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El complejo principal de histocompatibilidad en el herrerillo común (*Cyanistes caeruleus*): parasitismo, selección sexual e inmunogenética

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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EL COMPLEJO PRINCIPAL DE HISTOCOMPATIBILIDAD EN EL HERRERILLO COMÚN *(CYANISTES CAERULEUS)*: PARASITISMO, SELECCIÓN SEXUAL E INMUNOGENÉTICA

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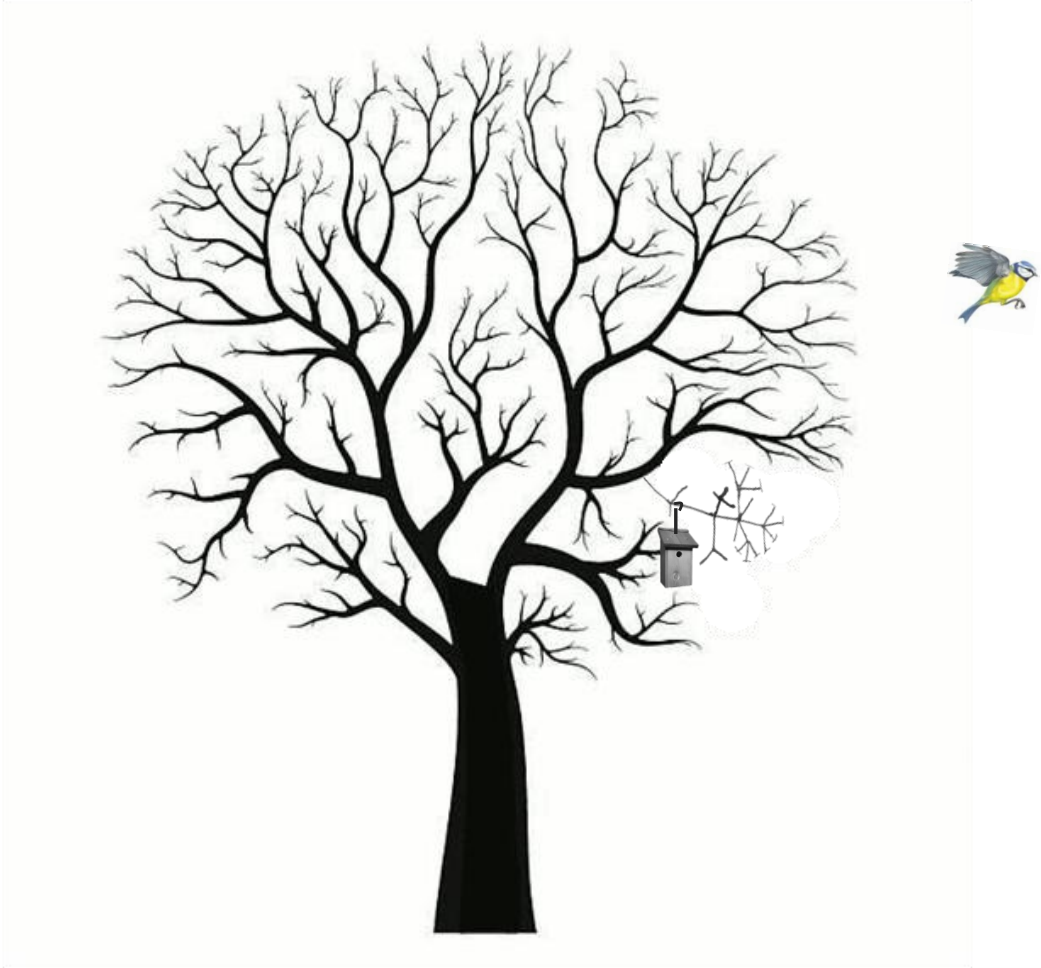

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Memoria presentada por el Licenciado Juan Rivero de Aguilar Cachafeiro para la obtención del grado de Doctor en Ciencias Biológicas, dirigida por el Dr. Santiago Merino Rodríguez del Museo Nacional de Ciencias Naturales-CSIC y el Dr. Francisco Javier Martínez González de la Universidad de Alcalá.

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El Doctorando VºBº del Director VºBº del Director

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pasamos en el campo, subiendo y bajando la montaña en el coche día si y día también escuchando esos discos que traías, que si no es por ellos nos hubiésemos dormido en cualquier curva. Aunque ahora estés en otros asuntos espero que por lo menos mantengas tu ojo crítico, aquel con el cual nos tenías a todos acostumbrados. Al mismo tiempo, por el despacho también rondaba Rodrigo, todo el día pegado al microscopio escrudiñando el mundo de los microorganismos. Que decir que con Rodrigo aún gustándole más "la escama que la pluma", siempre se ha ofrecido gustoso a ayudar con las aves y ofrecerse el primero ante cualquier problema que surgiese. Además constato en estos agradecimientos que la labor de Rodrigo en la socialización de los becarios del museo ha sido increíble, pues ahora se respira un ambiente muy agradable y se aprecia el compañerismo entre la gente que pulula por los pasillos. Espero que este compañerismo se mantenga incluso ante las adversidades de la carrera investigadora. Agradezco Rodri tu ayuda y compañía estos años y espero que saques adelante todos esos datos que tanto te has esmerado en conseguirlos. Más tarde llegó Elisa, la cual vino volando desde Inglaterra cual Barón Rojo. Desde entonces hemos compartido interminables horas de campo y discusiones estadísticas (todo el día "R que R"). También espero que salgan adelante todos esos datos que son muy prometedores y así podamos ahondar en el conocimiento del R-rillo común. También te agradezco tu compañía y buen humor en los momentos difíciles que siempre se agradece. Por lo reciente, también agradezco a Alazne por participar en la última temporada de campo y ayudarnos con los herrerillos. Espero que encuentres un buen tema de investigación y arranques en la carrera investigadora. Asombrosa tu capacidad de reconstruir la escena del crimen a partir de un grupillo de plumas encontradas en el campo. Y también que mantengas tu buen humor norteño (aún recuerdo el susto que me diste cuando gritaste que me envestía una vaca). También Paco gracias por echar una mano en el campo cuando has podido, sin tu ayuda ahora tendría más dolor de espalda del que tengo, que esas baterías pesaban un quintal.

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INTRODUCCIÓN

En la naturaleza los organismos establecen relaciones ecológicas de diferente tipo. Entre ellas, el parasitismo constituye una de las principales fuerzas selectivas en la evolución de las especies, pues afecta a la supervivencia de los hospedadores (Bush et al. 2001). En la relación entre parásito-hospedador el primero intenta obtener recursos de su hospedador mientras que éste intenta evitarlo. Este conflicto de intereses desemboca en una relación evolutiva dinámica entre los simbiontes (carrera de armamentos; Dawkins y Krebs 1979), desarrollándose ciertas adaptaciones comportamentales, anatómicas y fisiológicas por parte del hospedador encaminadas a evitar, impedir y/o expulsar al parásito; y contra-adaptaciones por parte del parásito para acceder, permanecer y explotar los recursos presentes en el hospedador. Entre las adaptaciones fisiológicas más relevantes generadas por los hospedadores para expulsar y/o destruir a los parásitos hay que destacar el sistema inmunitario. Aunque este sistema ya aparece en los organismos invertebrados, es en los vertebrados donde alcanza un mayor grado de sofisticación. La respuesta inmunológica en los vertebrados se divide en innata y adquirida (Regueiro et al. 2003). La primera tiene como misión la interceptación de parásitos cuando estos han sobrepasado las primeras barreras defensivas físicas del hospedador. Esta respuesta es inespecífica y se basa en el reconocimiento por parte de células fagocíticas de ciertos patrones moleculares conservados presentes en los parásitos. En cambio, la respuesta adquirida se basa en el reconocimiento específico de parásitos por parte de células especializadas (linfocitos). Así, a diferencia de la respuesta innata, se produce una memoria inmunológica donde la resistencia mejora notablemente si se repite la infección. Independientemente de si los parásitos infectan activamente a la célula o estos sean incorporados mediante fagocitosis, las proteínas pertenecientes a los parásitos son digeridas enzimáticamente en los fagosomas y, a continuación, pequeñas porciones peptídicas derivadas de la degradación de estas proteínas son exhibidas en la membrana celular junto con proteínas del propio hospedador. Estas proteínas del hospedador forman parte del complejo principal de histocompatibilidad (CMH o acrónimo ingles MHC). Su función radica en garantizar la presentación del antígeno a los linfocitos T, que son los encargados de activar la respuesta inmune adquirida (Piertney y Oliver 2006). Así, el MHC constituye una adquisición evolutiva novedosa para el sistema inmunitario (Klein 1986), constituyendo un salto cualitativo en el reconocimiento de los parásitos (Danchin et al. 2004; Kaufman 2010). El éxito del MHC en la detección de antígenos se debe a la particular estructura de las proteínas codificadas en sus genes.

Estructura y función del MHC

En los vertebrados, el MHC constituye una región cromosómica formada por genes que codifican diferentes moléculas involucradas en la activación de la respuesta inmunitaria (Piertney y Oliver 2006). Entre estos genes están los que codifican las proteínas reconocedoras

de antígeno (moléculas de MHC). Como hemos mencionado anteriormente, pequeños péptidos procedentes de los parásitos se unen a las moléculas del MHC para ser expuestos conjuntamente en la superficie celular. De esta forma, los antígenos son presentados a las células responsables de su reconocimiento, los linfocitos T (Janeway et al. 2001). En el supuesto de que el péptido expuesto sea derivado de un parásito y este sea reconocido como no propio, se desencadenará la respuesta inmunitaria. Esta particularidad convierte al MHC en un agente central del sistema inmunitario, pues la respuesta adquirida del hospedador está mediada a través de él. Debido a que la principal función de las moléculas de MHC es la presentación de péptidos, los agentes selectivos son principalmente los parásitos (Klein y O'Huigin 1994). De esta manera, las asociaciones observadas entre los genes de MHC y los diferentes parásitos ponen de manifiesto el papel que desempeñan estos genes en la respuesta inmune ante una determinada infección. Por lo tanto, el estudio del MHC es determinante para entender las interacciones entre los parásitos y sus hospedadores en las poblaciones naturales.

El estudio del MHC comienza con el descubrimiento de una determinada región genética mientras se realizaba un estudio sobre la respuesta antigénica en ratones (Gorer 1936). Con posterioridad al estudio, se confirma que esta región incluye genes codificadores de enzimas y moléculas estructurales necesarias para la activación y función de linfocitos T y B (Edwards et al. 1999). A partir de entonces, la caracterización de esta región en diferentes especies de vertebrados ha ido en aumento, revelando que el MHC forma una familia de genes variada y compleja (Hess y Edwards 2002). En las aves, el estudio del MHC comenzó con el descubrimiento de antígenos celulares en el gallo común (*Gallus gallus*) (Briles y McGibbon 1948). En esta especie el MHC se denominó complejo B y, a diferencia de lo que ocurre en los mamíferos, se caracteriza por poseer una región genética densamente empaquetada. De hecho, se denomino MHC "mínimo esencial" en referencia a su sencillez estructural y funcional (Kaufman et al. 1999). Por otro lado, y a diferencia de los mamíferos, el gallo común tiene dos regiones genéticas bien diferenciadas albergando genes del MHC, el complejo B relacionado con la estricta presentación peptídica y el complejo Rfp-Y asociado con el ensamblaje del péptido pero no con su presentación (Wakenell et al. 1996). A partir de los estudios realizados en el gallo común, se ha conseguido caracterizar el MHC (complejo B) en otras especies de aves, incluyendo tanto aves paseriformes como no paseriformes (Hess y Edwards 2002; Westerdahl 2007). Sobre la base de estas caracterizaciones se desprende la conclusión general de que a lo largo de la historia evolutiva de las aves se han ido produciendo duplicaciones de genes que han aumentado la complejidad de esta región, observándose diferencias entre especies tanto en el número de genes como en la longitud de los intrones, regiones del ADN que deben ser eliminadas de la transcripción primaria de ARN (Westerdahl 2007; Balakrishnan et al. 2010). Por otra parte, también se observa la pérdida de función en algunos genes (pseudogenes), así genes del MHC que en un momento dado son útiles en el reconocimiento de péptidos

derivados de los parásitos, se convierten en inservibles ante la aparición de nuevos linajes (Nei et al. 1997; Edwards y Hedrick 1998). Este hecho permite que un individuo presente una determinada variedad de moléculas del MHC que determinará finalmente su capacidad en la respuesta inmunitaria frente a los parásitos.

Desde el punto de vista de la propiedad de unión al péptido, la capacidad en el reconocimiento de péptidos extraños recaerá en gran medida en la estructura de la molécula del MHC. Las moléculas del MHC se clasifican en dos grupos dependiendo de la clase de gen que la codifica. Así las moléculas del MHC de clase I y de clase II son codificadas por los genes del MHC de clase I (MHC-I) y de clase II (MHC-II) respectivamente (Klein y Sato 2000). Las moléculas del MHC de clase I (glucoproteína con tres dominios denominados α1, α2 y α3) se expresan en asociación con la molécula microglobulina β2 en la superficie de todas las células nucleadas (Figura 1).

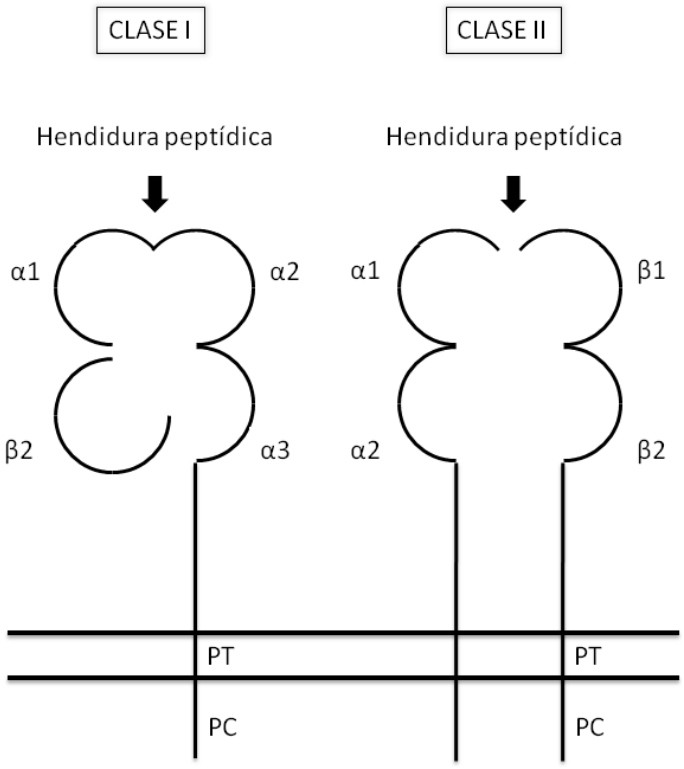

Figura 1. Representación de las moléculas del MHC de clase I y II. Porción transmembrana (PT), porción citoplasmática (PC).

Así en humanos hay tres loci genéticos de clase I (HLA-A, HLA-B y HLA-C) altamente polimórficos, con cerca de 100 alelos por cada locus. Esto conlleva a que debido a que los genes del MHC se expresan codominantemente, es decir, los dos alelos del mismo locus se expresan independientemente, hasta 6 moléculas de clase I diferentes pueden ser mostradas en la

superficie celular en un individuo heterocigoto. Estas moléculas presentan una hendidura estructural entre los dominios α1 y α2 que se encarga de la unión del péptido parasitario (hendidura peptídica) (Figura 1). Este péptido para ser reconocido debe tener entre ocho y nueve aminoácidos. Debido a ligeras variaciones estructurales en la hendidura peptídica de las diferentes formas alélicas, determinados péptidos parasitarios pueden tener mayor afinidad por determinadas moléculas del MHC de clase I (Falk et al. 1991; Piertney y Oliver 2006). Debido a esta afinidad diferencial, la respuesta inmunitaria variará en cada individuo al poseer una combinación diferente de moléculas del MHC. Adicionalmente a los tres loci principales se han encontrado otros loci (HLA-E, HLA-F, HLA-G y HLA-H) que codifican para moléculas de clase I pero que muestran variabilidad y distribución tisular limitada. Por el contrario, las moléculas del MHC de clase II se expresan solo en ciertos tipos celulares conocidos como células presentadoras de antígeno (células dendríticas, macrófagos, linfocitos B, algunos linfocitos T y algunas células especializadas del timo y el intestino). Al igual que las moléculas del MHC de clase I, presentan una hendidura estructural para acomodar el péptido parasitario (Figura 1). La hendidura peptídica se forma entre la cadena α1 y β1, acomodando péptidos de una longitud de entre 18 a 20 aminoácidos. En humanos estas moléculas son codificadas por genes de las regiones HLA-DP, HLA-DQ y HLA-DR. Así, por ejemplo, el locus de la región HLA-DP produce una cadena DP alfa y una DP beta que se combinarán para formar la hendidura peptídica. En las aves, al igual que en mamíferos, se observa también en las moléculas del MHC una hendidura para acomodar el péptido, confirmándose la función de presentación antigénica (Koch et al. 2007).

Las moléculas del MHC de clase I y II presentan antígenos derivados de parásitos que difieren en su capacidad de infectar células. Las moléculas del MHC de clase I presentan principalmente péptidos de parásitos intracelulares tales como virus, mientras que las moléculas del MHC de clase II presentan principalmente antígenos derivados de parásitos extracelulares como son bacterias, nematodos y cestodos (Klein y Figueroa 1986). En la presentación del antígeno vía MHC-I, las proteínas derivadas de los parásitos o parte de ellas son degradadas en el citosol, los péptidos resultantes son ensamblados con las moléculas del MHC de clase I y, posteriormente, son presentados en la membrana celular a través de un transporte desde el retículo endoplasmático pasando por el aparato de Golgi. Finalmente el péptido es reconocido por los linfocitos T CD8+ citotóxicos. Por el contrario, en la presentación del antígeno vía MHC-II, las proteínas derivadas de los parásitos tienen un origen extracelular, son incorporadas a la célula mediante procesos de fagocitosis y degradados en los fagosomas. Los péptidos obtenidos son entonces ensamblados con moléculas del MHC de clase II y, al igual que en la vía MHC-I, son transportadas a la superficie celular para su presentación a los linfocitos T CD4+ cooperadores. En ambos casos los linfocitos T no reconocen el antígeno de forma soluble sino asociados a las proteínas del MHC. Así, los linfocitos T citotóxicos destruyen directamente las

células infectadas, mientras que los linfocitos T cooperadores ayudan a los linfocitos B a producir anticuerpos y a los macrófagos a destruir los parásitos fagocitados por estos. Una vez los antígenos parasitarios han sido expuestos en la membrana de las células, los linfocitos T deben reconocerlos mediante un receptor de membrana denominado TCR. En principio, solo los antígenos parasitarios restringidos por moléculas del MHC podrán ser reconocidos para que la respuesta inmunitaria adquirida llegue finalmente a desencadenarse (Regueiro et al. 2003). Esto permite que la respuesta inmunitaria sea específica ante los antígenos extraños encontrados. En este sentido, durante el desarrollo de los linfocitos T se produce una selección clonal en función de las moléculas del MHC presentes en cada individuo, evitando de esta forma el reconocimiento y destrucción por estos de tejidos propios del hospedador (Doan et al. 2008).

MHC y parásitos

En principio, la alta diversidad alélica presente en el MHC se atribuyó a procesos de deriva genética y/o de las mutaciones neutrales o casi neutrales, pero la presencia de residuos aminoacídicos altamente polimórficos en la región presentadora de antígeno (acrónimo ingles PBR) de las moléculas de HLA-A y HLA-B dentro de la hendidura peptídica proporcionaron evidencias de que el polimorfismo no se genera solo debido a procesos neutrales (Hedrick et al. 1991). Al descartar esta opción, parece que la existencia de algún tipo de selección balanceadora podría ser el origen del polimorfismo alélico observado en las poblaciones naturales. En cada población existen diferentes alelos que constituyen el acervo genético necesario para llevar a cabo el reconocimiento antigénico. Cada individuo presente en una población porta una combinación de alelos diferente. Este hecho permite que en el supuesto de que un parásito evada el reconocimiento de una molécula del MHC de un determinado individuo, haya otro que presente un alelo capaz de detectar al parásito y eliminarlo. Así la viabilidad de las poblaciones dependerá finalmente de la diversidad alélica individual. Estudios de selección en el PBR confirman que el polimorfismo es principalmente mantenido por la presión selectiva ejercida por los parásitos, favoreciendo, por lo tanto, la diversidad en esta región (Piertney y Oliver 2006). En este sentido, se descubrió que la tasa de sustitución no-sinónima (es decir, cambios nucleotídicos que resultan en un cambio de aminoácido) en el PBR era mayor que la sinónima (cambios nucleotídicos que no resultan en un cambio de aminoácido), indicando que la selección ha actuado para aumentar la diversidad aminoacídica en esta región (Hughes y Nei 1988, 1989). Se han propuesto otros mecanismos generadores de diversidad en el MHC como la recombinación entre diferentes locus, la selección debida a la elección de pareja y las incompatibilidades materno-fetales (Penn y Potts 1999; Hess y Edwards 2002; Spurgin et al. 2011). Por otra parte, el hecho de que algunos alelos se mantengan en el acervo genético durante largos periodos de tiempo (polimorfismo trans-especie) se ha explicado como consecuencia de selección balanceadora (van Oosterhout 2009).

Dos hipótesis principales se han propuesto para explicar cómo los parásitos mantienen la alta diversidad en el MHC, estas son la "hipótesis de la ventaja del heterocigoto" (o ventaja sobredominante) y la "hipótesis de la ventaja de alelos poco frecuentes" (o selección negativa dependiente de la frecuencia) (Spurgin y Richardson 2010). La primera hipótesis postula que en una población expuesta a un determinado grupo de parásitos será ventajoso para un individuo ser heterocigoto para los genes del MHC, ya que tendrá más posibilidades de desencadenar una respuesta inmune que un individuo homocigoto (Doherty y Zinkernagel 1975). Esto es debido a que un individuo heterocigoto es capaz de presentar un mayor número de péptidos parasitarios. Un ejemplo que corrobora esta hipótesis se ha encontrado en humanos, ya que la enfermedad del SIDA causada por el virus del VIH-I progresa más rápidamente en individuos infectados homocigotos en el MHC-I (Carrington et al. 1999). Además, el grado de divergencia entre los alelos puede ser determinante en el reconocimiento antigénico, pues individuos con el mismo grado de heterocigosidad pero que porten alelos mas divergentes pueden presentar un espectro más amplio de péptidos (Hughes y Hughes 1995; Sommer 2005). Por lo tanto, el beneficio de la heterocigosidad individual dependerá de los alelos particulares que posea un individuo y del grado de solapamiento entre el repertorio de péptidos que puede presentar (Sepil et al. 2013). Por otro lado, la "hipótesis de la ventaja de alelos poco frecuentes" propone que la presencia de alelos raros o poco frecuentes (por ejemplo los generados recientemente) responderán mejor a las nuevas variantes de parásitos, ya que estos probablemente han evolucionado para evadir los alelos más comunes (Jeffery y Bangham 2000). De hecho, parece que los parásitos se adaptan a los genotipos más comunes del hospedador permitiendo la presencia de individuos en la población con baja carga parasitaria y con genotipos más raros (Lively y Dybdahl 2000). Si un alelo proporciona una mayor inmunidad frente a un parásito virulento, éste incrementará su frecuencia en la población, pero también lo hará la presión selectiva para que el parásito desarrolle mecanismos de evasión para evitar el reconocimiento del alelo más común. A medida que cambia la antigenicidad del parásito la eficacia biológica del alelo más abundante disminuye, pudiendo proporcionar al mismo tiempo una ventaja selectiva a cualquier alelo minoritario que determine un mejor reconocimiento del parásito. Los valores cíclicos de la eficacia biología determinada por diferentes alelos del MHC darán como resultado un mantenimiento de la diversidad genética. Esta selección dependiente de la frecuencia puede haber causado el aumento de frecuencia del alelo HLA-Bw*53 en la población de Gambia expuesta a malaria (Hill et al. 1991).

