07
4	A+B+C+E	6	139.59	2.00	0.06
5	A+B+C+F	6	139.59	2.00	0.06
(e) 2012					
1	A+B+C+D	6	83.52	0.00	0.12
2	A+B+C+D+F	7	83.83	0.30	0.10
3	B+C+D+F	6	84.27	0.75	0.08
4	A+B+C	5	84.43	0.91	0.08
5	B+C+D	5	84.49	0.97	0.07
6	A+B+C+D+E	7	84.94	1.41	0.06
7	B+C+D+E+F	7	84.97	1.44	0.06
8	B+C+D+E	6	85.09	1.56	0.05
9	A+B+C+D+E+F	8	85.16	1.64	0.05
(f) 2013					
1	A+B	4	77.95	0.00	0.11
2	B+E	4	78.23	0.28	0.10
3	E	3	79.00	1.04	0.07
4	A+B+F	5	79.26	1.31	0.06

Table S5 Tests for the effects of single locus heterozygosity (SLH) on probability of inter-annual survival for each study year. Table shows effect sizes and *P*-values. No test was significant after sequential Bonferroni correction.

	2008			2009			2010			2011			2012			2013		
Locus	Effect size	*Z*	*P*	Effect size	*Z*	*P*	Effect size	*Z*	*P*	Effect size	*Z*	*P*	Effect size	*Z*	*P*	Effect size	*Z*	*P*
PK11	-0.002	0.00	0.980	-0.079	0.68	0.410	0.056	0.35	0.553	-0.051	0.28	0.600	0.123	1.27	0.263	0.196	2.91	0.092
Pca3	0.075	0.60	0.442	0.053	0.32	0.572	0.110	1.43	0.235	-0.034	0.12	0.728	0.101	0.92	0.340	-0.135	1.36	0.247
Pca9	0.030	0.10	0.758	0.078	0.70	0.403	-0.084	0.82	0.366	-0.107	1.22	0.272	-0.020	0.03	0.853	0.070	0.36	0.550
Pocc1	0.131	1.82	0.180	0.105	1.23	0.270	0.079	0.73	0.395	0.003	0.00	0.975	0.137	1.68	0.198	0.150	1.67	0.201
Pocc6	-0.142	2.20	0.141	-0.143	2.37	0.127	-0.170	3.48	0.065	0.103	1.13	0.290	0.129	1.47	0.229	-0.101	0.74	0.393
Pat-MP2-43	0.036	0.14	0.712	-0.040	0.18	0.674	0.056	0.36	0.547	-0.038	0.15	0.698	-0.063	0.35	0.556	-0.091	0.61	0.439
PK12	-0.089	0.86	0.356	0.178	3.70	0.057	-0.043	0.21	0.646	-0.034	0.12	0.728	-0.005	0.00	0.965	0.048	0.17	0.686
Ase18	-0.056	0.34	0.564	0.144	2.43	0.122	0.024	0.06	0.802	0.039	0.15	0.699	0.105	0.91	0.343	-0.139	1.29	0.260
Pdoμ5	0.016	0.03	0.874	0.013	0.02	0.897	0.075	0.59	0.445	0.039	0.14	0.711	-0.049	0.18	0.675	0.139	1.35	0.250
Pca7	0.048	0.24	0.624	0.078	0.67	0.416	-0.075	0.66	0.417	-0.099	1.03	0.313	0.144	1.77	0.186	0.026	0.05	0.825
Pca4	0.104	1.14	0.288	-0.057	0.36	0.549	0.068	0.54	0.464	-0.242	6.45	0.013	0.121	1.27	0.263	-0.014	0.01	0.904
Pca2	0.021	0.05	0.831	0.110	1.37	0.244	-0.070	0.57	0.450	-0.291	9.46	0.003	-0.060	0.29	0.590	0.101	0.71	0.404
Mcyμ4	0.151	2.47	0.119	0.054	0.32	0.575	0.142	2.35	0.128	-0.027	0.08	0.783	0.046	0.17	0.680	-0.014	0.01	0.904
Pca8	-0.185	3.69	0.057	0.085	0.80	0.374	0.004	0.00	0.968	-0.042	0.18	0.674	0.032	0.08	0.771	-0.142	1.49	0.226
CcaTgu28	-0.087	0.81	0.371	0.006	0.00	0.949	0.002	0.00	0.980	0.008	0.01	0.940	0.194	2.92	0.092	-0.013	0.01	0.922
PiJ14	0.064	0.45	0.506	-0.006	0.00	0.953	-0.148	2.61	0.109	0.037	0.14	0.711	0.025	0.05	0.823	0.197	2.90	0.093
TG05-053	0.010	0.01	0.922	-0.019	0.04	0.845	0.169	3.34	0.070	-0.110	1.23	0.271	-0.050	0.20	0.654	-0.115	0.96	0.331
TG05-046	0.115	1.37	0.245	-0.076	0.61	0.435	0.022	0.05	0.818	0.055	0.29	0.594	0.076	0.46	0.500	0.071	0.35	0.555
CcaTgu15	0.10	1.23	0.269	0.114	1.39	0.241	0.095	1.03	0.312	0.094	0.88	0.350	-0.051	0.22	0.640	0.048	0.16	0.688
Tg13-017	0.037	0.14	0.713	0.046	0.21	0.650	0.099	1.07	0.304	-0.052	0.25	0.618	0.051	0.21	0.646	-0.284	5.97	0.017
CcaTgu19	-0.044	0.20	0.652	0.024	0.06	0.806	-0.188	4.16	0.044	-0.124	1.52	0.220	-0.130	1.20	0.277	0.075	0.39	0.532
CcaTgu11	-0.090	0.87	0.352	0.041	0.18	0.673	0.103	1.23	0.270	0.042	0.17	0.679	-0.071	0.36	0.551	0.079	0.44	0.510
CcaTgu8	-0.136	1.86	0.175	-0.068	0.48	0.489	0.072	0.56	0.455	-0.078	0.58	0.448	0.084	0.49	0.484	0.042	0.12	0.732
Tgu07	-0.115	1.43	0.235	-0.103	1.18	0.279	-0.021	0.05	0.821	-0.035	0.12	0.729	0.120	1.04	0.310	-0.011	0.01	0.924
CcaTgu14	-0.034	0.13	0.723	0.149	2.45	0.121	-0.015	0.02	0.877	0.035	0.12	0.729	0.131	1.32	0.253	0.074	0.38	0.538
CcaTgu7	-0.077	0.60	0.442	-0.011	0.01	0.916	0.079	0.68	0.411	-0.121	1.38	0.244	-0.056	0.24	0.623	-0.160	1.79	0.186

SUPPLEMENTARY REFERENCES

Bensch S, Price T, Kohn J. 1997. Isolation and characterization of microsatellite loci in a *Phylloscopus* warbler. *Mol. Ecol.* 6: 91-92.

Dawson DA, Chittock J, Jehle R, Whitlock A, Nogueira D, Pellatt J, Birkhead T, Burke T. 2005. Identification of 13 polymorphic microsatellite loci in the zebra finch, *Taeniopygia guttata* (Passeridae, Aves). *Mol. Ecol. Notes* 5: 298-301.

Dawson DA, Horsburgh GJ, Küpper C, Stewart IRK, Ball AD, Durrant KL, Hansson B, Bacon I, Bird S, Klein A, Krupa AP, Lee JW, Martín-Gálvez D, Simeoni M, Smith G, Spurgin LG, Burke T. 2010. New methods to identify conserved microsatellite loci and develop primer sets of high cross-species utility - as demonstrated for birds. *Mol. Ecol. Resour.* 10: 75-494.

Double MC, Dawson D, Burke T, Cockburn A. 1997. Finding the fathers in the least faithful bird: A microsatellite-based genotyping system for the superb fairy-wren *Malurus cyaneus*. *Mol. Ecol.* 6: 691-693.

Griffith SC, Stewart IRK, Dawson DA, Owens IPF, Burke T. 1999. Contrasting levels of extra-pair paternity in mainland and island populations of the house sparrow (*Passer domesticus*): is there an "island effect"? *Biol. J. Linn. Soc.* 68: 303-316.

Olano-Marin J, Dawson DA, Girg A, Hansson B, Ljungqvist M, Kempenaers B, Mueller JC. 2010. A genome-wide set of 106 microsatellite markers for the blue tit (*Cyanistes caeruleus*). *Mol. Ecol. Resour.* 10: 516-532.

Otter K, Ratcliffe L, Michaud D, Boag PT. 1998. Do female black-capped chickadees prefer high-ranking males as extra-pair partners? *Behav. Ecol. Sociobiol.* 43 25-36.

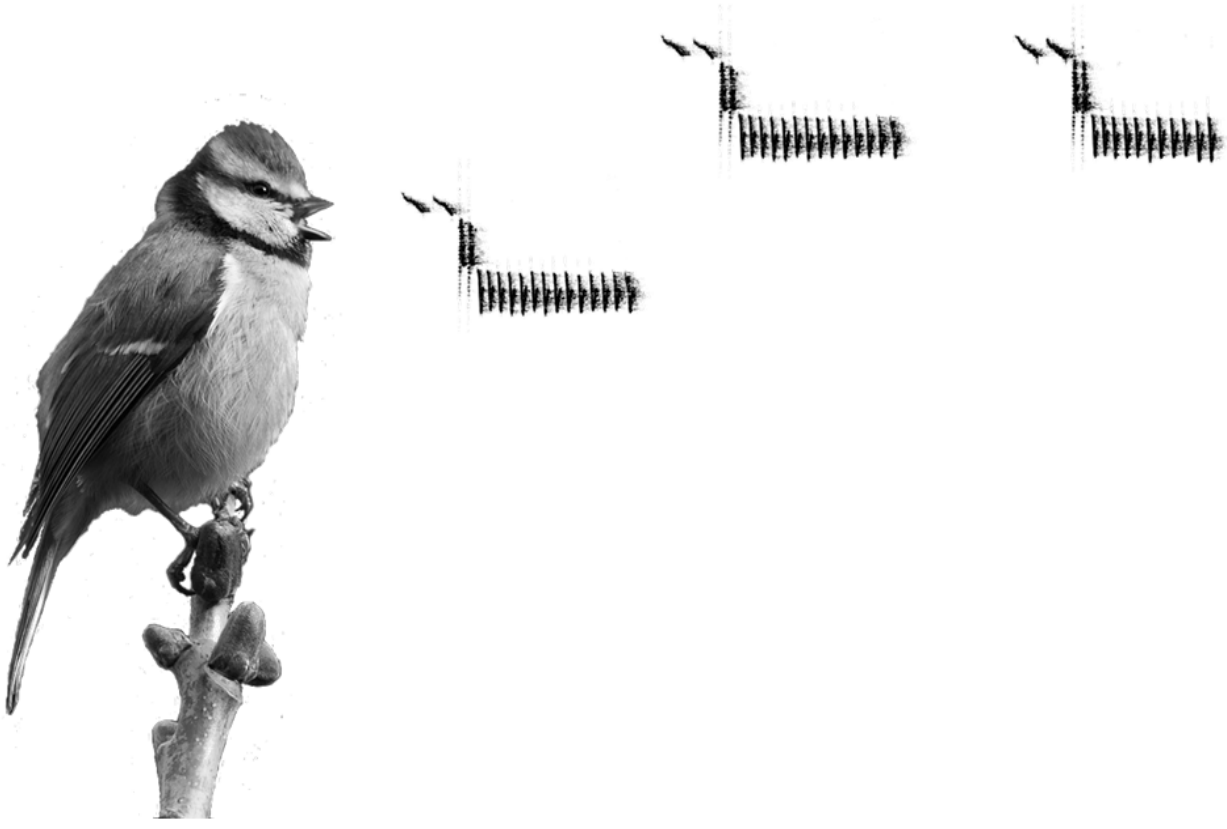
Richardson DS, Jury FL, Dawson DA, Salgueiro P, Komdeur J, Burke T. 2000. Fifty Seychelles warbler (*Acrocephalus sechellensis*) microsatellite loci polymorphic in Sylviidae species and their cross-species amplification in other passerine birds. *Mol. Ecol.* 9: 2226-2231.

Slate J, Hale M, Birkhead TR. 2007. Simple sequence repeats in zebra finch (*Taeniopygia guttata*) expressed sequence tags: a new resource for evolutionary genetic studies of passerines. *BMC Genom.* 8: 52.

6. Multiple sexual ornaments signal heterozygosity in male blue tits

Ferrer ES, García-Navas V, Bueno-Enciso J, Sanz JJ & Ortego J

Biological Journal of the Linnean Society, 114: (2015) In press



Multiple sexual ornaments signal heterozygosity in male blue tits

Abstract

Higher individual genetic quality has been hypothesized to be associated with the expression of conspicuous ornaments. However, the relationship between multicomponent sexual signals and heterozygosity is poorly understood. In this study, we examined whether different ornaments, including song (repertoire size and bout length) and plumage coloration (yellow breast and blue crown), reflect individual genetic diversity in male blue tits (*Cyanistes caeruleus*). We estimated genetic diversity using 26 microsatellite markers that were classified as putatively functional (12 loci) and neutral (14 loci). We found that yellow breast carotenoid chroma, blue crown brightness, bout length and body condition were positively associated with heterozygosity at functional loci, but not with genetic diversity estimated at all typed loci or the subset of neutral markers. The lack of strong single locus effects and the presence of identity disequilibrium in our population suggest that the observed heterozygosity-phenotype associations are driven by loci widely distributed across the genome. The predominant role of putatively functional loci evidences that the expression of secondary sexual characters is more tightly reflected by heterozygosity at genomic regions containing coding genes that are being actively expressed, a fact that may make ornamental traits more reliable indicators of the genetic quality of individuals. Overall, this study shows that multiple secondary sexual characters reflect male genetic diversity and lends support to the good-genes-as-heterozygosity hypothesis.

INTRODUCTION

Mate choice based on elaborated sexual ornaments is an important focus of study in behavioral and evolutionary research. The expression of secondary sexual traits often entails high costs, which implies that individuals (generally males) face a trade-off between investing in these ornaments and allocating resources towards other necessary physiological processes (Andersson 1994). Thus, only superior males will be able to develop and maintain these conspicuous traits without jeopardizing their viability and, as a result, ornaments become reliable and honest signals of individual quality (Zahavi 1975; Getty 1998). Female preferences for ornamented males are maintained as a result of the benefits derived from such selective behavior. Females may choose attractive males for direct benefits in terms of either increased parental care (Hoelzer 1989; Kokko 1998; Senar *et al.* 2002) or enhanced fertility (Sheldon 1994; Helfenstein *et al.* 2010). Such a preference for more ornamented males may also result in indirect additive

genetic benefits if they are able to produce offspring of superior genetic quality through the transmission of good alleles or fewer deleterious alleles (Von Schantz *et al.* 1996; Fromhage *et al.* 2009; Cutrera *et al.* 2012). Another possibility is that ornaments reflect male heterozygosity ("good-genes-as-heterozygosity hypothesis"; Brown 1997), a genetic trait that has often been found to positively affect fitness due to overdominance and a reduced chance that deleterious recessive alleles will be expressed (reviewed in Chapman *et al.* 2009; Szulkin *et al.* 2010). Selection on highly ornamented and heterozygous males may increase female fitness directly, e.g. via increased provisioning effort of more heterozygous partners (e.g., García-Navas, *et al.* 2009), or indirectly, via non-additive genetics benefits such as the production of more heterozygous descendants (reviewed in Kempenaers 2007). The latter can be possible when allele frequencies are asymmetric (Mitton *et al.* 1993; Nietlisbach *et al.* in press; e.g. Reid *et al.* 2006; Roberts *et al.* 2006; Ortego *et al.* 2009). Under this circumstance, the most common in multi-allelic loci, more heterozygous parents produce more heterozygous offspring (i.e., heterozygosity becomes "heritable" *sensu* Mitton *et al.* 1993).

Information conveyed by different ornaments can be complementary ("multiple messages" hypothesis) or redundant ("back-up signal" hypothesis) (reviewed in Candolin 2003). According to the "multiple messages" hypothesis, different ornaments can provide information about different aspects of mate quality and, evaluated together, these traits reflect overall quality (Møller & Pomiankowski 1993). Meanwhile, multiple back-up cues (i.e., traits that reflect the same quality with some error) may facilitate mate assessment and/or make it more difficult for mates to misrepresent their quality (Johnstone 1996, 1997). Back-up signals are thought to be less common than multiple messages as the majority of studies have found multiple traits to be uncorrelated (e.g., Marchetti 1998; but see Hegyi *et al.* 2015). However, there is little available information about the relationship between the expression of secondary sexual traits and individual genetic diversity and most studies on this topic have focused only on one or few traits (e.g., Foerster *et al.* 2003; Marshall *et al.* 2003; Reid *et al.* 2005; but see Bolund *et al.* 2010; Leclaire *et al.* 2011 for exceptions). Thus, more studies testing the good-genes-as-heterozygosity hypothesis across multiple secondary sexual traits can help to elucidate whether a single ("multiple messages" hypothesis) or several ("back-up signal" hypothesis) ornaments are signalling individual genetic diversity.

In the present study, we use Mediterranean blue tit (*Cyanistes caeruleus*) as a model system to investigate whether different ornaments reflect male heterozygosity. In particular, we used a total of 26 microsatellite markers to estimate individual genetic diversity and analyse its association with male physical condition, body size and the expression of multiple secondary sexual traits (yellow breast

coloration, blue crown coloration and song characteristics). Further, we employed two different arrays of markers classified as neutral (14 loci) or functional (12 loci) by considering whether the genomic region where the markers are located is transcribed to RNA (*sensu* Olano-Marin *et al.* 2011a, b; see also Da Silva *et al.* 2009; Küpper *et al.* 2010; Laine *et al.* 2012). This allowed us to test for the first time potential differences in the relationships between the above described traits and these subsets of markers, which may reflect different biological processes (Szulkin & David 2011; e.g., Olano-Marin *et al.* 2011a, b; Ferrer *et al.* 2014). The specific goals of this study are to (*i*) analyse the relationship between heterozygosity and the expression of secondary sexual traits and determine whether individual genetic diversity is reflected by a single ("multiple messages" hypothesis) or several ("back-up signal" hypothesis) ornaments (Candolin 2003); (*ii*) test if this relationship varies depending on whether functional or neutral loci are considered. Furthermore, (*iii*) we examined whether the observed associations between phenotype and heterozygosity reflect a genome wide effect ("general effect hypothesis"; Weir & Cockerham 1973; David 1998) or strong linkage disequilibrium between the employed loci and genes involved in the expression of the studied traits ("local effect hypothesis"; David, 1998; Hansson *et al.* 2001; Hansson & Westerberg 2002). In particular, we expect neutral markers to cause these associations either by general effects (Weir & Cockerham 1973; David 1998) or local effects if they happen to be linked to functional loci (David 1998; Hansson *et al.* 2001; Hansson & Westerberg 2002; Balloux *et al.* 2004), but we hypothesize that direct or strong local effects are more likely to be caused by functional markers (Olano-Marin *et al.* 2011a, b; Laine *et al.* 2012).