También, la selección ejercida por los parásitos puede variar tanto temporalmente como localmente reflejándose en una selección diferencial de alelos específicos (Loiseau et al. 2009). Así un alelo puede asociarse con la resistencia a un determinado parásito en una población pero a la vez ser neutral en otra (Bonneaud et al. 2006). O un mismo alelo puede conferir resistencia a un parásito y susceptibilidad a otro, así complicando el discernimiento de las asociaciones

entre alelos específicos y una determinada infección (Loiseau et al. 2008; Spurgin y Richardson 2010).

Evidencias sobre la existencia de asociaciones entre determinados alelos del MHC y la resistencia / susceptibilidad a una determinada enfermedad han sido principalmente observadas en cautividad. Por ejemplo, en el gallo común el alelo B21 se asoció con una mayor resistencia al herpesvirus causante de la enfermedad de Marek (Briles et al. 1977) y en el ratón el locus H-2D se asoció con una resistencia a la infección del virus de Theiler (Azoulay-Cayla et al. 2001). En poblaciones naturales, se han encontrado también asociaciones entre alelos del MHC y la susceptibilidad / resistencia a determinadas infecciones (Hill et al. 1991; Sommer 2005; Piertney y Oliver 2006; Spurgin y Richardson 2010; Westerdahl et al. 2012). En el caso de las aves se han investigado principalmente asociaciones con respecto a parásitos sanguíneos (Bonneaud et al. 2005; Westerdahl et al. 2005; Loiseau et al. 2011; Radwan et al. 2012; Sepil et al. 2013) y los resultados ponen en evidencia el papel que desempeñan determinados alelos y/o la diversidad general del MHC del individuo para hacer frente a estas infecciones. En el caso de los estudios realizados en poblaciones naturales para diferenciar el estatus de cada alelo (resistente o susceptible) es relevante incorporar los datos tanto de la presencia (infectado o no infectado) como de la intensidad de infección (parasitemia) (Westerdahl et al. 2012). Por ejemplo, un alelo puede conferir resistencia ya sea eliminando el parásito, o manteniéndolo a baja intensidad de infección o, por el contrario, conferir susceptibilidad si una vez infectado se mantiene una alta intensidad de infección.

En el caso de selección por parte de los parásitos se espera observar cambios en las frecuencias alélicas dentro de una población a lo largo del tiempo. En diferentes estudios realizados en vertebrados se han observado dichos cambios relacionados con la presencia de alelos específicos de resistencia (Spurgin y Richardson 2010; Eizaguirre et al. 2012). En dichos estudios se pone en evidencia el papel de la selección dependiente de la frecuencia, ya que los alelos asociados a resistencia aumentan en frecuencia al conferir ventaja frente a otros alelos menos ventajosos. Por ejemplo, en el carricero tordal (*Acrocephalus arundinaceus*) se ha comprobado que la presión selectiva ejercida por el parásito *Plasmodium* a lo largo del tiempo se relaciona con cambios en la frecuencia de ciertos alelos (Westerdahl et al. 2004).

MHC y selección sexual

La teoría de la selección sexual fue propuesta formalmente por Charles Darwin en "El origen de las especies por medio de la selección natural" y más tarde desarrollada en "El origen del hombre" (Darwin 1859, 1871). Darwin propuso que la competencia entre individuos del mismo sexo por el acceso a individuos del sexo contrario llevaría a la evolución de rasgos particulares. Además, también se esperaría una selección por parte del sexo contrario en base a esos rasgos. Así, la selección sexual explicaría la evolución de los denominados caracteres

sexuales secundarios, es decir, aquellos no directamente implicados en la reproducción. En la competencia conespecífica (principalmente entre machos) los caracteres exagerados, por ejemplo la cornamenta de los ciervos, están directamente involucrados en el acceso por conseguir hembras, ya que confieren una ventaja en la confrontación directa contra otro individuo. Por lo contrario, en la selección intrasexual, por ejemplo la cola del pavo real, los rasgos exagerados en vez de estar relacionados con una confrontación directa entre machos, más bien sugiere una selección por parte de las hembras. A veces discernir si un carácter actúa como una señal frente a conspecíficos o frente al sexo contrario es complicado pues puede darse el caso de que actúe frente a ambos. Esto puede ser lo que ocurre con el canto de las aves, ya que aparte de señalizar el territorio para que otros individuos no accedan a él también puede servir para atraer a la pareja.

Así, dentro de la teoría de la selección sexual la elección de pareja es determinante en la evolución de las poblaciones, afectando la capacidad de producir o no descendencia. Darwin propuso que el desarrollo y mantenimiento de caracteres fenotípicos exagerados en los machos, aún siendo éstos perjudiciales para su portador, conferiría algún tipo de ventaja si con ello se consigue ser seleccionado como pareja por individuos del sexo contrario. Por ejemplo, en las aves, si las hembras tienen preferencia, por machos con colas más largas, estos tendrán más posibilidades de ser seleccionados. Más tarde, se propuso que si los machos que tienen ese rasgo son los preferidos por las hembras, entonces en su descendencia se trasmitirá la preferencia de las hembras por elegir machos con ese rasgo y sus hijos heredarían también ese rasgo (Fisher 1930). Pero en este caso con el paso del tiempo los machos perderían variación en ese rasgo y se perdería la variabilidad necesaria para que se produzca selección por parte de las hembras, incluso podría darse el caso de que el rasgo finalmente llegase a ser perjudicial. Entonces se propusieron varias hipótesis dentro de lo que se conoce la teoría del hándicap, en la cual sólo los individuos de mayor calidad pueden expresar rasgos exagerados pues su producción y mantenimiento son energéticamente costosos (Zahavi 1975, 1977). En esta teoría se incluiría el efecto por parte de los parásitos ya que al ser perjudiciales solo aquellos individuos que son capaces de contrarrestar sus efectos serán capaces de destinar recursos suficientes a la producción y mantenimiento de caracteres exagerados y, así, se mantendría la varianza fenotípica necesaria para que las hembras puedan diferenciar entre los machos de más calidad (Hamilton y Zuk 1982; Møller et al. 1999). En esta teoría el sistema inmunitario juega un papel importante, debido a su implicación en la detección y eliminación de los parásitos. El mantenimiento energético para su correcto funcionamiento sería tan elevado que solo los individuos de máxima calidad serán los que tendrán más posibilidades de desarrollar esos caracteres y ser finalmente seleccionados. En este caso la condición individual sería determinante (Zelano y Edwards 2002).

Dentro de la selección de pareja se han propuesto dos hipótesis en relación a los beneficios obtenidos por la hembra y su descendencia, ya sean estos directos o indirectos (Møller et al. 1999; Schmid-Hempel 2011). Una es la llamada hipótesis "del buen padre", en la cual la hembra elige a su pareja para conseguir beneficios directos, ya sea por ejemplo en forma de buen territorio de cría, cuidado parental, o un macho libre de parásitos. Otra teoría es la denominada "de los buenos genes", en la cual se propone que la hembra escoge machos que confieran beneficios indirectos para su descendencia, como es la transmisión de genes de resistencia. En el contexto de esta última teoría puede darse una elección de pareja sobre la base del MHC. En el caso de la mayoría de las aves, la hembra puede seleccionar un macho compatible con respecto a sus genes, es decir que el macho tenga alelos diferentes, o por el contrario seleccionar a uno con el mejor genotipo, independientemente del genotipo de la hembra. En este caso seleccionaría individuos con alelos específicos ventajosos o los más diversos en el MHC. Como resultado de este tipo de emparejamientos se esperarían en la descendencia un aumento de la heterocigosidad, la protección contra parásitos que se adaptan a los genotipos parentales, la prevención de endogamia o una optimización de la diversidad genética (Møller 1994; Neff y Pitcher 2005; Piertney y Oliver 2006). En vertebrados se han encontrado evidencias de estas hipótesis, incluso en conjunto, pues posiblemente los individuos más diversos albergan alelos raros más frecuentemente. En las aves, el emparejamiento con respecto al MHC se ha investigado en el carricero tordal *Acrocephalus arundinaceus*, el carricero de Seychelles *Acrocephalus sechellensis*, en el papamoscas collarino *Ficedula albicollis*, la agachadiza real *Gallinago media*, el mascarita común *Geothlypis trichas*, el petrel azulado *Halobaena caerulea*, el carbonero común *Parus major*, el gorrión común *Passer domesticus*, el gorrión sabanero común *Passerculus sandwichensis*, el pavo real *Pavo cristatus*, el faisán común *Phasianus colchicus* y el pingüino de Magallanes *Spheniscus magellanicus*, (Von Schantz et al. 1997; Freeman-Gallant et al. 2003; Ekblom et al. 2004; Westerdahl 2004; Richardson et al. 2005; Bonneaud et al. 2006; Hale et al. 2009; Knafler et al. 2012; Radwan et al. 2012; Strandh et al. 2012; Dunn et al. 2013; Sepil et al. 2013). En general los resultados evidencian el papel de las hipótesis de selección sexual anteriormente citadas. En este sentido, las hembras se emparejan con individuos heterocigotos o con alelos específicos que permiten maximizar su eficacia biológica y asegurar la supervivencia de su descendencia. En las aves, la elección de pareja se ha relacionado con el tipo de estatus reproductor. En especies donde el macho no ejerce cuidados parentales los beneficios genéticos indirectos son más evidentes. Pero en especies monógamas, como el herrerillo común, en donde el cuidado parental por ambos sexos es determinante en la viabilidad de la descendencia, la ventaja obtenida por la obtención de genes de resistencia se complementa con los beneficios directos obtenidos por la hembra al seleccionar a un buen padre, que sea eficaz por ejemplo en proveer alimento a la descendencia.

En el herrerillo común, a diferencia de especies donde el dimorfismo sexual es muy pronunciado, el tamaño y plumaje del macho y la hembra son muy parecidos (Svensson 2011). Ambos comparten una coloración amarilla del pecho que viene determinada por la ingesta de carotenos (principalmente luteina y xantina), los cuales son abundantes en la dieta (Andersson y Prager 2005). El color del pecho de esta especie se ha relacionado con la calidad del individuo, indicado por el esfuerzo reproductor o por la capacidad de encontrar alimento (Senar et al. 2002; García-Navas et al. 2012). De esta manera, un color de pecho brillante estaría señalando al sexo contrario su calidad para reproducirse. En el herrerillo común, aunque macho y hembra tienen un plumaje visualmente parecido, todavía existe un dimorfismo en regiones específicas del plumaje (la corona) en el espectro del ultravioleta. De hecho, existen evidencias de que las hembras escogen a los machos basándose también en este ornamento (Sheldon et al. 1999). En este contexto la elección de pareja en esta especie depende de decisiones que deben pasar por evaluar entre otras cosas el color del plumaje, que viene determinado por diferentes factores, como es la acumulación de pigmentos o el estatus de infección (Møller et al. 1999; Zelano y Edwards 2002).

Parásitos sanguíneos

Los parásitos sanguíneos, debido a la relativa facilidad de muestreo, son actualmente un campo muy productivo de investigación dentro de la ecología evolutiva, pues permiten estudiar los efectos de la selección natural en poblaciones naturales. Los parásitos tienen diferentes grados de especificidad parasitaria, variando ésta a través de sus ciclos biológicos. Por lo tanto, el conocimiento de la biología de los hospedadores es también esencial para establecer modelos de estudio adecuados. En nuestro caso, el herrerillo común está considerado una especie modelo para estudios de la ecología evolutiva y en especial de las relaciones parásito-hospedador. En esta especie se han identificado diversas especies de parásitos sanguíneos, incluyen parásitos del género *Haemoproteus*, *Leucocytozoon o Plasmodium* (Valkiūnas 2005; Atkinson et al. 2008). Estos parásitos pertenecen al grupo de los haemosporidios (Sporozoa: Haemosporidia), un grupo dentro del filo Aplicomplexa que usan insectos dípteros hematófagos como vectores (Valkiūnas 2005). Mediante experimentación se ha demostrado que estos parásitos provocan efectos negativos en su hospedador aviar, afectando la condición y supervivencia de los mismos (Merino et al. 2000; Marzal et al. 2005; Knowles et al. 2010; Martínez-de la Puente et al. 2010). La transmisión del parásito se produce principalmente en el periodo reproductor del hospedador vertebrado, el cual suele coincidir con las condiciones meteorológicas adecuadas que permiten la máxima actividad de los vectores (Valkiūnas 2005). La evolución de la infección dentro del hospedador se caracteriza por un aumento inicial de la parasitemia (número de parásitos en sangre), hasta que el sistema inmunitario logra eliminar al parásito o reducirlo. Cuando el parasito persiste por largos periodos de tiempo con una intensidad baja la infección es

considerada crónica. En momentos que requieren un esfuerzo energético elevado, como es el caso de la época reproductora, los individuos con infecciones crónicas experimentan un aumento de la parasitemia. Posiblemente, este hecho sea fruto del compromiso energético que se genera entre el sistema inmune y el sistema reproductor del hospedador, facilitando, en cualquier caso, las posibilidades de transmisión del parasito a un hospedador susceptible.

Especie de estudio

El herrerillo común es un ave insectívora paseriforme perteneciente a la familia Paridae (del Hoyo et al. 2009). Tiene una distribución holártica con predilección por diferentes ambientes, desde bosques caducifolios hasta aéreas desérticas (Cramp y Perrins 1998). Forma un grupo taxonómico constituido por la especie nominal y diferentes subespecies, las cuales están repartidas por toda su área de distribución. Ciertas variaciones fenotípicas permiten diferenciar las subespecies pero en ocasiones las diferencias son tan sutiles que podrían ser consecuencia de adaptaciones latitudinales o locales (Svensson 2011). En la Península Ibérica es posible encontrar dos subespecies, la subespecie *caeruleus* que se distribuye principalmente por toda Europa con límite meridional en la Península Ibérica y la subespecie *ogliaestrae* con una distribución principalmente ibérica pudiendo alcanzar el sur de Francia. La distribución geográfica actual es el resultado de procesos climáticos durante la última glaciación (Kvist et al. 2004; Illera et al. 2011).

El herrerillo común presenta predilección por utilizar las cajas nido para su reproducción, por lo tanto la captura de individuos y la toma de muestras es relativamente fácil. Tiene un ciclo de reproducción característico en el cual el macho y la hembra sacan conjuntamente adelante a los polluelos (Cramp y Perrins 1998). La hembra es la encargada de la construcción del nido, el cual está formado principalmente por musgo tapizado con hierba, plumas o pelos. Las nidadas suelen tener una media de 9 polluelos (9,17 huevos de tamaño de puesta medio para la población bajo estudio entre 1999 y 2012) aunque varía a lo largo de la temporada de cría y de la distribución geográfica de la especie. Los pollos son alimentados por ambos padres, principalmente con orugas y en menor medida arácnidos. Los pollos abandonan el nido pasados alrededor de veinte días tras la fecha de eclosión.

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OBJETIVOS

1. Estudiar las asociaciones entre la diversidad y presencia de alelos del MHC-I y varias especies de parásitos sanguíneos intracelulares en el herrerillo común. (Capítulo1)

2. Estudiar el efecto de la infección por distintos parásitos sanguíneos sobre un ornamento sexual: el color amarillo del pecho del herrerillo común. (Capítulo 2).

3. Determinar si la selección de pareja está relacionada con la diversidad y presencia de alelos del MHC-I en el herrerillo común. (Capítulo 3).

4. Determinar el polimorfismo y caracterizar parcialmente los alelos del MHC-II en el herrerillo común. (Capítulo 4).

RESULTADOS PRINCIPALES

Capítulo 1: Estudio de las asociaciones entre la diversidad y presencia de alelos del MHC-I y varias especies de parásitos sanguíneos intracelulares en el herrerillo común.

En este capítulo se investigaron las asociaciones existentes entre los genes del MHC-I y los parásitos sanguíneos *Haemoproteus majoris* y *Leucocytozoon* sp. en una población reproductora de herrerillo común localizada en el centro de la península ibérica. Para ello, se determinó la presencia e intensidad de infección por ambos parásitos, así como la diversidad alélica del MHC-I. A continuación, se exploró si la diversidad alélica o la presencia de alelos específicos determinaban la presencia e intensidad de infección de los haemosporidios mencionados anteriormente. La prevalencia de ambos parásitos fue elevada y la intensidad de infección varió entre individuos (Tabla 1). En la población objeto de estudio se detectaron un total de siete alelos. En estudios previos realizados con individuos pertenecientes a varias poblaciones de la misma especie, cuatro de ellos (Paca UA*104, Paca UA*108, Paca UA*114 y Paca UA*117) mostraron selección en el PBR (Schut et al. 2011), confirmando su posible involucración en la presentación de péptidos parasitarios. Además de estos alelos, se detectaron tres alelos inéditos: Paca UA*236, Paca UA*249 y Paca UA*274. La diversidad alélica individual del MHC varió entre 1 y 5 alelos.

Cuando se estudiaron las asociaciones entre la diversidad del MHC y los parásitos sanguíneos no se observaron diferencias significativas entre la presencia de la infección y los alelos del MHC-I. Sin embargo, se observó una asociación significativamente positiva con la intensidad de infección por parásitos del género *Leucocytozoon* (Figura 1). Así, una mayor diversidad alélica estuvo asociada a parasitemias mas elevadas. Además, el sexo y la edad de los individuos parecen estar involucrados en esta asociación, ya que la asociación se cumple especialmente para individuos jóvenes o para machos. Además se observó una interacción entre la diversidad del MHC, el sexo y la edad, de forma que la asociación positiva entre diversidad y parasitemia por *Leucocytozoon* se mantiene especialmente entre los machos jóvenes.

Por otro lado, se observó una asociación significativamente positiva entre la intensidad de infección por *Leucocytozoon* y la presencia de los alelos Paca UA*104 o Paca UA*108 (Figura 2). En este caso, el sexo y la edad también tuvieron un efecto sobre esta asociación. El grupo de los individuos macho y el de los individuos jóvenes, portadores del alelo Paca UA*104 o Paca UA*108, presentaron una parasitemia mayor que las hembras o los adultos portadores, respectivamente. Adicionalmente, el análisis de la interacción sexo/edad mostró que los machos portadores, ya sean jóvenes o adultos, presentaron parasitemias mas elevadas. Además, el alelo Paca UA*117, aunque no se asoció por si solo con la intensidad de infección por *Leucocytozoon*, se observó que las hembras portadoras de este alelo presentaron una

intensidad de infección mayor que las hembras carentes del mismo o que los machos tanto sin son portadores o no. Además, los individuos con el MHC más diverso presentaron con más frecuencia los alelos Paca*104 y Paca*108. Sin embargo, excluyendo estos alelos del análisis, la asociación entre la diversidad alélica y la infección por *Leucocytozoon* se mantuvo. Por lo tanto, tanto la diversidad alélica como la presencia de alelos específicos, parecen ser determinantes en las asociaciones comentadas anteriormente.

Finalmente, se investigaron los cambios en las frecuencias alélicas entre dos años consecutivos. Como resultado se observó un aumento en la frecuencia del alelo Paca*104 en el segundo año. Además, ningún alelo encontrado en baja frecuencia el primer año aumentó de frecuencia al año siguiente.

Parásito	Machos (n= 68)			Hembras (n= 74)			Total		
	IM (ES)	(rango)	P %	IM (ES)	(rango)	P %	IM (ES)	(rango)	P %
Leucocytozoon sp.	1.56 (0.58)	0 – 39	89.3	1.67 (0.44)	0 – 23	94.5	1.62 (0.58)	0 - 39	91.9
Haemoproteus majoris	20.95 (3.15)	0 – 115	89.2	20.35 (3.41)	0 – 138	81.1	20.63 (2.32)	0 - 138	85.0

Tabla 1. Intensidad y prevalencia de infección por parásitos sanguíneos en machos y hembras del herrerillo común. IM = Intensidad media. P = Prevalencia. ES = Error estándar.

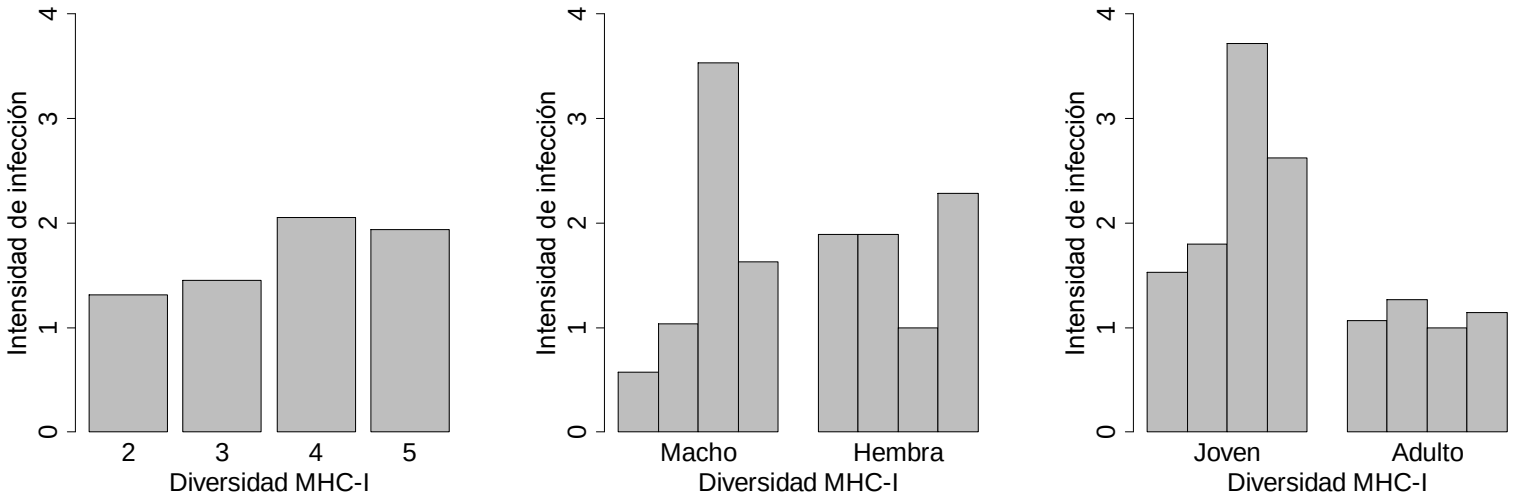

Figura 1. Relación entre la intensidad de infección por *Leucocytozoon* en el herrerillo común y la diversidad del MHC-I. Se muestran también los resultados en función del sexo y la edad de los individuos. El nivel de diversidad del MHC-I = 2 fue creado a partir de la unión de los niveles diversidad MHC-I=1 y 2 debido al bajo número de individuos en dichas categorías.

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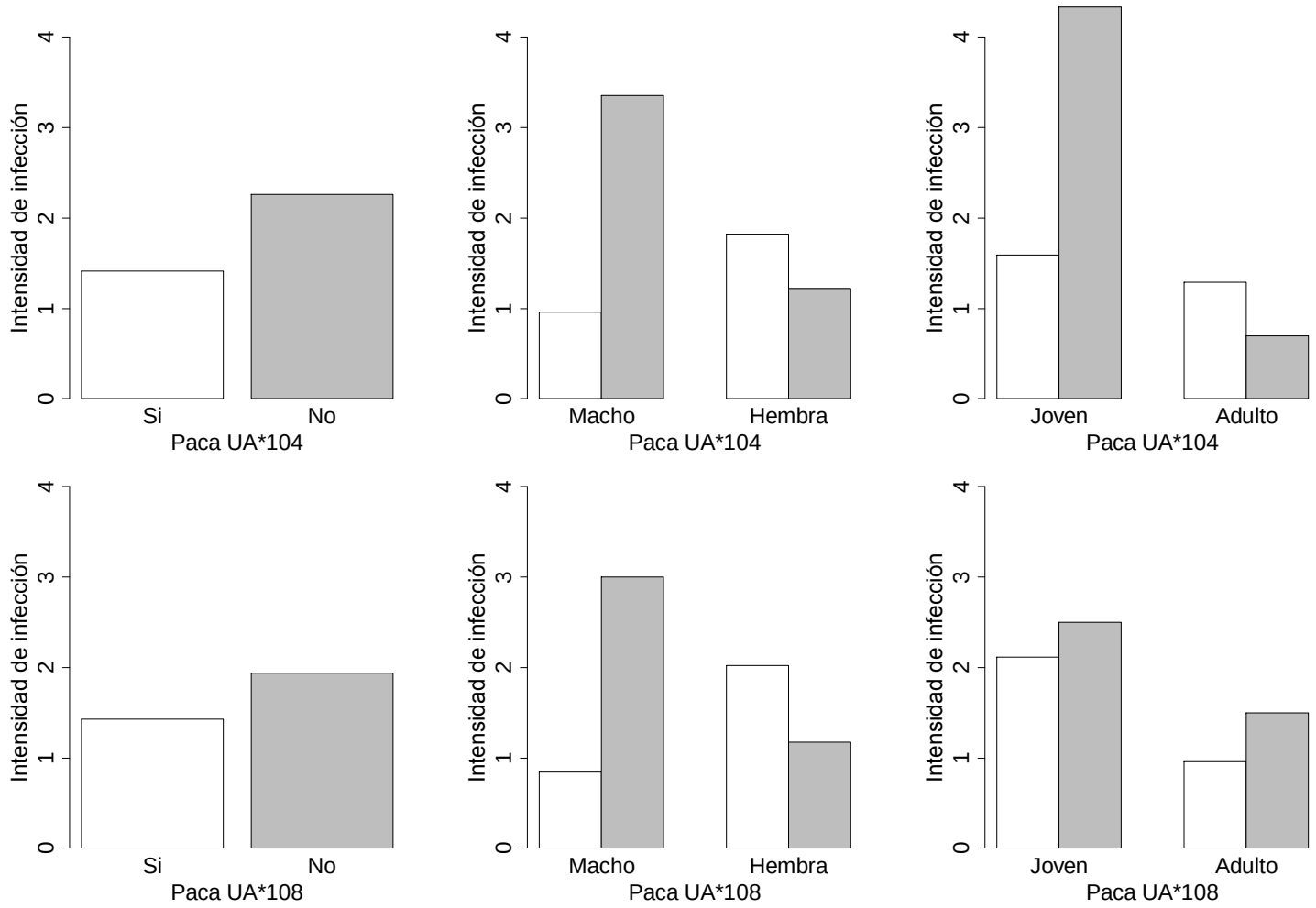

Figura 2. Relaciones entre la intensidad de infección por *Leucocytozoon* en el herrerillo común y la presencia o no del alelo del MHC-I Paca UA*104 y Paca UA*108. Se muestran también los resultados en función del sexo y la edad de los individuos.

Capítulo 2: Estudio del efecto ejercido por la infección por distintos parásitos sanguíneos sobre un ornamento sexual: el color amarillo del pecho del herrerillo común.

En este capítulo se investigó el papel de los carotenos como indicadores del estatus de infección en una población de herrerillo común. Para ello, se estudió en cada individuo y durante dos años, la relación entre la riqueza de diferentes parásitos sanguíneos y el color del plumaje del pecho. Además, también se estudiaron estas asociaciones con respecto a diferentes parámetros fisiológicos como el nivel de las proteínas de estrés y el de las immunoglobulinas. El color de las plumas del pecho es adquirido mediante la incorporación de carotenos a partir de la dieta. Como estos nutrientes también intervienen en otros procesos fisiológicos, incluido el sistema inmunitario, se espera que la presencia de los parásitos desencadene una redistribución de este recurso nutricional hacia el sistema inmunitario en detrimento de su depósito en el plumaje del ave. Es decir, los individuos más parasitados destinaran más carotenos al sistema inmunitario y, por lo tanto, presentaran plumajes más pálidos.

Los resultados obtenidos corroboraron esta hipótesis. El aumento de la riqueza parasitaria disminuyó la intensidad del color amarillo del plumaje del pecho (Figura 3). Así, los individuos parasitados por más de una especie mostraron un plumaje más pálido que los individuos no parasitados o los que solo presentaron una especie de parásito. También se encontraron diferencias entre años.

Por otro lado, no se encontró un efecto significativo por si solo de la fecha de muestreo, el sexo o la edad sobre el color, pero sí que se encontraron interacciones entre el sexo y la edad, o el sexo y el tratamiento. Además, el color del pecho no estuvo relacionado con el nivel de inmunoglobulinas. Sin embargo, el nivel de la proteína de estrés HSP70 fue mayor en los individuos con un plumaje más pálido (Figura 4). Finalmente, el nivel de la proteína de estrés HSP60 no estuvo relacionado con el color.

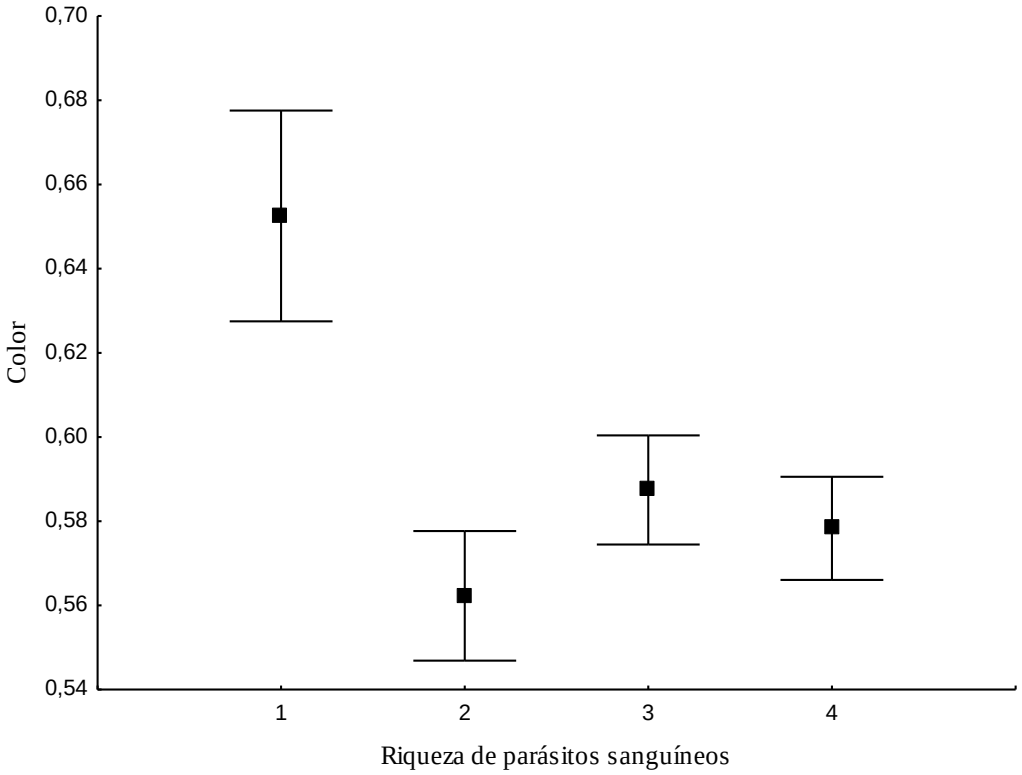

Figura 3. Variación en el color del plumaje del pecho en el herrerillo común en relación a la riqueza de parásitos sanguíneos. Las barras indican el error estándar.

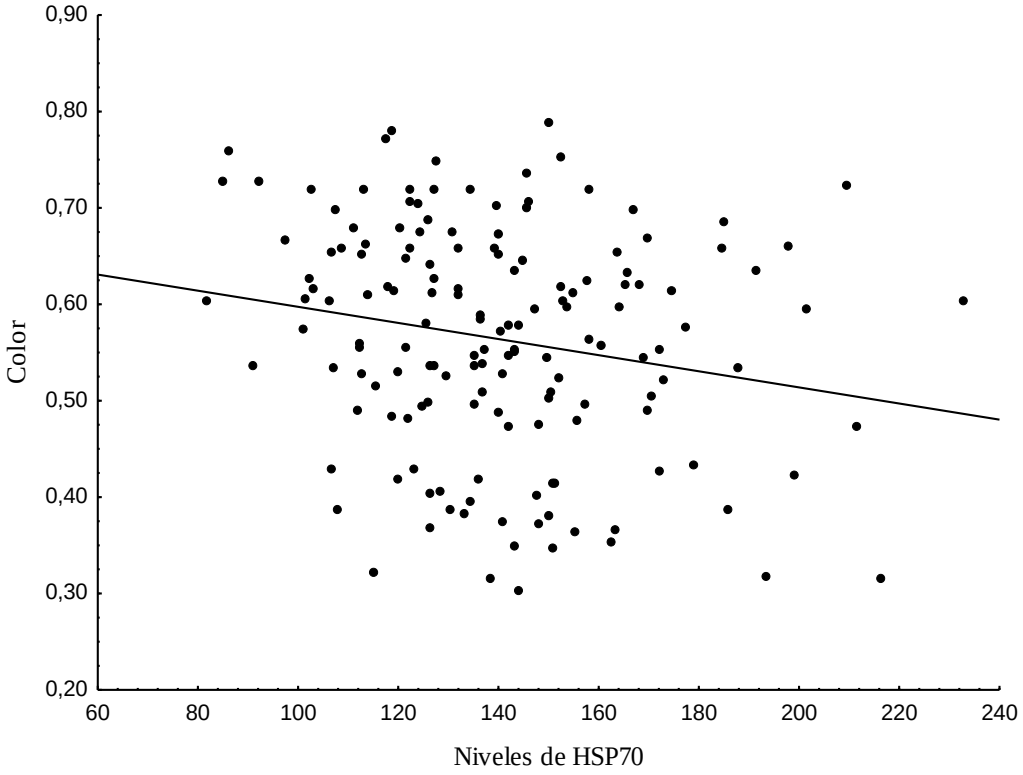

Figure 4. Relación entre los niveles de la proteína de estrés HSP70 y el color del plumaje en el herrerillo común.

Capítulo 3: Estudio de la selección de pareja en relación con la diversidad y presencia de alelos del MHC-I en el herrerillo común.

En este capítulo se investigó si la selección de pareja en una población de herrerillo común estaba relacionada con los alelos del MHC-I presente en los individuos. Al mismo tiempo se exploró si el color del plumaje del pecho jugaba algún papel en este proceso. Para llevar a cabo el estudio, se determinó tanto la diversidad del MHC-I de los miembros de cada pareja como el porcentaje de alelos compartidos en cada pareja. El resultado principal de los análisis mostró una relación significativamente positiva entre la diversidad alélica de los machos y la diversidad alélica de las hembras que forman cada pareja (Figura 5). Es decir, las hembras que presentaron haplotipos más diversos se emparejaron con los machos más diversos. Por otro lado, el estudio de la similitud de alelos indicó un cierto grado de diferencia dentro de cada pareja (es decir, en el porcentaje de alelos compartidos), pero cuando se compararon los emparejamientos con una distribución de emparejamientos al azar no se observaron diferencias significativas (Figura 6). Por lo tanto, no parece existir evidencias sobre el emparejamiento basado en la compatibilidad del MHC-I.

Por otra parte, no se detectó ninguna asociación entre la diversidad del MHC-I de los individuos con la intensidad del color del plumaje. Sin embargo, las hembras con menor intensidad de color se emparejaron con machos más diversos (Figura 7).

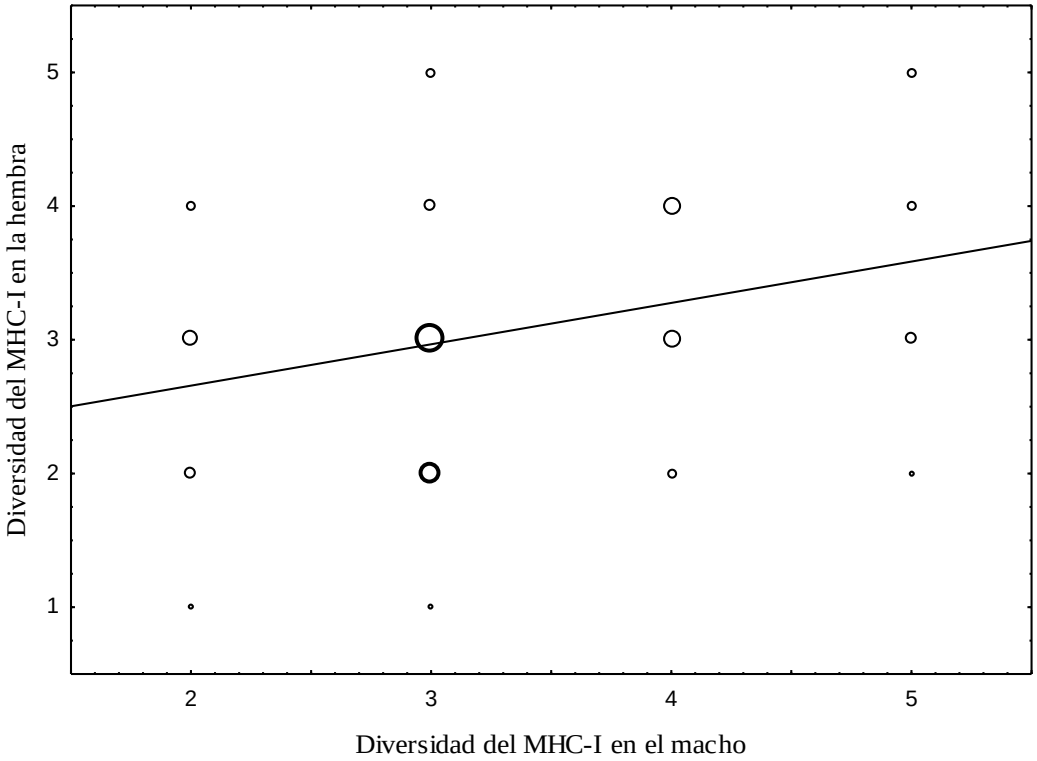

Figura 5. Asociación entre la diversidad del MHC-I entre machos y hembras. El tamaño del círculo es proporcional a la frecuencia observada.

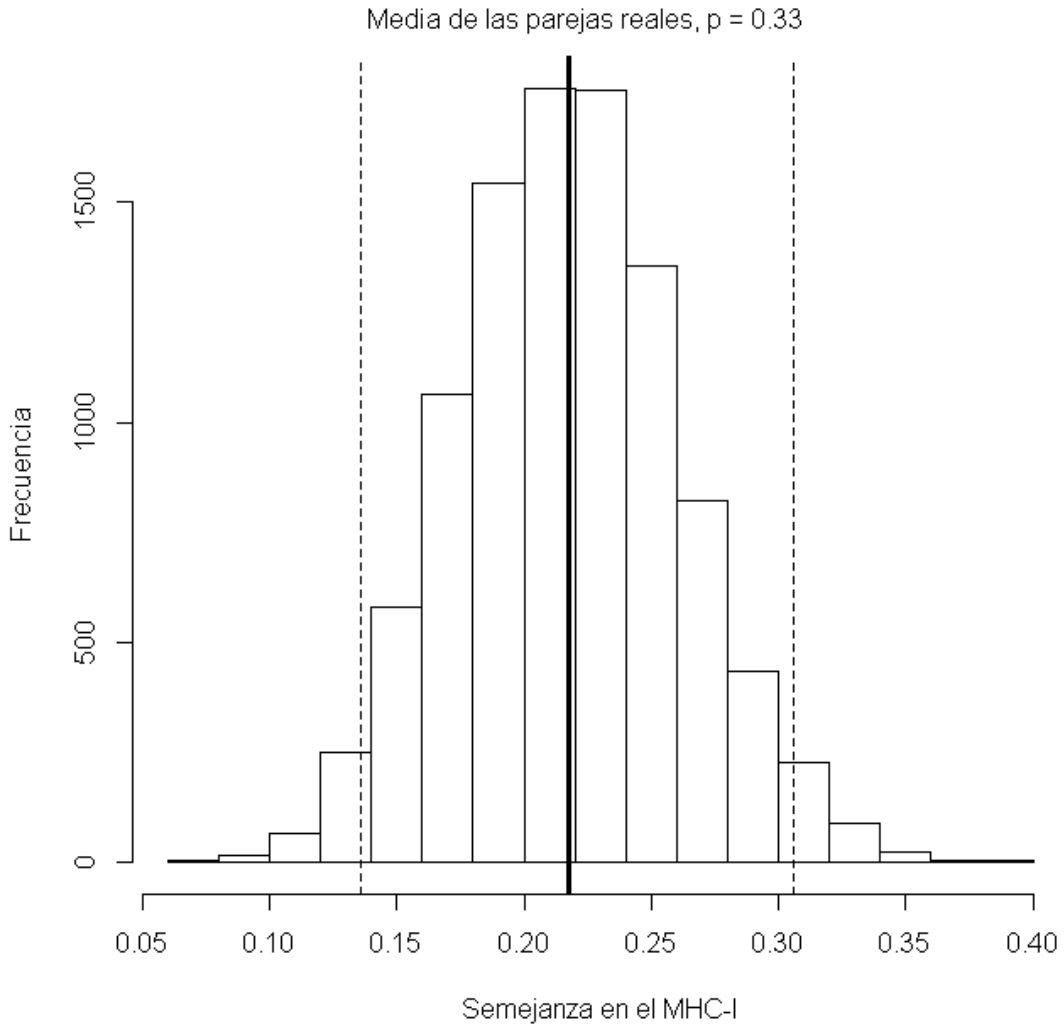

Figura 6. Comparación entre el valor medio de similitud del MHC-I de las parejas reales frente a una distribución de emparejamiento al azar. El valor medio de las parejas reales está representado por una línea vertical. Las líneas entrecortadas laterales representan el intervalo de confianza al 95 % de las parejas reales.

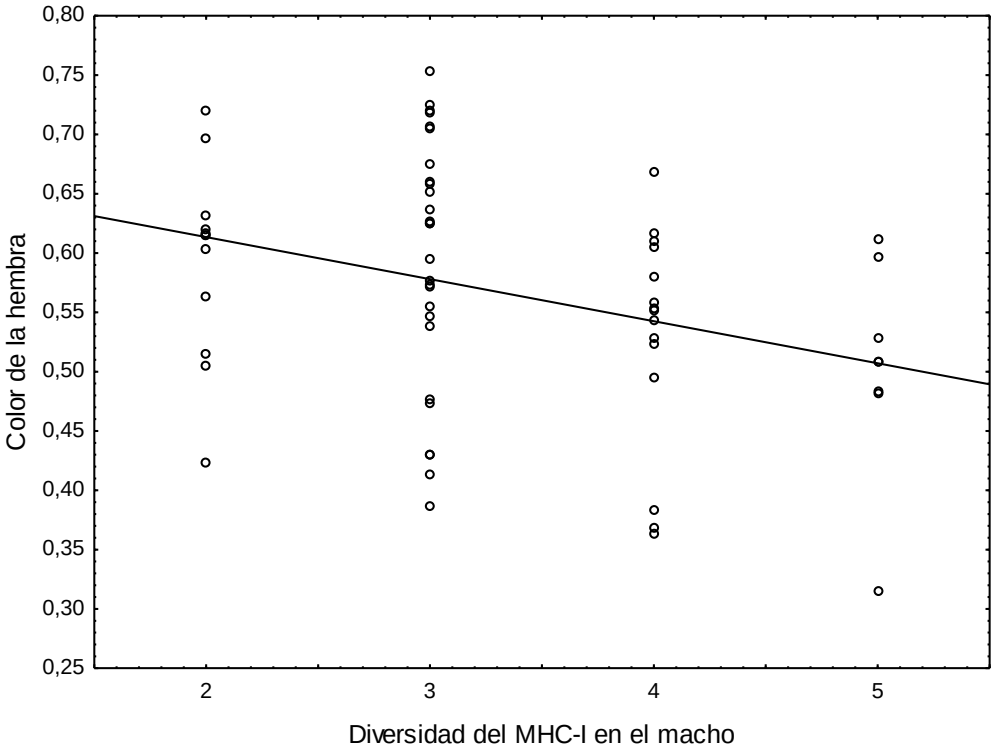

Figura 7. Asociación entre la diversidad del MHC-I y el color del plumaje de las hembras.

Capítulo 4*:* Determinación del polimorfismo y caracterización parcial de los alelos del MHC-II en el herrerillo común.

Los datos obtenidos por secuenciación y por análisis de restricción de 22 individuos de tres localidades europeas (España, Holanda y Suecia) sugieren que el número de genes del MHC II están presentes en una horquilla de 2 a 4 genes (Figura 8**)**. El número de loci detectado es relativamente bajo comparado con el que presentan otras especies de aves, variando entre 2 y 20 loci. Este resultado estuvo apoyado independientemente por las dos metodologías moleculares empleadas. Además, a partir de ADN y ARN se verificaron 217 secuencias entre las cuales se detectaron 13 alelos diferentes. Las secuencias no presentaron codones de terminación confirmando la ausencia de pseudogenes en la muestra.

Por otro lado, se realizó un análisis filogenético incluyendo los alelos detectados en el herrerillo común junto a los de otras especies de aves. Esta metodología se ha empleado anteriormente para establecer en que grupo se sitúan los alelos detectados *de novo*. Si un alelo queda posicionado en un determinado grupo se supone que todos los alelos del grupo son afines. Por lo tanto, si los alelos de un grupo han demostrado su funcionalidad en la presentación de antígenos, se espera que el nuevo alelo posicionado en este mismo grupo desempeñe la misma función. El resultado del análisis filogenético indicó que los alelos del herrerillo no son afines con los situados en el grupo principal, entre los cuales se encuentran alelos relacionados con la presentación de antígenos (Figura 9). Tampoco son afines a otros grupos de alelos, por lo que parece que corresponden a genes que hasta el momento no habían sido detectados.

Adicionalmente, investigamos la hipotética presencia de signos de selección en la región secuenciada. Para ello, empleamos dos metodologías para detectar selección positiva, una basada en el conocimiento *a priori* de las posiciones relacionadas con la unión peptidíca y la otra en modelos de selección independiente de dicho conocimiento *a priori*. Sólo de esta última forma logramos detectar posiciones aminoacídicas bajo selección positiva aunque ésta fue débil. Por otra parte, también se investigó la capacidad de retención alélica entre especies como señal de selección a lo largo de la evolución de los loci. Sin embargo, no se detectó ningún caso de evolución trans-especie en los alelos del herrerillo común.

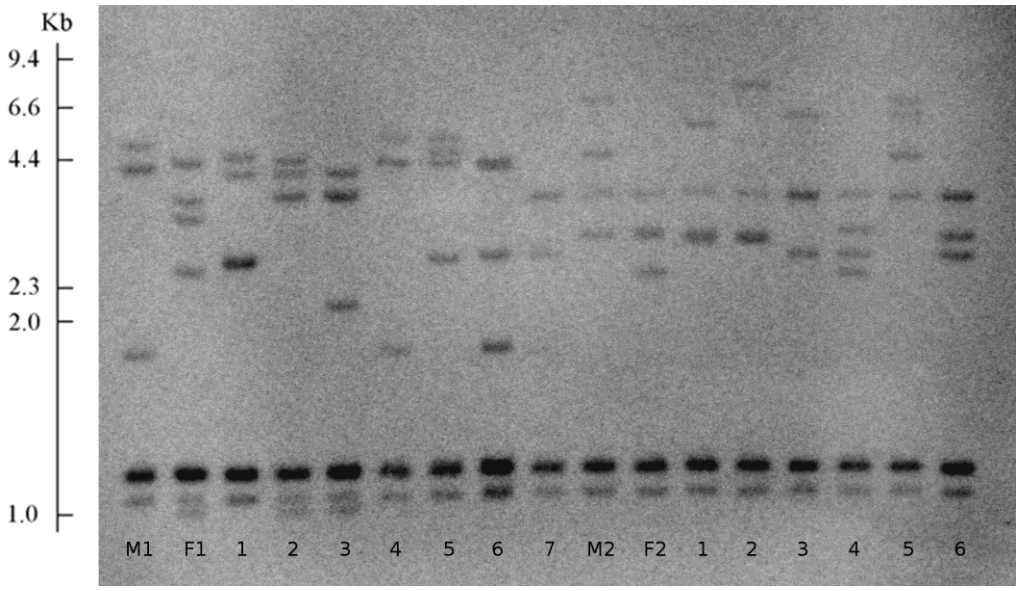

Figura 8. Patrones de RFLP con la enzima de restricción Pvu II tras hibridar con la sonda del exón 2 de MHC-II de un herrerillo común sobre 17 individuos de dos familias de aves suecas. M= madre, F= padre, los números corresponden a la descendencia. Familia 1 = individuos desde M1 a 6. Familia 2 = individuos desde M2 a 13. Se encontraron entre 4-7 fragmentos por individuo en el rango de tamaño entre 1-9 kb.