MATERIALS AND METHODS

Study site and general field methods

The study area is located in San Pablo de los Montes, Toledo province (central Spain; 39º31´N, 4º21´W), and comprises two nearby (< 2 km) forest patches ("Majadillas" and "Arroyo del Marchés") dominated by Pyrenean oak (*Quercus pyrenaica*). During the 2012 breeding season, we obtained basic reproductive parameters from 50 breeding pairs. Parents were captured by means of spring traps when feeding nestlings 8-9 days old. All adults birds were identified with metal rings, sexed and aged according to Svensson (1992) as juveniles (yearlings) or experienced breeders (second-year and older birds). Birds were weighed to the nearest 0.1 g using an electronic portable balance, and their wing length was measured to the nearest 1 mm using a top-ruler. Blood samples (≤ 25 μL) were taken from the brachial vein of adults and stored on FTA reagent loaded cards (Whatman Bioscience, Florham Park, NJ, USA) until needed for genetic analyses.

Microsatellite genotyping and basic genetic statistics

We genotyped a total of 50 male blue tits using a panel of 26 polymorphic microsatellite markers (see Supporting Information, Table S1). These markers were classified as presumably functional or neutral as described by Olano-Marin *et al.* (2011a, b) (Table S1). DNA extraction, microsatellite amplification and genotyping and tests for linkage disequilibrium (LD) between each pair of loci and deviations from Hardy-Weinberg equilibrium (HWE) were performed as described in Ferrer *et al.* (2014). We investigated genetic differentiation between the two sampling locations by calculating the pair-wise F_{ST}-value and testing its significance with a Fisher's exact test after 10000 permutations as implemented in ARLEQUIN 3.1 (Excoffier *et al.* 2005).

Heterozygosity estimates and identity disequilibrium

We used homozygosity by locus (*HL*) to estimate individual genetic diversity (Aparicio *et al.* 2006). The *HL* index represents homozygosity instead of heterozygosity, and we used the inverse of *HL* (i.e., 1-*HL*) as an estimate of individual heterozygosity. *HL* values were calculated using CERNICALIN, an Excel spreadsheet available on request. We used two methods to analyse the presence of identity disequilibrium (ID) and test whether heterozygosity measured at our set of microsatellite loci was representative of genome-wide inbreeding. We calculated heterozygosity-heterozygosity correlations (HHC) following Balloux *et al.* (2004). We used the R package "rhh" to run 1000 randomizations of the markers and estimate the average HHC coefficient (*r*) and the 95% confidence intervals (Alho *et al.* 2010). Moreover, we calculated the parameter g^2, a central measure of identity disequilibrium that quantifies the excess of double heterozygotes at two loci relative to the expectation under random association (David *et al.* 2007). This estimate is constant for any pair of loci considered and only depends on the mean and variance of inbreeding in the population (David *et al.* 2007; Szulkin *et al.* 2010). We used the RMES software to calculate g^2 and test whether this parameter differed significantly from zero (David *et al.* 2007).

Song data

We recorded 50 male blue tits at dawn chorus using Song Meter SMS2 (Wildlife Acoustics Inc., Maynard, MA, USA) and Olympus DM-650 (Olympus Corp., Beijing, China) digital recording devices. Males were recorded during their female's fertile period (two days before egg laying until one day before the last egg was laid). Audio recording devices were set up in close proximity (<1m) to the focal nestbox and programmed to record between 04:30-09:00 am during two consecutive days in order to reduce the possibility of obtaining inaccurate recordings. Even so, we did not get any clear dawn chorus recording for 8

individuals and they were not considered for further analyses. Dawn chorus was considered finished when the male did not sing for more than 5 min (Poesel *et al.* 2001). All recordings were analysed by two observers (ESF, JBE) using the same criteria. We used AUDACITY 2.0.0 (http://audacity.sourceforge.net) to filter and remove background noise and RAVEN PRO 1.5 (http://www.birds.cornell.edu/raven) to measure song variables. A total of 43 different song types (strophes repeated and constituting a bout) were identified in this population, of which one was sung by 40 males (i.e., 95% of the analysed individuals). The length of this song type, the most common one in the study population, was measured using RAVEN PRO 1.5 (Dreiss *et al.* 2006; Murphy *et al.* 2008). We also calculated individual repertoire size. In this case, we only considered chorus that contained more than 70 strophes, the number of strophes required to achieve 95 % confidence that the complete individual repertoire was recorded (Dreiss *et al.* 2006). Song recordings from 39 males met such criteria and were selected to examine repertoire size.

Colour data

Plumage reflectance measurements were taken from the blue crown and yellow breast of 49 male blue tits. However, some spectral measurements failed and some individuals showed little or no blue plumage on the crown probably due to fights with other conspecifics. As a result, blue crown and yellow breast coloration data from 20 and 13 individuals, respectively, could not be used in subsequent analyses. Colour data were collected in the field using an Ocean Optics USB2000 (Ocean Optics Inc., Dunedin, FL, USA) spectrophotometer (range 250-800 nm) with ultraviolet (deuterium) and visible (tungsten-halogen) lamps and a bifurcated 400-μm fibre-optic probe. The fibre-optic probe both provided illumination and obtained light reflected from the sample in a reading area of about 1 mm^2. The measurements were taken at a 90º angle to the sample. All measurements were relative to a white WS-1-SS Spectralon tablet (Ocean Optics) and the system was frequently calibrated. For each individual, we took three different measurements of yellow breast and blue crown coloration and averaged the values obtained from the three readings. Reflectance curves were determined by calculating the median of the percentage reflectance in 10 nm intervals, from 320 nm to 700 nm, the full spectral range that can be perceived by birds (Cuthill *et al.* 2000). We calculated three standard colourimetrics variables for breast: yellow breast carotenoid chroma, calculated as the difference in reflectance (R) at the wavelengths of the two main carotenoids, lutein and zeaxanthin ($(R_{700}-R_{450})/ R_{700}$) (Andersson & Prager 2006); yellow breast brightness, calculated as total reflectance in the range 320-700 nm; and yellow breast hue, calculated as wavelength of peak reflectance λ (R_{max}). In addition to the last two variables, we also calculated chroma ($(R_{max}-R_{min})/R_{average}$) and UV-chroma ($R_{320–}$

$_{400}$ / $R_{320–700}$) for the blue crown. Analyses for blue crown chroma are not presented because this variable was highly correlated with blue crown UV-Chroma ($r > 0.93$). Further, analyses for hue are not presented because hue was highly correlated with brightness for both the yellow breast and blue crown ($r > 0.98$). We obtained qualitatively identical results for these parameters and those with which they were correlated (data not shown).

Statistical analyses: multilocus effects

We used an information-theoretic model selection approach to analyse the association between individual heterozygosity and song and plumage coloration parameters described above (Burnham & Anderson 1998). For each dependent variable we constructed two separate general linear models (GLMs), one including as predictor variable individual heterozygosity (i.e., 1-*HL*) calculated for all loci (HL_{Total}) and another including as predictor variables heterozygosity estimated for the subsets of neutral ($HL_{Neutral}$) and functional ($HL_{Functional}$) markers. Note that heterozygosity estimated at the subset of neutral markers was not correlated with heterozygosity at the subset of functional markers ($r = 0.10$, $P = 0.478$; see also Olano-Marin *et al.* 2011a for a similar result). Study plot and male age were included as fixed factors in all the models. Given that the expression of some ornaments is condition-dependent (e.g., Scheuber *et al.* 2003; Peters *et al.* 2008; Griggio *et al.* 2009), we included body condition (estimated as the residuals of a linear regression of body mass on wing length) as a covariate in the models for all the studied secondary sexual traits. The model for bout length included the time an individual had been singing before switching to the common song, as this could influence bout length due to fatigue. Models for both repertoire size and bout length also included recording date as a covariate because habitat structure differs between early and late spring due to the development of tree foliage and this could potentially influence the transmission of sound and the singing strategy of individuals (Boncoraglio & Saino 2007). We ranked the resulting models following a model-selection approach on the basis of the Akaike's information criterion corrected for small sample size (AICc; Burnham & Anderson 1998). AICc values for each model were rescaled (ΔAICc) calculating the difference between the AICc value of each model and the minimum AICc obtained among all competing models (i.e. the best model has ΔAICc = 0). Models with ΔAICc ≤ 2 were considered equivalent (Burnham & Anderson, 1998). In cases where model selection as a function of AICc did not give a single model, we performed an averaging of equivalent models (i.e. models with ΔAICc ≤ 2; Burnham & Anderson 2002). We calculated the mean of the predictor estimators, their unconditional standard errors (USE) and confidence intervals (CI), and the relative importance of each variable in the final averaged model (Σ ωi, the sum of Akaike

weights of models with ΔAICc ≤ 2 in which the variable was included). Parameter estimates were considered significant when their 95% CI did not span zero (Burnham & Anderson 2002). Model selection and averaging was performed using the R package lme4 and AICcmodavg (R Core Team 2012). Finally, we examined correlations between all the studied secondary sexual characters and body condition using Pearson rank correlations. Basic statistics (mean ± S.E. and range) for the studied phenotypic traits are summarized in Table S2 (see Supporting Information).

Statistical analyses: single locus effects

First, we analysed the effect of single locus heterozygosity (*SLH*) by fitting one GLM per locus and secondary sexual trait. Effect size was calculated for each locus as the partial correlation coefficient obtained from its respective model (Nakagawa & Cuthill 2007). Second, we examined whether *MLH* explained more variance than *SLH* following the approach described in Szulkin *et al.* (2010). We performed *F*-test ratio tests to compare models including *MLH* with those in which we replaced *MLH* with "normalized" *SLH* at all markers (Szulkin *et al.* 2010). Finally, we used a GLM to analyse whether absolute effect sizes of single locus heterozygosities were associated with marker variability (allelic richness and observed and expected heterozygosity, included as covariates in different models) and differed between neutral and putatively functional loci (marker category was included as a fixed factor) (e.g., Olano-Marin *et al.* 2011a, b; Ruiz-López *et al.* 2012; Ferrer *et al.* 2014).

RESULTS

Basic genetic statistics, genetic differentiation and identity disequilibrium

Observed heterozygosity at each locus ranged from 0.34 to 0.97, with 3-26 alleles per locus (see Supporting Information Table S1). Neutral loci had higher allele richness than functional loci ($F_{1, 24} = 4.90$, $P = 0.036$), but the subsets of loci did not significantly differ in observed (H_O) ($F_{1, 24} = 2.09$, $P = 0.160$) or expected heterozygosity (H_E) (one-way ANOVA: $F_{1, 24} = 2.58$, $P = 0.120$). After applying sequential Bonferroni corrections to compensate for multiple statistical tests, only loci *Tgu07* and *CcaTgu14* showed significant deviations from Hardy-Weinberg equilibrium (HWE) in one study plot ("Majadillas"). Significant linkage disequilibrium (LD) was detected for loci *Tgu07*/*PK12* and *Tgu07*/*Ase18* in "Arroyo del Marchés" locality after sequential Bonferroni corrections. Pair-wise significant, indicating that individuals from the two studied localities are not genetically differentiated (all markers: $F_{ST} = 0.006$, $P = 0.099$; neutral markers: $F_{ST} = 0.008$, $P = 0.070$; functional markers: $F_{ST} = -0.000$, $P = 0.448$). We found significant (i.e., 95% quantiles did not cross zero) and positive heterozygosity-heterozygosity correlations

Table 1 General linear models (GLMs) for (a) repertoire size, (b) bout length, (c) yellow breast brightness, (d) yellow breast carotenoid chroma, (e) blue crown brightness, (f) blue crown UV-chroma, (g) wing length, and (h) body mass. A single model with ΔAICc ≤ 2 was obtained for bout length. For the rest of the studied variables we performed model averaging of the best ranked equivalent models (ΔAICc ≤ 2) to obtain parameter estimates and unconditional standard errors (USE) (see Supporting Information, Table S3). Variables are sorted according with their relative importance based on the sum of Akaike weights (Σ ωi) of those models with ΔAICc ≤ 2 in which the variable was present. Bold type indicates significant variables, i.e. variables for which their unconditional 95 % confidence interval (CI) did not cross zero.

	Estimate ± USE	Σ ωi	Lower 95% CI	Upper 95% CI
(a) *Repertoire size*				
Study plot	-2.25 ± 0.79	0.57	**-3.79**	**-0.71**
Body condition	-1.00 ± 0.69	0.23	-2.35	0.35
$HL_{Neutral}$	-3.51 ± 2.76	0.15	-8.92	1.89
Recording date	-0.03 ± 0.03	0.12	-0.09	0.03
$HL_{Functional}$	-3.24 ± 2.71	0.08	-8.56	2.08
(b) *Bout length*				
$HL_{Functional}$	200.61 ± 81.59	0.25	**40.69**	**360.54**
Study plot	56.33 ± 21.15	0.25	**14.87**	**97.78**
Recording date	1.87 ± 0.80	0.25	**0.30**	**3.44**
Body condition	17.79 ± 19.19	0.25	-19.82	55.4
(c) *Yellow breast brightness*				
$HL_{Functional}$	26.51 ± 149.64	0.53	-266.77	319.80
Age	42.01 ± 37.35	0.15	-31-19	115.21
Body condition	-34.53 ± 43.35	0.11	-119.50	50.44
(d) *Yellow breast carotenoid chroma*				
$HL_{Functional}$	0.53 ± 0.27	0.26	**0.01**	**1.06**
Body condition	0.11 ± 0.07	0.26	-0.03	0.25
Age	-0.10 ± 0.07	0.25	-0.23	0.03
Study plot	-0.08 ± 0.08	0.08	-0.24	0.07
$HL_{Neutral}$	-0.31 ± 0.28	0.06	-0.85	0.23
(e) *Blue crown brightness*				
$HL_{Functional}$	363.83 ± 155.86	0.37	**58.34**	**669.32**
Body condition	-89.61 ± 49.07	0.22	-185.79	6.57
(f) *Blue crown UV-chroma*				
Body condition	0.01 ± 0.01	0.50	**0.01**	**0.01**
$HL_{Functional}$	-0.01 ± 0.01	0.35	-0.01	0.01
Age	0.01 ± 0.01	0.10	-0.01	0.01
(g) *Wing length*				
Age	0.95 ± 0.41	0.75	**0.14**	**1.76**
$HL_{Functional}$	-1.32 ± 1.65	0.75	-4.56	1.92
$HL_{Neutral}$	3.57 ± 1.80	0.52	-0.01	7.10
(h) *Body mass*				
$HL_{Functional}$	1.14 ± 0.55	0.57	**0.06**	**2.22**
Wing length	0.11 ± 0.04	0.57	**0.03**	**0.20**
$HL_{Neutral}$	-0.65 ± 0.59	0.16	-1.80	0.50

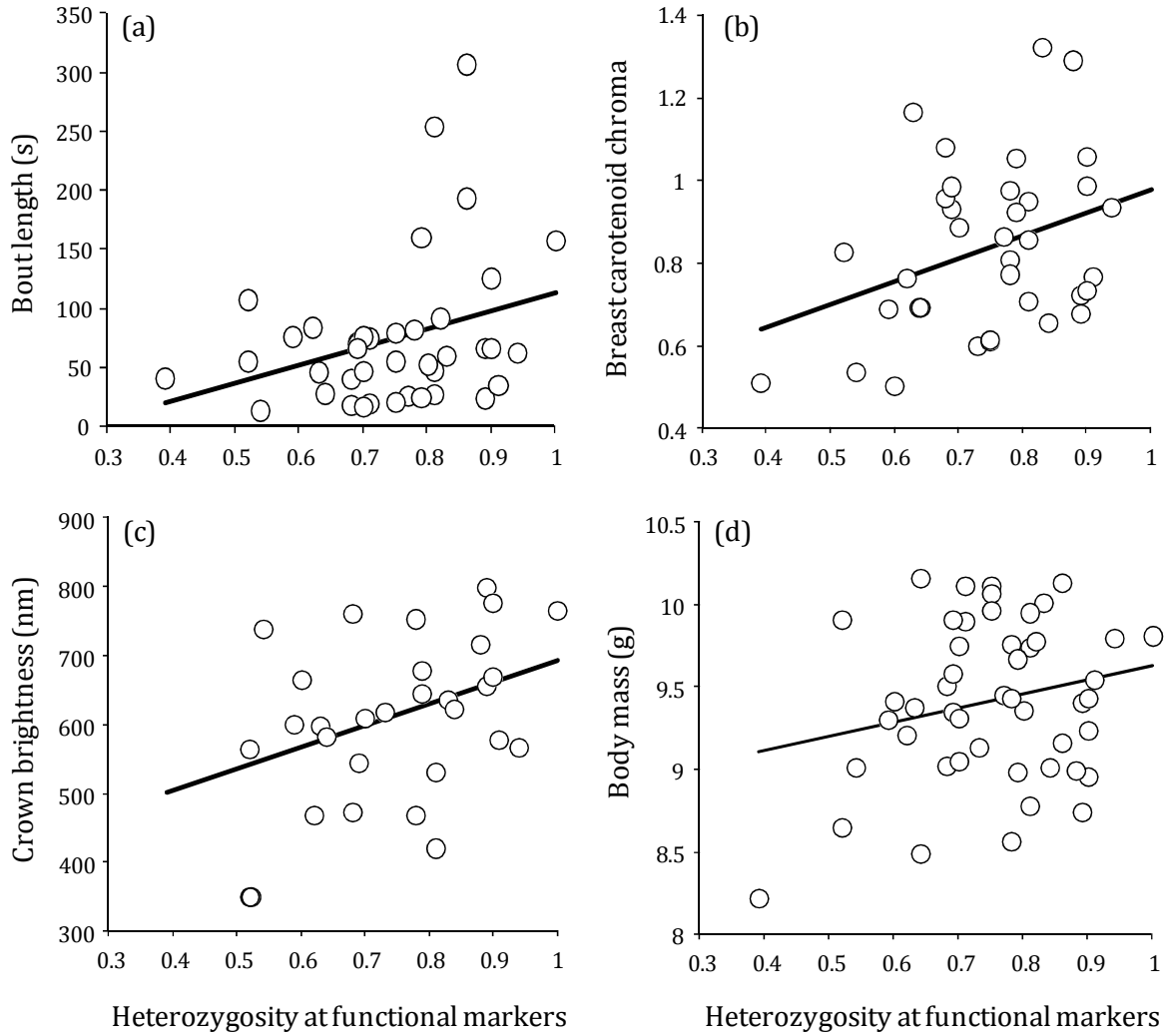


Figure 1 Relationship between multilocus heterozygosity at functional loci (1-$HL_{Functional}$) and (a) bout length, (b) yellow carotenoid chroma, (c) blue crown brightness, and (d) body mass.