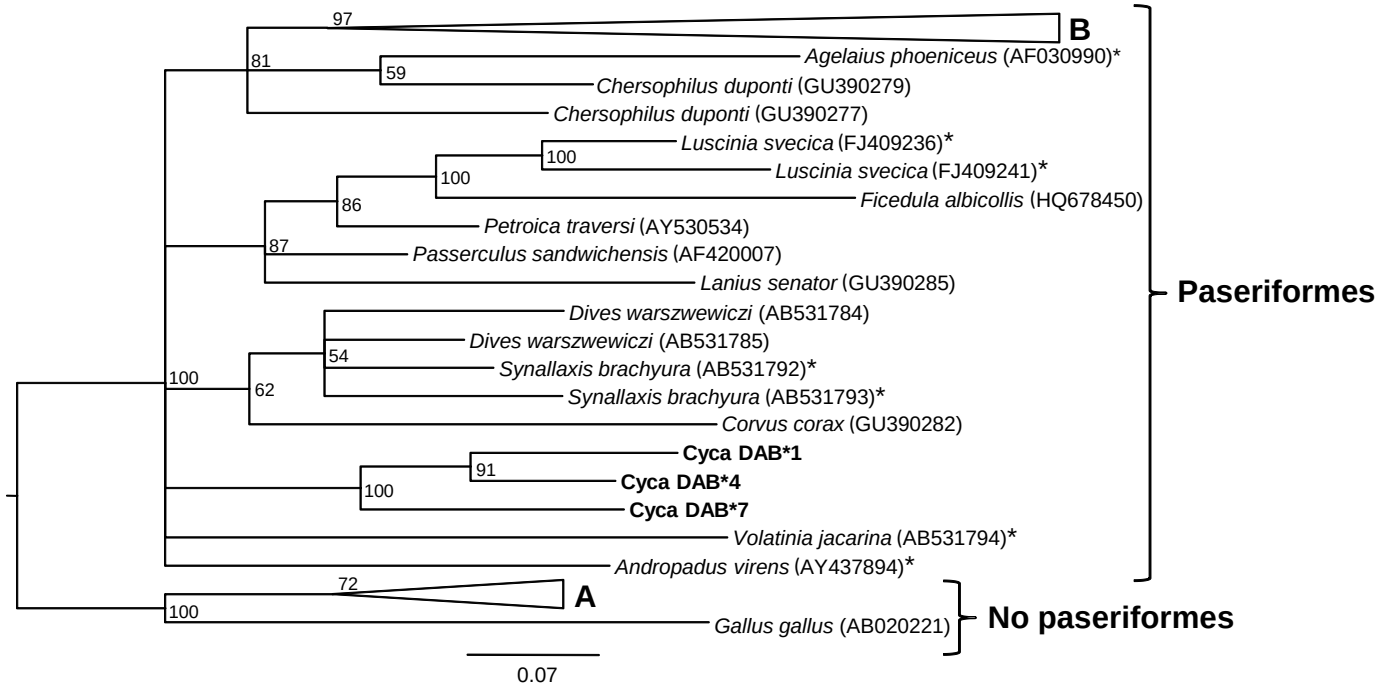

Figura 9. Filogenia bayesiana basada en secuencias correspondientes al exón 2 del MHC-II (159 pb) del herrerillo común junto a otros paseriformes y no paseriformes. Números en las ramas = probabilidad posterior. * = secuencias no funcionales. Códigos a continuación del nombre común = números de accesos del GenBank. A = grupo formado por secuencias obtenidas de diferentes aves paseriformes. B = grupo formado por secuencias obtenidas de diferentes aves no paseriformes.

DISCUSIÓN INTEGRADORA

En este trabajo de investigación se ha explorado el papel jugado por el MHC en diferentes aspectos relacionados con la ecología del herrerillo común. Actualmente, el MHC es considerado un área de estudio en el ámbito genético muy interesante dentro de la ecología evolutiva. Ello se debe al papel central que juega en la respuesta inmunitaria adaptativa, permitiendo estudiar cuestiones relacionadas con el parasitismo, la inmunología, el comportamiento y la evolución de las especies. Diferentes estudios han demostrado que la evolución de los genes del MHC esta mediada por la presión ejercida por los parásitos y la selección sexual. Por ello, en este trabajo se ha indagado sobre las relaciones existentes entre el MHC del herrerillo común, los parásitos sanguíneos y distintos aspectos relacionados con la selección sexual, como es el color del plumaje o la elección de pareja. En este sentido se exploró la relación entre la diversidad y presencia de alelos específicos del MHC-I y las prevalencia e intensidad de infección por diversas especies de parásitos sanguíneos para posteriormente comprobar el potencial efecto de dichos parásitos sobre un carácter sexual secundario y explorar la posible relación entre el MHC-I y la selección de pareja. Finalmente se realizó una caracterización parcial del MHC-II en el herrerillo común que abre la puerta a posteriores investigaciones para conocer el papel que desempeña en la relación entre estas aves y los parásitos que las infectan. A continuación se discuten con mayor profundidad estos resultados.

En primer lugar se exploraron las asociaciones entre el MHC-I con respecto a dos géneros de parásitos sanguíneos que se encuentran comúnmente infectando al herrerillo común, *Haemoproteus* y *Leucocytozoon*. Para ello, se determinó la prevalencia e intensidad de infección de ambos parásitos mediante técnicas microscópicas y moleculares. Los datos revelaron valores elevados de la prevalencia de ambos parásitos, así como una cierta variabilidad en la intensidad de infección entre los individuos. Aunque no se detectó ninguna asociación del MHC-I con la presencia de infección de ambos haemosporidios, la intensidad de infección por *Leucocytozoon* se asoció con alelos específicos o con la diversidad del MHC-I. Los individuos que portan ciertos alelos (Paca*104 o Paca*108) o los individuos con haplotipos del MHC-I más diversos fueron los que mostraron infecciones de mayor intensidad por este parásito. Este hecho podría indicar que los individuos que presentan estas características en su MHC-I son más susceptibles a este parásito. En un contexto evolutivo (carrera de armamentos), este parásito podría haber desarrollado un mecanismo de evasión del reconocimiento de las moléculas del MHC-I, ya que no hemos podido detectar alelos resistentes frente al mismo. Para realizar una correcta determinación del papel jugado por un determinado alelo en la susceptibilidad o resistencia de una infección es necesario incorporar en el análisis la presencia o ausencia de infección, y así comprobar si la presencia de un determinado alelo aumenta las probabilidades de estar infectado

(Westerdahl et al. 2012). En este caso, si los individuos que portan un determinado alelo suelen estar infectados entonces se podría concluir que el alelo determina susceptibilidad a la infección. En este trabajo no se ha encontrado ninguna asociación del MHC con la presencia de infección por *Leucocytozoon* aunque sí con la intensidad de la misma, corroborando la idea de la existencia de una cierta predisposición a padecer mayores parasitemias en los individuos que presentan ciertos alelos del MHC-I o que tienen una alta diversidad alélica. Sin embargo, es necesario documentar una relación clara entre estos alelos/diversidad y la presencia de infección para confirmar que confieren susceptibilidad a *Leucocytozoon*. Por otra parte, hay que tener en cuenta que estos alelos podrían conferir resistencia a otras enfermedades más virulentas (por ejemplo, víricas o bacterianas) que ejercen una mayor presión selectiva en la especie, siendo la susceptibilidad a *Leucocytozoon* un efecto secundario asumible. Si esta conjetura es real, nos indicaría que las infecciones por este haemosporidio serían relativamente benignas. De hecho, la gran prevalencia y baja parasitemia presente en la población indican un estado crónico de esta parasitosis en el herrerillo. Además, el aumento de la parasitemia solo ocurre en momentos críticos (ej: época reproductora), en los cuales existe un compromiso energético entre los diferentes sistemas fisiológicos y una alteración hormonal en el ave. El esfuerzo reproductor podría afectar los recursos destinados al mantenimiento del sistema inmunitario provocando el aumento estacional de la parasitemia. En otras poblaciones europeas de herrerillo también se ha observado un aumento en la intensidad de infección en el momento de la reproducción, lo que favorece la transmisión del parasito entre individuos susceptibles. Por otro lado, factores como el sexo y la edad se han relacionado con la probabilidad y nivel de parasitación en aves. Así las hembras y los individuos más viejos suelen tener una mayor inmunocompetencia que los machos o los individuos jóvenes (Martínez-de la Puente et al. 2007; Lobato et al. 2008). Debido a las diferentes estrategias reproductivas asociadas a cada sexo se espera observar diferencias sexuales en la intensidad de infección. En el caso de individuos jóvenes estos se enfrentan de diferente manera al evento reproductor, ya que debido a su menor experiencia la capacidad para defender un buen territorio o encontrar pareja también aumenta las probabilidades de infección. Además el sistema inmunitario de las aves jóvenes puede no estar todavía plenamente desarrollado o bien no haberse expuesto todavía a una cantidad suficiente de infecciones como para funcionar a pleno rendimiento por lo que podría mostrar una mayor susceptibilidad a la infección (Valkiūnas 2005). De hecho, en el presente estudio los machos jóvenes son el grupo de individuos que mostraron una mayor probabilidad de sufrir altas parasitemias.

Por otro lado, considerando el efecto perjudicial observado en esta población donde parásitos como *Haemoproteus* o *Leucocytozoon* reducen el éxito reproductor, no es extraño encontrar algún tipo de asociación entre la susceptibilidad y algún alelo de MHC. Debido a la naturaleza multigénica del MHC, existe la posibilidad de que individuos portadores de un alelo resistente a un determinado parásito presenten al mismo tiempo alelos asociados con

susceptibilidad hacia otros. Esto se debe a que los beneficios obtenidos al tener un alelo resistente a un parásito compensan el efecto perjudicial de albergar al mismo tiempo un alelo susceptible. Esta situación se espera en individuos infectados por diferentes especies de parásitos que varían en su virulencia. En poblaciones naturales de aves se ha observado que el mismo alelo puede asociarse de manera antagónica dependiendo de la comunidad de parásitos que afecta al hospedador, así un mismo alelo puede estar relacionado con susceptibilidad en una población y con resistencia en otra (Loiseau et al. 2008). Esto pone en evidencia la importancia de la heterogeneidad de la distribución de las comunidades de parásitos, ya sea esta local o temporal. Por lo tanto, en los estudios que aborden las relaciones entre los parásitos y el MHC sería importante incluir toda la parasitofauna del hospedador ya que se espera un efecto conjunto de todos ellos.

En esta tesis también se ha investigado el posible efecto ejercido por los parásitos sanguíneos en la selección de alelos específicos a lo largo del tiempo. En una determinada población, se espera observar un cambio en las frecuencias alélicas debido a variaciones en la presión selectiva debida a los parásitos. En las poblaciones naturales los parásitos se distribuyen heterogéneamente y sufren variaciones temporales debido a diferentes factores como pueden ser las condiciones ambientales (Merino y Potti 1996; Martínez-de la Puente et al. 2009; Merino y Martínez 2011). En este sentido, hemos explorado el cambio en las frecuencias alélicas del MHC-I en dos años consecutivos. Los resultados indican que el alelo Paca UA*104, anteriormente relacionado con parasitemias elevadas por *Leucocytozoon*, aumentó en frecuencia en el grupo constituido por los individuos recapturados en el segundo año. Así, el cambio en la frecuencia de este alelo, asociado a susceptibilidad, apoyaría la hipótesis de la presencia de una cierta tolerancia a este parásito en la población, aún habiéndose observado ciertos efectos perjudiciales con anterioridad (Merino et al. 2000). Por otro lado, no detectamos aumentos en las frecuencias de alelos minoritarios en la población en el año siguiente. Los alelos raros son la base de la "hipótesis de alelos poco frecuentes". Estos alelos podrían seleccionarse en algún momento evolutivo si determinasen resistencia ante ciertos linajes de parásitos que escapan al reconociendo de las moléculas de MHC más comunes. De esta manera, se esperaría un aumento en su frecuencia a lo largo del tiempo. Los cambios en las frecuencias alélicas pueden suceder en cortos periodos de tiempo, incluso de un año para otro (Westerdahl et al. 2004), así el resultado observado puede deberse perfectamente al efecto del parasitismo. Este tipo de variaciones en las frecuencias alélicas se han observado en diferentes vertebrados, apoyando el papel que juegan los parásitos en su evolución (Spurgin y Richardson 2010).

En esta tesis también se ha abordado el estudio del efecto del parasitismo sobre la expresión de ornamentos. La presión selectiva ejercida por los parásitos sobre sus hospedadores se ha relacionado con la expresión de ciertos ornamentos en las aves, como es el color del plumaje (Hamilton y Zuk 1982; Møller 1994). El color de las aves está considerado como un

ornamento costoso de mantener, por ello un plumaje lustroso sería reflejo de una buena condición general. En concreto, analizamos la asociación entre la riqueza de parásitos y el color del plumaje del pecho. El color amarillo del herrerillo común es un buen indicador de la condición general del individuo puesto que, al depender de la ingesta de alimentos ricos en carotenos, un plumaje intenso y brillante indicaría una alta capacidad para encontrar alimento. Al ser el color una señal directa de la capacidad individual para conseguir alimento, las hembras podrían utilizar el color del plumaje como indicador de la calidad del macho. En especies monógamas, como es el caso del herrerillo común, en las que es necesaria la cooperación del macho para sacar adelante a la descendencia, sería importante para la hembra elegir un macho que tenga capacidad para proporcionar alimento. Basando su elección en este ornamento, la hembra obtendría información valiosa sobre la calidad del macho. Por otra parte, como los carotenos son necesarios para el correcto funcionamiento del sistema inmune (Svensson y Wong 2011), una infección podría desencadenar una mayor demanda de esta molécula para mantener la inmunocompetencia en detrimento de su depósito en las plumas. Por lo tanto, los individuos más parasitados mostrarían un color más pálido que los individuos menos infectados. En este estudio se ha observado este patrón, ya que los individuos con una mayor riqueza de parásitos fueron también los más pálidos. En este sentido, se evidencia el papel que juegan los parásitos en el desarrollo de las señales honestas. Adicionalmente, las proteínas de estrés también parecen estar involucradas en esta relación. Los individuos que exhibían colores más intensos presentaban menores niveles de proteínas de estrés. Como los niveles elevados de las proteínas de estrés están relacionados con el aumento de los procesos metabólicos, los individuos con menos brillo debido a la infección podrían presentar elevados los procesos anabólicos y catabólicos relacionados con el funcionamiento del sistema inmunitario.

Sobre la base del anterior resultado, se investigó la relación entre el MHC-I y la elección de pareja, observándose una asociación positiva entre la diversidad alélica del macho y la de la hembra. Un emparejamiento en función de la diversidad del MHC apoyaría la "hipótesis de los buenos genes". Esta hipótesis sugiere que el emparejamiento entre individuos que presentan una alta diversidad del MHC, o alelos ventajosos, conferiría beneficios genéticos indirectos a la descendencia por el aumento de la heterocigosidad de la descendencia (Brown y Eklund 1994; Neff y Pitcher 2005). Este hecho incrementaría la probabilidad en la detección y eliminación de los parásitos, ya que aumenta la probabilidad de albergar alelos ventajosos. El emparejamiento basado en la diversidad alélica del MHC se ha observado en diferentes vertebrados incluidas las aves. Sin embargo, la evolución dependiente de la frecuencia alélica también ha podido jugar un papel relevante en las interacciones parasitarias, ya que la presencia de un solo alelo ha podido conferir resistencia a enfermedades virulentas. Por otra parte, la selección de pareja se ha relacionado con una optimización de la diversidad alélica en la descendencia y no tanto en una alta diversidad. De hecho, un número óptimo de alelos también

se ha relacionado con una mayor resistencia a enfermedades (Reusch et al. 2001; Wegner et al. 2003). Por lo tanto, las hembras seleccionarían a los machos con una diversidad alélica, que en relación a la suya propia, confiera un número óptimo de alelos a la descendencia. Es decir, la presencia de un número intermedio, óptimo de alelos, sería en este caso la opción más ventajosa para los individuos.

Sin embargo, los resultados obtenidos en el presente estudio no apoyan la hipótesis de que las hembras seleccionen parejas con haplotipos diferentes a los que ellas presentan. De hecho, existen evidencias de que los individuos de la pareja, aun teniendo cierto grado de diferenciación en los haplotipos del MHC, muestran un emparejamiento similar al de una población con emparejamiento al azar. En este sentido, el emparejamiento de individuos que comparten los mismos alelos puede ser una estrategia para asegurar en la descendencia la presencia de alelos que son funcionales en el reconocimiento de parásitos actuales (Bonneaud et al. 2006). No obstante, al mismo tiempo también aumentaría el riesgo de que nuevos antígenos escapen al reconocimiento de los alelos más comunes. Por lo tanto, contar con alelos potencialmente ventajosos sería determinante en la lucha contra nuevas variantes de los parásitos. Esta posibilidad se ha observado en la población objeto de estudio, detectándose varios alelos en baja frecuencia que podrían ser seleccionados ante la aparición de un nuevo linaje o parásito. Sin embargo, ninguno de estos alelos aumentó en frecuencia en el periodo de un año. Este dato indica que estos alelos no parecen estar seleccionados por ningún parásito o que éste no ejerce una presión selectiva demasiado intensa.

En relación al color del pecho y la diversidad alélica del MHC-I, los análisis demostraron la ausencia de relación entre estas dos variables a pesar de que el color del pecho está afectado por la riqueza de parásitos sanguíneos. Si la diversidad alélica del MHC se hubiese asociado positivamente con la resistencia a la infección, podríamos haber esperado la existencia de alguna relación significativa entre las variables. Por el contrario, los análisis mostraron que las hembras menos coloridas se emparejaron con los machos que presentaban mayor diversidad alélica del MHC-I. Debido a que el color está relacionado con la carga de parásitos, este apareamiento podría interpretarse como un intento por parte de las hembras de peor calidad de emparejarse con machos de mayor calidad si es que esta se ve reflejada en una mayor diversidad en el MHC. Sin embargo, la asociación entre diversidad del MHC y susceptibilidad a *Leucocytozoon* hacen dudar de esa asociación entre calidad y diversidad en MHC. No obstante, la intensidad de infección es bastante baja en la población, pudiendo indicar este hecho que los individuos más diversos sean en realidad de alta calidad, ya que presentan en cierta medida tolerancia a las infecciones crónicas por este parásito. En este sentido la relación encontrada entre la susceptibilidad a *Leucocytozoon* y la mayor diversidad de MHC puede ser en realidad indicadora de una cierta resistencia a la presencia del parásito de forma que los individuos que en los individuos menos diversos la infección sea letal y no se capturen individuos con baja

diversidad y elevadas intensidades de infección. Abundando en la relación MHC / selección de pareja, la diversidad del MHC se ha asociado con la presencia de copulas extra-pareja en el herrerillo común. Así las hembras de más calidad son más promiscuas cuando su pareja social tiene baja calidad genética (Dreiss et al. 2008). Finalmente, la ausencia de relación entre la diversidad alélica y el color del pecho podría indicar la existencia de otros caracteres sexuales secundarios relacionados con la selección de pareja. En este sentido, la reflectancia de la luz ultravioleta en la corona de la cabeza podría jugar un papel señalizador importante para las hembras a la hora de elegir pareja (Sheldon et al. 1999).

A lo largo de este trabajo se ha indagado sobre el papel jugado por los genes del MHC-I en distintos aspectos ecológicos del herrerillo común. El MHC-I esta principalmente involucrado en la presentación de antígenos derivados de parásitos intracelulares. Sin embargo, no hay que descartar una implicación directa del MHC-II en los aspectos ecológicos analizados, tanto en la elección de pareja como en la lucha frente a los parásitos intracelulares mencionados. Por ello, decidimos caracterizar la diversidad alélica del MHC-II como paso previo a la realización de futuros estudios que determinen el papel jugado por este complejo proteico en los aspectos ecológicos comentados en esta tesis. Además, como los genes del MHC-II codifican proteínas principalmente especializadas en la presentación de antígenos extracelulares, también se podrá explorar el papel jugado por algunos parásitos sanguíneos extracelulares detectados en el herrerillo común como *Trypanosoma* o microfilarias.

La caracterización del MHC-II indica que este complejo está formado por un número de genes que varía entre cuatro y siete, variación debida a los diferentes resultados obtenidos al aplicar distintas técnicas de restricción y secuenciación molecular. Además de la determinación del número de genes presentes, también se inspeccionó la diversidad alélica, mostrando valores entre medios y bajos respecto a los hallados en otras especies de aves. Debido a las presiones selectivas sufridas durante el proceso evolutivo, cada especie tiene su particularidad en el número y diversidad genética. En el caso del herrerillo común se ha propuesto que la confinación sufrida por esta especie en refugios glaciares ha podido ser el agente principal que determinó su configuración genética actual (Kvist et al. 2004). Teniendo en cuenta esta hipótesis, el MHC habría sufrido una pérdida de variabilidad que se reflejaría en los valores actuales de la diversidad alélica presentes en esta especie, siendo ésta baja o intermedia. Este hecho, es decir, la baja diversidad alélica respecto a otras especies de aves paseriformes, también ha sido confirmada para los genes de clase I (Schut et al. 2011). Aunque los resultados obtenidos apuntan a un MHC-II no muy diverso, las limitaciones técnicas debidas al diseño de los cebadores utilizados en la obtención de las secuencias moleculares podrían infravalorar la diversidad alélica presente en esta especie. Sin embargo, los estudios realizados hasta el momento sobre la diversidad del MHC en otras especies no están exentos de problemas similares.

Por otra parte, el análisis filogenético de los alelos del MHC-II detectados en el herrerillo común indica la existencia de una agrupación particular respecto a los alelos de otras especies. En concreto, el clado formado por los alelos del herrerillo común aparece en una posición basal respecto a la gran mayoría de alelos caracterizados en otras especies. Este hecho podría indicar que el herrerillo común ha evolucionado en un contexto diferente al resto de estas especies o que los alelos caracterizados en esta tesis pertenecen a un grupo particular aun no detectado en el resto de especies por cuestiones metodológicas. El hallazgo de un grupo de alelos en el pinzón cebra *Taeniopygia guttata* con características filogenéticas similares al clado formado por los alelos del herrerillo común podría indicar que la metodología empleada en la detección de los mismos juega un papel relevante en este asunto. Alternativamente, como en otras aves los alelos del MHC-II se encuentran agrupados en dos clados principales con diferente grado de polimorfismo, los alelos caracterizados en este estudio podrían estar relacionados con el clado que presenta alelos con un polimorfismo bajo no relacionados con la presentación de antígenos. Aunque los análisis realizados con los alelos del herrerillo común demostraron la presencia de procesos selectivos relacionados con el MHC-II, la magnitud de la selección fue débil. Sin embargo, la obtención de las secuencias alélicas a partir de ARN y la utilización de cebadores diseñados para amplificar genes funcionales implicados en la presentación de antígenos, sugiere que los alelos detectados en el presente estudio son funcionales.

En conclusión, los alelos del MHC-I parecen tener un papel relevante en la susceptibilidad del herrerillo común hacia las infecciones provocadas por el haemosporidio *Leucocytozoon*, así como en la elección de pareja. Sin embargo, las limitaciones metodológicas actuales a la hora de detectar toda la diversidad alélica nos hacen sospechar sobre la existencia de otros alelos implicados en fenómenos de resistencia a determinadas infecciones. En este sentido, la caracterización parcial del MHC-II, llevada a cabo en esta tesis, podría ayudar a desentrañar el papel jugado por este complejo en el proceso coevolutivo entre parásitos y hospedadores.

CONCLUSIONES

- El herrerillo común presenta alelos del MHC-I que confieren susceptibilidad a las infecciones causadas por el parásito *Leucocytozoon*. Los individuos con mayor diversidad alélica o los que portan los alelos Paca UA*104 o Paca UA*108 presentaron una mayor parasitemia por este parásito.

- Los individuos de herrerillo común con mayor riqueza de parásitos sanguíneos presentaron un color amarillo del pecho más pálido.

- La diversidad alélica del MHC-I juega un papel en el emparejamiento del herrerillo común, ya que las hembras suelen seleccionar machos con una diversidad alélica similar a la que ellas presentan.

- En la población bajo estudio de herrerillo común la selección de pareja no está relacionada con la compatibilidad alélica, ya que el porcentaje de alelos compartidos por la pareja no difiere del de un emparejamiento al azar.

- En el herrerillo común el número de genes del MHC-II se situó entre cuatro y siete, siendo la diversidad alélica media/baja respecto a la presentada por otras especies de aves.