(HHC) between different subsets of loci, suggesting that genetic diversity estimated at our set of markers is representative of genome-wide heterozygosity (all markers: r= 0.356, 95% CI = 0.185-0.547; neutral markers: r = 0.209, 95% CI = 0.034-0.362; functional markers: r = 0.257, 95% CI = 0.106-0.421). However, this was not supported by analyses based on the parameter g^2, which did not significantly differ from zero for all markers (g^2 = -0.003, P = 0.765) or when the subsets of neutral (g^2 = 0.002, P = 0.348) and functional markers (g^2 = -0.007, P = 0.750) were analysed separately.

Multilocus effects

Our most parsimonious models showed that repertoire size was higher in "Arroyo del Marchés" than in "Majadillas" locality, but it was not significantly associated with any heterozygosity estimate (Table 1 and Table S3-5). Strophe bout length increased with recording date and was higher in "Majadillas" than in "Arroyo del Marchés" locality (Table 1 and Table S3-5). We also

found a positive relationship between bout length (Table 1; Fig. 1a), yellow breast carotenoid chroma (Table 1; Fig. 1b), and blue crown brightness (Table 1; Fig. 1c) and heterozygosity estimated at the subset of functional loci, but these variables were not significantly associated with heterozygosity estimated at the subset of neutral loci (Table 1 and Table S3) or at all typed markers (Table S4-5). Yellow breast brightness and blue crown UV-chroma were not associated with any estimate of individual genetic diversity (Table 1 and Table S3-5). Blue crown UV-chroma was the only variable positively associated with body condition (Table 1 and Table S3-5). Wing length was not associated with any estimate of individual genetic diversity (Table 1 and Table S3-5). After correcting for wing length, body mass was also positively associated with heterozygosity estimated at the subset of functional markers (Table 1 and Table S3; Fig. 1d). However, body mass was not associated with heterozygosity calculated at all markers or the subset of neutral loci (Table 1 and Table S3-5). When examining the interdependence of studied traits, we only found a significant relationship between blue crown brightness and yellow breast brightness (Table 2).

Single locus effects

We did not find significant differences in the variance explained by the models including multilocus heterozygosity (*MLH*) compared to the models including single locus heterozygosity (*SLH*) considering any subset of loci (all $Ps > 0.05$). For each trait, the direction of *SLH* effects did not differ significantly for the subsets of neutral and functional markers (all $Ps > 0.05$). Absolute effect sizes of *SLH* did not differ between the subsets of neutral and functional loci and were not associated with allelic richness or observed or expected heterozygosity in any trait (all $Ps > 0.05$) (see Supporting Information, Fig. S1 and Table S6).

DISCUSSION

Our results suggest that more heterozygous individuals may be able to produce more conspicuous ornaments and support the hypothesis that secondary sexual traits can mirror the genetic quality of its bearer (Brown 1997). The fact that ornamentation is associated with individual genetic diversity across multiple secondary sexual traits can also explain the evolution of directional mate preferences as suggested by the good genes as heterozygosity hypothesis (Brown 1997; Kempenaers 2007). Our results support the "back-up signal" hypothesis and suggest that different ornaments indicate redundant information about an aspect of individual quality, in our case individual genetic diversity, that may allow a more accurate assessment of mate quality based on the same aspect (Candolin, 2003). Several previous studies have found a positive relationship between heterozygosity and the expression of a single sexual ornament (Aparicio *et al.* 2001; Foerster *et al.* 2003; Marshall *et al.* 2003;

Table 2 Pearson rank correlations between the studied secondary sexual characters and body condition in male blue tits. Correlation coefficients (below the diagonal) and significance values (above the diagonal) are shown. Asterisks denote variables statistically significant after sequential Bonferroni correction.

Trait	Repertoire size	Bout length	Yellow brightness	Yellow chroma	Blue brightness	Blue UV-chroma	Body condition
Repertoire size	-	0.021	0.598	0.893	0.707	0.598	0.268
Bout length	-0.384	-	0.053	0.338	0.672	0.344	0.119
Yellow brightness	0.109	-0.376	-	0.662	0.001*	0.059	0.687
Yellow carotenoid	0.028	0.192	-0.075	-	0.960	0.409	0.135
Blue brightness	0.085	-0.098	0.987	0.010	-	0.030	0.358
Blue UV-chroma	-0.119	-0.217	-0.375	0.169	-0.404	-	0.037
Body condition	-0.182	-0.254	-0.069	0.254	-0.177	0.389	-

Seddon *et al.* 2004; Reid *et al.* 2005; Araya-Ajoy *et al.* 2009; Pérez-González *et al.* 2010), but only a few have simultaneously considered multiple secondary sexual traits (Bolund *et al.* 2010; Zajitschek & Brooks 2010; Leclaire *et al.* 2011), and none of these studies analysed whether associations between ornamentation and heterozygosity differed between neutral versus putatively functional markers.

Heterozygosity and ornamentation

Previous studies have found an association between different song parameters and individual genetic diversity or inbreeding (Marshall *et al.* 2003; Seddon *et al.* 2004; Reid *et al.* 2005; Bolund *et al.* 2010). Marshall *et al.* (2003) and Reid *et al.* (2005) reported a link between song complexity and heterozygosity in sedge warblers (*Acrocephalus schoenobaenus*) and song sparrows (*Melospiza melodia*), respectively. They interpreted their results as indicating that learning and brain capacity are affected by inbreeding and this may cause a reduced ability to memorize song. Seddon *et al.* (2004) showed that more heterozygous males of the subdesert mesite (*Monias benschi*) produce trills of longer duration and lower pitch, while Bolund *et al.* (2010) found that song rate was negatively affected by inbreeding in zebra finches (*Taeniopygia guttata*). We found that repertoire size was not associated with heterozygosity, suggesting that this parameter could be only influenced by morphometric, environmental, and social conditions in our study species (Doutrelant *et al.* 2000; Johannessen *et al.* 2006). However, more heterozygous male blue tits sang longer bouts than homozygous ones. Thus, bout length may be a reliable indicator of genetic diversity that could be used by females in mate choice decisions as suggested in a previous study on this species (Dreiss *et al.* 2006).

Regarding plumage coloration, previous studies on blue tits suggest a

relationship between crown coloration and individual attractiveness (e.g., Andersson *et al.* 1998; Sheldon *et al.* 1999). We found that crown brightness is positively associated with heterozygosity, a pattern that has been consistently reported by studies performed in different populations of blue tits (Foerster *et al.* 2003; García-Navas *et al.* 2009). Our study has shown for the first time that male blue tits with higher yellow breast carotenoid chroma values have higher heterozygosity levels than less chromatic individuals. Past research indicates that carotenoid-based plumage reflects individual quality in a variety of birds (e.g., Jawor & Breitwisch 2004; Senar *et al.* 2008) and is subjected to sexual selection (Badyaev & Hill 2002; Jawor *et al.* 2003). Although some have argued that colour traits based on carotenoids reflect foraging ability and territory quality rather than genetic quality (Hõrak *et al.* 2000; Pagani-Núñez *et al.* 2014), recent studies have shown that carotenoid-pigmented ornaments have a heritable component (Evans & Sheldon 2012; Vergara *et al.* 2015). In blue tits, yellow breast coloration reflects individual health and parasitism status (del Cerro *et al.* 2010) and has been associated with provisioning ability (García-Navas *et al.* 2012) and foraging capacity (Senar & Quesada 2006). Male heterozygosity is positively associated with nestling feeding rates in blue tits (García-Navas *et al.* 2009), suggesting that the higher performance of more pigmented individuals could be reflecting the greater foraging capacity and/or ability to acquire a better territory and assimilate resources of more heterozygous individuals. Previous studies have also shown a relationship between carotenoid-based coloration and heterozygosity in other species, suggesting that these ornaments can also be reliable signals to assess the genetic quality of potential partners (e.g., Oosterhout *et al.* 2003; Bolund *et al.* 2010; Leclaire *et al.* 2011, Herdegen *et al.* 2014).

Body condition was positively associated with individual genetic diversity, a relationship that has been previously reported in other organisms and suggests that heterozygosity influences the capacity to obtain and assimilate resources (Lens *et al.* 2000; Pujolar *et al.* 2005; Bolund *et al.* 2010; Herdegen *et al.* 2013). However, the ornamental traits associated with heterozygosity were not correlated with either the age or the physical condition of individuals. The latter may be consequence of the index used for determining body condition is a poor estimate of general physical condition or it might only reflect some aspects of the individual`s physiological state. Alternatively, if secondary sexual characters associated with individual heterozygosity mostly convey information about overall genetic quality, then, they may not be strongly influenced by environment or the physical condition of individuals (Scheuber *et al.* 2003; Freeman-Gallant *et al.* 2010). Thus, different proximate mechanisms can explain the observed associations between individual genetic diversity and the expression of secondary sexual characters. Highly heterozygous individuals could display

more conspicuous ornaments if genes directly involved in their development exhibit overdominance or are affected by deleterious or partly deleterious recessive alleles that have a reduced chance of being expressed in genetically more diverse individuals (Charlesworth & Charlesworth 1987; Falconer & Mackay 1996). However, this would require that many genes are involved in the expression of secondary sexual characters so that they can collectively capture the effects of genome-wide heterozygosity (Aparicio *et al.* 2007). Another possibility is that more heterozygous individuals show a higher resistance to parasites and diseases (Acevedo-Whitehouse *et al.* 2003), superior physiological response to stress and/or increased cellular homeostasis (Mitton & Grant 1984), aspects that might have not been captured by our index of physical body condition and that are likely to reduce the costs of producing elaborated secondary sexual characters (Van Oosterhout *et al.* 2003).

Identity disequilibrium, functional *vs.* neutral markers and local effects

Correlations between heterozygosity and phenotype or fitness-related traits are expected to be detected in populations that experience genetic drift, bottlenecks, non-random mating or population admixture, processes that cause variance in inbreeding and increase identity disequilibrium (ID) (Szulkin *et al.* 2010). Although we failed to detect significant g^2 values, we found positive heterozygosity-heterozygosity correlations (HHCs), suggesting that genetic diversity estimated at our different sets of markers may be representative of genome-wide heterozygosity in this population (Balloux *et al.* 2004; see also Kardos *et al.* 2014). The very limited power to detect ID when variance in inbreeding is low and the number of employed loci is relatively small (<100 markers), the typical situation in most studies in natural populations, may have resulted in we have been able to detect ID with one method but not with the other (Kardos *et al.* 2014; Miller & Coltman, 2014). Accordingly, a recent meta-analysis by Miller & Coltman (2014) showed that only ~20% of microsatellite-based studies found significant g^2 values. However, it should be considered that non-significant g^2 values (or HHCs) do not necessarily imply that the detection of correlations between heterozygosity and fitness or phenotypic traits are not due inbreeding (or a genome-wide effect), given that the studied traits are likely to capture the effect of potentially many more loci than the number of typed markers (see Szulkin *et al.* 2010).

Most studies in natural populations have employed neutral markers to analyse the association between heterozygosity and fitness or phenotype, as their higher polymorphism is expected to better capture the effects of genome-wide inbreeding (Slate *et al.* 2004). However, we only detected significant associations between heterozygosity and the expression of ornaments across the panel of functional markers, despite the fact that our functional

markers showed slightly lower polymorphism than our neutral markers (see also Olano-Marin *et al.* 2011a; Ferrer *et al.* 2014). This suggests that reduced heterozygosity at functional regions of the genome may be more relevant in the expression of secondary sexual characters, which may make these ornamental traits more reliable indicators of the genetic quality of individuals given that only functional genomic regions are translated into phenotypic differences. Further, we did not detect significant single locus effects and the employed functional loci are distributed across nine chromosomes and are located within or in close vicinity to coding genes involved in different physiological processes (see Table 1 in Olano-Marin *et al.* 2011a). Different genes are also expected to be involved in the expression of the different studied ornaments (e.g., related to plumage coloration or song elaboration), which suggests that the observed associations between heterozygosity at functional loci and the expression of secondary sexual traits are driven by loci widely distributed across the genome and not due to the particular set of markers chosen or their specific functions. Our results contrast with previous microsatellite-based studies that have found different roles of neutral and putatively functional markers in observed correlations between heterozygosity and fitness or phenotype (e.g., Olano-Marin *et al.* 2011a, b; Laine *et al.* 2012; Ferrer *et al.* 2014). Several authors have reported stronger correlations with specific microsatellite loci, suggesting the presence of strong local effects (Da Silva *et al.* 2009; Küpper *et al.* 2010; Olano-Marin *et al.* 2011ab; Laine *et al.* 2012; García-Navas *et al.* 2014), whereas others have found that heterozygosity at neutral markers is more strongly associated with the studied traits than heterozygosity at functional markers in absence of relevant single locus effects (Olano-Marin *et al.* 2011a; Ferrer *et al.* 2014). Finally, some studies have found a different contribution of functional/neutral markers and general/local effects depending on the studied trait (Küpper *et al.* 2010; Olano-Marin *et al.* 2011b; Laine *et al.* 2012). It should also be considered that heterozygosity at neutral markers was not correlated with heterozygosity estimated at functional markers, a result reported in previous studies that may reflect the fact that the two sets of markers are impacted by selective processes in a different manner (Olano-Marin *et al.* 2011b; Szulkin & David 2011; Ferrer *et al.* 2014). Natural selection across different life stages acting against individuals genetically less diverse at functional loci could contribute to partially decoupling levels of genetic diversity in selectively neutral and functional genomic regions. Mate choice could also play a role in these differences, for instance if individuals select mates more different (compatible) from themselves at multiple functional but not neutral loci (Yamazaki & Beauchamp 2007). In this case, neutral loci would be expected to more accurately reflect inbreeding. However, functional loci are also likely to reflect genome-wide inbreeding to some extent and they could develop further identity disequilibrium due

to variance among individuals in mate choice decisions that can be context-dependent and influenced by different factors such as the availability of potential mates, age or the phenotypic or genotypic quality of individuals (Lie *et al.* 2010). Thus, contrasting influences of sexual and natural selection on neutral versus functional loci may cause these loci to show different associations with phenotype and fitness-related traits, even in the absence of strong local effects, potentially explaining the discrepancy between our study and some past research (Olano-Marin *et al.* 2011b; see also Hansson & Westerberg 2008). Overall, this and previous work indicate that the expected association between phenotype or fitness-related traits and heterozygosity at functional/neutral markers is difficult to predict, highly dependent on the studied trait and, when the association is mostly driven by variability at putatively functional markers, does not necessarily have to be the result of local effects (Szulkin & David 2011).

Conclusions

In summary, we found that more heterozygous males showed increased expression of secondary sexual traits and body condition. Males with a higher level of carotenoid chroma on the yellow breast, a brighter blue crown, longer song bouts, and higher body condition were more heterozygous, indicating that genetic diversity can be reflected across multiple traits that are likely to be used by females during mate choice decisions. The strength of selection may increase if mate choice based on traits that reflect the same attribute facilitates mate assessment and skews mate choice toward males that express high levels of multiple types of ornamentation. In our study population, both song and different colour attributes reflect male heterozygosity, which may increase female's ability to accurately identify a high-quality partner, thus reducing the costs of mate choice in accordance with the "back-up signal" hypothesis (Candolin 2003). However, we did not find correlations between most ornaments, which may be due to our relatively small sample sizes or because the studied traits being produced in different parts of the annual cycle (e.g., plumage moult in summer-autumn and singing in spring), reacting to other influential factors at different rates (fast response for singing *vs.* slow for coloration; Birkhead *et al.* 1998) or being involved in different processes (e.g. female choice *vs.* intrasexual competition; Candolin 2003; e.g. Andersson *et al.* 2002; Freeman-Gallant *et al.* 2010). The lack of strong local effects and the presence of identity disequilibrium in our population suggest that genome-wide heterozygosity is the most likely mechanism behind the observed heterozygosity-phenotype associations, whereas the predominant role of putatively functional loci indicates that the expression of secondary sexual characters is more tightly reflected by heterozygosity at genomic regions containing coding genes that are being actively expressed. The implementation of

candidate-gene approaches, considering loci with functions related with the trait of interest, and the application of high-throughput sequencing technology to get accurate estimates of genome-wide inbreeding based on thousands of loci will help to greatly increase our understanding of the role of genetic diversity in the expression of secondary sexual characters and disentangle the underlying mechanisms (Fitzpatrick *et al.* 2005; Walsh *et al.* 2011; Hoffman *et al.* 2014; Zuk & Balenger 2014).

ACKNOWLEDGEMENTS

We thank the Council of San Pablo de los Montes for allowing us to work there and Diego Gil for lending his recording equipment and resolving our doubts about song data analysis. We are indebted to Pedro J. Cordero for allowing us to carry out the genetic analyses in his lab and to Conchi Cáliz for her advice with genotyping. Three anonymous referees provided useful discussion and valuable comments on an earlier draft of this manuscript. Funding was provided by Ministerio de Ciencia e Innovación (CGL2010-21933-C02-01) and Junta de Comunidades de Castilla-La Mancha and European Social Fund (POIC10-0269-7632). ESF and JBE are both supported by a doctoral scholarship from Junta de Comunidades de Castilla-La Mancha-European Social Fund, VGN is supported by a Forschungskredit of the University of Zurich (FK-14-103) and JO is supported by a research contract funded by Severo Ochoa Program (SEV-2012-0262).

REFERENCES

Acevedo-Whitehouse K, Gulland F, Greig D, Amos W. 2003. Disease susceptibility in California sea lions. *Nature*, 422: 35.

Alho JS, Välimäki K, Merilä J. 2010. Rhh: an R extension for estimating multilocus heterozygosity and heterozygosity-heterozygosity correlation. *Mol. Ecol. Resour.* 10: 720-722.

Andersson M. 1994. *Sexual Selection*. Princeton: Princeton University Press.

Andersson S, Ornborg J, Andersson M. 1998. Ultraviolet sexual dimorphism and assortative mating in blue tits. *Proc. R. Soc. B-Biol. Sci.* 265: 445-450.

Andersson S, Pryke SR, Ornborg J, Lawes MJ, Andersson M. 2002. Multiple receivers, multiple ornaments, and a trade-off between agonistic and epigamic signaling in a widowbird. *Am. Nat.* 160: 683-91.

Andersson S, Prager M. 2006. Quantifying colors. Pp. 41-89 *in* Hill GE, McGraw KJ (eds.). *Bird Coloration, vol. 1: Mechanisms and Measurements*. Cambridge: Harvard University Press.

Aparicio JM, Cordero PJ, Veiga JP. 2001. A test of the hypothesis of mate choice based on heterozygosity in the spotless starling. *Anim. Behav.* 62: 1001-1006.

Aparicio JM, Ortego J, Cordero PJ. 2006. What should we weigh to estimate heterozygosity, alleles or loci? *Mol. Ecol.* 15: 4659-4665.

Aparicio JM, Ortego J, Cordero PJ. 2007. Can a simple algebraic analysis predict markers–genome heterozygosity correlations? *J. Hered.* 98: 93-96.

Araya-Ajoy Y-M, Chaves-Campos J, Kalko EKV, DeWoody JA. 2009. High-pitched notes during vocal contests signal genetic diversity in ocellated antbirds. *PLoS One* 4: e8137.

Badyaev AV, Hill GE. 2002. Paternal care as a conditional strategy: Distinct reproductive tactics associated with elaboration of plumage ornamentation in the house finch. *Behav. Ecol.* 13: 591-597.

Balloux F, Amos W, Coulson T. 2004. Does heterozygosity estimate inbreeding in real populations? *Mol. Ecol.* 13: 3021-3031.

Birkhead TR, Fletcher F, Pellatt EJ. 1998. Sexual selection in the zebra finch *Taeniopygia guttata*: condition, sex traits and immune capacity. *Behav. Ecol. Sociob.* 44: 179-191.

Bolund E, Martin K, Kempenaers B, Forstmeier W. 2010. Inbreeding depression of sexually selected traits and attractiveness in the zebra finch. *Anim. Behav.* 79: 947-955.

Boncoraglio G, Saino N. 2007. Habitat structure and the evolution of bird song: a meta-analysis of the evidence for the acoustic adaptation hypothesis. *Funct. Ecol.* 21: 134-142.

Brown JL. 1997. A theory of mate choice based on heterozygosity. *Behav. Ecol.* 8: 60-65.

Burnham KP, Anderson DR. 1998. *Model selection and inference: a practical information-theoretic approach*. New York: Springer-Verlag.

Burnham KP, Anderson DR. 2002 *Model selection and multi-model inference: a practical information-theoretic approach*. New York, NY: Springer-Verlag.

Candolin U. 2003. The use of multiple cues in mate choice. *Biol. Rev.* 78: 575-595.

Chapman JR, Nakagawa S, Coltman DW, Slate J, Sheldon BC. 2009. A quantitative review of heterozygosity- fitness correlations in animal populations. *Mol. Ecol.* 18: 2746-2765.

Charlesworth D, Charlesworth B. 1987. Inbreeding depression and its evolutionary consequences. *Annu. Rev. Ecol. Evol. Syst.* 18: 237-268.

Cuthill IC, Partridge JC, Bennett ATD, Church SC, Hart NS, Hunt S. 2000. Ultraviolet vision in birds. *Adv. Stud. Behav.* 29: 159-214.

Cutrera AP, Fanjul MS, Zenuto RR. 2012. Females prefer good genes: MHC-associated mate choice in wild and captive tuco-tucos *Anim. Behav.* 83: 847-856.

Da Silva A, Gaillard JM, Yoccoz NG, Hewison AJM, Galan M, Coulson T, Allaine D, Vial L, Delorme D, Van Laere G, Klein F, Luikart G. 2009. Heterozygosity-fitness correlations revealed by neutral and candidate gene markers in roe deer from a long-term study. *Evolution* 63: 403-417.

David P. 1998. Heterozygosity-fitness correlations: new perspectives on old problems. *Heredity* 80: 531-537.

David P, Pujol B, Viard F, Castella V, Goudet J. 2007. Reliable selfing rate estimates from imperfect population genetic data. *Mol. Ecol.* 16: 2474-2487.

del Cerro S, Merino S, Martínez-de la Puente J, Lobato E, Ruiz-de-Castañeda R, Rivero-de Aguilar J, Martínez J, Morales J, Tomás G, Moreno J. 2010. Carotenoid-based plumage colouration is associated with blood parasite richness and stress protein levels in blue tits (*Cyanistes caeruleus*). *Oecologia* 162: 825-835.

Doutrelant C, Gregoire A, Grnac N, Gomez D, Lambrechts MM, Perret P. 2000. Blue tit song repertoire size, male quality and interspecific competition. *J. Avian Biol.* 31: 360-366.

Dreiss A, Richard M, Moyen F, White J, Møller AP, Danchin E. 2006. Sex ratio and male sexual characters in a population of blue tits, *Parus caeruleus*. *Behav. Ecol.* 17: 13-19.

Excoffier L, Laval G, Schneider S. 2005. Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evol. Bioinf.* 1: 47-50.

Evans SR, Sheldon BC. 2012. Quantitative genetics of a carotenoid-based color: heritability and persistent natal environmental effects in the great tit. *Am. Nat.* 179: 79-94.

Falconer DS, Mackay TFC. 1996. *Introduction to Quantitative Genetics, 3rd edn*. Essex: Longman.

Ferrer ES, García-Navas V, Sanz JJ, Ortego J. 2014. Individual genetic diversity and probability of infection by avian malaria parasites in blue tits (*Cyanistes caeruleus*). *J. Evol. Biol.* 27: 2468-2482.

Fitzpatrick MJ, Ben-Shahar Y, Smid HM, Vet LEM, Robinson GE, Sokolowski MB. 2005. Candidate genes for behavioural ecology. *Trends Ecol. Evol.* 20: 96-104.

Foerster K, Delhey K, Johnsen A, Lifjeld JT, Kempenaers B. 2003. Females increase offspring heterozygosity and fitness through extra-pair matings. *Nature* 425: 714-717.

Freeman-Gallant CR, Taff CC, Morin DF, Dunn PO, Whittingham LA, Tsang SM. 2010. Sexual selection, multiple male ornaments, and age- and condition-dependent signaling in the common yellowthroat. *Evolution* 64: 1007-1017.

Fromhage L, Kokko H, Reid JM. 2009. Evolution of mate choice for genome-wide heterozygosity. *Evolution* 63: 684-694.

García-Navas V, Ortego J, Sanz J J. 2009. Heterozygosity-based assortative mating in blue tits (*Cyanistes caeruleus*): implications for the evolution of mate choice. *Proc. R. Soc. B-Biol. Sci.* 276: 2931-2940

García-Navas V, Ferrer ES, Sanz JJ. 2012. Plumage yellowness predicts foraging ability in the blue tit *Cyanistes caeruleus*. *Biol. J. Linn. Soc.* 106: 418-429.

García-Navas V, Cáliz-Campal C, Ferrer ES, Sanz JJ, Ortego J. 2014. Heterozygosity at a single locus explains a large proportion of variation in two fitness-related traits in great tits: a general or a local effect? *J. Evol. Biol.* 27: 2807-2819.

Getty T. 1998. Reliable signalling need not be a handicap. *Anim. Behav.* 56: 253-255.

Griggio M, Serra L, Licheri D, Campomori C, Pilastro A. 2009. Moult speed affects structural feather ornaments in the blue tit. *J. Evol. Biol* 22: 782-792.

Hansson B, Bensch S, Hasselquist D, Åkesson M. 2001. Microsatellite diversity predicts recruitment in sibling great reed warblers. *Proc. R. Soc. B-Biol. Sci.* 268: 1287-1291.

Hansson B, Westerberg L. 2002. On the correlation between heterozygosity and fitness in natural populations. *Mol. Ecol.* 11: 2467-2474.

Hansson B, Westerberg L. 2008. Heterozygosity-fitness correlations within inbreeding classes: local or genome-wide effects? *Conserv. Genet.* 9: 73-83.

Hegyi G, Laczi M, Nagy G, Szász E, Kötél D, Török J. 2015. Stable correlation structure among multiple plumage colour traits: can they work as a single signal? *Biol. J. Linn. Soc.* 114: 92-108.

Helfenstein F, Losdat S, Moller A, Blount J D, Richner H. 2010. Sperm of colourful males are better protected against oxidative stress. *Ecol. Lett.* 13: 213-222.

Herdegen M, Dudka K, Radwan J. 2014. Heterozygosity and orange coloration are associated in the guppy (*Poecilia reticulata*). *J. Evol. Biol.* 27: 220-225.

Herdegen M, Nadachowska-Brzyska K, Konowalik A, Babik W, Radwan J. 2013. Heterozygosity, sexual ornament and body size in the crested newt. *J. Zool.* 291: 146-153.

Hoelzer GA. 1989. The good parent process of sexual selection. *Anim. Behav.* 38: 1067-1078.

Hoffman JI, Simpson F, David P, Rijks JM, Kuiken T, Thorne MAS, Lacy RC, Dasmahapatra KK. 2014. High-throughput sequencing reveals inbreeding depression in a natural population. *Proc. Natl. Acad. Sci. U.S.A.* 111: 3775-3780.

Hõrak P, Vellau H, Ots I, Møller AP. 2000. Growth conditions affect carotenoid-based plumage coloration of great tit nestlings. *Naturwissenschaften* 87: 460-464.

Jawor JM, Breitwisch R. 2004. Multiple ornaments in male northern cardinals, *Cardinalis cardinalis*, as indicators of condition. *Ethology* 110: 113-126.

Jawor JM, Linville SU, Beall SM, Breitwisch R. 2003. Assortative mating by multiple ornaments in northern cardinals, *Cardinalis cardinalis*. *Behav. Ecol.* 14: 515-520.

Johannessen LE, Slagsvold T, Hansen BT. 2006. Effects of social rearing conditions on song structure and repertoire size: experimental evidence from the field. *Anim. Behav.* 72: 83-95.

Johnstone RA. 1996. Multiple displays in animal communication: "backup signals" and "multiple messages". *Philos. T. Roy. Soc. B-Biol. Sci.* 351: 329-338.

Johnstone RA. 1997. The evolution of animal signals. Pp. 155-178 *in* Krebs JR, Davies NB (eds). *Behavioural Ecology: An Evolutionary Approach*. Oxford: Blackwell.

Kardos M, Allendorf FW, Luikart G. 2014. Evaluating the role of inbreeding depression in heterozygosity-fitness correlations: how useful are tests for identity disequilibrium? *Mol. Ecol. Resour.* 14: 519-530.

Kempenaers B. 2007. Mate choice and genetic quality: A review of the heterozygosity theory. *Adv. Stud. Behav.* 37: 189-278

Kokko, H. 1998. Should advertising parental care be honest? *Proc. R. Soc. B-Biol. Sci.* 265: 1871-1878

Küpper C, Kosztolanyi A, Augustin J, Dawson DA, Burke T, Szekely T. 2010. Heterozygosity-fitness correlations of conserved microsatellite markers in Kentish plovers *Charadrius alexandrinus*. *Mol. Ecol.* 19: 5172-5185.

Laine VN, Herczeg G, Shikano T, Primmer CR. 2012. Heterozygosity-behaviour correlations in nine-spined stickleback (*Pungitius pungitius*) populations: contrasting effects at random and functional loci. *Mol. Ecol.* 21: 4872-4884.

Leclaire S, White J, Arnoux E, Faivre B, Vetter N, Hatch SA, Danchin E. 2011. Integument coloration signals reproductive success, heterozygosity, and antioxidant levels in chick-rearing black-legged kittiwakes. *Naturwissenschaften* 98: 773-782.

Lens L, Van Dongen S, Galbusera P, Schenck T, Matthysen E, Van de Casteele T. 2000. Developmental instability and inbreeding in natural bird populations exposed to different levels of habitat disturbance. *J. Evol. Biol.* 13: 889-896.

Lie HC, Simmons LW, Rhodes G. 2010. Genetic dissimilarity, genetic diversity, and mate preferences in humans. *Evol. Hum. Behav.* 31: 48-58.

Marchetti K. 1998. The evolution of multiple male traits in the yellow-browed leaf warbler. *Anim. Behav.* 55: 361-376.

Marshall RC, Buchanan KL, Catchpole CK. 2003. Sexual selection and individual

genetic diversity in a songbird. *Proc. R. Soc. B-Biol. Sci.* 270: S248-S250.

Miller JM, Coltman DW. 2014. Assessment of identity disequilibrium and its relation to empirical heterozygosity fitness correlations: a meta-analysis. *Mol. Ecol.* 23: 1899-1909.

Mitton JB, Grant MC. 1984. Associations among protein heterozygosity, growth-rate, and developmental homeostasis. *Annu. Rev. Ecol. Evol. Syst.* 15: 479-499.

Mitton JB, Schuster WSF, Cothran EG, De Fries JC. 1993. The correlation between the individual heterozygosity of parents and their offspring. *Heredity* 71: 59-63.

Møller AP, Pomiankowski A. 1993. Why have birds got multiple sexual ornaments? *Behav. Ecol. Sociobiol.* 32: 167-176.

Murphy MT, Sexton K, Dolan AC, Redmond LJ. 2008. Dawn song of the eastern kingbird: an honest signal of male quality? *Anim. Behav.* 75: 1075-1084.

Nakagawa S, Cuthill IC. 2007. Effect size, confidence interval and statistical significance: a practical guide for biologists. *Biol. Rev.* 82: 591-605.

Nietlisbach P, Keller LF, Postma E. 2015. Genetic variance components and heritability of heterozygosity under inbreeding. *Heredity*, in press.

Olano-Marin J, Mueller JC, Kempenaers B. 2011a. Correlations between heterozygosity and reproductive success in the blue tit (*Cyanistes caeruleus*): an analysis of inbreeding and single locus effects. *Evolution* 65: 175-3194.

Olano-Marin J, Mueller JC, Kempenaers B. 2011b. Heterozygosity and survival in blue tits (*Cyanistes caeruleus*): contrasting effects of presumably functional and neutral loci. *Mol. Ecol.* 20: 4028-4041.

Ortego J, Calabuig G, Bonal R, Muñoz A, Aparicio JM, Cordero PJ. 2009. Temporal variation of heterozygosity-based assortative mating and related benefits in a lesser kestrel population. *J. Evol. Biol.* 22: 2488-2495.

Pagani-Núñez E, Uribe F, Hernández-Gómez S, Muñoz G, Senar JC. 2014. Habitat structure and prey composition generate contrasting effects on carotenoid-based coloration of great tit *Parus major* nestlings. *Biol. J. Linn. Soc.* 113: 547-555.

Pérez-González J, Carranza J, Torres-Porras J, Fernández-García JL. 2010. Low heterozygosity at microsatellite markers in Iberian red deer with small antlers. *J. Hered.* 101: 553-561.

Peters A, Delhey K, Andersson S, van Noordwijk H, Förschler M. 2008. Condition-dependence of multiple carotenoid-based plumage traits: an experimental study. *Funct. Ecol.* 22: 831-839.

Poesel A, Foerster K, Kempenaers B. 2001. The dawn song of the blue tit *Parus caeruleus* and its role in sexual selection. *Ethology* 107: 521-531.

Pujolar JM, Maes GE, Vancoillie C, Volckaert FAM. 2005. Growth rate correlates to individual heterozygosity in the European eel, *Anguilla anguilla* L. *Evolution* 59: 189-199.

R Core Team. 2012. *R: a language and environment for statistical computing*. Version 3.0.2. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.r-project.org.

Reid JM, Arcese P, Cassidy A, Marr AB, Smith JNM, Keller LF. 2005. Hamilton and Zuk meet heterozygosity? Song repertoire size indicates inbreeding and immunity in song sparrows (*Melospiza melodia*). *Proc. R. Soc. B-Biol. Sci.* 272: 481-487.

Reid JM, Arcese P, Keller LF. 2006. Intrinsic parent-offspring correlation in inbreeding level in a song sparrow (*Melospiza melodia*) population open to immigration. *Am. Nat.* 168: 1-13.

Roberts SC, Hale ML, Petrie M. 2006. Correlations between heterozygosity and measures of genetic similarity: implications for understanding mate choice. *J. Evol. Biol.* 19: 558-569.

Ruiz-López MJ, Monello RJ, Gompper ME, Eggert LS. 2012. The effect and relative importance of neutral genetic diversity for predicting parasitism varies across parasite taxa. *PLoS One* 7: e45404.

Scheuber H, Jacot A, Brinkhof MWG. 2003. Condition dependence of a multicomponent sexual signal in the field cricket *Gryllus campestris*. *Anim. Behav.* 65: 721-727.

Seddon N, Amos W, Mulder RA, Tobias JA. 2004. Male heterozygosity predicts territory size, song structure and reproductive success in a cooperatively breeding bird. *Proc. R. Soc. B-Biol. Sci.* 271: 1823-1829.

Senar JC, Figuerola J, Pascual J. 2002. Brighter yellow blue tits make better parents. *Proc. R. Soc. B-Biol. Sci.* 269: 257-261.

Senar JC, Negro JJ, Quesada J, Ruiz I, Garrido J. 2008. Two pieces of information in a single trait? The yellow breast of the great tit (*Parus major*) reflects both pigment acquisition and body condition. *Behaviour* 145: 1195-1210.

Senar JC, Quesada J. 2006. Absolute and relative signals: a comparison between melanin- and carotenoid-based patches. *Behaviour* 143: 589-595.

Sheldon BC. 1994. Male phenotype, fertility and the pursuit of extra-apir copulation by female birds. *Proc. R. Soc. B-Biol. Sci.* 257: 25-30.

Sheldon BC, Andersson S, Griffith SC, Ornborg J, Sendecka J. 1999. Ultraviolet colour variation influences blue tit sex ratios. *Nature* 402: 874-877.

Slate J, David P, Dodds KG, Veenvliet BA, Glass BC, Broad TE, McEwan JC. 2004. Understanding the relationship between the inbreeding coefficient and multilocus

heterozygosity: theoretical expectations and empirical data. *Heredity* 93: 255-265.