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Capítulo 1

Asociaciones entre los genes del MHC-I e infecciones de parásitos sanguíneos en una población de herrerillo común del centro España

Los genes del complejo principal de histocompatibilidad (MHC) juegan un papel fundamental en la respuesta inmune adaptativa contra los parásitos. En vertebrados, se han observado asociaciones ya sea de resistencia o susceptibilidad entre alelos de MHC e infecciones de parásitos, incluyendo las aves, ya sea en estudios en cautividad o en poblaciones naturales. En este trabajo, hemos investigado tales asociaciones en una población natural de una ave paseriforme, el herrerillo común *Cyanistes caeruleus*, en el centro de España. La población está adversamente afectada por una comunidad diversa de parásitos sanguíneos incluyendo parásitos del genero *Haemoproteus* y *Leucocytozoon*. Los genes del MHC-I fueron analizados para investigar las asociaciones entre la diversidad del MHC y/o alelos específicos con la presencia e intensidad de infección de ambos parásitos. Los análisis estadísticos pusieron de manifiesto que los individuos con mayor diversidad de MHC o aquellos que portaban los alelos específicos Paca UA*104 o Paca UA*108 tuvieron mayor intensidad de infección por *Leucocytozoon*, aunque no se detectó una relación significativa con la prevalencia de este parásito. Paradójicamente, los individuos recapturados un año después mostraron una elevada diversidad alélica y presentaron el alelo Paca UA*104 con mayor frecuencia, indicando que la asociación con *Leucocytozoon* no tuvo consecuencias deletéreas. Además los machos o los jóvenes portadores del los alelos Paca UA*104 o Paca UA*108 fueron los que tuvieron mayor intensidad de infección por dicho parásito. No se observaron asociaciones significativas con la prevalencia o la intensidad de infección por *Haemoproteus*. En conclusión, (i) la relación entre MHC-I y la susceptibilidad a *Leucocytozoon* sería probablemente un efecto colateral de la selección de alelos dirigida bajo la presión de un agente patógeno virulento desconocido y (ii) otros factores como el sexo o la edad jugarían un importante papel a la hora de determinar la patogenicidad del parásito.

Associations among MHC-I genes and blood parasites infections in a blue tit population from central Spain

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Major histocompatibility complex (MHC) genes are central for the adaptive immune response against parasites. Several resistance/susceptibility associations between specific MHC alleles and parasites have been found in vertebrates, including birds, both from captivity and wild populations. Here, we investigate such associations in a natural population of a passerine bird, the blue tit *Cyanistes caerulue*s, in central Spain. A diverse community of blood parasites including *Haemoproteus* and *Leucocytozoon* species adversely affects this bird population. MHC-I genes were analysed to search for associations among MHC diversity and/or specific MHC alleles, and parasite prevalence and intensity of infection. The statistical analyses revealed that individuals with elevated MHC diversity or carrying specific alleles (Paca UA*104 or Paca UA*108) had higher intensity of infection by *Leucocytozoon* but no significant relationship was detected with *Leucocytozoon* prevalence. Paradoxically, the group of individuals recruited one year later had higher allelic diversity and presented the allele Paca UA*104 in higher frequency, indicating that the association with *Leucocytozoon* had not deleterious consequences. In addition, the males or young birds with Paca UA*104 or Paca UA*108 alleles were more prone to suffer from high intensity of infection. No significant associations with prevalence or intensity of infection were observed for *Haemoproteus*. In conclusion, (i) the relationship between MHC-I and susceptibility to *Leucocytozoon* would be a side effect of the allele selection conducted by an unknown virulent pathogen and (ii) other factors such as sex and age would play an important role in determining pathogenicity.

Introduction

In vertebrates, the major histocompatibility complex (MHC) constitutes a genetic region with genes involved in the immune response. In this region, the MHC class I (MHC-I) genes code for proteins that present parasite-derived peptides to effectors cells of the immune system (Zinkernagel, 1979; Klein, 1986; Hughes & Yeager, 1998; Sommer, 2005). If the presented peptide is recognised to be parasite-derived, the adaptive immune response is triggered (Hughes & Yeager, 1998). Different MHC molecules bind a diverse range of peptides, therefore chances on antigen recognition will depend on individual MHC diversity (defined as the number of MHC alleles) or specific MHC alleles (Altuvia & Margalit, 2004). Thus, a form of balancing selection from parasites would maintain an elevated MHC allele polymorphism to counteract parasites infections (Doherty & Zinkernagel, 1975; Takahata & Nei, 1990; Hedrick, 1998; Bernatchez & Landry, 2003; Milinski, 2006; Spurgin & Richardson, 2010). The alternation of parasites lineages or changes in parasites prevalence/intensity would select for different MHC alleles in host populations, turning an allele to common if it confer advantages to hosts and a common allele into rare when loosing parasite antigen recognition (Parham & Ohta, 1996). Thus, parasites are expected to affect MHC allele frequencies over time (Westerdahl *et al.*, 2004; Spurgin & Richardson, 2010). Therefore, antigen recognition by MHC molecules constitutes an evolutionary target to be avoided by parasites (Hedrick, 2002; Spurgin & Richardson, 2010).

Associations between MHC alleles and resistance/susceptibility to parasites have been found in vertebrates, including both correlational/experimental and captive/free-living population studies (Piertney & Oliver, 2006; Westerdahl, 2007; Spurgin & Richardson, 2010). In some cases, strong associations involving MHC diversity or specific MHC alleles have been found, whereas in others, only weak associations or no such associations were found (see Jeffery & Bangham, 2000). Contrasting outcomes have been explained as the result of different approaches such as the inclusion of prevalence and/or intensity of infection (Westerdahl *et al.*, 2012) or spatial/temporal host-parasite dynamics (Hedrick, 2002; Loiseau *et al.*, 2009). In birds, strong evidence of associations among parasites and avian MHC genes have been observed

mainly in studies in non-passerines chickens *Gallus gallus domesticus* and other galliforms, involving resistance and susceptibility for example to infection by Marek's disease and Rous sarcoma virus (Zekarias *et al.*, 2002). Moreover, MHC genes appear as being determinant in fighting off infections against bacteria, coccidia and helminths (Schou *et al.*, 2010). In wild populations, the effects from parasites have been investigated in passerines including Haemosporidia parasites (Atkinson *et al.*, 2008). Thus, in great reed warblers *Acrocephalus arundinaceus*, an association between MHC-I and the infection by an avian *Plasmodium* lineage was found, suggesting that either a large number and/or a specific MHC allele conferred protection against a lethal infection (Westerdahl *et al.*, 2005, 2012). In house sparrows *Passer domesticus*, some MHC-I alleles were associated with both resistance and susceptibility to malaria infections (Bonneaud *et al.*, 2006; Loiseau *et al.*, 2008, 2011) and antagonistic effects in terms of susceptibility/resistance towards two malaria parasites were also found (Loiseau *et al.*, 2011). In great tits *Parus major*, it was found support of a role from MHC-I genes on survival rates and differential associations with lifetime reproductive success, as well as on infections by Haemosporidia parasites (Sepil *et al.*, 2012, 2013). At the same time, in collared flycatchers *Ficedula albicollis*, MHC-II diversity was negatively associated with probability of infection by malaria infections although its influence on lifetime reproductive success was weak (Radwan *et al.*, 2012).

In this study, we look for associations between MHC-I genes and malaria-like parasites *Haemoproteus* and *Leucocytozoon* in a blue tit population from central Spain. Both parasites are protozoan members of the phylum Apicomplexa (order Haemosporida). They are transmitted mainly by haematophagous biting midges in the genus *Culicoides* (Ceratopogonidae) and black flies (Simuliidae), respectively (Valkiūnas, 2005; Atkinson *et al.*, 2008). These parasites have detrimental effects on blue tits fitness and their pathogenicity has been supported by experimental manipulation of parasite loads (Merino *et al.*, 2000; Knowles *et al.*, 2010; Martínez-de la Puente *et al.*, 2010b). The transmission is similar in both parasites. It starts when the arthropod vector feeds on host's blood and the infecting stages (sporozoites) are inoculated into blood stream, reaching different cells types. After asexual reproduction a new parasite stage

(merozoites) penetrates circulating red cells (Atkinson *et al.*, 2008). MHC-I molecules are expressed on all body cells surface, including red blood cells. Therefore the antigen presentation would be successful if host bears an MHC molecule that recognise that parasite-derived antigens. Merozoites are finally transformed in gametocytes and sexual reproduction of the parasite is produced in the gut of the insect vector when feed on host blood. In central Spain the transmission occurs mainly during spring and summer, coinciding with hosts breeding season. Flies are affected by environmental factors like wind speed and temperature, with effects on the prevalence and abundance of parasites (Martínez-de la Puente *et al.*, 2009a). Thus, it is expected that a MHC allele polymorphism will be maintained to provide enough allelic variability to respond to local or temporal changes in parasites. In the present study, we have specifically obtained data on prevalence and intensity of infection of *Haemoproteus* and *Leucocytozoon* to determine the extant associations between parasitism / MHC-I and the hypothetical change in MHC allele frequencies from recaptured individuals one year later.

Material and methods

During the spring of 2004 and 2005 we studied a blue tit population breeding in nest-boxes in a Pyrenean oak forest in Valsaín (Segovia, central Spain, 40º53' N, 4º01' W). Nest-boxes were periodically checked to record hatching date and when chicks were three days old, adults were trapped in nest-boxes, ringed (if necessary), and classified as young (second calendar year, 2Y) or adults (third calendar year or older, 3Y+) based on plumage characteristics (Svensson, 2011). At the moment of capture a blood sample (50 - 100 μL) was obtained by brachial puncture. One drop of blood was immediately smeared on a slide, air dried and, later in the laboratory, fixed with absolute ethanol within the same day. Blood smears were stained with Giemsa stain (1/10 v/v) for 45 min at the end of the birds breeding season. The rest of blood was stored in a cold box during the day and at the laboratory frozen at -80º C until posterior molecular analysis.

Parasite detection

Blood smears were examined by microscopy to determine blood parasite's intensity of infection as the number of parasites per 2000 erythrocytes for *Haemoproteu*s *majoris* and *Leucocytozoon* sp. (see Merino et al. 1997). All slides were examined by the same person (J.M.P.). Prevalence was first determined by using direct microscopy and when a negative result for any of the parasite genera was obtained a complementary molecular polymerase chain reaction (PCR) analysis was performed. For this analysis a fragment of the cytochrome b gene of parasites was amplified using the primers and PCR conditions described in del Cerro et al. (2010). To that end, total genomic DNA was isolated from each blood sample by using the UltraClean DNA BloodSpin kit (MO BIO laboratories, Inc., California) following manufacturer's protocol. Individuals were identified as infected or not infected by observing the bands of appropriate size on an ethidium bromide-stained 2% agarose gel.

Screening of MHC-I alleles

We investigate blue tit MHC-I exon 3 which encodes for the most variable part of the MHC class I molecule, involved in the antigen presentation of intracellular parasites to T cells. An individual screening of MHC-I alleles was done by applying the Reference Strand Conformation Analysis method (RSCA) adapted to the blue tit (Schut, 2012). This method use a fluorescein-labelled reference (FLR) strand primer to detect blue tit MHC alleles from an individual with up to one pair base resolution. The method allows determining the MHC diversity and specifying MHC alleles per individual inspecting the peak pattern in a molecular sequencer (Argüello *et al.*, 1998). Since MHC genes are expressed codominantly, individuals with several MHC alleles are probably more heterozygotes than individuals with a low number of MHC alleles. This measure of MHC allelic diversity is approximate, because alleles constitute a subsample of MHC-I alleles detected in the blue tit (Schut *et al.*, 2011). This subsample corresponded to the most diverse cluster of sequences found in this species, those with signals of balancing selection. Therefore the method is detecting the diversity of the most likely MHC alleles involved in peptide presentation. This method has been applied to several avian species (Hale *et al.*, 2009; Brouwer *et al.*, 2010; Strand & Höglund, 2011) and constitutes

a reliable method to measure MHC diversity (see also Babik, 2010). Although we refer to "MHC alleles", actually they did not span the entire exon 3 and the assignment to specific loci was not possible. The FLRs were tested before genotyping to ensure that MHC alleles were discriminated perfectly. The whole method is detailed in Schut (2012). Samples were analysed in an ABI 310 capillary sequencer (Applied Biosystems), using a non-denaturing polymer. GeneMapper 4.0 software was used to visualize peak patterns. Each individual had his own pattern of peaks allowing us to determine MHC allele diversity and MHC allele composition. To control for reliability and repeatability among samples a control individual was included in each run. All individuals were screened once with FLR-GT6 and FLR-GT11. The peak pattern obtained from FLR-GT11 did not work for 34 individuals, thus we decided to include in the analyses only the MHC alleles detected with the primer FLR-GT6. A Spearman's correlation test was performed with the individuals where both FLRs worked, confirming that the MHC diversity detected by using only FLR-GT6 was correlated with the diversity detected by using both FLRs ($R = 0.86$, $N = 42$, $P < 0.0001$).

Statistical analysis

Generalised linear models (GLZ) were used to study associations between MHC genes and the prevalence and intensity of infection by different parasites. Associations among MHC diversity and prevalence or intensity of infection were explored by using a logistic regression (using prevalence of infection as dependent variable) or assuming a Poisson distribution with a log-link function (using intensity of infection as dependent variable) with MHC diversity, bird sex and age as factors. In both analyses, due to the presence of few cases in the factor level MHC diversity=1 (four cases) the MHC diversity variable was categorized by merging MHC diversity categories 1 and 2. On the other hand, associations among specific MHC alleles and prevalence or intensity of infection were explored as indicated for MHC diversity but using specific MHC alleles instead of MHC diversity as factor. Main effects and interactions among factors were included in all models. Sex and age were included as factors as it is well known that these variables can influence immunological responses and susceptibility to infections by

blood parasites (Valkiūnas, 2005; Martínez-de la Puente *et al.*, 2007; Atkinson *et al.*, 2008; Lobato *et al.*, 2008). Model selection was performed automatically by AIC stepwise followed by fine tuning by dropping variables from the model by mean of a likelihood ratio test (Zuur *et al.*, 2009). In all models, when over-dispersion was observed, then a quasi-Poisson function or an alternative negative binomial function was applied. Competing distributions were selected by a likelihood ratio test. All tests were performed independently for each parasite. Finally, changes in MHC allele frequency and MHC diversity between years were also investigated. To that end, year was selected as factor and MHC allele presence/absence or MHC diversity as dependent variable. Only individuals breeding in 2004 and recruited individuals in 2005 were included in the analyses.

For all analyses it was checked that the model fitted the data by using a Hosmer-Lemeshow test. All tests were performed independently for each parasite. All analyses were performed in R statistical software (R-project, 2012) and MASS package (Venables & Ripley, 2002).

Results

MHC diversity and MHC alleles

A total of 68 males (31 young and 37 older) and 74 females (29 young and 45 older) from 2004 were screened for parasite infections (Table 1). MHC allele diversity varied among individuals, ranging from one to five MHC alleles per individual. The average (± SE) number of alleles per individual was 3.2 ± 0.08, and there were no significant differences between males and females ($Z = 0.12$, $P = 0.88$) or between young and adults ($Z = -1.42$, $P = 0.09$). Specific MHC alleles were also determined according to Schut (2012) and correspond to Paca UA*104, Paca UA*108, Paca UA*114 and Paca UA*117. In addition three new MHC alleles were found: Paca UA*236, Paca UA*249 and Paca UA*274. MHC allele frequencies varied among alleles (Table 2). When comparing the five most frequent MHC alleles, frequencies did not differ significantly for males and females (all $P > 0.3$), except for Paca UA*249, for which males had a higher frequency than females ($Z = 2.19$, $P = 0.02$). Also frequencies did not differed

significantly between young and adults (all P > 0.1). MHC allele frequencies associated to MHC diversity are shown in Table S1.

Changes in MHC allele frequency between years were investigated by comparing MHC allele frequency from all individuals in 2004 and recruited individuals in 2005 (Table 2). A frequency increasing from 2004 to 2005 was observed for the Paca UA*104 allele (F = 4.26, P = 0.04). All other alleles did not vary in frequency (All P > 0.1). Also, MHC diversity showed a tendency to increase between years (F = 3.05, P = 0.08).

MHC diversity and parasite infections

No significant associations were detected between MHC diversity and infection by *Haemoproteus majoris* parasites, neither for prevalence nor for intensity of infection (see Table S2 and Table S3). However, MHC diversity was significantly related to intensity of infection by *Leucocytozoon* (Table 3), while there was no effect on prevalence (Table S2). In infections implying *Leucocytozoon,* individuals with MHC diversity=4 were more intensely infected than those with lower MHC diversity (Table 3, Fig. 1). Post-hoc tests indicated that birds with MHC diversity=4 were more intensely infected by *Leucocytozoon* than birds with MHC diversity=2 (Tukey HSD test: P = 0.018) and birds with MHC diversity=3 (Tukey HSD test: P = 0.03). No significant differences were observed between other levels of MHC diversity (Tukey HSD test: P > 0.1). In addition, some significant interactions with sex and age were observed in infections involving *Leucocytozoon*, with males with MHC diversity=4 being more intensely infected than males or females with other MHC diversity. Moreover, young individuals with MHC diversity=4 showed a higher intensity of infection compared to young individuals with lower MHC diversity and adults with other MHC diversity.

MHC alleles and parasite infections

No significant associations were detected between specific MHC alleles and infection by *Haemoproteus majoris* parasites, neither for prevalence nor for intensity of infection (Table S4 and Table S5). However, there were associations among specific MHC alleles and the

intensity of infection by *Leucocytozoon* (Table 4) while there was no significant association with prevalence (Table S4). Thus MHC alleles Paca UA*104 and Paca UA*108 were significantly and positively related to intensity of infection by *Leucocytozoon* (Fig. 2). In addition, some significant interactions with sex and age emerged implying that males and young individuals with allele Paca UA*104 or Paca UA*108 were those with higher intensities of infection by *Leucocytozoon*. Moreover, the analysis of the significant interaction "Paca UA*108 allele X sex X age" indicated that young females with the allele Paca UA*108 have lower intensity of infection (Fig.3). In addition, the analysis of the significant interaction "Paca UA*117 allele X sex X age" revealed that young females with the allele Paca UA*117 presented the highest intensities of infection by *Leucocytozoon* in the sample (see Table 4 and Fig.3).

MHC diversity, specific MHC alleles and parasite infections

As MHC diversity was related with intensity of infection by *Leucocytozoon*, we explored the possibility that this association was due to the presence of some specific alleles. To that end, we performed a negative binomial GLZ using the MHC diversity recalculated after removing the alleles Paca UA*104 and Paca UA*108. Both alleles were associated to higher intensities of infection by *Leucocytozoon* and also were found in high frequency in diverse individuals (Table S1), therefore it could be possible that these alleles affected the association involving MHC diversity. The model selection started from a model including the main effects (the new MHC diversity variable and the presence/absence of Paca UA*104 or Paca UA*108) and the interactions Paca UA*104 X MHC diversity or Paca UA*108 X MHC diversity. Intensity of infection by *Leucocytozoon* was introduced as dependent variable in both models. In the final model involving Paca UA*104, MHC diversity was significant (Table S6) and Paca UA*104 marginally significant (P = 0.059). However, in the model involving Paca UA*108, both MHC diversity and Paca UA*108 were significant (Table S6).

Discussion

Looking for associations between MHC diversity and/or specific MHC alleles and blood parasites in wild populations is always a difficult task due to the many factors involved in determining the prevalence and intensity of infections. Each parasite differs in their life history characteristics, including life cycles implying different vectors that can be affected by different biotic and abiotic variables (Martínez-de la Puente *et al.*, 2009a, b, 2010b). In addition, virulence may oscillate considerably among parasites depending on characteristics of both hosts and parasites (Toft & Karter, 1990). Also, the relationship between hosts and parasites is shaped by a coevolutionary arms race process which may render fast changes in selection pressures due to the forces exerted between hosts and parasites (Ewald, 1994).

In this complex scenario, we failed to find significant relationships between intensity of infection by *Haemoproteus majoris* and MHC diversity or specific alleles. In a recent study, Westerdahl et al. (2013) found a negative association between a specific MHC-I allele (allele 242) and intensity of infection by *Haemoproteus majoris*, indicating quantitative resistance to this infection. Since one RSCA peak could represent more than a single allele, we were conservative in our analyses and only included those alleles that were clearly resolved. Thus, we removed this potential allele from the analyses. Anyway, we explored this association and allele 242 was not related to resistance to *Haemoproteus majoris* (data not shown). However, we found that individuals with high MHC diversity or specific MHC alleles had a higher intensity of infection by *Leucocytozoon*. This result does not support the relationship between high/optimal MHC diversity and higher chance of parasite antigen recognition (Edwards & Hedrick, 1998; Hughes & Yeager, 1998), but instead these associations suggest parasite susceptibility. One hypothetical explanation for this fact would be that individuals with a more diverse/optimal MHC, lack specific MHC allele that confer resistance against *Leucocytozoon* parasites (see Spurgin & Richardson, 2010). In this situation, potential advantageous MHC alleles are those found in low frequencies (rare). These alleles could recognise parasite lineages which evade the recognition by common MHC alleles (Ejsmond *et al.*, 2010), becoming frequent as selection by the parasite increases (Sommer, 2005). On the other hand, the associations with alleles determining susceptibility / resistance should be checked using both

prevalence and intensity of infection (Westerdahl *et al.*, 2012). In this sense, the later authors sort the MHC alleles as a function of the level of resistance. First, MHC alleles which confer protection against an infection either removing or avoiding the parasite (qualitative resistance) should be exclusively related to uninfected individuals, but we did not detect this type of allele in our blue tit population. However, the low number of uninfected individuals present in the population and the methodologically limitation to obtain the complete MHC allele diversity should be taken into account before to exclude completely the occurrence of rare alleles offering qualitative resistance. Second, MHC alleles determining susceptibility should be exclusively related to infected individuals. In this study, the group of individuals with MHC alleles Paca UA*104, Paca UA*108 or Paca UA*117 had similar prevalence than the group of individuals without these alleles. And the individuals with higher intensity of infection by *Leucocytozoon* had at least one of these specific MHC alleles. Thus, it would be possible that they play a role as susceptible MHC alleles. Third, MHC alleles conferring quantitative resistance should be related to low intensity of infection.

Leucocytozoon is a common parasite infecting birds with deleterious effects on host fitness in some host/parasite systems (Merino *et al.*, 2000; Valkiūnas, 2005; Atkinson *et al.*, 2008). Being *Leucocytozoon* detrimental, why should blue tits maintain a high frequency of MHC alleles that allows susceptibility to this parasite? Several alternatives could explain this situation. First, the parasite could have overcome the antigen recognition associated with the most abundant MHC alleles (coevolutionary process). In this case, those alleles will be scarcer in next host generations if the virulence of the parasite is high. As a consequence, new or rare resistant alleles present in the population will increase their frequency. No MHC allele found in low frequency increased its frequency between years and their scarcity makes it impossible for us to show statistically whether they confer some kind of resistance to *Leucocytozoon*. For example, we checked this possibility for the allele Paca UA*274 and a tendency towards conferring resistance against *Leucocytozoon* appears ($P = 0.08$), but due to its low frequency (3.5 %) this result should be taken cautiously. Thus, sample size is evidenced as a limiting factor to detect rare alleles in wild studies. Second, susceptible alleles related to *Leucocytozoon*

could offer resistance against an unknown more virulent disease as previously suggested by other host/parasite systems (McClelland *et al.*, 2003; Loiseau *et al.*, 2008). That is, protective effects against more virulent parasites compensate the negative effect produced by *Leucocytozoon*. In support of this hypothesis, the frequency of allele Paca UA*108, associated with higher intensity of infection, increased in recaptured individuals one year later.

However, the MHC haplotypes presented by the individuals probably only predispose to disease, being other physiological factors determinant in the disease development. In fact, life cycle, sex, and age have been related to the inability of the immune response to control infections (Atkinson *et al.*, 2008; McClelland & Smith, 2011). In this sense, *Leucocytozoon* causes a disease with characteristic clinical phases, first an acute phase and later a latent or chronic infection (Valkiūnas, 2005). The chronic infection is reached when the immune system reduces the parasitemia to low levels and hosts show little or no signs of infection. However, relapses occur during host breeding period (seasonal relapse) when physiological stress is high (Loye & Zuk, 1991), or there are changes in hormonal levels (Valkiūnas, 2005). Thus, stress or changes in hormone levels could interfere with the immune response and an apparent susceptibility to the parasite will emerge even having a resistant allele against the parasite exist (Råberg *et al.*, 1998). In fact, cost of parasitism reduces the amount of resources available to be allocated to immune defence, making maintenance of innate and acquired resistance or activation difficult (Atkinson *et al.*, 2008). In addition, susceptibility associated with testosterone has been thoroughly described for males (reviewed in e.g. Nava-Castro *et al.*, 2012) and lower immunocompetence has been described for males and yearlings (Martínez-de la Puente *et al.*, 2007; Lobato *et al.*, 2008). In this sense, we found relationships between susceptible MHC alleles and intensity of infection by *Leucocytozoon* depending on host sex and age. Male or young individuals with the alleles Paca UA*104 and Paca UA*108 presented higher intensity of infection in the blue tit population, corroborating that other factors are involved in the susceptibility/resistance to infections. However, the group formed by young females with the allele Paca UA*117 showed the highest intensity of infection, indicating that

although there is a general observation of higher infections in males, the associations involving MHC alleles related to susceptibility affects both males and females.

In conclusion, the statistical models explained an important percentage of the variance in the intensity of infection by *Leucocytozoon*, being the diversity of the MHC-I and some specific MHC alleles the main variables involved in the infection. The high prevalence, the low intensity of infection, and the increase of the frequency of the susceptible allele Paca UA*104 from year to year suggest that the parasite does not exert an excessive selective pressure on the blue tit population. Thus, the occurrence of the susceptible alleles in this host/parasite system would be the side effect of selective pressure exerted by other more virulent pathogen.

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Table 1. Intensity and prevalence of infection of blood parasites found in blue tit males and females. MI = Mean intensity. P = Prevalence. SE = Standard error.

Parasite	Male (n= 68)			Female (n= 74)			Total		
	MI (SE)	(range)	P %	MI (SE)	(range)	P %	MI (SE)	(range)	P %
Leucocytozoon sp.	1.56 (0.58)	0 – 39	89.3	1.67 (0.44)	0 – 23	94.5	1.62 (0.58)	0 - 39	91.9
Haemoproteus majoris	20.95 (3.15)	0 – 115	89.2	20.35 (3.41)	0 – 138	81.1	20.63 (2.32)	0 - 138	85.0

Table 2. MHC allele frequencies in 2004 and 2005. Only recruited individuals were included in 2005.

	Paca UA*104	Paca UA*108	Paca UA*114	Paca UA*117	Paca UA*236	Paca UA*249	Paca UA*274
2004	0.25	0.35	0.23	0.25	0.03	0.34	0.03
2005	0.44	0.41	0.37	0.14	0.08	0.24	0.08

Accession numbers: Paca UA*104 (JF742767), Paca UA*108 (JF742771), Paca UA*114 (JF742777), and Paca UA*117 (JF742780). Paca UA*236, Paca UA*249 and Paca UA*274 are new alleles and no sequencing was performed after RSCA screening.

Table 3. Results from GLZ analysis between MHC diversity and intensity of infection by *Leucocytozoon*, after AIC stepwise model selection. SE = Standard error. D^2 = Explained deviance of the model. MHC diversity, sex and age were introduced as factors. Only variables retained by the final model are shown.

Final model	Estimate	SE	Z-value	P-value	D^2
Intercept	-0.69	0.64	-1.07	0.28	20.2
MHC diversity 3	0.51	0.79	0.64	0.51	
MHC diversity 4	2.86	0.83	3.42	< 0.001	
MHC diversity 5	1.38	0.88	1.57	0.11	
Female	1.58	0.77	2.03	0.04	
Adult	0.28	0.94	0.30	0.75	
MHC diversity 3 : Female	-0.22	1.01	-0.22	0.82	
MHC diversity 4 : Female	-3.87	1.07	-3.59	< 0.001	
MHC diversity 5 : Female	-0.98	1.22	-0.8	0.42	
MHC diversity 3 : Adult	0.04	1.10	0.04	0.96	
MHC diversity 4 : Adult	-2.56	1.18	-2.15	0.03	
MHC diversity 5 : Adult	-0.98	1.42	-0.69	0.49	
Female : Adult	-0.89	1.14	-0.78	0.43	
MHC diversity 3: Female : Adult	-0.32	1.39	-0.23	0.81	
MHC diversity 4: Female : Adult	3.37	1.51	2.22	0.02	
MHC diversity 5: Female : Adult	0.51	1.87	0.27	0.78	

Table 4. Results from GLZ analyses between specific MHC alleles and intensity of infection by *Leucocytozoon*, after AIC stepwise model selection. D^2 = Explained deviance of the model. MHC allele, sex and age were introduced as factors for each parasite. Only variables retained by the final model are shown.

MHC allele	Final model	Estimate	Std. Error	Z-value	P-value	D^2
Paca UA*104	Intercept	0.06	0.27	0.23	0.81	17.6
	Paca UA*104	1.64	0.50	3.25	< 0.001	
	Female	0.63	0.29	2.14	0.03	
	Adult	-0.17	0.29	-0.59	0.55	
	Paca UA*104 : Female	-1.36	0.58	-2.31	0.02	
	Paca UA*104 : Adult	-1.51	0.59	-2.57	0.01	
Paca UA*108	Intercept	-0.32	0.39	-0.83	0.4	21.1
	Paca UA*108	1.85	0.54	3.42	< 0.001	
	Female	1.57	0.49	3.19	< 0.01	
	Adult	0.24	0.49	0.5	0.61	
	Paca UA*108 : Female	-4.12	0.87	-4.73	< 0.001	
	Paca UA*108 : Adult	-1.6	0.77	-2.05	0.03	
	Female : Adult	-1.5	0.65	-2.3	0.02	
	Paca UA*108 : Female : Adult	4.37	1.12	3.88	< 0.001	
Paca UA*114	Intercept	0.82	0.19	4.29	< 0.0001	5.2
	Female	-0.69	0.26	-2.63	< 0.0001	
Paca UA*117	Intercept	0.76	0.26	2.9	< 0.01	20.4
	Paca UA*117	0.21	0.80	0.26	0.79	
	Female	-1.37	0.46	-2.96	< 0.01	
	Adult	-0.89	0.41	-2.17	0.02	
	Paca UA*117 : Female	2.45	1.001	2.45	0.01	
	Paca UA*117 : Adult	0.13	0.96	0.14	0.88	
	Female : Adult	1.78	0.61	2.9	< 0.01	
	Paca UA*117 : Female : Adult	-3.005	1.24	-2.41	0.015	

Paca UA*249	Intercept	0.27	0.32	0.85	0.39	9.7
	Paca UA*249	0.84	0.45	1.85	0.064	
	Female	-0.26	0.33	0.78	0.43	
	Adult	-0.33	0.32	-1.02	0.3	
	Paca UA*249: Female	0.2	0.55	0.36	0.71	
	Paca UA*249: Adult	0.2	0.55	0.36	0.71	

Figure 1. Results from the negative binomial model of the intensity of infection by *Leucocytozoon* in blue tits, in relation to MHC-I diversity. MHC diversity=2 level was created by merging MHC diversity categories 1 and 2 due to the low number of individuals under category 1.

Figure 2. Results from the negative binomial model of the intensity of infection by *Leucocytozoon* in blue tits, in relation to presence of MHC allele Paca UA*104 and MHC allele Paca UA*108.

Figure 3. Triple interaction from the negative binomial model of the intensity of infection by *Leucocytozoon* in blue tits, in relation to presence of MHC allele Paca UA*108 and MHC allele Paca UA*117.

Figure 1

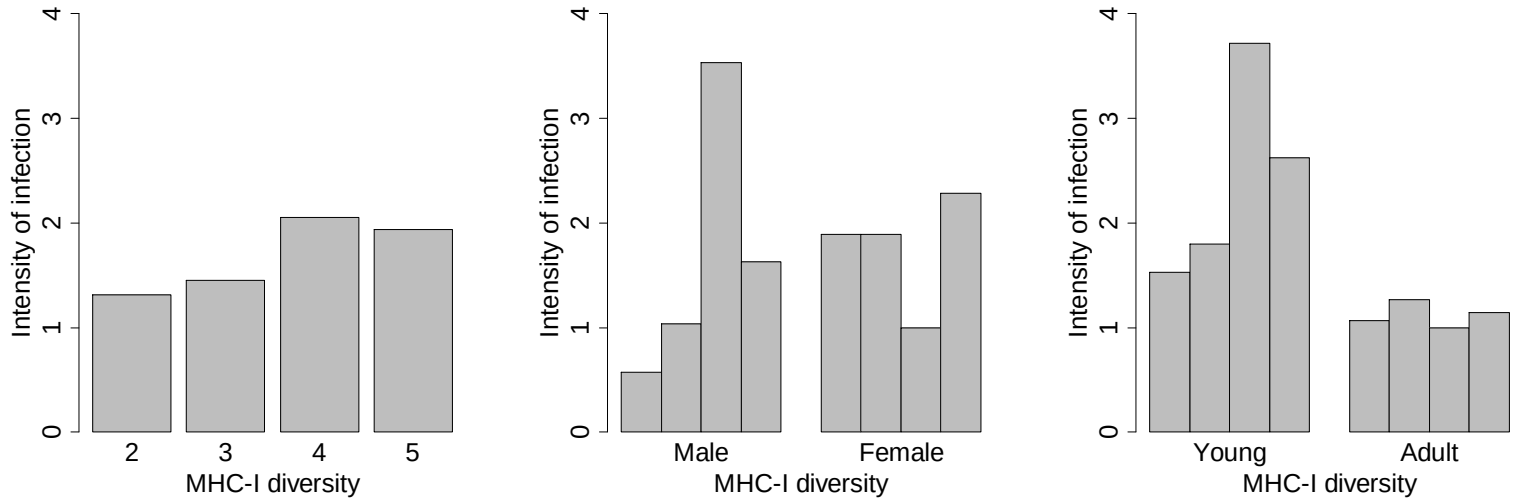

Intensity of infection
0
1
2
3
4
2
3
4
5
MHC-I diversity
Intensity of infection
0
1
2
3
4
Male
Female
MHC-I diversity
Intensity of infection
0
1
2
3
4
Young
Adult
MHC-I diversity

Figure 2

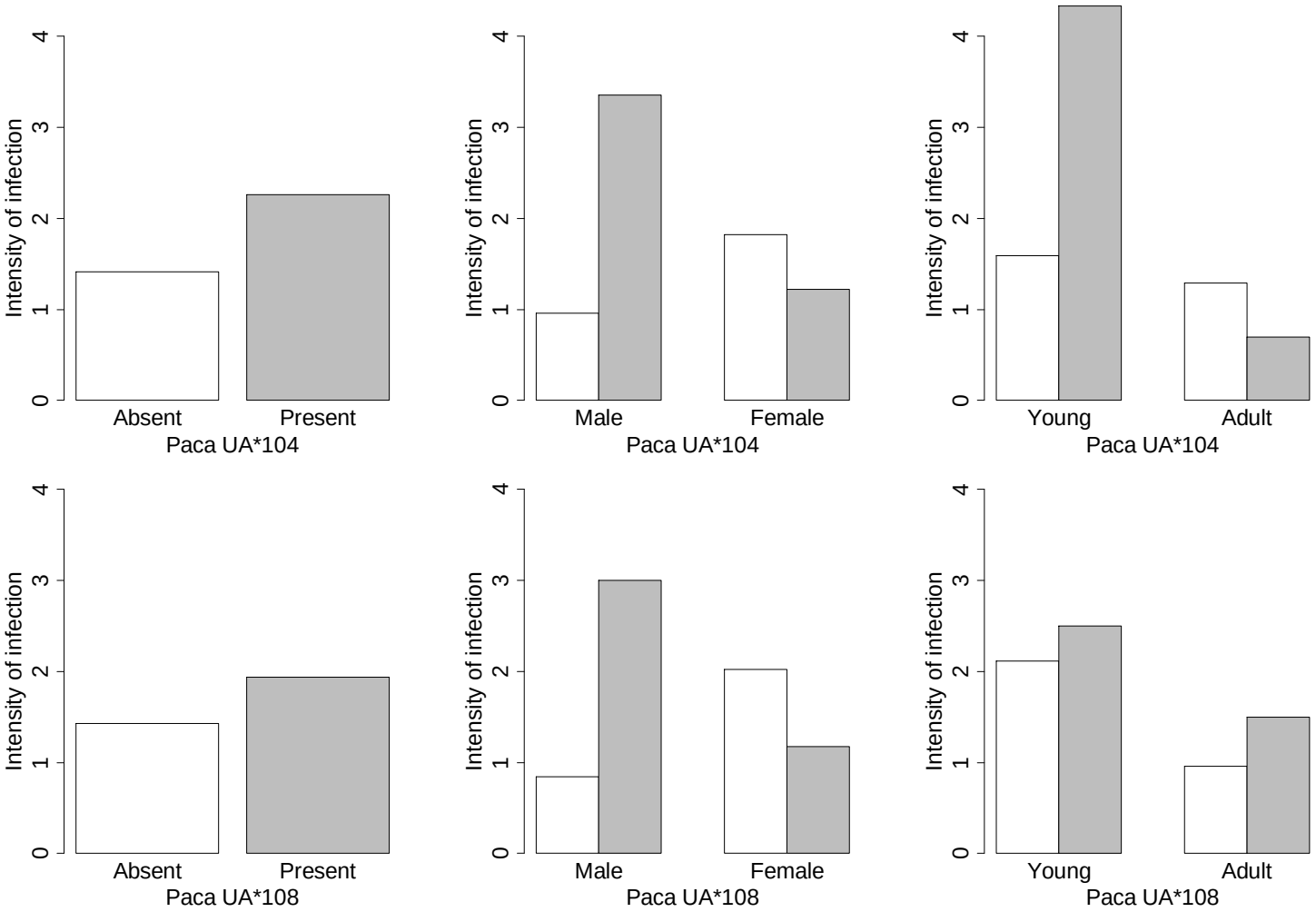
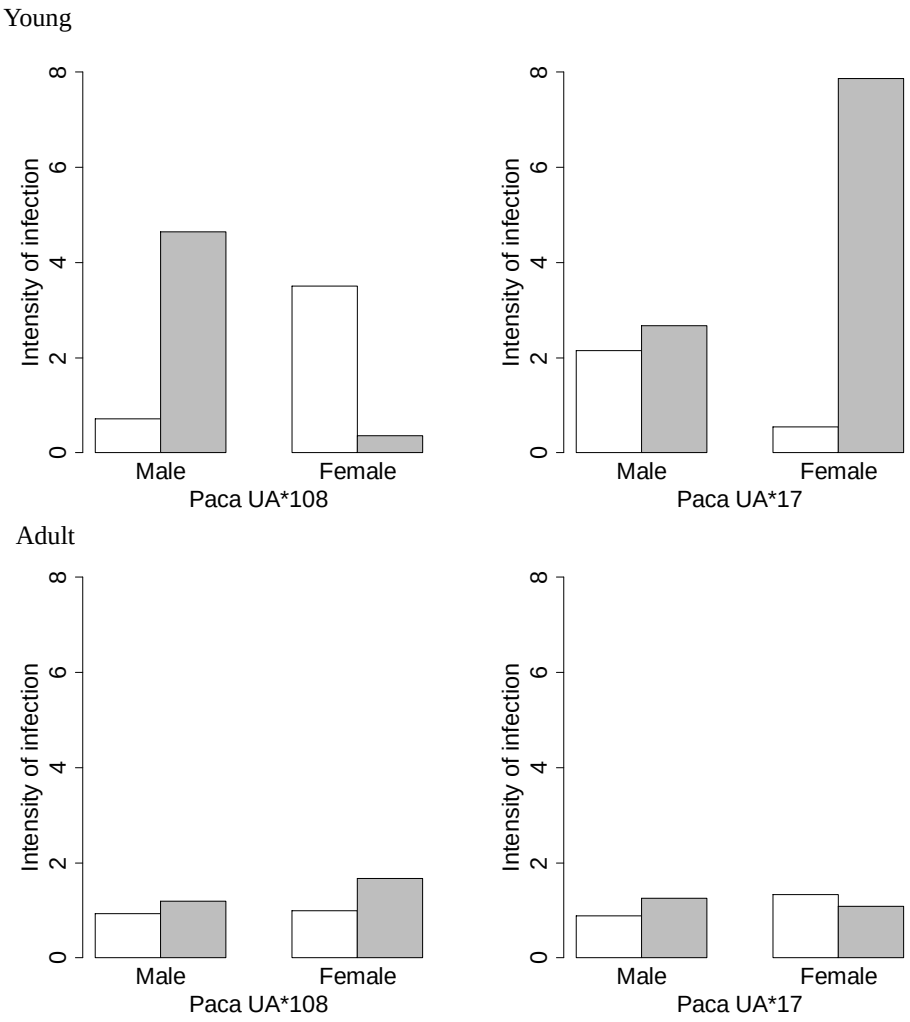

Intensity of infection
0
1
2
3
4
Absent
Present
Paca UA*104
Male
Female
Paca UA*104
Young
Adult
Paca UA*104
Paca UA*108
Paca UA*108
Paca UA*108

Figure 3


Young
Intensity of infection
0
2
4
6
8
Male
Female
Paca UA*108
Intensity of infection
0
2
4
6
8
Male
Female
Paca UA*17
Adult
Intensity of infection
0
2
4
6
8
Male
Female
Paca UA*108
Intensity of infection
0
2
4
6
8
Male
Female
Paca UA*17

Supporting information

Table S1. MHC allele frequencies associated to MHC diversity level. χ^2 test were performed to detect differences between observed and expected frequencies.

		Paca UA*104[a]		Paca UA*108[b]		Paca UA*114[c]		Paca UA*117[d]		Paca UA*249[e]	
		Absent	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Present
MHC diversity 2[†]	Count	31	1	28	4	26	6	28	4	25	7
	Expected count	24.1	7.9	20.5	11.5	24.1	7.9	24.4	7.6	21.1	10.9
MHC diversity 3	Count	49	9	37	21	46	12	45	13	35	23
	Expected count	43.6	14.4	37.1	20.9	43.6	14.4	44.2	13.8	38.3	19.7
MHC diversity 4	Count	21	15	19	16	27	9	24	11	24	12
	Expected count	27.1	8.9	22.4	12.6	27.1	8.9	26.7	8.3	23.7	12.3
MHC diversity 5	Count	5	10	5	9	7	8	9	5	9	6
	Expected count	11.3	3.7	9.0	5.0	11.3	3.7	10.7	3.3	9.9	5.1

a. $\chi^2 = 30.3$, df = 3, $P < 0.0001$
b. $\chi^2 = 13.9$, df = 3, $P = 0.003$
c. $\chi^2 = 7.6$, df = 3, $P = 0.053$
d. $\chi^2 = 4.5$, df = 3, $P = 0.2$
e. $\chi^2 = 4.5$, df = 3, $P = 0.36$
[†]. MHC diversity=2 variable was categorized by merging MHC diversity categories 1 and 2.

Table S2. Results from GLZ analyses between MHC diversity and prevalence of infection by *Leucocytozoon* and *Haemoproteus*, after AIC stepwise model selection. SE = Standard error. D^2 = Explained deviance of the model. MHC diversity, sex and age were introduced as factors for each parasite. Only variables retained by the final model are shown.

Parasite	Final model	Estimate	SE	Z-value	P-value	D^2
Leucocytozoon	Intercept	1.88	0.49	3.83	< 0.001	2.6
	Female	0.70	0.65	1.07	0.28	
	Adult	0.50	0.63	0.72	0.42	
Haemoproteus	Intercept	1.73	0.23	7.32	0.57	0

Table S3. Results from GLZ analyses between MHC diversity and intensity of infection by *Haemoproteus*, after AIC stepwise model selection. SE = Standard error. D^2 = Explained deviance of the model. MHC diversity, sex and age were introduced as factors. Only variables retained by the final model are shown.

Final model	Estimate	SE	Z-value	P-value	D^2
Intercept	3.42	0.21	16.07	< 0.0001	5.1
Adult	-0.81	0.28	-2.89	< 0.01	

Table S4. Results from GLZ analyses between specific MHC alleles and prevalence of infection by *Leucocytozoon* and *Haemoproteus*. SE = Standard error. D^2 = Explained deviance of the model. MHC allele, sex and age were introduced as factors. Only variables retained by the final model are shown.

Parasite	MHC allele	Final model	Estimate	SE	Z-value	P-value	D^2
Leucocytozoon	Paca UA*104	Intercept	2.46	0.31	7.8	< 0.0001	0
	Paca UA*108	Intercept	2.44	0.31	7.78	< 0.0001	0
	Paca UA*114	Intercept	2.44	0.31	7.78	< 0.0001	0
	Paca UA*117	Intercept	2.44	0.31	7.78	< 0.0001	0
	Paca UA*249	Intercept	2.46	0.31	7.8	< 0.0001	0
Haemoproteus	Paca UA*104	Intercept	1.73	0.23	7.32	< 0.0001	0
	Paca UA*108	Intercept	1.71	0.23	7.24	< 0.0001	0
	Paca UA*114	Intercept	1.73	0.23	7.32	< 0.0001	0
	Paca UA*117	Intercept	1.71	0.23	7.24	< 0.0001	0
	Paca UA*249	Intercept	2.57	0.5	5.15	< 0.0001	4.4
		Cyca*249	-0.87	0.49	-1.75	0.08	
		Cyca*249	-0.87	0.49	-1.75	0.08	

Table S5. Results from GLZ analyses between specific MHC alleles and intensity of infection by *Haemoproteus*, after AIC stepwise model selection. SE = Standard error. D^2 = Explained deviance of the model. MHC allele, sex and age were introduced as factors. Only variables retained by the final model are shown.

MHC allele	Final model	Estimate	SE	Z-value	P-value	D^2
Paca UA*104	Intercept	3.42	0.21	16.06	< 0.0001	5.1
	Adult	-0.81	0.28	-2.89	< 0.01	
Paca UA*108	Intercept	3.42	0.21	16.06	< 0.0001	5.1
	Adult	-0.81	0.28	-2.89	< 0.01	
Paca UA*114	Intercept	3.51	0.22	15.9	< 0.0001	6.9
	Paca*114	-0.53	0.32	-1.65	0.09	
	Adult	-0.78	0.27	-2.82	< 0.01	
Paca UA*117	Intercept	3.42	0.21	16.06	< 0.0001	5.1
	Adult	-0.81	0.28	-2.89	< 0.01	
	Intercept	3.42	0.21	16.06	< 0.0001	
Paca UA*249	Intercept	-0.81	0.28	-2.89	< 0.01	0

Table S6. Results from GLZ analyses between MHC diversity, Paca UA*4 and Paca UA*8 and intensity of infection by *Leucocytozoon*, after AIC stepwise model selection. SE = Standard error. D^2 = Explained deviance of the model. In the table is observed a reduction in the MHC diversity to levels to 0, 1 and 2, due to the elimination of Cyca*4 and Cyca*8. MHC diversity was calculated as the sum of only alleles Paca UA*14, Paca UA*17 and Paca UA*249.