Szulkin M, Bierne N, David P. 2010. Heterozygosity-fitness correlations: a time for reappraisal. *Evolution* 64: 1202-1217.

Szulkin M, David P. 2011. Negative heterozygosity-fitness correlations observed with microsatellites located in functional areas of the genome. *Mol. Ecol.* 20: 3949-3952.

van Oosterhout C, Trigg RE, Carvalho GR, Magurran AE, Hauser L, Shaw PW. 2003. Inbreeding depression and genetic load of sexually selected traits: how the guppy lost its spots. *J. Evol. Biol.* 16: 273-281.

Vergara P, Fargallo JA, Martínez-Padilla J. 2015. Genetic basis and fitness correlates of dynamic carotenoid-based ornamental coloration in male and female common kestrels *Falco tinnunculus*. *J. Evol. Biol.* 28: 146-154 in press.

Von Schantz T, Wittzell H, Göransson G, Grahn M, Persson K. 1996. MHC genotype and male ornamentation: Genetic evidence for the Hamilton-Zuk model. *Proc. R. Soc. B-Biol. Sci.* 263: 265-271.

Walsh N, Dale J, McGraw KJ, Pointer MA, Mundy NI. 2011. Candidate genes for carotenoid coloration in vertebrate and their expression profiles in the carotenoid-containing plumage and bill of a wild bird. *Proc. R. Soc. B-Biol. Sci.* 279: 58-66.

Weir B, Cockerham CC. 1973. Mixed self and random mating at two loci. *Genet.Res.* 21: 247-262.

Yamazaki K, Beauchamp GK. 2007. Genetic basis for MHC-dependent mate choice. *Adv. Genet.* 59: 129-45.

Zahavi A. 1975. Mate selection - a selection for a handicap. *J. Theor. Biol.* 53: 205-214.

Zajitschek SRK, Brooks RC. 2010. Inbreeding depression in male traits and preference for outbred males in *Poecilia reticulata*. *Behav. Ecol.* 21: 884-891.

Zuk M, Balenger S. 2014. Behavioral ecology and genomics: new directions, or just a more detailed map? *Behav. Ecol.* 25: 1277-1282.

SUPPORTING INFORMATION

Table S1 Panel of 26 microsatellite markers used in this study. The following information is given: chromosome location in the zebra finch genome, category (neutral or functional), number of alleles (*K*), expected heterozygosity (H_E), observed heterozygosity (H_O), annealing temperature (T), and reference for each locus.

Locus	Chromosome	Category	*K*	H_E	H_O	T (ºC)	Reference
PK11	Unassigned	Neutral	7	0.77	0.80	52	GenBank Acc. no.: AF041465
Pca3	4	Neutral	18	0.90	0.98	55	Dawson *et al.*, 2000
Pca9	7	Neutral	11	0.71	0.70	62	Dawson *et al.*, 2000
Pocc1	7	Neutral	11	0.86	0.93	55	Bensch *et al.*, 1997
Pocc6	2	Neutral	16	0.85	0.73	62	Bensch *et al.*, 1997
Pat-MP2-43	2	Neutral	7	0.48	0.45	59	Otter *et al.*, 1998
PK12	5	Neutral	14	0.79	0.85	62	GenBank Acc. no.: AF041466
Ase18	3	Neutral	13	0.85	0.88	60	Richardson *et al.*, 2000
Pdoμ5	4	Neutral	17	0.87	0.86	46	Griffith *et al.*, 1999
Pca7	1	Neutral	13	0.88	0.88	60	Dawson *et al.*, 2000
Pca4	8	Neutral	9	0.74	0.69	60	Dawson *et al.*, 2000
Pca2	Unassigned	Neutral	9	0.76	0.73	60	Dawson *et al.*, 2000
Mcyμ4	5	Neutral	13	0.83	0.84	50	Double *et al.*, 1997
Pca8	2	Neutral	26	0.94	0.88	53	Dawson *et al.*, 2000
CcaTgu28	23_random	Functional	8	0.64	0.67	60	Olano-Marin *et al.*, 2010
PiJ14	7	Functional	12	0.88	0.89	60	Olano-Marin *et al.*, 2010
TG05-053	5	Functional	8	0.75	0.74	55	Dawson *et al.*, 2009
TG05-046	5	Functional	3	0.50	0.34	55	Dawson *et al.*, 2009
CcaTgu15	5	Functional	4	0.66	0.82	60	Olano-Marin *et al.*, 2010
Tg13-017	13	Functional	14	0.87	0.84	60	Dawson *et al.*, 2010
CcaTgu19	10	Functional	21	0.92	0.96	60	Olano-Marin *et al.*, 2010
CcaTgu11	3	Functional	6	0.74	0.71	60	Olano-Marin *et al.* 2010
CcaTgu8	2	Functional	3	0.58	0.52	63	Olano-Marin *et al.* 2010
Tgu07	6	Functional	6	0.68	0.76	55	Slate *et al.* 2007
CcaTgu14	5	Functional	13	0.78	0.73	55	Olano-Marin *et al.* 2010
CcaTgu7	2	Functional	5	0.72	0.59	55	Olano-Marin *et al.* 2010

Table S2 Basic statistics (mean ± S.E. and range) for the different phenotypic traits analysed in the present study.

Trait	Mean ± S.E.	Range
Repertoire size	4.13 ± 0.35	1.00-10.00
Bout length	74.23 ± 9.97	12.96-307.03
Yellow breast brightness	655.45 ± 17.66	432.85-827.66
Yellow breast carotenoid chroma	0.84 ± 0.03	0.50-1.31
Blue crown brightness	615.43 ± 20.86	350.08-798.95
Blue crown UV-chroma	0.24 ± 0.01	0.23-0.24
Wing length	62.29 ± 0.22	59.00-66.00
Body mass	9.43 ± 0.07	8.22-10.16

Table S3 Model selection to assess the association of the studied phenotypic traits [(a) repertoire size, (b) bout length, (c) yellow breast yellow brightness, (d) yellow breast yellow carotenoid chroma, (e) blue crown brightness, (f) blue crown UV-chroma, (g) wing length, and (h) body mass] with genetic [A: heterozygosity estimated at the subset of neutral loci, $HL_{Neutral}$; B: heterozygosity estimated at the subset of functional loci, $HL_{Functional}$] and non-genetic terms [C: study plot; D: age; E: condition; F: recording date; G: singing time; H: wing length]. The genetic and non-genetic terms tested for each dependent variable are indicated in brackets. Only best ranked equivalent models with $\Delta AICc \leq 2$ are shown.

Model no.	Model	*K*	AIC_c	ΔAIC_c	ωi
(a) *Repertoire size* [A, B, C, D, E, F]					
1	C	3	170.21	0.00	0.12
2	C+E	4	170.69	0.48	0.10
3	A+C+E	5	171.13	0.92	0.08
4	B+C	4	171.18	0.97	0.08
5	A+C	4	171.33	1.12	0.07
6	C+F	4	171.45	1.24	0.07
7	C+E+F	5	172.07	1.86	0.05
(b) *Bout length* [A, B, C, D, E, F, G]					
1	B+C+E+F	6	427.57	0.00	0.25
(c) *Yellow breast brightness* [A, B, C, D, E]					
1	B	3	431.82	0.00	0.27
2	B+D	4	433.02	1.20	0.15
3	B+E	4	433.69	1.87	0.11
(d) *Yellow breast carotenoid chroma* [A, B, C, D, E]					
1	B	3	-10.33	0.00	0.10
2	D+E	4	-10.22	0.10	0.10
3	E	3	-9.63	0.70	0.07
4	D	3	-9.35	0.97	0.06
5	A+B	4	-9.10	1.23	0.06
6	B+D	4	-8.92	1.41	0.05
7	B+E	4	-8.76	1.57	0.05
8	C+D+E	5	-8.59	1.74	0.04
9	C	3	-8.48	1.85	0.04
(e) *Blue crown brightness* [A, B, C, D, E]					
1	B+E	4	356.94	0.00	0.22
2	B	3	357.73	0.79	0.15
(f) *Blue crown UV-chroma* [A, B, C, D, E]					
1	B+E	4	-258.72	0.00	0.25
2	E	3	-257.77	0.94	0.15
3	B+D+E	5	-256.99	1.72	0.10
(g) *Wing length* [A, B, C, D]					
1	A+B+D	5	180.89	0.00	0.52
2	B+D	4	182.52	1.63	0.23
(h) *Body mass* [A, B, C, D, H]					
1	B+H	4	65.24	0.00	0.29
2	A+B+H	5	66.43	1.18	0.16
3	B+C+H	5	67.06	1.82	0.12

K, number of parameters in the model; AIC_c, corrected Akaike's information criterion (AIC) value; ΔAIC_c, difference in AIC_c value from that of the strongest model; ωi, AIC_c weight.

Table S4 Model selection to assess the association of the studied phenotypic traits [(a) repertoire size, (b) bout length, (c) yellow breast brightness, (d) yellow breast carotenoid chroma, (e) blue crown brightness, (f) blue crown UV-chroma, (g) wing length, and (h) body mass] with genetic [A: heterozygosity estimated at all loci, HL_{Total}] and non-genetic terms [B: study plot; C: age; D: condition; E: recording date; F: singing time; G: wing length]. The genetic and non-genetic terms tested for each dependent variable are indicated in brackets. Only best ranked equivalent models ($\Delta AICc \leq 2$) are shown.

Model no.	Model	*K*	AIC_c	ΔAIC_c	ωi
(a) *Repertoire size* [A, B, C, D, E]					
1	B	3	170.21	0.00	0.15
2	A+B	4	170.47	0.26	0.13
3	B+D	4	170.69	0.48	0.12
4	A+B+D	5	171.00	0.79	0.10
5	B+E	4	171.45	1.24	0.08
6	A+B+E	5	171.67	1.47	0.07
7	B+D+E	5	172.07	1.86	0.06
(b) *Bout length* [A, B, C, D, E, F]					
1	B+D+E	5	431.15	0.00	0.14
2	D+E	4	431.28	0.14	0.13
3	A+B+D+E	6	431.44	0.29	0.12
4	C+D+E	5	432.04	0.89	0.09
5	B+C+D+E	6	432.58	1.43	0.07
6	A+D+E	5	433.15	2.00	0.05
(c) *Yellow breast brightness* [A, B, C, D]					
1	C	3	442.07	0.00	0.21
2	A	3	443.13	1.06	0.12
3	B	3	443.16	1.09	0.12
4	D	3	443.46	1.39	0.10
5	A+C	4	444.06	2.00	0.08
(d) *Yellow breast carotenoid chroma* [A, B, C, D]					
1	C+D	4	-10.22	0.00	0.19
2	D	3	-9.63	0.59	0.14
3	C	3	-9.35	0.87	0.12
4	B+C+D	5	-8.59	1.63	0.08
5	B	3	-8.48	1.74	0.08
6	B+C	4	-8.24	1.98	0.07
(e) *Blue crown brightness* [A, B, C, D]					
1	A	3	359.06	0.00	0.21
2	A+D	4	359.41	0.35	0.17
3	A+C	4	360.96	1.90	0.08
4	C	3	361.03	1.97	0.08
5	B	3	361.04	1.99	0.08
(f) *Blue crown UV-chroma* [A, B, C, D]					
1	D	3	-257.77	0.00	0.26
2	A+D	4	-257.68	0.10	0.24
3	A+C+D	5	-256.01	1.76	0.11
4	C+D	4	-255.83	1.94	0.10
(g) *Wing length* [A, B, C]					
2	C	3	-189.75	0.00	0.48
(h) *Body mass* [A, B, C, G]					
1	A+B	3	71.51	0.00	0.19
2	C	3	72.2	0.69	0.14
3	A+G	4	72.83	1.32	0.10
4	C+G	4	72.97	1.47	0.09
5	A	3	73.00	1.50	0.09

K, number of parameters in the model; AIC_c, corrected Akaike's information criterion (AIC) value; ΔAIC_c, difference in AIC_c value from that of the strongest model; ωi, AIC_c weight.

Table S5 General linear models (GLMs) for (a) repertoire size, (b) bout length, (c) yellow breast brightness, (d) yellow breast carotenoid chroma, (e) blue crown brightness, (f) blue crown UV-chroma, (g) wing length, and (h) body mass. A single model with ΔAICc ≤ 2 was obtained for wing length. For the rest of the studied variables we performed model averaging of the best ranked equivalent models (ΔAICc ≤ 2) to obtain parameter estimates and unconditional standard errors (USE) (see Table S4). Variables are sorted according with their relative importance based on the sum of Akaike weights (Σ ωi) of those models with ΔAICc ≤ 2 in which the variable was present. Bold type indicates significant variables, i.e. variables for which their unconditional 95 % confidence interval (CI) did not cross zero.

	Estimate ± USE	Σ ωi	Lower 95% CI	Upper 95% CI
(a) *Repertoire size*				
Study plot	-2.29 ± 0.79	0.71	**-3.84**	**-0.73**
HL_{Total}	-5.10 ± 3.48	0.30	-11.91	1.71
Body condition	-0.94 ± 0.68	0.28	-2.27	0.39
Recording date	-0.03 ± 0.03	0.21	-0.09	0.03
(b) *Bout length*				
Recording date	2.29 ± 0.89	0.60	**0.54**	**4.04**
Body condition	33.57 ± 19.35	0.60	-4.35	71.48
Study plot	35.34 ± 21.17	0.33	-6.16	76.83
HL_{Total}	127.87 ± 102.42	0.17	-72.86	328.60
Age	-24.77 ± 20.30	0.16	-64.57	15.02
(c) *Yellow breast brightness*				
Age	43.21 ± 35.19	0.29	-25.75	112.18
HL_{Total}	129.06 ± 184.26	0.20	-232.09	490.21
Study plot	-28.80 ± 42.81	0.12	-112.71	55.11
Body condition	-15.20 ± 37.47	0.10	-88.64	58.23
(d) *Yellow breast carotenoid chroma*				
Age	-0.11 ± 0.07	0.46	-0.24	0.02
Body condition	0.12 ± 0.07	0.23	-0.02	0.25
Study plot	-0.09 ± 0.08	0.23	-0.24	0.07
(e) *Blue crown brightness*				
HL_{Total}	390.07 ± 215.84	0.46	-32.97	813.11
Body condition	-74.46 ± 50.15	0.17	-172.76	23.84
Age	38.57 ± 41.60	0.08	-42.97	120.12
Study plot	-51.43 ± 51.51	0.08	-152.38	49.52
(f) *Blue crown UV-chroma*				
Body condition	0.01 ±0.01	0.71	**0.01**	**0.01**
HL_{Total}	-0.01 ± 0.01	0.35	-0.02	0.01
Age	0.01 ±0.01	0.21	-0.01	0.01
(g) *Wing length*				
Age	0.84 ± 0.44	0.48	-0.02	1.69
(h) *Body mass*				
HL_{Total}	0.75 ± 0.75	0.38	-0.72	2.21
Age	0.16 ± 0.14	0.23	-0.12	0.44
Wing length	0.07 ± 0.04	0.19	-0.02	0.15

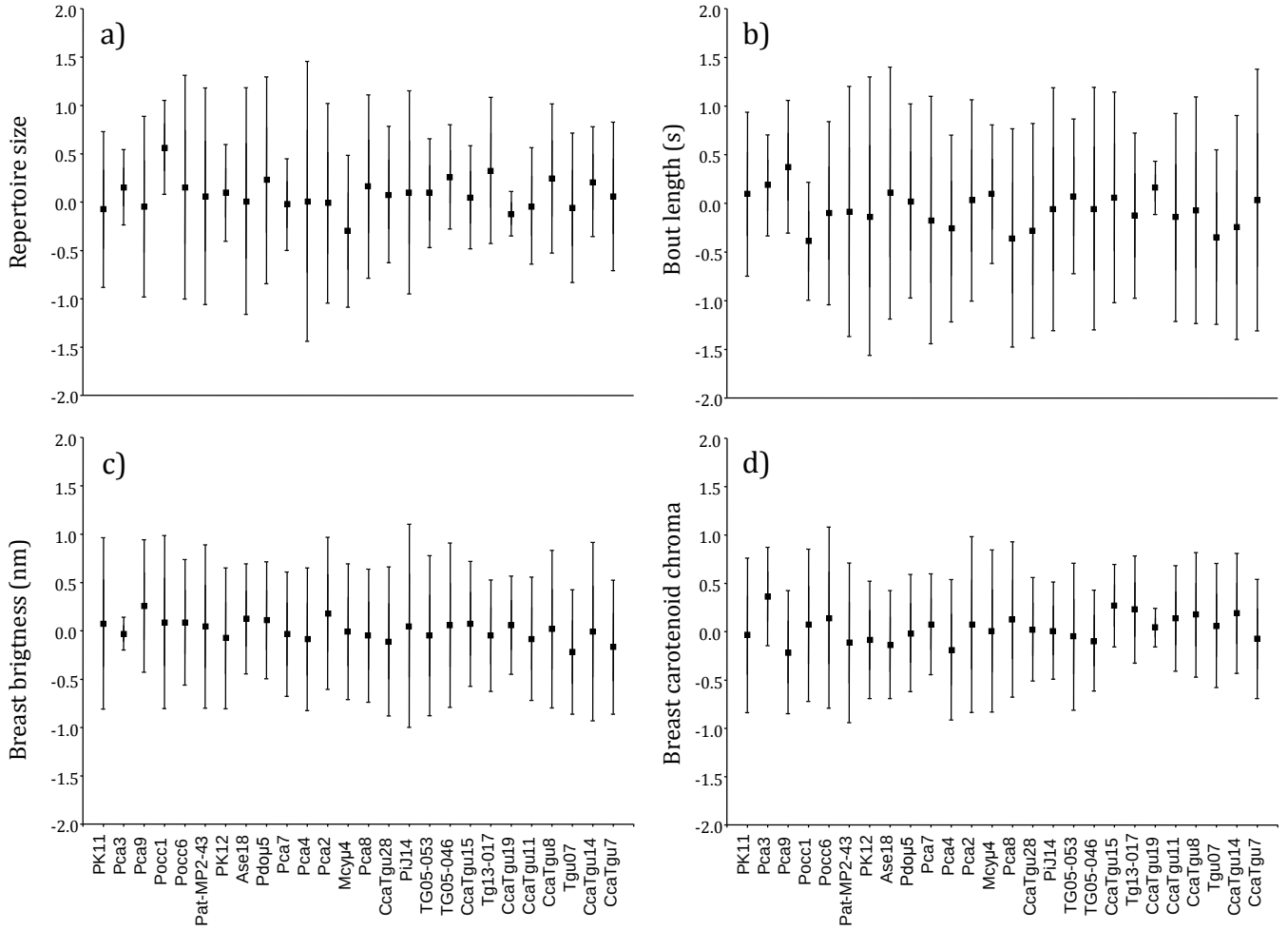
Table S6 Tests for the effects of single locus heterozygosity (SLH) on the studied traits. Table shows effect sizes and *P*-values. No test was significant after sequential Bonferroni correction.