Factors	Final model	Estimate	SE	Z-value	P-value	D^2
MHC diversity + Paca UA*104 + sex + age + MHC diversity : Paca UA*104	Intercept	-0.39	0.25	-1.51	0.13	15.5
	Paca UA*104	0.54	0.29	1.88	0.059	
	MHC diversity 1	0.68	0.305	2.22	0.026	
	MHC diversity 2	1.45	0.37	3.09	< 0.0001	
MHC diversity + Paca UA*108 + sex + age + MHC diversity : Paca UA*108	Intercept	-0.41	0.31	-1.33	0.18	21.7
	Paca UA*108	0.75	0.27	2.71	< 0.01	
	MHC diversity 1	0.75	0.301	2.5	0.012	
	MHC diversity 2	1.65	0.38	4.28	< 0.0001	
	Age	-0.40	0.25	-1.57	0.11	

Capítulo 2

La coloración del plumaje dependiente de los carotenos está relacionada con la riqueza de parásitos sanguíneos y los niveles de proteínas de estrés en el herrerillo común (*Cyanistes caeruleus*)

Los carotenos son moléculas que no pueden ser sintetizadas por las aves y por lo tanto, deben ser adquiridas a través de la dieta. Estos pigmentos, aparte de conferir colores rojizos y amarillos cuando son depositados en las plumas, parecen actuar como estimuladores de la respuesta inmune y como antioxidantes. Por lo tanto, solo los individuos más sanos serán capaces de expresar ornamentos dependientes de los carotenos sin comprometer otras funciones fisiológicas. Varios estudios han encontrado que las aves infectadas por parásitos tienen un color de plumaje mas pálido que los individuos no infectados, pero hasta ahora no se ha estudiado el posible efecto de infecciones múltiples de parásitos sanguíneos sobre el color del plumaje. Al comparar el color amarillo del plumaje del pecho en el herrerillo común, *Cyanistes caeruleus*, entre aves infectadas por diferentes géneros de parásitos sanguíneos, encontramos que los individuos infectados por más de un género fueron más pálidos que los que estuvieron infectados por un sólo parasito. Además, hemos examinado el papel potencial del color del plumaje como un indicador de la salud, con respecto a la condición general del ave, el nivel de inmunoglobulinas y las HSPs (proteínas de estrés). Nuestros resultados indican que las aves más coloridas presentan un menor nivel de HSP70 con respecto a las aves más pálidas, pero no encontramos una asociación significativa con la condición general ni con el nivel de inmunoglobulinas. En general, estos resultados apoyan el papel de los colores dependientes de los carotenos como indicador de salud en el herrerillo.

Carotenoid-based plumage colouration is associated with blood parasite richness and stress protein levels in blue tits (*Cyanistes caeruleus*)

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Carotenoids are molecules that birds are not able to synthesize and therefore, must be acquired through their diet. These pigments, besides their function giving red and yellow colours when deposited in feathers, seem to act as immune-stimulators and antioxidants in the organism. Hence, only the healthiest individuals would be able to express carotenoid-based ornaments to a larger extent without compromising their physiological functions. Various studies have reported that birds infected by parasites are paler than those uninfected, but, to our knowledge, none of them assessed the possible effect of multiple infections by blood parasites on plumage colour. By comparing the yellow colour in the breast plumage of blue tits, *Cyanistes caeruleus*, between birds infected by different number of blood parasite genera, we found that those birds infected by more than one genus were paler than those parasitized just by one. In addition, we have examined the potential role of carotenoid-based plumage colour of blue tits as a long-term indicator of other parameters of health status, such as body condition and immunoglobulin and HSPs (heat shock proteins) levels. Our results indicate that brightly coloured birds had lower HSP70 levels than pale birds, but we did not find any significant association between colour and body condition or immunoglobulin levels. Overall, these results support the role of carotenoid-based colours as indicators of health status in blue tits.

Introduction

Numerous observational and experimental studies have shown that female birds prefer males with the brightest and most intense plumage colours (e.g. Zuk *et al.* 1990, Hill 1991, MacDougall and Montgomerie 2003), although the proximate reasons for this choice are not fully understood. One of the potential selective pressures proposed to explain the evolution of female mate choice is that plumage colour may be signaling health status and parasite resistance (Hamilton and Zuk 1982); thus, females would acquire selective advantages by mating with colourful males. Several hypotheses have been developed in this respect. The influential hypothesis of Hamilton and Zuk (1982) poses that females choosing a brightly coloured male may gain benefits for their nestlings in form of heritable resistance to parasites. Also, females may avoid parasite transmission to themselves and their nestlings (Clayton 1990) or they may benefit from a higher parental effort performed by a healthy male (Read 1990). Despite the role of colourful plumage has especially been studied in males, it is known that in several species females also show conspicuous plumages. Traditionally, it has been assumed that this might be due to a genetic correlation with male ornamentation, thereby suggesting that female colouration was not functional (Lande 1980). However, males may suffer costs associated with reproduction and a way to reduce these costs would be choosing a showy female if showiness signals her good quality (Amundsen 2000). Indeed, evidence is accumulating on the adaptive function of female ornamentation (see Kraaijeveld *et al.* 2007 for a review).

Carotenoid-based colours are very common among birds (Goodwin 1984). It has been proposed that the honesty of carotenoid-based ornaments as quality signals is based on the multiple functions of carotenoids in the organism. Besides being deposited in feathers and thus giving yellow and red colours to bird's plumages, they seem to participate as immune-stimulators and antioxidants in the organism (Lozano 1994, Møller *et al.* 2000). Among their functions we can cite their role as scavengers of free radicals and immunosuppressive peroxides (e.g. Chew 1993, Surai and Speake 1998, von Schantz *et al.* 1999, Møller *et al.* 2000; but see Costantini and Møller 2008), as well as immune-enhancers in the production of lymphocytes, the phagocytic ability of neutrophils and macrophages, and tumor immunity (Møller *et al.* 2000,

Surai *et al.* 2001). In addition, carotenoids are pigments that vertebrates cannot synthesize *de novo*, so they must be obtained through the diet (Hill 1992, Olson and Owens 1998). Hence, according to the handicap hypothesis (Zahavi 1975), only the high quality individuals (e.g. those with higher resistance to parasites and/or foraging ability) would be capable of being intensely coloured without compromising the quantity of carotenoids allocated to other physiological functions.

The negative effects of parasitism on plumage colours have been reported in many studies (Brawner *et al.* 2000, McGraw and Hill 2000, Hõrak *et al.* 2001, 2004, Figuerola *et al.* 2005, Martínez-Padilla *et al.* 2007, Baeta *et al.* 2008), those birds infected by parasites being normally paler than unparasitized ones. However, most of the studies on the effects of parasitism on plumage colours are based on only one parasite species, whereas, to our knowledge, there is no work examining the possible effect of joint infections by multiple blood parasites on carotenoid-based plumage colour. This kind of study may help to better understand the role of carotenoid-based plumage colourations as quality signals, since birds in the wild are normally infected by different parasites species simultaneously as reported in previous studies (Merino *et al.* 2000, Valkiūnas *et al.* 2003). Furthermore, it has been shown that hosts could suffer harmful effects, like anaemia, loss of body mass and reduction of survival, due to multiple infections (Evans and Otter 1998, Graham *et al.* 2005, Davidar and Morton 2006, Marzal *et al.* 2008).

The aim of this study was evaluating the role of a carotenoid-based trait, the yellow breast plumage colouration of blue tits (*Cyanistes caeruleus*), as a long-term indicator of health status in a population commonly infected by several species of blood parasites (Merino *et al.* 2000). The blue tit is a small (11 g) insectivorous passerine, very common in Europe. It is a slightly sexually dichromatic species, males being more intensely coloured than females (Cramp 1998). In blue tit males, yellow carotenoid-based colouration has been shown to indicate parental investment (Senar *et al.* 2002), while in females it reflects their reproductive capacity (Doutrelant *et al.* 2008). Moreover, there is assortative mating by this trait (Hidalgo-García 2006, Ferns and Hinsley 2008), which suggests that yellow plumage colouration might be

sexually selected in both sexes. The health status variables were parasite richness, body condition index and the levels of total immunoglobulins and stress proteins (HSPs). Immunoglobulins play an important role in the specific humoral immune response of vertebrates, being responsible for antigen recognition (Roitt *et al.* 1996). Measures of humoral immune response are becoming widely used in many ecological studies (Norris and Evans 2000, Martínez-de la Puente *et al.* 2007a, Tomás *et al.* 2007), and fitness costs of immune defence against parasites and trade-offs in the face of limited resources have been reported (Deerenberg *et al.* 1997, Moreno *et al.* 1999, Soler *et al.* 2002). Likewise, studies on the ecological and evolutionary role of the HSP-mediated stress response have been published in recent years (see Sørensen *et al.* 2003 for a review). HSPs are molecules that maintain cellular homeostasis by responding to a wide array of stressors, like heat (Gehring and Wehner 1995), toxins (Mariño *et al.* 1999), oxidant compounds (Martínez *et al.* 1999a), cold (Martínez *et al.* 2001) and parasites (Merino *et al.* 1998, Martínez *et al.* 1999b, Tomás *et al.* 2005).

We expected that intensely coloured birds were infected by fewer blood parasite genera. Also, colourful birds would be in better body condition and would show higher levels of immunoglobulins (immunocompetence) and fewer stress protein levels.

Materials and methods

The study was carried out during the 2004 and 2007 breeding seasons in a Pyrenean oak (*Quercus pyrenaica*) forest located in Valsaín, central Spain (Segovia province, 40º53'N, 4º01'W, 1200 m a. s. l.), where a population of blue tits breeds in wooden nest boxes. This population is being studied since 1991 (e.g. Merino *et al.* 2000, Martínez-de la Puente *et al.* 2006). Every year, nest boxes are periodically inspected to determine the laying date (day 1 = April 1st), clutch size, hatching date and fledging success.

In both study years, adult birds were trapped in nest boxes twice. In the first capture (when nestlings were 3 days old) they were ringed if necessary, weighed with an electronic balance to the nearest 0.1 g and sampled for blood from the brachial vein (see below). Besides, 0.1 mg of an antimalarial drug, primaquine, diluted in 0.1 ml of saline solution or the same

volume of saline solution was injected subcutaneously in the abdominal region for another experimental purpose (see Martínez-de la Puente *et al.* 2006, 2007b, Tomás *et al.* 2008). In the second capture (10 days later), tarsus length was measured with a digital caliper to the nearest 0.01 mm. Birds were sexed and classified as yearling or older (≥ 2 years) according to plumage characteristics as described by Svensson (1992). During the second capture, breast yellow plumage colour was objectively measured using a portable photospectrometer (Minolta, CM-2600d), that covers the range of wavelengths between 360 and 740 nm. To take colour measures, the photospectrometer was perpendicularly placed on one flank of the breast feather surface. From the raw spectral reflectance data that we obtained, we computed the "carotenoid chroma" $(R_{700}-R_{450})/R_{700}$ for each individual (Andersson and Prager 2005). This is the relative difference in reflectance between the wavelengths of minimum (700 nm) and maximum (450 nm) absorbance of the two main carotenoids in blue tit plumage (lutein and zeaxanthin). This measure has the advantage of being a strong correlate of perceived chroma and also being the best spectrometric estimate of actual carotenoid concentration (Andersson and Prager 2005). Two measurements were made of the yellow plumage colour of each individual during 2004, using the mean of both values of carotenoid chroma in the analyses because of the high repeatability of measurements (R_i=0.67, p=0.0018; Figuerola *et al.* 1999). In 2007, we just recorded one measurement of plumage colour for each individual. We have not measured the UV reflectance of the yellow plumage colouration of blue tits (Hunt *et al.* 1998; Örnborg 2002), however, due to carotenoid-based plumage colouration and UV reflectance of plumage in blue tits indicate reproductive capacity (Senar *et al.* 2002, Doutrelant *et al.* 2008) and survival (Sheldon *et al.* 1999) respectively, in short, good quality and/or condition, both colourations may be probably correlated. In addition, our main aim was to shed light on the role of the carotenoid-based plumage colouration as a signal of good health status so it does not seem particularly important the mismeasure of UV colour.

For the molecular detection of blood parasites (*Haemoproteus*, *Leucocytozoon*, *Plasmodium*, *Lankesterella* and *Trypanosoma*), a drop of blood from birds of 2004 was stored at -20ºC until processed whereas samples of 2007 were stored in FTA cards (Whatman

International Ltd. UK). DNA was extracted using a commercial kit (UltraClean DNA BloodSpin Kit, MO BIO laboratories, California, USA) or the protocol described in Merino *et al.* (2008) in the case of FTA cards to immediately amplify cytochrome B gene using the primers described in table 1. PCR reactions consisted of 25 µl reaction volumes containing 20 ng template DNA, 50 mM KCl, 10mM Tris-HCl, 1.5 $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, California). The reactions were cycled at the following parameters using a thermal cycler (MasterCycler Personal, Eppendorf): 94°C for 10 min (polymerase activation), 40 cycles at 95°C for 40 sec, annealing temperature for 1 min (see table 1), 72°C for 1 min, and a final extension at 72°C for 10 min. We mainly used data from molecular detection in the analyses, but when this was not possible (due to small blood sample or technical problems to detect parasites with molecular techniques as in the case of *Trypanosoma*) we used data obtained from microscopical observations. For this purpose, another drop of blood was immediately smeared upon extraction and air-dried to later check for the presence of parasites under optical microscope. These samples were fixed in absolute ethanol and stained with Giemsa stain for 45 min. Half of a smear was scanned at 200X to search for large parasites such as *Trypanosoma* and *Leucocytozoon*, whereas small intra-erythrocytic parasites, such as *Haemoproteus*, *Lankesterella* and *Plasmodium* were detected in the other half of the smear at 1000X with the oil immersion objective (see Merino *et al.* 1997).

Only in 2004, we obtained the intensity of infection by *Haemoproteus* as the number of parasites per 2000 erythrocytes (Merino *et al.* 1997) and the rest of the blood extracted was centrifuged (2000 g, 5 min) with a portable centrifuge (Labnet, catalogue no. 1201-220V, Woodbridge, NJ, USA) being cellular and plasma fractions separated. The cellular fraction was used to determine two different heat shock proteins (HSP) levels, HSP60 and HSP70, through Western blot following the protocol described in Tomás *et al.* (2004). The plasmatic fraction was used to determine immunoglobulin levels through a direct ELISA (Enzyme-Linked Immunosorbent Assay) using a polyclonal rabbit antichicken IgG conjugated with peroxidase

(Sigma A-9046, St Louis, MO, USA). For details on the methodology see Martínez *et al.* (2003). Body condition index was calculated as weight/tarsus length.

Treatment with primaquine reduces intensity of infection by *Haemoproteus* and prevalence of infection by *Leucocytozoon* in blue tits (Merino *et al.* 2000). To avoid any confounding effect of this treatment (e.g. on blood parasite infection), data from the first capture was used in the analyses, which was available for all variables except plumage colour and tarsus length (obtained in the second capture). It should be also noted that in 2003 and 2006, i.e. the two breeding seasons previous to those included in the present study (when birds moulted the plumage that was measured in the present study), no bird was treated with primaquine. This precludes that any relationship found between blood parasitaemias and plumage colour of birds can be associated with previous primaquine treatments.

To study the effect of the variables (categorical variables: year, sex, age, treatment, parasite richness; continuous variables: date of measurement, body condition index, levels of immunoglobulin, HSP60 and HSP70) on plumage colour (carotenoid chroma), general linear models GLMs (Statistica 2001, StatSoft) were used. For simplicity only two-way interactions between categorical variables were included. The full models are reported, as recommended by Whittinghan *et al.* (2006). To study the variation of *Haemoproteus* intensity of infection in accordance to blood parasite richness we used a general linear model GLM (Statistica 2001, StatSoft), controlling for sex and age of birds. All the variables showed a normal distribution except date of measurement and *Haemoproteus* intensity of infection. Those variables were logarithmically transformed to satisfy assumptions of normality.

Results

After removing cases of birds recaptured in 2007 (5 birds) to avoid pseudoreplication, we kept samples from 166 birds (85 females, 30 yearlings and 55 adults, and 81 males, 34 yearlings and 47 adults) in 2004 and 57 birds (29 females, 9 yearlings and 20 adults and 28 males, 14 yearlings and 14 adults) in 2007.

The prevalence of blood parasites was considerably high, being *Haemoproteus* the most frequent blood parasite (84% in 2004 and 81% in 2007), followed by *Leucocytozoon* (87% in 2004 and 57% in 2007). Other blood parasites were also present but with lower prevalences: *Trypanosoma* (29% in 2004 and 19% in 2007), *Lankesterella* (45% in 2004 and 38% in 2007) and *Plasmodium* (65% in 2004 and 36% in 2007). We assigned birds to four groups depending on the number of parasites they were infected with: 1) one blood parasite genera: 21 birds (10 in 2004 and 11 in 2007); 2) two blood parasite genera: 57 birds (33 in 2004 and 24 in 2007); 3) three blood parasite genera: 74 birds (60 in 2004 and 14 in 2007); 4) four or five blood parasites genera: 69 (49 in 2004 and 7 in 2007 with four different parasites and 12 in 2004 and 1 in 2007 infected by 5 parasites). Only one bird of 2007 was unparasitized and was not included in the analyses.

The results of the analysis relating carotenoid chroma with blood parasite richness and body condition are presented in table 2. We did not find a significant effect of year, date of measurement, treatment, sex or age on carotenoid chroma. However, we found an effect of the sex x age and sex x treatment interactions on colour. Young females were significatively paler than adult females and young males, whereas untreated males were significatively more colourful than females and treated males. On the other hand, multiple blood parasite infections were more likely to be present in paler birds (those with lower carotenoid chroma) as compared to those parasitized by just one parasite genus. Post-hoc comparisons determined that carotenoid chroma drastically decreased in birds infected by more than one blood parasite (Fig. 1, Fisher LSD: $p<0.05$), but not between birds infected with 2, 3 or 4 genera. In addition, we could find an effect of year x blood parasite richness on carotenoid chroma (Fig. 2). However, carotenoid chroma was not significantly related to body condition or to any of the other two-way interactions (Table 2). The final model explains 13% of variation in carotenoid chroma ($F_{8, 211} = 5.08$, $p<0.001$).

The analysis corresponding to data from 2004 is presented in table 3. In 2004, carotenoid chroma was not significantly related to immunoglobulin levels, although higher levels of HSP70 in blood were significantly associated with paler colour (less carotenoid

chroma) in birds (Fig. 3). In contrast, we did not find a significant relationship between HSP60 levels and carotenoid chroma. The final model explains 11% of variation in carotenoid chroma ($F_{6, 148} = 4.222$, $p<0.001$).

Discussion

Sex and age differences of carotenoid-based colouration of blue tits have been reported in some populations (Figuerola *et al.* 1999, Hidalgo-García 2006) but not in others (Ferns and Hinsley 2008). In this study, adult birds from both sexes showed similar values of carotenoid chroma, whereas male young birds were significatively more colourful than female young birds. Additionally, we did not find age differences in colour of males but we did in colour of females, young females being paler than adults. Therefore, it seems that blue tits are more or less dimorphic in the yellow plumage of the breast depending on population considered. This fact may be due to the partial dependence of carotenoid-based plumage colour on environmental factors (Hadfield and Owens 2006), that is, factors such as habitat and population density may contribute to the presence of sex and age differences.

On the other hand, we also found an effect of primaquine on carotenoid chroma in relation to sex. Untreated males showed significatively higher values of carotenoid chroma than treated males and females. We treated birds with primaquine months after deposition of carotenoids in feathers during moult so we did not expect a direct effect of this drug on bird's colour. It could be that this was a spurious effect, that is, colour of untreated males was higher even previously to treatment; however, another possibility could be that increased provisioning rates of treated birds (Tomás *et al.* 2007) induced a higher abrasion of males' plumages due to rubbing against nest boxes, resulting in paler colours. Studies taking into account colouration before treatment are needed to clarify the effect of primaquine on carotenoid-based plumage colour.

The most novel result of this study is the effect of multiple blood parasite infections on carotenoid chroma. Birds parasitized by a single genus of blood parasite had higher carotenoid chroma and thus showed a more colourful plumage than birds parasitized by more than one

parasite. In spite of the temporal separation between moult and the reproductive season (when we took colour measurements), the relationship between parasite richness and carotenoid chroma could be expected. Birds being able to develop a conspicuous plumage during moult could show a better health status in the next spring, especially because most of these blood parasite infections are chronic with birds suffering relapses while breeding (Valkiūnas 2005). Birds in poor condition would be less efficient acquiring carotenoid-rich food (Hill 1991, 1992; Senar *et al.* 2002) and consequently they would exhibit a paler plumage. A previous experiment in the study population showed that medicated females with primaquine did not vary in body condition during the breeding season, whereas it was reduced for control females, suggesting that blood parasites have negative effects on female condition (Merino *et al.* 2000). In addition, Tomás *et al.* (2007) showed that females treated with high doses of the antimalarial drug were able to increase their reproductive effort in comparison to control females and those treated with low doses of the drug. These results support the existence of harmful effects induced by parasitism, parasitized birds being in poor condition and therefore showing a paler colour. Birds in the poorest conditions are probably more susceptible to be infected by multiple parasites (Beldomenico *et al.* 2008) which could partially explain the paler colour in birds with multiple infections.

Furthermore, in accordance to the trade-off in carotenoid allocation between deposition of these pigments into the feathers and their participation in physiological functions as immune-stimulators and antioxidants, parasitized birds would have less carotenoids for pigmentation. A non-excluding mechanism would be that carotenoid-based traits are signalling levels of other non-pigmentary antioxidants that might protect carotenoids from free radical attacks and make them available for sexual advertisements (Hartley and Kennedy 2004). Additionally, apart from the passive "protection mechanism" (Hartley and Kennedy 2004), the allocation of colourless antioxidants to sexual signalling may promote an active mechanism to increase the amount of pigments (Pérez *et al.* 2008). Another hypothesis suggests a trade-off between lipids being used for energy generation or for absorption and/or transportation of carotenoids (Fitze *et al.* 2007). Whatever the mechanism implied, it appears that carotenoid-based plumage colouration signals

health status in our blue tit population, as multiple infections affect negatively the showiness of the yellow breast plumage. Negative effects of multiple infections by blood parasites in birds have been also found in other studies (Davidar and Morton 2006; Marzal et al. 2008). This could be due to additive effects of infection by different blood parasite genera. If this happens, our results would indicate that carotenoid-based colour of birds does not reflect the additive effect of the third and following blood parasite genera or it could be that there would not be an additive effect of the third and following blood parasite genera on the host. Another possibility is that infection by a second genus could induce competition for resources between different parasites (Frank 1996) or activation of the immune response (Read and Taylor 2000), which would induce parasites to become more virulent than if they infected hosts separately (Frank 1996). It is assumed that parasites which grow more rapidly inflict more damage to their hosts and they are competitively superior to less harmful parasites (see de Roode *et al.* 2005). The intensity of infection by *Haemoproteus* (the most frequent blood parasite in each category) in our blue tit population was not different when infecting a host alone or with other blood parasites ($F_{3, 114}$= 1.847, p=0.143). It could be that competition between parasites did not occur or whether it did, the effect on host would be similar irrespective of the number of blood parasites involved, thus implying that a higher competition may be also detrimental for parasites. In fact, as in additive effects, it could be that the effects of competition between three and following blood parasite genera did not appear on carotenoid-based colour. Nevertheless, it would be interesting to consider the intensities of the rest of the blood parasites involved to go into the interactions between parasites in a host and their effects.

On the other hand, we found an inter-annual variation in the effect of multiple infections on carotenoid chroma. In 2007, birds parasitized by two and three blood parasite genera were significatively more colourful than birds infected by two and three blood parasite genera in 2004. Carotenoids must be acquired through the diet (Olson and Owens 1998), being caterpillars the major source of these pigments for blue tits (Slagsvold and Lifjeld 1985). Thus, a year may be "favourable" for birds because there is a higher abundance of food (greater carotenoid availability) and/or a lower abundance of parasites, and consequently, more

carotenoids would be available to be deposited into birds' feathers giving them colour. Hence, it was not surprising to find inter-annual variation in carotenoid chroma of birds in relation to blood parasite richness. Likewise, Hõrak *et al.* (2000) showed that great tit nestlings which grew up in a "bad" year had paler yellow breasts than nestlings which grew up in a "good" year. Different environmental conditions can also be found between habitats, explaining a great part of the variation in colour of birds from diverse quality habitats. Blue tits inhabiting structurally complex forests obtain more caterpillars (Ferns and Hinsley 2008), and consequently present higher chroma values than blue tits from poor quality forests (Arriero and Fargallo 2006). Our results suggest that birds of 2007 infected by 2 or 3 blood parasite genera, in late 2006 (when they moulted their plumage) had higher carotenoid availability for pigmentation than birds of 2004 infected by 2 or 3 blood parasite genera in late 2003. However, birds infected by 4 or 5 blood parasite genera presented similar values of carotenoid chroma in both years, suggesting that highly parasitized birds are the palest birds independently of goodness of environmental conditions.