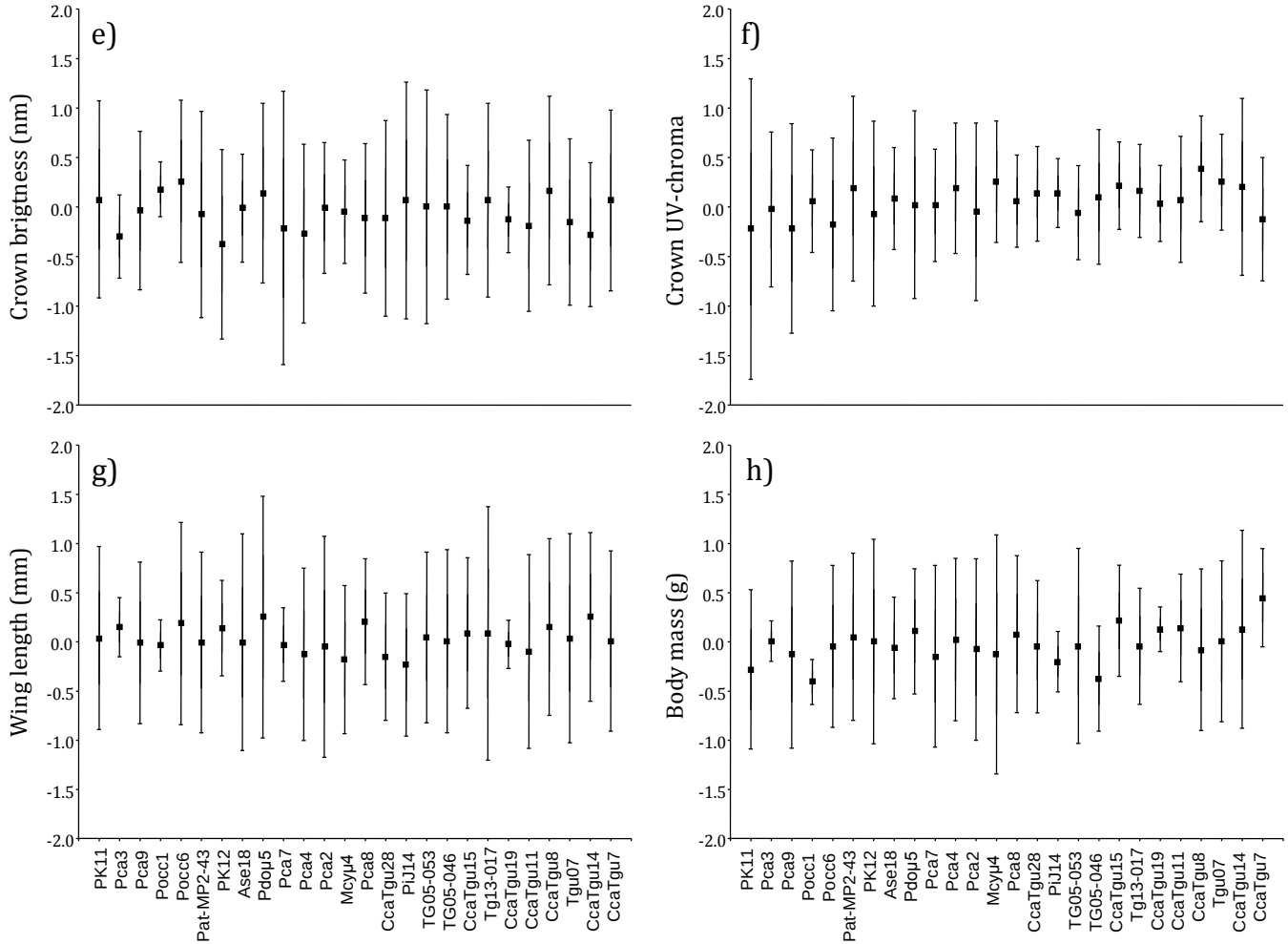
	Repertoire size			*Bout length*			*Yellow breast brightness*			*Yellow breast carotenoid chroma*		
Locus	**Effect size**	***F***	***P***	**Effect size**	***F***	***P***	**Effect size**	***F***	***P***	**Effect size**	***F***	***P***
PK11	0.052	0.09	0.758	-0.303	3.64	0.064	0.008	0.00	0.962	-0.293	3.20	0.082
Pca3	-0.220	1.83	0.184	0.027	0.02	0.874	0.091	0.28	0.600	0.011	0.01	0.951
Pca9	-0.061	0.13	0.726	-0.311	3.85	0.057	-0.030	0.03	0.863	-0.178	1.11	0.299
Pocc1	0.135	0.66	0.419	0.068	0.16	0.687	-0.024	0.02	0.887	-0.115	0.45	0.504
Pocc6	0.242	2.24	0.143	-0.134	0.42	0.424	0.168	0.99	0.326	-0.011	0.01	0.950
Pat-MP2-43	0.000	0.00	0.999	0.202	1.52	0.224	0.015	0.00	0.930	0.000	0.01	1.000
PK12	-0.453	9.28	0.004	-0.039	0.05	0.816	0.101	0.34	0.559	0.063	0.13	0.717
Ase18	-0.001	0.00	0.996	0.066	0.15	0.692	0.019	0.01	0.913	-0.082	0.23	0.634
Pdoμ5	0.139	0.70	0.406	0.072	0.18	0.667	0.360	5.07	0.031	0.113	0.43	0.513
Pca7	-0.235	2.09	0.156	0.063	0.14	0.708	-0.008	0.00	0.961	-0.149	0.76	0.387
Pca4	-0.253	2.46	0.125	0.208	1.62	0.210	-0.103	0.36	0.551	0.047	0.07	0.786
Pca2	0.001	0.00	0.997	-0.067	0.16	0.690	-0.002	0.00	0.991	-0.011	0.01	0.947
Mcyμ4	-0.079	0.22	0.638	0.337	4.60	0.039	-0.256	2.38	0.132	-0.136	0.64	0.427
Pca8	-0.157	0.90	0.347	0.081	0.24	0.627	0.174	1.06	0.309	0.103	0.36	0.551
CcaTgu28	-0.113	0.46	0.499	0.148	0.8	0.376	-0.134	0.62	0.435	-0.010	0.01	0.956
PiJ14	0.144	0.75	0.39	0.114	0.45	0.503	-0.217	1.68	0.203	-0.167	0.94	0.338
TG05-053	0.035	0.04	0.836	-0.018	0.01	0.913	-0.001	0.00	0.996	0.040	0.05	0.816
TG05-046	-0.004	0.00	0.979	0.037	0.04	0.827	0.026	0.02	0.881	-0.337	4.36	0.044
CcaTgu15	-0.157	0.90	0.347	0.220	1.83	0.185	0.057	0.11	0.743	0.260	2.47	0.125
Tg13-017	0.058	0.12	0.728	0.158	0.92	0.343	0.138	0.66	0.421	-0.033	0.03	0.849
CcaTgu19	-0.103	0.38	0.538	0.032	0.03	0.851	-0.023	0.01	0.893	0.089	0.27	0.605
CcaTgu11	-0.230	2.01	0.165	0.110	0.44	0.509	-0.089	0.27	0.605	0.148	0.76	0.388
CcaTgu8	0.166	1.02	0.319	0.435	8.38	0.006	0.161	0.90	0.348	-0.075	0.19	0.662
Tgu07	-0.157	0.90	0.347	0.253	2.45	0.126	0.004	0.00	0.981	0.022	0.01	0.898
CcaTgu14	-0.316	4.00	0.053	0.136	0.67	0.417	0.253	2.32	0.137	0.358	5.00	0.032
CcaTgu7	0.108	0.42	0.519	-0.173	1.11	0.299	-0.036	0.04	0.834	0.442	8.27	0.007

Continued

	Blue crown brightness			*Blue crown UV-Chroma*			*Wing length*			*Body mass*		
Locus	**Effect size**	***F***	***P***	**Effect size**	***F***	***P***	**Effect size**	***F***	***P***	**Effect size**	***F***	***P***
PK11	-0.080	0.17	0.679	0.123	0.40	0.532	0.092	0.41	0.523	-0.035	0.05	0.813
Pca3	0.087	0.20	0.654	0.147	0.57	0.454	-0.019	0.18	0.895	0.245	2.93	0.093
Pca9	-0.063	0.10	0.744	0.339	3.37	0.078	0.243	3.01	0.089	-0.204	2.00	0.164
Pocc1	0.454	7.01	0.013	-0.298	2.53	0.123	0.127	0.79	0.379	0.071	0.23	0.633
Pocc6	-0.107	0.31	0.582	-0.071	0.13	0.720	0.095	0.43	0.511	0.161	1.22	0.274
Pat-MP2-43	0.090	0.22	0.643	0.000	0.00	0.998	0.049	0.11	0.734	-0.109	0.55	0.461
PK12	0.062	0.10	0.750	-0.182	0.88	0.355	-0.072	0.25	0.617	-0.071	0.23	0.633
Ase18	0.037	0.03	0.848	0.113	0.33	0.565	0.107	0.56	0.457	-0.127	0.75	0.389
Pdoμ5	0.258	1.92	0.177	0.059	0.09	0.764	0.107	0.56	0.457	0.015	0.01	0.920
Pca7	-0.045	0.05	0.818	-0.235	1.51	0.229	0.009	0.00	0.948	0.105	0.50	0.479
Pca4	0.139	0.53	0.471	-0.266	1.97	0.172	-0.109	0.50	0.483	-0.197	1.85	0.180
Pca2	-0.009	0.01	0.965	0.110	0.31	0.578	0.211	2.24	0.141	0.041	0.07	0.783
Mcyμ4	-0.310	2.87	0.102	0.129	0.44	0.512	-0.006	0.00	0.966	0.007	0.01	0.961
Pca8	0.094	0.23	0.629	-0.486	8.04	0.009	-0.030	0.04	0.837	0.145	0.99	0.324
CcaTgu28	0.105	0.30	0.587	-0.308	2.72	0.111	-0.115	0.64	0.425	0.024	0.02	0.872
PiJ14	0.113	0.35	0.559	0.030	0.02	0.880	0.019	0.01	0.895	0.031	0.04	0.838
TG05-053	0.082	0.18	0.673	0.075	0.14	0.706	-0.001	0.00	0.997	-0.091	0.38	0.539
TG05-046	0.258	1.92	0.176	-0.048	0.05	0.810	0.083	0.33	0.564	-0.077	0.27	0.601
CcaTgu15	0.043	0.05	0.825	0.074	0.14	0.708	0.070	0.23	0.629	0.278	3.85	0.056
Tg13-017	0.300	2.67	0.113	-0.082	0.17	0.680	-0.072	0.25	0.617	0.184	1.62	0.209
CcaTgu19	-0.090	0.22	0.641	0.113	0.33	0.567	0.038	0.06	0.795	0.050	0.11	0.738
CcaTgu11	-0.053	0.07	0.784	-0.144	0.55	0.464	-0.055	0.14	0.704	0.141	0.93	0.338
CcaTgu8	0.281	2.30	0.140	-0.050	0.06	0.800	0.067	0.21	0.644	0.143	0.96	0.331
Tgu07	-0.035	0.03	0.858	-0.431	5.94	0.022	-0.239	2.91	0.094	0.048	0.10	0.745
CcaTgu14	0.234	1.56	0.222	-0.240	1.58	0.219	-0.001	0.00	0.997	0.227	2.49	0.121
CcaTgu7	0.089	0.21	0.646	-0.074	0.14	0.707	-0.163	1.31	0.257	-0.116	0.62	0.433

Figure S1 Effect sizes and 95% confidence intervals of single-locus heterozygosity (SLH) for a) repertoire size, b) bout length, c) yellow breast brightness, d) yellow breast carotenoid chroma, e) blue crown brightness, f) blue crown UV-chroma, g) wing length and h) body mass.




e)
f)
g)
h)
Crown brigtness (nm)
Crown UV-chroma
Wing length (mm)
Body mass (g)
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
PK11
Pca3
Pca9
Pocc1
Pocc6
Pat-MP2-43
PK12
Ase18
Pdoμ5
Pca7
Pca4
Pca2
Mcyμ4
Pca8
CcaTgu28
Pij14
TG05-053
TG05-046
CcaTgu15
Tg13-017
CcaTgu19
CcaTgu11
CcaTgu8
Tgu07
CcaTgu14
CcaTgu7

SUPPLEMENTARY REFERENCES

Bensch S, Price T, Kohn J. 1997. Isolation and characterization of microsatellite loci in a *Phylloscopus* warbler. *Mol. Ecol.* 6: 91-92.

Dawson DA, Chittock J, Jehle R, Whitlock A, Nogueira D, Pellatt J, Birkhead T, Burke T. 2005. Identification of 13 polymorphic microsatellite loci in the zebra finch, *Taeniopygia guttata* (Passeridae, Aves). *Mol. Ecol. Notes* 5: 298-301.

Dawson DA, Horsburgh GJ, Küpper C, Stewart IRK, Ball AD, Durrant KL, Hansson B, Bacon I, Bird S, Klein A, Krupa AP, Lee JW, Martín-Gálvez D, Simeoni M, Smith G, Spurgin LG, Burke T. 2010. New methods to identify conserved microsatellite loci and develop primer sets of high cross-species utility - as demonstrated for birds. *Mol. Ecol. Resour.* 10: 475-494.

Double MC, Dawson D, Burke T, Cockburn A. 1997. Finding the fathers in the least faithful bird: A microsatellite-based genotyping system for the superb fairy-wren *Malurus cyaneus*. *Mol. Ecol.* 6 :691-693.

Griffith SC, Stewart IRK, Dawson DA, Owens IPF, Burke T. 1999. Contrasting levels of extra-pair paternity in mainland and island populations of the house sparrow (*Passer domesticus*): is there an "island effect"? *Biol. J. Linn. Soc.* 68: 303-316.

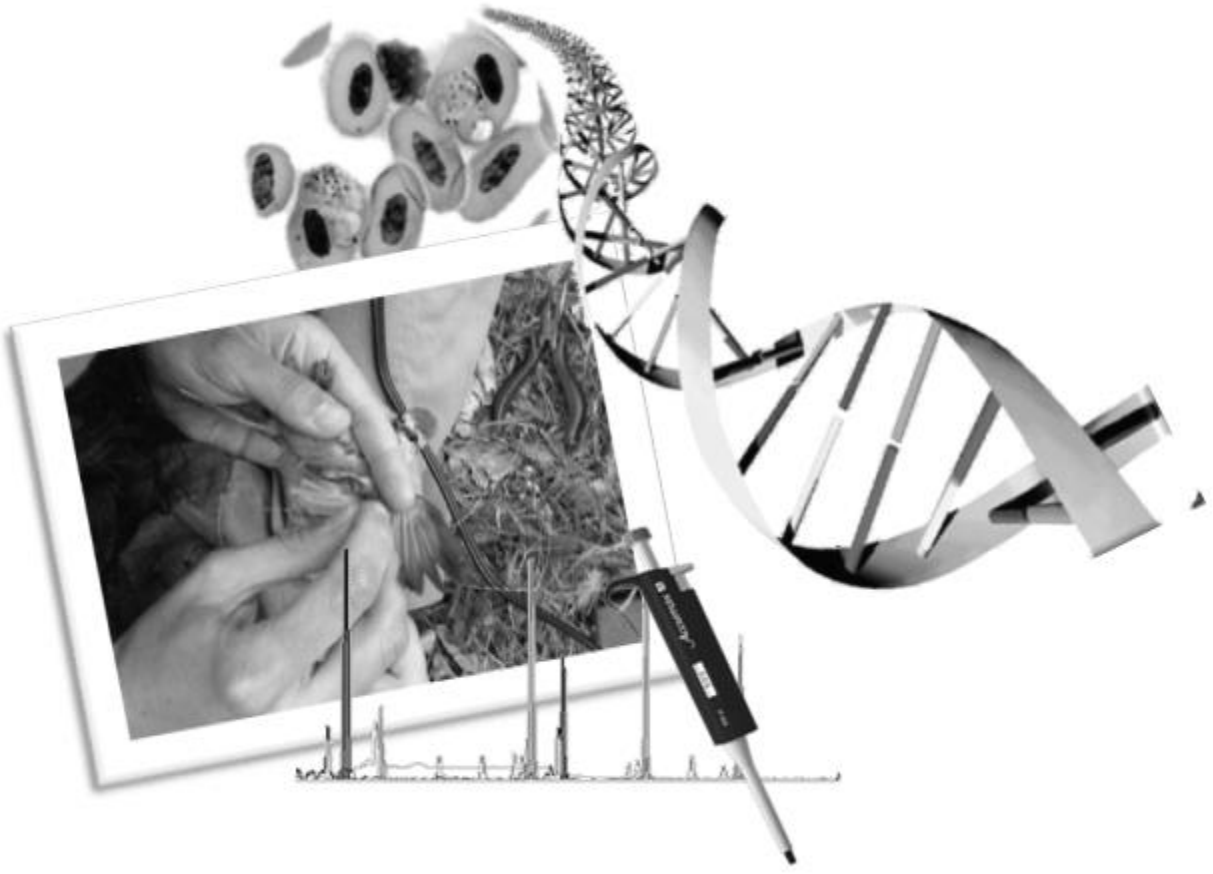
Olano-Marin J, Dawson DA, Girg A, Hansson B, Ljungqvist M, Kempenaers B, Mueller JC. 2010. A genome-wide set of 106 microsatellite markers for the blue tit (*Cyanistes caeruleus*). *Mol. Ecol. Resour.* 10: 516-532.

Otter K, Ratcliffe L, Michaud D, Boag PT. 1998. Do female black-capped chickadees prefer high-ranking males as extra-pair partners? *Behav. Ecol. Sociobiol.* 43: 25-36.

Richardson DS, Jury FL, Dawson DA, Salgueiro P, Komdeur J, Burke T. 2000. Fifty Seychelles warbler (*Acrocephalus sechellensis*) microsatellite loci polymorphic in Sylviidae species and their cross-species amplification in other passerine birds. *Mol. Ecol.* 9: 2226-2231.

Slate J, Hale M, Birkhead TR. 2007. Simple sequence repeats in zebra finch (*Taeniopygia guttata*) expressed sequence tags: a new resource for evolutionary genetic studies of passerines. *BMC Genom.* 8: 52.

Discusión integradora



Para comprender mejor los factores que determinan la diferenciación genética de las poblaciones es necesario estudiar la relación existente entre flujo genético, estructura demográfica y el componente geográfico y ambiental con el que interaccionan los individuos. El herrerillo común es un modelo ideal para estudiar cómo las particularidades del entorno y de la especie pueden influir en la diferenciación genética de las poblaciones, debido a que es un ave mayormente sedentaria que en nuestra área de estudio se distribuye en un paisaje muy heterogéneo, en el que sus hábitats se encuentran altamente fragmentados. El herrerillo común presenta estrategias de dispersión que difieren entre sexos (**Capítulo I**). Los machos muestran un marcado comportamiento filopátrico, con movimientos dispersivos muy limitados espacialmente que sugieren que las constricciones comportamentales en la búsqueda y defensa del territorio condicionan las distancias de dispersión. En cambio, las hembras exhiben unas distancias de dispersión mayores de lo esperado por azar dentro de las zonas natales. Estos patrones de dispersión se reflejan en una estructuración genética significativa en machos, pero no en hembras, sugiriendo que la fragmentación del hábitat podría tener un mayor impacto en hembras que en machos, ya que éstos muestran una baja dispersión incluso dentro de las zonas natales que presentan hábitats con distribución continua.

Considerar la composición y configuración del paisaje puede contribuir a mejorar notablemente nuestro conocimiento sobre los factores que modulan la estructuración genética de las poblaciones (McRae 2006; Koen *et al.* 2012; Wang *et al.* 2013; Pflueger & Balkenhol 2014). En este sentido, los modelos de resistencia mostraron que el modelo nulo (equivalente a la distancia geográfica) explicaba la diferenciación genética estimada con marcadores neutros y con el conjunto de marcadores neutros y funcionales, mientras que la diferenciación genética estimada con marcadores funcionales fue mejor explicada por el modelo que atribuía una mayor resistencia a la vegetación natural que a las zonas fundamentalmente constituidas por campos de cultivo (**Capítulo II**). Esto indica que la fragmentación del hábitat podría forzar a los individuos a dispersarse largas distancias atravesando hábitats desfavorables motivados por la búsqueda de un lugar óptimo para la reproducción (Wiens 2001; Harrison *et al.* 2013). La estructura genética estimada con el panel de marcadores neutros era más marcada que con el subconjunto de marcadores funcionales, diferencias que podrían ser debidas a las distintas presiones evolutivas a las que son sometidos ambos grupos de marcadores. Los loci funcionales podrían estar sujetos a selección estabilizadora, lo que explicaría el menor nivel de diferenciación genética estimada con este grupo de marcadores. Finalmente, no se encontraron diferencias genéticas entre poblaciones debidas al tipo de hábitat o a la altitud. A diferencia de lo descrito en estudios realizados en las poblaciones de herrerillo de Córcega, el tipo de hábitat parece no haber originado

adaptaciones locales que hayan contribuido a la diferenciación de nuestras poblaciones (Porlier *et al.* 2012). En este mismo sentido, la altitud tampoco explicaba los patrones de diferenciación genética, de modo que las diferencias fenológicas observadas en poblaciones localizadas en distintos rangos altitudinales podrían ser atribuibles a la capacidad plástica de la especie en lugar de tener una base genética como resultado de procesos de adaptación local. No obstante, no podemos descartar el aislamiento por ambiente (IBE) entre nuestras poblaciones ya que únicamente analizamos el tipo de hábitat (Porlier *et al.* 2012) y la altitud (Thomassen *et al.* 2010) y otros factores, como diferencias en parasitismo o en la disponibilidad de diferentes nutrientes, podrían generar diferentes presiones selectivas que potencialmente provocasen adaptaciones locales y contribuyeran a reducir el flujo genético entre las poblaciones (Garroway *et al.* 2013).

La caracterización genética de los parásitos de malaria aviar de las poblaciones de estudio reveló la existencia de un total de seis linajes, uno de los cuales no había sido descrito previamente para ninguna especie de ave y dos que se registraron por primera vez parasitando al herrerillo común (**Capítulo III**). Sin embargo, un solo linaje (SGS1) de la morfoespecie *Plasmodium relictum* fue el que estaba involucrado en la gran mayoría de las infecciones detectadas. Por otro lado, encontramos notables diferencias de prevalencia entre las distintas poblaciones analizadas y que son comparables a las observadas en un estudio realizado a escala europea para la misma especie (Szöllosi *et al.* 2011). Diferencias entre las distintas zonas de estudio en la abundancia de hábitats óptimos para el desarrollo de los vectores transmisores y/o en la composición de la comunidad de otros hospedadores podrían haber originado diferencias en las tasas de transmisión del parásito y en los niveles de prevalencia entre poblaciones. Además, estas diferencias en las prevalencias de malaria aviar también podrían mantenerse como consecuencia de la baja capacidad de dispersión del herrerillo común (**Capítulo I**), algo que provocaría que los niveles de prevalencia reflejasen fielmente las tasas locales de transmisión del parásito. Los parásitos de malaria aviar pueden modular de manera importante la diversidad genética del hospedador (**Capítulo IV**). En concreto se observó que los individuos con niveles de heterocigosidad intermedia estimada con marcadores neutros presentaron una mayor probabilidad de ser parasitados por el linaje SGS1 de *P. relictum*. No se encontraron efectos locales significativos, lo que indica que esta asociación es posiblemente debida a un efecto general. La probabilidad de ser parasitado se espera que esté influenciada por múltiples procesos fisiológicos lo que podría explicar que este tipo de relaciones sea detectada principalmente con marcadores neutros que parecen reflejar mejor la heterocigosidad del conjunto del genoma (Olano-Marin *et al.* 2011a, b). Relaciones cuadráticas similares entre parasitismo y diversidad genética han sido descritas en otros sistemas de estudio en los que no se detectaron tampoco efectos locales significativos, pero en los que se ha

sugerido que la combinación de pequeños efectos locales positivos y negativos podrían explicar estas relaciones no lineales entre parasitismo y heterocigosidad (Blanchet *et al.* 2009; Ruiz-López *et al.* 2012). Alternativamente, la purga genética de alelos deletéreos recesivos en individuos muy homocigotos (Dudash *et al.* 1997) podría también estar detrás de estas relaciones cuadráticas (Neff 2004; Küpper *et al.* 2010). Es también destacable que estos resultados fueron similares en las tres zonas de estudio, lo que indica que las poblaciones de herrerillo común responden de manera semejante a las enfermedades infeccionas en diferentes contextos espaciales y con diferentes presiones de parasitismo (**Capítulo III**).

Las diferencias espacio-temporales en las condiciones ambientales pueden influir en la detección de las relaciones entre diversidad genética y eficacia biológica (Lesbarreres *et al.* 2005; Da Silva *et al.* 2006; Annavi *et al.* 2014) y explicar los diferentes resultados obtenidos en estudios realizados en distintas poblaciones de la misma especie e incluso entre años en una misma población (Chapman *et al.* 2009). Aunque en el **Capítulo IV** no se encontraron diferencias en estas relaciones entre poblaciones sometidas a distintos niveles de presión de parasitismo, el **Capítulo V** muestra que las condiciones meteorológicas específicas de cada año sí que pueden influir de modo notable en la relación entre heterocigosidad y probabilidad de supervivencia interanual. Se observó que la intensidad de la selección en contra de individuos homocigotos era mayor en años con una gran abundancia de precipitaciones. Esta abundancia de precipitaciones podría influir en la condición física y poner en peligro la viabilidad de los individuos en la medida en que provoca un aumento de los requerimientos energéticos necesarios para la termorregulación, reduce la maniobrabilidad en el vuelo (Wilson *et al.* 2004) y dificulta notablemente la búsqueda de alimento por la menor actividad de las presas durante días lluviosos (Arlettaz *et al.* 2010). Cuando las condiciones ambientales son desfavorables (alta precipitación), los individuos más heterocigotos presentan mayores probabilidades de supervivencia posiblemente debido a su mayor capacidad para hacer frente a esas situaciones estresantes. Sin embargo, cuando las condiciones son más favorables (menor precipitación) los individuos inmigrantes más heterocigotos (García-Navas *et al.* 2014) o aquellos exogámicos resultantes de cruces entre inmigrantes y locales parecen mostrar una menor eficacia biológica, algo que podría ser debido a su desventaja competitiva y/o menor adaptación a las condiciones locales en comparación con los individuos residentes (Dias & Blondel 1996; Hansson *et al.* 2004). Aunque la dirección de la asociación entre supervivencia y heterocigosidad era dependiente del contexto ambiental, la selección en contra de los individuos homocigotos era predominante en la mayoría de los años. Este proceso, junto con el moderado grado de heredabilidad de la heterocigosidad (García-Navas *et al.* 2009), parece haber contribuido al incremento de la diversidad genética de la población a lo largo de los

seis años de estudio, sugiriendo un cambio micro-evolutivo en respuesta a la selección (Kaeuffer *et al.* 2007; Forcada & Hoffman 2014).

Aunque el contexto ambiental es importante, generalmente existe una relación positiva entre heterocigosidad y eficacia biológica (Kempenaers 2007; Olano-Marin *et al.* 2011a, b). Por lo tanto, los individuos más heterocigotos deberían ser seleccionados como pareja debido a que en promedio mostrarán una mayor calidad y eficacia biológica que los más homocigotos (Kempenaers 2007). En este sentido, el **Capítulo VI** mostró una relación positiva entre el desarrollo de múltiples ornamentos sexuales secundarios y la diversidad genética en machos. Los individuos más heterocigotos desarrollaban señales sexuales conspicuas (plumaje y canto), algo que podría ser debido a que genes directamente implicados en su desarrollo presentan sobredominancia o una cierta frecuencia de alelos deletéreos recesivos que son expresados en homocigosis (Charlesworth & Charlesworth 1987; Falconer & Mackay 1996). Alternativamente, los individuos más heterocigotos podrían mostrar una mejor respuesta fisiológica al estrés o inmunitaria frente a enfermedades y parásitos que les confiriese una mayor capacidad para destinar recursos a otros procesos como el desarrollo de caracteres sexuales secundarios (Van Oosterhout *et al.* 2003). En su conjunto, estos resultados sugieren que la diversidad genética es reflejada a través de múltiples rasgos sexuales, lo que permitiría incrementar la fiabilidad de las señales y reducir la energía y el tiempo empleados por las hembras para evaluar la calidad genética de sus potenciales parejas (Candolín 2003).

REFERENCIAS

Annavi G, Newman C, Buesching CD*, et al.* 2014. Heterozygosity-fitness correlations in a wild mammal population: accounting for parental and environmental effects. *Ecol. Evol.* 4: 2594-2609.

Arlettaz R, Schaad M, Reichlin TS, Schaub M. 2010. Impact of weather and climate variation on hoopoe reproductive ecology and population growth. *J. Ornithol.* 151: 889-899

Blanchet S, Rey O, Berthier P, Lek S, Loot G. 2009. Evidence of parasite-mediated disruptive selection on genetic diversity in a wild fish population. *Mol. Ecol.* 18: 1112-1123.

Candolin U. 2003. The use of multiple cues in mate choice. *Biol. Rev.* 78: 575-595.

Chapman JR, Nakagawa S, Coltman DW, Slate J, Sheldon BC. 2009. A quantitative review of heterozygosity-fitness correlations in animal populations. *Mol. Ecol.* 18: 2746-2765.

Charlesworth D, Charlesworth B. 1987. Inbreeding depression and its evolutionary consequences. *Annu. Rev. Ecol. Evol. Syst.* 18**:** 237-268.

Da Silva A, Luikart G, Yoccoz NG, Cohas A, Allaine D. 2006. Genetic diversity-

fitness correlation revealed by microsatellite analyses in European alpine marmots (*Marmota marmota*). *Conserv. Genet.* 7: 371-382.

Dias PC, Blondel J. 1996. Local specialization and maladaptation in the Mediterranean blue tit (*Parus caeruleus*). *Oecologia* 107: 79-86.

Dudash MR, Carr DE, Fenster CB. 1997. Five generations of enforced selfing and outcrossing in *Mimulus guttatus*: Inbreeding depression variation at the population and family level. *Evolution* 51: 54-65.

Falconer DS, Mackay TFC. 1996. *Introduction to quantitative genetics*, 3rd edn. Essex: Longman.

Forcada J, Hoffman JI. 2014. Climate change selects for heterozygosity in a declining fur seal population. *Nature* 511: 462-465.

García-Navas V, Ferrer ES, Sanz JJ, Ortego J. 2014. The role of immigration and local adaptation on fine-scale genotypic and phenotypic population divergence in a less mobile passerine. *J. Evol. Biol.* 27: 1590-1603.

García-Navas V, Ortego J, Sanz JJ. 2009. Heterozygosity-based assortative mating in blue tits (*Cyanistes caeruleus*): implications for the evolution of mate choice. *Proc. R. Soc. B-Biol. Sci.* 276: 2931-2940.

Garroway CJ, Radersma R, Sepil I, Santure AW, De Cauwer I, Slate J, Sheldon BC. 2013. Fine-scale genetic structure in a wild bird population: The role of limited dispersal and environmentally based selection as causal factors. *Evolution* 67: 3488-3500.

Hansson B, Bensch S, Hasselquist D. 2004. Lifetime fitness of short- and long-distance dispersing great reed warblers. *Evolution* 58: 2546-2557.

Harrisson KA, Pavlova A, Amos JN, Takeuchi N, Lill A, Radford JQ, Sunnucks P. 2013. Disrupted fine-scale population processes in fragmented landscapes despite large-scale genetic connectivity for a widespread and common cooperative breeder: the superb fairy-wren (*Malurus cyaneus). J. Anim. Ecol.* 82: 322-333.

Kaeuffer R, Coltman DW, Chapuis J-L, Pontier D, Reale D. 2007. Unexpected heterozygosity in an island mouflon population founded by a single pair of individuals. *Proc. R. Soc. B-Biol. Sci.* 274: 527-533.

Kempenaers B. 2007. Mate choice and genetic quality: A review of the heterozygosity theory. *Adv. Study Behav.* 37: 189-278.

Koen EI, Bowman J, Walpole AA. 2012. The effect of cost surface parameterization on landscape resistance estimates. *Mol. Ecol.* 12:686-696.

Küpper C, Kosztolanyi A, Augustin J, Dawson DA, Burke T, Szekely T. 2010. Heterozygosity-fitness correlations of conserved microsatellite markers in Kentish plovers *Charadrius alexandrinus*. *Mol. Ecol.* 19: 5172-5185

Lesbarreres D, Primmer CR, Laurila A, Merila J. 2005. Environmental and population dependency of genetic variability-fitness correlations in *Rana temporaria*. *Mol. Ecol.* 14: 311-323.

McRae BH. 2006. Isolation by resistance. *Evolution* 60: 1551-1561.

Neff BD. 2004. Stabilizing selection on genomic divergence in a wild fish population. *Proc. Natl. Acad. Sci. U. S. A.* 101: 2381-2385.

Olano-Marin J, Mueller JC, Kempenaers B. 2011a. Correlations between heterozygosity and reproductive success in the blue tit (*Cyanistes caeruleus*): an analysis of inbreeding and single locus effects. *Evolution* 65: 3175-3194.

Olano-Marin J, Mueller JC, Kempenaers B. 2011b. Heterozygosity and survival in blue tits (*Cyanistes caeruleus*): contrasting effects of presumably functional and neutral loci. *Mol. Ecol.* 20: 4028-4041.

Pflueger FJ, Balkenhol N. 2014. A plea for simultaneously considering matrix quality and local environmental conditions when analysing landscape impacts on effective dispersal. *Mol. Ecol.* 23: 2146-2156.

Porlier M, Garant D, Perret P, Charmantier A. 2012. Habitat-linked population genetic differentiation in the blue tit *Cyanistes caeruleus*. *J. Hered.* 103: 781-791.

Ruiz-López MJ, Monello RJ, Gompper ME, Eggert LS. 2012. The effect and relative importance of neutral genetic diversity for predicting parasitism varies across parasite taxa. *PLoS One* 7: e45404.

Szöllösi E, Cichon M, Eens M *et al.* 2011. Determinants of distribution and prevalence of avian malaria in blue tit populations across Europe: separating host and parasite effects. *J. Evol. Biol.* 24: 2014-2024

Thomassen HA, Cheviron ZA, Freedman AH, Harrigan RJ, Wayne RK, Smith TB. 2010. Spatial modelling and landscape-level approaches for visualizing intra-specific variation. *Mol. Ecol.* 19: 3532-3548.

van Oosterhout C, Trigg RE, Carvalho GR, *et al.* 2003. Inbreeding depression and genetic load of sexually selected traits: how the guppy lost its spots. *J. Evol. Biol.* 16: 273-281.

Wang IJ, Glor RE, Losos JB. 2013. Quantifying the roles of ecology and geography in spatial genetic divergence. *Ecol. Lett.* 16: 175-182.

Wiens JA. 2001. The landscape context of dispersal. Pp. 96-99 *in* Clobert J, Danchin E, Dhondt AA, Nichols JD (eds). *Dispersal*. New York, NY: Oxford University Press.

Wilson GR, Cooper SJ, Gessaman JA. 2004. The effects of temperature and artificial rain on the metabolism of American kestrels (*Falco sparverius*). *Comp. Biochem. Phys. A*. 139: 389-394.

Conclusiones

1. El herrerillo común muestra diferencias entre sexos en las distancias de dispersión. Las menores distancias de dispersión en machos se ven reflejadas en una mayor estructura y diferenciación genética en este sexo en comparación con las hembras.

2. Las poblaciones estudiadas presentan cierto grado de estructuración genética y ésta es reflejada de modo más patente por marcadores neutros que funcionales. Las poblaciones no muestran aislamiento genético en base a los dos componentes ambientales analizados (hábitat y altitud). Los análisis de aislamiento por resistencia indican que la fragmentación del hábitat puede forzar a los individuos a atravesar hábitats desfavorables para alcanzar un lugar óptimo de reproducción, algo que podría ser en parte responsable de la marcada mezcla genética observada en el conjunto de las poblaciones de estudio.

3. Las poblaciones estudiadas están parasitadas por un total de seis linajes (SGS1, GRW11, COLL1, DELURB4, GRW04 y BLUTI10) de malaria aviar pertenecientes al género *Plasmodium*. El linaje SGS1 estuvo involucrado en la mayor parte de las infecciones detectadas y el linaje BLUTI10 no había sido aislado en ninguna especie de ave hasta el momento. Se observaron diferencias significativas en la prevalencia del linaje SGS1 entre las distintas poblaciones que podrían explicarse por diferencias en la abundancia de vectores y la baja dispersión de los individuos.

4. Se observó una relación cuadrática entre la probabilidad de infección por malaria aviar (linaje SGS1) y heterocigosidad estimada con marcadores neutros, de modo que individuos con niveles intermedios de diversidad genética presentan mayores probabilidades de estar parasitados. La ausencia de efectos locales sugiere que esta asociación es consecuencia de la heterocigosidad del conjunto del genoma. Esta relación fue similar entre poblaciones que presentaban diferentes presiones de parasitismo.

5. Existe una relación entre la probabilidad de supervivencia interanual y la diversidad genética individual que parece ser explicada por la heterocigosidad del conjunto del genoma y variaba en magnitud e intensidad entre años. La intensidad de la relación entre supervivencia y heterocigosidad está positivamente relacionada con la precipitación anual, lo que indica la importancia del contexto ambiental para explicar las relaciones entre diversidad genética y eficacia biológica. Finalmente, se ha observado un aumento de la heterocigosidad a lo largo de los años que podría ser consecuencia de la selección generalizada en contra de individuos homocigotos y la moderada heredabilidad de la heterocigosidad.

6. Los machos de herrerillo más heterocigotos presentan píleos más brillantes, pechos con altos índices de carotenoide-croma, cantos de mayor duración y una mejor condición física. Estas asociaciones fueron detectadas únicamente con el grupo de marcadores funcionales y parecen reflejar un efecto general del genoma, lo que sugiere que regiones funcionales podrían estar mayormente implicadas en la expresión de caracteres sexuales secundarios. Estos resultados indican que los ornamentos pueden ser utilizados por las hembras para evaluar de forma rápida y fiable la calidad genética de sus potenciales parejas.

Resúmenes

RESÚMENES

Capítulo I- La estructura genética refleja los movimientos de dispersión natal a diferentes escalas espaciales en el herrerillo común, *Cyanistes caeruleus*

El estudio de las consecuencias genéticas de la dispersión es un tema central en las investigaciones sobre comportamiento, conservación y evolución. Sin embargo, pocos estudios han considerado de forma simultánea los movimientos de dispersión de individuos marcados y los patrones de flujo genético. En el presente trabajo se analizó la relación entre el comportamiento de dispersión y el flujo genético en cuatro poblaciones de herrerillo común con diferente grado de conectividad. Para este propósito, se monitorizaron las poblaciones establecidas en cuatro parches de reproducción y se utilizaron datos genotípicos y de captura-marcaje-recaptura para estudiar las consecuencias genéticas de la dispersión a diferentes escalas espaciales. Los datos de los movimientos de dispersión natal revelaron que tanto los machos como las hembras se dispersan menos de lo esperado por azar en las dos escalas espaciales mayores consideradas, que incluían todo el área de estudio y las dos principales zonas. Sin embargo, la distancia de dispersión natal fue menor de lo esperado por azar dentro del parche natal para los machos, mientras que un patrón opuesto fue encontrado para las hembras. Los marcadores microsatélites indicaron un flujo genético limitado entre las localidades estudiadas y un patrón de aislamiento por distancia que fue particularmente fuerte a una escala espacial amplia considerando parches de reproducción geográficamente distantes. Por último, la fuerte filopatría de los machos fue reflejada en una mayor estructura genética y un menor grado de mezcla genética en este sexo. En conjunto, nuestro estudio apunta a que tanto la fragmentación como la dispersión limitada pueden haber contribuido a reducir el flujo genético entre poblaciones a diferentes escalas espaciales y demuestra una concordancia entre los patrones inferidos con datos genéticos y mediante estudios de captura-marcaje-recaptura.

Capítulo II- Influencia de la configuración del paisaje y el ambiente en la estructura genética poblacional de un paseriforme sedentario: nuevas perspectivas aportadas por el uso de loci putativamente funcionales y neutros

El estudio de los factores que estructuran la variación genética puede ayudar a inferir los procesos adaptativos y neutrales que determinan las trayectorias demográficas y evolutivas de las poblaciones naturales. En este trabajo se analiza el papel del aislamiento por distancia (IBD), el aislamiento por resistencia (IBR, definido por la composición del paisaje), y el aislamiento por ambiente (IBE, estimado como las diferencias de hábitat y altitud) en la estructuración de la variación genética de 25 poblaciones de herrerillo común (*Cyanistes caeruleus*). Genotipamos 1385 individuos con dos subconjuntos de loci, clasificados como neutros (14 loci) y supuestamente funcionales (12 loci) en función de si la región genómica donde se localizan está siendo activamente transcrita a ARN. La diferenciación genética de las poblaciones fue detectada principalmente mediante el panel de marcadores neutros. Los análisis genéticos del paisaje mostraron un patrón de IBD para todos los loci y el panel de

marcadores neutros, pero la diferenciación genética estimada en loci funcionales fue sólo explicada por modelos IBR que asignaban una alta resistencia a la vegetación natural y una baja resistencia a las tierras agrícolas. Por último, la ausencia de IBE sugiere una ausencia de presiones de selección divergentes asociadas a las diferencias de hábitat y altitud. En conjunto, este estudio muestra que marcadores ubicados en diferentes regiones del genoma pueden ofrecer diferentes perspectivas sobre los patrones del flujo genético a escala de paisaje en poblaciones naturales.

Capítulo III- Caracterización molecular de parásitos de malaria aviar en tres poblaciones Mediterráneas de herrerillo común (*Cyanistes caeruleus*)

En este trabajo caracterizamos genéticamente parásitos de malaria (Protozoa) en tres poblaciones mediterráneas de herrerillo común (*Cyanistes caeruleus*) en el centro de España. Un total de 853 individuos reproductores fueron analizados para la detección de parásitos del género *Plasmodium* y *Haemoproteus* utilizando una eficiente técnica de reacción en cadena polimerasa que amplifica un segmento del gen mitocondrial citocromo *b* de estos parásitos. Encontramos seis linajes de *Plasmodium* (SGS1, GRW11, COLL1, DELURB4, GRW04 y BLUTI10) que parasitaban las poblaciones estudiadas, pero no detectamos ninguna infección por *Haemoproteu*s. Uno de los linajes detectados (BLUTI10) no había sido descrito anteriormente en ninguna especie de ave y, por otro lado, este estudio es el primero en detectar los linajes DELURB4 y GRW04 en herrerillo común. El linaje SGS1 (perteneciente a la morfoespecie *Plasmodium relictum*) fue el más común (una prevalencia general del 24 %), mientras que el resto de los linajes mostraron una incidencia mucho más baja (< 4 %). Sólo una pequeña fracción (12.2%) de las amplificaciones positivas para el linaje más común (SGS1) fueron detectadas en frotis sanguíneos usando microscopía óptica y en todos los casos las intensidades de infección fueron muy bajas (media±error estándar: 2.0±1.4 parásitos/2000 eritrocitos). También se encontró una alta variabilidad inter-poblacional en los patrones de prevalencia (rango: 12-41% para el linaje SGS1), lo que sugiere diferencias importantes en la tasas de transmisión de parásitos incluso entre localidades geográficamente próximas.

Capítulo IV- Diversidad genética individual y probabilidad de infección por parásitos de malaria aviar en herrerillo común (*Cyanistes caeruleus*)

Comprender la importancia de la diversidad genética de los hospedadores para hacer frente a los parásitos y las enfermedades infecciosas es un objetivo fundamental en la biología evolutiva. En este trabajo se estudió la relación entre la probabilidad de infección por malaria aviar (*Plasmodium relictum*) y la diversidad genética individual en tres poblaciones de herrerillo común (*Cyanistes caeruleus*) que difieren enormemente en la prevalencia de este parásito. Para este propósito, se analizaron las infecciones de malaria aviar y se genotiparon 789 herrerillos con 26 marcadores microsatélites. Se utilizaron dos conjuntos de marcadores diferentes: 14 loci clasificados como neutrales y 12 loci clasificados como supuestamente funcionales. Se encontró una relación significativa entre la probabilidad de infección y la diversidad genética del hospedador estimada por el subconjunto de marcadores neutros que no fue explicada

por efectos locales y no difería entre las poblaciones estudiadas. Esta relación no fue lineal, de modo que la probabilidad de la infección aumentaba hasta valores de homocigosidad por locus (*HL*) de alrededor de 0.15, se estabilizaba en valores de *HL* de entre 0.15 a 0.40 para, finalmente, disminuir entre una pequeña proporción de individuos altamente homocigotos (*HL* > 0.4). No se encontró evidencia de desequilibrio de identidad significativo, algo que puede ser resultado de una baja varianza en los niveles de endogamia en las poblaciones de estudio y/o consecuencia del pequeño poder de nuestro conjunto de marcadores para detectarlo. La relación cuadrática negativa observada podría ser explicada por una combinación de sutiles efectos locales positivos y negativos y/o un umbral de saturación en la asociación entre la probabilidad de infección y la diversidad genética del hospedador en combinación con el aumento de la resistencia a los parásitos en los individuos altamente homocigotos. En general, nuestro estudio pone de relieve que los parásitos juegan un papel importante en el mantenimiento de la variación genética de sus hospedadores y sugiere que el uso de un mayor número de marcadores neutros puede ser más apropiado para el estudio de la correlaciones entre heterocigosidad y eficacia biológica.

Capítulo V- La fuerza de selección sobre la heterocigosidad en regiones genómicas funcionales y neutras aumenta con la dureza de las condiciones ambientales

Se ha sugerido que el grado de depresión por endogamia y la magnitud de las correlaciones entre heterocigosidad y eficacia biológica (HFC) dependen del contexto ambiental en el que se analizan. Sin embargo, hay poca evidencia disponible para poblaciones naturales. En el presente estudio, se combinan datos moleculares y de captura-marcaje-recaptura en una población de herrerillo común (*Cyanistes caeruleus*) para (i) analizar la relación entre la heterocigosidad y la probabilidad de supervivencia interanual y (ii) comprobar si el estrés ambiental impuesto por temperaturas fisiológicas subóptimas y elevadas precipitaciones influyen en la magnitud de las HFC y la intensidad de la selección sobre la heterocigosidad. Para abordar estas cuestiones se emplearon dos conjuntos diferentes de marcadores: 14 loci clasificados como neutros y 12 loci clasificados como supuestamente funcionales. Se encontró una relación significativa entre la diversidad genética individual y la probabilidad de supervivencia de los adultos que fue mayormente explicada por la heterocigosidad del conjunto del genoma. La intensidad de la selección sobre la heterocigosidad difería entre años y estaba positivamente asociada con la precipitación acumulada. La heterocigosidad media anual aumentó a lo largo de los años, lo que indica una respuesta micro-evolutiva a la selección durante el período de estudio. Por último, loci neutros y funcionales presentaron tendencias similares, pero el primer grupo de marcadores mostró un efecto más fuerte y reflejó mejor la heterocigosidad del conjunto del genoma. En su conjunto, estos resultados indican la necesidad de considerar el papel de la heterogeneidad ambiental como un factor clave a la hora de analizar las consecuencias de la diversidad genética individual en la eficacia biológica.

Capítulo VI- Múltiples ornamentos sexuales reflejan la heterocigosidad en machos de herrerillo común

Se ha sugerido que la expresión de conspicuas señales ornamentales está asociada a una mayor calidad genética individual. Sin embargo, la relación entre múltiples componentes sexuales y la heterocigosidad ha sido escasamente estudiada. En este trabajo, se ha examinado si diferentes ornamentos, incluyendo diferentes aspectos del canto (tamaño de repertorio y longitud del canto) y la coloración del plumaje (pecho amarillo y píleo azul), reflejan la diversidad genética individual en machos de herrerillo común (*Cyanistes caeruleus*). La diversidad genética fue estimada empleando 26 marcadores microsatélites clasificados como supuestamente funcionales (12 loci) y neutros (14 loci). Se encontró que el carotenoide-croma del pecho amarillo, el brillo del píleo azul, la longitud del canto y la condición física estuvieron positivamente asociados con la heterocigosidad estimada con loci funcionales, pero no con la diversidad genética estimada en todos los loci o el subconjunto de marcadores neutros. La ausencia de fuertes efectos locales y la presencia de desequilibrio de identidad en nuestra población sugieren que la asociación observada entre ornamentos y heterocigosidad es mediada por loci ampliamente distribuidos en el genoma. El papel predominante de los loci funcionales sugiere que la expresión de caracteres sexuales secundarios está mayormente mediada por la heterocigosidad en regiones genómicas que están expresándose activamente, hecho que puede hacer que los rasgos ornamentales sean indicadores fiables de la calidad genética de los individuos. Los resultados de este estudio muestran que múltiples caracteres sexuales secundarios reflejan la diversidad genética del macho y apoyan la hipótesis de los "buenos genes" basada en la heterocigosidad.

Agradecimientos

Agradecimientos

En primer lugar me gustaría dar las gracias a mis dos directores de tesis. A Joaquín Ortego por descubrirme el mundo de la ecología molecular, por tu ayuda y apoyo a lo largo de todos estos años, por tus críticas constructivas y los conocimientos que me has transmitido. Y por supuesto por los buenos momentos entre pipeteos, revisiones, cambiolines, teatrillos y los famosos picados de alelos que hacían a cualquiera ¡*los ojos chiribitas*! A Juan José Sanz por acogerme en su grupo y darme una oportunidad tras ese primer día algo accidentado en Quintos de Mora. Por la emoción que transmites durante las jornadas de campo, siempre alegre e incansable, "*la primavera ya está aquí*". Por tu optimismo y ayuda todos estos años que ha facilitado este trabajo.

A Vicente García-Navas por enseñarme a extraer sangre de pajaritos en el merendero, por tu ayuda en los largos días de laboratorio en Ciudad Real y ser un gran compañero siempre dispuesto a resolver todas mis dudas. A Javier Bueno por saber escuchar y empezar conmigo esta aventura entre herrerillos y carboneros. Por los descansos en lo alto de la Morra compartiendo un KitKat, por las paradas en Ventas para ojear los cuadernos, en definitiva por los buenos momentos que hemos disfrutado. Tú eres el siguiente, mucho ánimo.

A Pedro Cordero por abrirme las puertas de su laboratorio y recibirme siempre con una sonrisa. También a Conchi Cáliz por los consejos y las risas en el laboratorio que animaban el día a día, y a Víctor Noguerales por ser tan atento y estar siempre dispuesto a ayudar.

A todas las personas que permitieron que este trabajo se desarrollase en Quintos de Mora y en el Parque Nacional de Cabañeros. Especialmente a la gente de San Pablo de los Montes que siempre nos recibía con alegría y de la que he aprendido mucho. Particularmente a Álvaro, Neftalí y Pablo que nos han facilitado el trabajo en el campo y siempre se podía contar con ellos.

A Marta, porque contigo empezó esta aventura mucho antes de los calurosos viajes a Ciudad Real en el Ford Ka. Por ser una gran amiga y compañera de fatiga, por poder contar contigo ante cualquier problema, por la complicidad con la que nos podemos comunicar sin mediar palabra. A Jesús, por ser un magnifico compañero que me ha enseñado más de lo que puede imaginar dentro y fuera de esas cuatro paredes del altillo.

A Meji, porque tú eres una pieza fundamental sobre la que se sustenta este trabajo. Por ser la persona con la que compartía mi pasión por la naturaleza, desde lo que aprendíamos juntas en la Encarta hasta los aullidos, bramidos y otros sonidos que memorizabas y me enseñabas cada día en el colegio, ¡*el mono aullador*! Gracias por ser una persona tan especial. A M3, María, Marta y Pilar por

estar siempre que os he necesitado, por los buenos momentos que hemos pasado juntas ¡y los que quedan! Por tener la certeza de que mi vida estaría incompleta si vosotras no formaseis parte de ella. A los amig@s de la universidad, pero en especial a Elena, Esther, Lucía, Mariconsu y Meri por vuestra comprensión a mis continuas ausencias, por alegrarme la vida y hacérmela más fácil, por ser unas amigas increíbles con las que puedo contar siempre. A los perdiceños y aledaños que no son pocos y que continuamente me han animado y apoyado ante cualquier problema. ¡Muchas gracias a todos!

Y como no a mi familia, responsable directa de todos mis logros. A mis abuelos, por vuestro cariño y apoyo, por los días en Burguillos recogiendo almendras, cerezas o membrillos que me acercaron a la naturaleza de una manera especial, sin vosotros nada habría sido posible. A mis tíos, Félix y Loli, Jesús, Miguel, Mari y David porque habéis sido más que hermanos mayores, aconsejándome y ayudándome siempre que lo he necesitado. Todos vosotros habéis sido fundamentales en todo lo que ha sucedido en mi vida, gracias por estar ahí. A mis primos Claudia, Sergio, Diego, Lucía, Sofía y Pablo por la alegría que siempre me transmitís y que me distrae del trabajo cotidiano. A Charo y Alberto por vuestro apoyo y comprensión todos estos años.

A mi hermana, por los ánimos y la confianza depositada en mí, por estar siempre dispuesta a escuchar mis desvaríos y ayudarme en todo lo que necesito, gracias por todo. Y por supuesto a la piedra angular de mi vida, mi madre. Por tu fortaleza y cariño, por enseñarme a levantarme más fuerte en cada caída, porque todo lo que soy es por ti y tú eres parte de este trabajo. Por estar siempre disponible a revisar cajas nido y emocionarte más que yo al ver un nido con o sin pollitos, por enseñarme a disfrutar de la naturaleza y de la vida.

Y finalmente a Tito, la persona que me mantiene en calma y paz. Son tantas las cosas que debo agradecerte que estas frases quedarán incompletas al hablar de ti. Por tu infinita comprensión ante mis ausencias en los meses de primavera y otoño, por saber escuchar y entender mi frustración, por animarme e ilusionarme en los malos momentos. Por compartir las alegrías y convertir las penas en risas haciendo que todo fuese más fácil a tu lado. Por ser el mejor compañero de vida que nadie puede tener y mi principal apoyo. Este trabajo es también tuyo.

UNIVERSIDAD DE
CASTILLA-LA MANCHA