Since carotenoid chroma of plumage reflected to a certain degree the blood parasite richness within a host, we could also expect it to reflect body condition, and immunoglobulin and HSPs levels. Although Senar *et al.* (2003) found that carotenoid-based colour of the breast of the great tit *Parus major* was correlated with the nutritional condition as estimated by the rate of tail growth and several authors have reported positive correlations between carotenoid-based colours and immune function (Dufva and Allander 1995, McGraw and Ardia 2003, Alonso-Álvarez *et al.* 2004), we could not find any association between carotenoid chroma and immunoglobulin levels or body condition. However, birds with lower carotenoid concentration in feathers had higher HSP70 levels. These proteins prevent cellular homeostasis alteration and are involved in many different functions like protein synthesis, folding and transport, as well as in degradation of misfolded, non-functional proteins (Morimoto 1991). These proteins are implicated in responses to a wide array of stressors (Gehring and Wehner 1995, Martínez *et al.* 1999a) including blood parasites (Merino *et al.* 1998, Tomás *et al.* 2005). Thus the relationship between HSP70 and plumage colour may be in part related to the presence of infection or an

infection-associated stress. The fact that blood parasite infections are chronic and maintained in birds for several months and even years with periods of relapses (Valkiūnas 2005) may help to understand the existence of these associations between long term stress indicators as HSP levels and plumage colours.

Overall, based on the full data set, carotenoid-based plumage colouration seems to be a long-term indicator of health status in this population of blue tits. Results found in this study reveal negative effects of multiple infections by blood parasites on carotenoid-based plumage colour. Further experiments are needed to better understand the effects of multiple parasite infections on hosts and the mechanisms underlying carotenoid-based colouration.

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Table 1. Sequences and annealing temperatures of the used primers.

Primer	**Annealing**	**Parasite**	**Sequence (5' → 3')**
LDLd	58	*Leucocytozoon*	CAT TCY ACW GGT GCA TCT TT
LDRd	58	*Leucocytozoon*	CTG GAT GWG ATA ATG GWG CA
PLAS-F	60	*Plasmodium*	GTA ACA GCT TTT ATG GGT TAC
4292Rw [1]	60	*Plasmodium*	TGG AAC AAT ATG TAR AGG AGT
HML	58	*Haemoproteus*	GCT ACT GGT GCT ACA TTT GT
HMR	58	*Haemoproteus*	CCT AAA GGA TTA GAG CTA CC
S-755 [2]	60	*Trypanosma*	CTA CGA ACC CTT TAA CAG CA
S-823 [2]	60	*Trypanosma*	CGA AYA ACT GCY CTA TCA GC
Hep900F	58	*Lankesterella*	GTC AGA GGT GAA ATT CTT AGA TTT G
Hep1615R	58	*Lankesterella*	AAA GGG CAG GGA CGT AAT C

[1] Primer previously published by Beadell *et al.* (2004).

[2] Primers previously published by Maslov *et al.* (1996).

Table 2. Results of the GLM analysis exploring the relationships of parasite richness and body condition with carotenoid chroma of blue tit breast plumage in 2004 and 2007. The potential effects of year, date of measurement, sex, age and treatment were controlled for. Full and minimal models are presented.

			Full model		Minimal model
Effect	**d.f.**	**F**	**p**	**F**	**p**
Year	1, 188	3.164	0.077		
Date	1, 188	0.272	0.602		
Sex	1, 188	1.979	0.161		
Age	1, 188	0.0007	0.980		
Treatment	1, 188	2.478	0.117		
Body condition index	1, 188	0.013	0.908		
Blood parasite richness	3, 188	4.218	**0.006**	6.321	**<0.001**
Year x sex	1, 188	0.485	0.487		
Year x age	1, 188	0.511	0.476		
Sex x age	1, 188	5.336	**0.022**	6.299	**0.013**
Year x treatment	1, 188	0.115	0.735		
Sex x treatment	1, 188	8.560	**0.004**	7.486	**0.007**
Age x treatment	1, 188	1.915	0.168		
Year x blood parasite richness	3, 188	3.332	**0.021**	5.447	**0.001**
Sex x blood parasite richness	3, 188	0.634	0.595		
Age x blood parasite richness	3, 188	0.117	0.950		
Treatment x blood parasite richness	3, 188	1.135	0.336		

Table 3. Results of GLM analysis performed to explore the association of levels of immunoglobulins and stress proteins with carotenoid chroma of blue tit breast plumage in 2004. The potential effects of date of measurement, treatment, sex and age were controlled for. Full and minimal models are presented.

		Full model		Minimal model	
Effect	**d.f.**	**F**	**p**	**F**	**p**
Sex	1, 123	0.956	0.330		
Age	1, 123	0.010	0.921		
Treatment	1, 123	2.268	0.135		
Body condition index	1, 123	0.019	0.890		
Date	1, 123	0.069	0.794		
Blood parasite richness	3, 123	3.117	**0.029**	2.969	**0.034**
Immunoglobulin levels	1, 123	0.094	0.760		
HSP60 levels	1, 123	0.975	0.325		
HSP70 levels	1, 123	6.008	**0.015**	6.025	**0.015**
Sex x age	1, 123	3.955	**0.049**	4.869	**0.029**
Sex x treatment	1, 123	4.289	**0.040**	4.330	**0.039**
Age x treatment	1, 123	1.513	0.221		
Sex x blood parasite richness	3, 123	0.405	0.749		
Age x blood parasite richness	3, 123	0.800	0.496		
Treatment x blood parasite richness	3, 123	1.729	0.164		

Legends to figures:

Figure 1. Carotenoid chroma variation in relation to blood parasite richness. Bars indicate standard error.

Figure 2. Carotenoid chroma variation in relation to year x blood parasite richness interaction. Rounded symbols correspond to data from 2004 and triangles to 2007. Bars indicate 0.95 confidence bars.

Figure 3. Relationship between HSP70 levels and carotenoid chroma of breast plumage colour in blue tits of 2004.

Fig. 1:

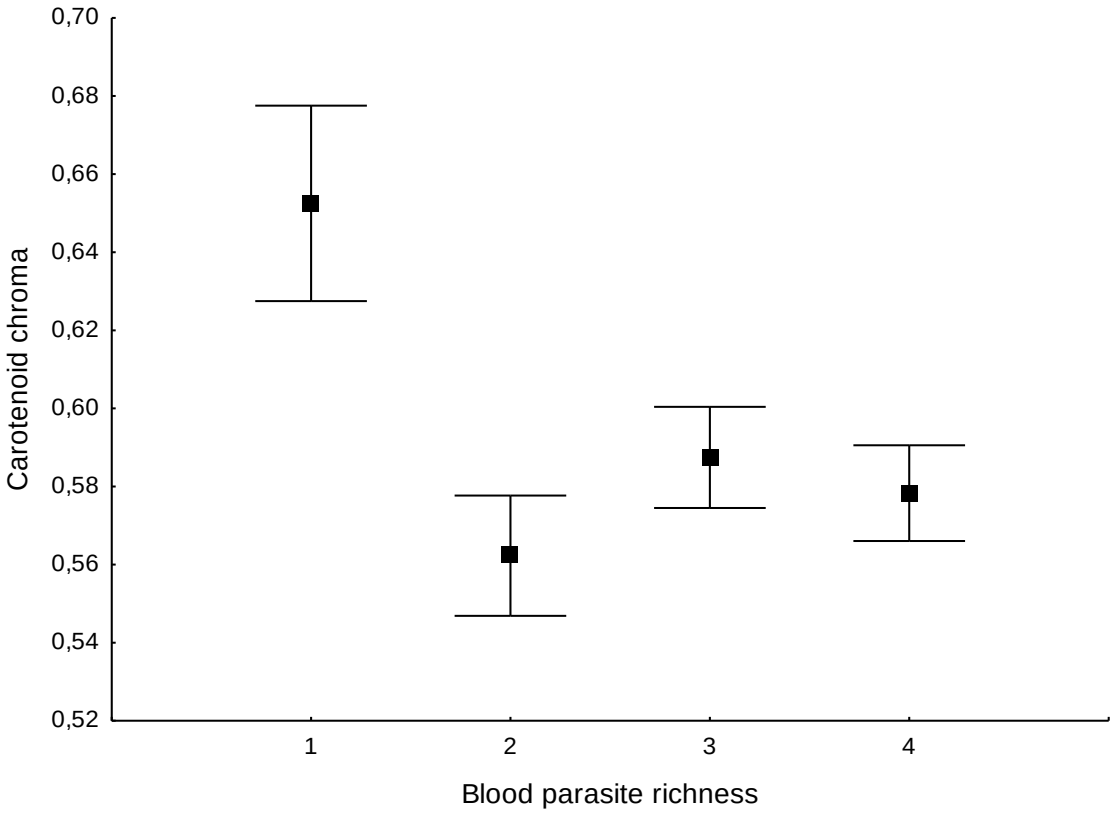

0,70
0,68
0,66
0,64
0,62
0,60
0,58
0,56
0,54
0,52
Carotenoid chroma
1
2
3
4
Blood parasite richness

Fig. 2:

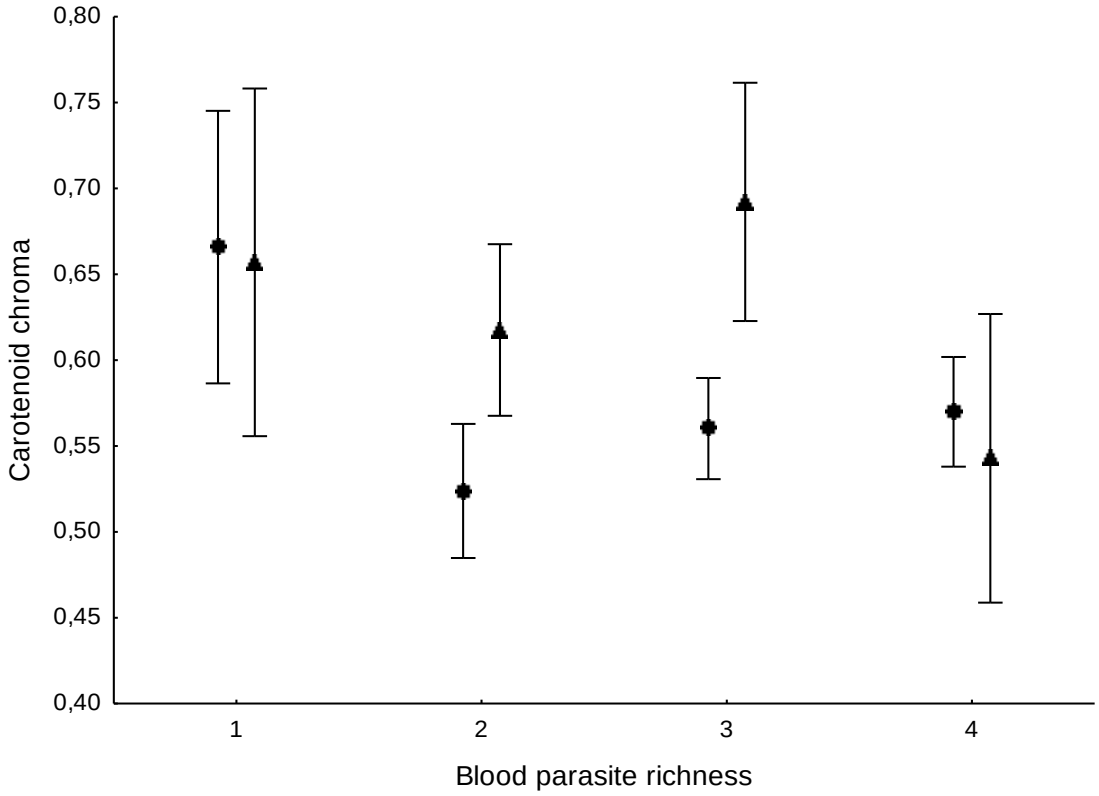
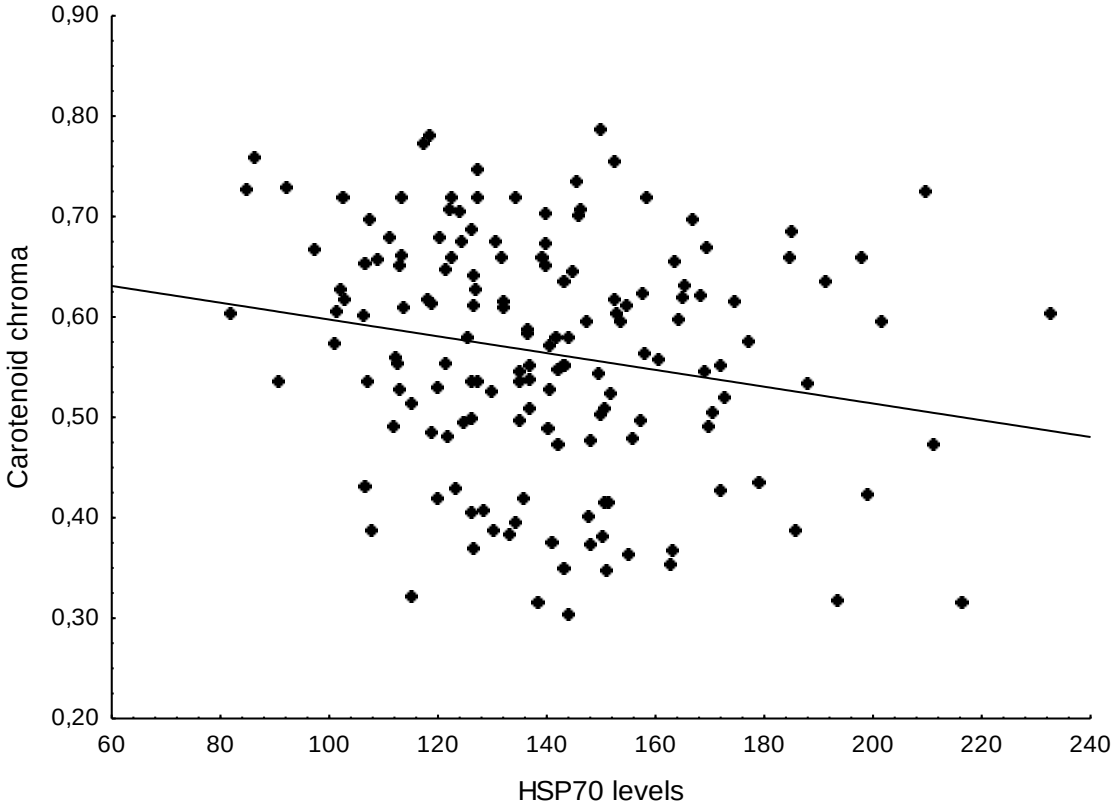

0,80
0,75
0,70
0,65
0,60
0,55
0,50
0,45
0,40
Carotenoid chroma
1
2
3
4
Blood parasite richness

Fig. 3:


0,90
0,80
0,70
0,60
0,50
0,40
0,30
0,20
Carotenoid chroma
60
80
100
120
140
160
180
200
220
240
HSP70 levels

Capítulo 3

MHC-I y selección de pareja en una población natural de herrerillo común en el centro de España

Los genes del complejo principal de histocompatibilidad (MHC) codifican para proteínas involucradas en el reconocimiento de antígenos derivados de los parásitos. La diversidad general en el MHC o alelos específicos se ha relacionado con una mayor resistencia y susceptibilidad a infecciones causadas por parásitos. Por lo tanto, la elección de pareja basada en el MHC podría actuar como un mecanismo de selección sexual para incrementar/optimizar la eficacia biológica de la descendencia. En este trabajo hemos estudiado una población de herrerillo común en el centro de España para investigar si la elección de pareja está relacionada con alelos específicos o con la diversidad general del MHC. Para ello se han investigado los alelos de MHC correspondientes a la región de unión del péptido (PBR) de la molécula de clase I, la cual está involucrada en el reconocimiento de parásitos sanguíneos intracelulares. Encontramos emparejamiento concordante en base a la diversidad del MHC. Es decir, las hembras estuvieron emparejadas con machos que tuvieron una diversidad del MHC similar. También, se observó que las parejas compartieron alelos similares. Además, las hembras más pálidas estaban emparejadas con machos con un MHC más diverso. También, el patrón de emparejamiento en base a la compatibilidad de los genes del MHC no fue estadísticamente diferente de un emparejamiento al azar. Nuestros resultados apoyan un sistema de emparejamiento concordante donde los individuos se emparejan con individuos con un MHC similar al propio. Este resultado parece lógico en una especie en donde ambos progenitores invierten en el cuidado de la descendencia y donde ambos miembros de la pareja se evalúan mutuamente. La asociación entre el color y la diversidad del MHC podría estar relacionada con la búsqueda de machos de mayor calidad por parte de las hembras, ya que la diversidad en el MHC se ha relacionado con una mayor probabilidad de reconocimiento de antígenos derivados de los parásitos.

MHC-I and mate choice in a natural blue tit population from central Spain

J. Rivero-de Aguilar, H. Westerdahl, J. Martínez-de la Puente, G. Tomás, J. Martínez, S. Merino

Major histocompatibility complex (MHC) genes encode proteins involved on parasite antigen recognition. MHC diversity and specific MHC alleles has been related to both resistance and susceptibility to parasite infections. Therefore, MHC-based mate choice could act as a sexual mechanism to increase/optimize offspring fitness. In this study we investigate a blue tit population in central Spain to search for associations between MHC-I and mate choice. We also investigate breast yellow plumage colour as a signal of infection status affecting mate choice. To that end, we screened MHC-I alleles corresponding to the peptide binding region (PBR). We found assortative mating according to MHC-I diversity. Females were paired with males that had similar MHC-I diversity. In addition, the apparent MHC compatibility-based mate choice pattern was not statistically different from a random pairing sample. These results point out to the occurrence of a mating system where individuals select pairs similar to each other in MHC-I genes. This finding is in accordance with host/parasite systems where parents invest in parental care and both members of the pair evaluate the quality of its mate. Furthermore, the less colourful females where paired to the more MHC diverse males, suggesting a female searching for high quality males.

Introduction

Several hypotheses implying parasitism and immune defences have been suggested to explain the evolutionary forces driving sexual selection (Schmid-Hempel, 2011). Among them, the "genetic compatibility hypothesis" proposes that the choosing sex (usually the female), in order to increase offspring fitness would select mates compatible/dissimilar at fitness-related genes, i.e. those belonging to the major histocompatibility complex (MHC) (Brown & Eklund, 1994; Neff & Pitcher, 2005). The underlying explanation is that females mating with a MHC-dissimilar male would increase offspring MHC diversity, improving the chances on parasite recognition by their offsprings. In this situation and being mate choice dependent on female's MHC genotype, females should discern between males with similar or dissimilar MHC genotypes (Stearns & Koella, 2008). Alternatively, females could select mates either for highly MHC diversity or for those with advantageous MHC genotypes ("good genes hypothesis") (Griggio *et al.*, 2011; Dunn *et al.*, 2013). Highly MHC diverse individuals will be able to recognise a wider spectrum of parasites, whereas individuals with advantageous MHC genotypes will face better the infection to specific parasites (Apanius *et al.*, 1997; Setchell & Huchard, 2010; Dunn *et al.*, 2013).

On the other hand, much empirical evidence has shown in birds that female choice rely on male secondary sexual traits (e.g. plumage colour), whose expression could be affected by parasites (Hamilton & Zuk, 1982; Møller *et al.*, 1999). Thus, males being capable of expressing highly elaborated ornaments which are costly to maintain could be signalling high genetic quality to the females (Zahavi, 1975; Møller *et al.*, 1999). These males potentially have high MHC diversity and/or advantageous MHC haplotypes (Piertney & Oliver, 2006). However, choosy females could also obtain direct benefits from males instead of indirect ones. For example, females could avoid infections from contagious parasites, breed in a good quality territory or obtain better food supply ("good parent hypothesis") by mating with healthy individuals (Møller, 1994).

Under this scenario, evidence of MHC-based mate choice has been observed in several vertebrates, including non-natural populations of birds, fish, and lizards (Setchell & Huchard,

2010). More data are necessary from wild populations where natural selection related to infections is relevant (Møller, 1994; Valkiūnas, 2005; Atkinson *et al.*, 2008). In birds, MHC-based mate choice has been investigated in several species rendering different findings. For example, no evidence of MHC-dissimilar mate choice was observed for MHC-I genes neither in great reed warblers *Acrocephalus arundinaceus*, nor great snipes *Gallinago media* nor in blue petrels *Halobaena caerulea* (Ekblom *et al.*, 2004; Westerdahl, 2004; Strandh *et al.*, 2012). However, evidence of MHC assortative mate choice was observed in house sparrows *Passer domesticus*, where females discriminated among males when formed pairs, avoiding pairing with males too dissimilar and with low MHC diversity (Bonneaud *et al.*, 2006). In the same study MHC diversity was positively correlated among pairs, but no relation was found involving phenotypic traits (breast patch) as a cue for choosing partners. In addition, two MHC alleles were found to be significantly associated, suggesting the evolution of co-adapted genes probably selected by parasites. In ring-necked pheasants *Phasianus colchicus* clear relationships between MHC-I genotype and spur length and age, body size and viability were found (von Schantz *et al.*, 1996; 1997) and in great tits *Parus major* MHC supertypes (related to peptide binding properties) were associated to survival and lifetime reproductive success (Sepil *et al.*, 2013). Contrarily, in common yellowthroats *Geothlypis trichas* MHC-I genes were not related to ornaments, parasitism and survival (Dunn *et al.*, 2013). Finally, in Seychelles warblers *Acrocephalus sechellensis* females that paired with low diverse males tried to gain extra-pair fertilisations and elevated diversity was found related to juvenile survival (Richardson *et al.*, 2005; Brouwer *et al.*, 2010). In addition, different MHC-based mate choice strategies are non-mutually exclusive and can coexist in a given population (e.g. targeting compatible or particular genotypes) (Setchell & Huchard, 2010; Dunn *et al.*, 2013).

In this study we have investigated MHC-based mate choice in a population of blue tits *Cyanistes caeruleus* breeding in central Spain. The blue tit is a social monogamous species commonly used as model species in mate choice and carotenoid-based ornamental plumages studies (Delhey *et al.*, 2010). Both sexes have a yellow breast carotenoid-based plumage and are considered sexually dimorphic for this trait (Figuerola *et al.*, 1999). Carotenoid-based

ornaments have been related to mate choice in birds, and its expression has been found related to physiological and immune functions, so that they can be considered as honest signals indicating health status or individual quality (Zahavi, 1975; Svensson & Wong, 2011). In our study population, individuals are commonly infected by different genera of blood parasites, such as *Plasmodium*, *Haemoproteus*, *Leucocytozoon*, *Lankesterella* and microfilaria, which have detrimental effects on host breeding success or survival (Merino *et al.*, 2000; Tomás *et al.*, 2005; Martínez-de la Puente *et al.*, 2010a,b). The cost imposed by these parasites has been related with the expression of carotenoid chroma in plumage in the blue tit (del Cerro *et al.*, 2010). Therefore, we investigated the relationship between MHC-I genes and breast plumage colour of breeding pairs.

Material and methods

Study species

During spring 2004 a blue tit nest-box breeding population was studied in a Pyrenean oak forest in Valsaín, Segovia, central Spain (40º53' N, 4º01'W). Nest-boxes were inspected from 1 of April to determine laying date and hatching date. At day 3 after hatching day (hatching date = 0), adults were trapped and a blood sample was taken from the brachial vein of each individual after punched with a needle. Blood was collected with a capillary tube (50 - 100 µl) and kept in a cold bag during the field day and later in the laboratory centrifuged (5 min at 12000 rpm) to separate serum from cells. Finally, blood samples were stored at -80º C for posterior molecular analysis. Total genomic DNA was isolated from each blood sample by using the UltraClean DNA BloodSpin kit (MO BIO laboratories, Inc., California) following manufacturer's protocol.

MHC diversity

MHC diversity was investigated by the reference strand conformation analysis (RSCA) method (Arguello *et al.*, 1998) adapted for the blue tit (Schut, 2012). RSCA is a PCR based technique that uses a fluorescently labelled reference strand (FLR) to separate individual MHC

alleles. In brief, the hybridization product from blue tit genomic DNA and a great tit clone is ran in a molecular sequencer resolving peaks that corresponds to MHC alleles. Details of the whole method are explained in Schut (2012). This method allowed determining the polymorphic MHC-I allele group detected in the blue tit, which is expected to be involved in antigen presentation (Schut *et al.*, 2011). MHC diversity was investigated by using one reference great tit *Parus major* clone (FLR-GT6). Thus, only alleles perfectly identified were included in the analyses. The number of MHC alleles detected is related to MHC diversity, thus individuals with several MHC alleles are probably more heterozygotes than individuals with a low number of MHC alleles (Arguello *et al.*, 1998).

Colour measurements

Individuals included in the study belonged to the dataset analysed in del Cerro et al. (2010), where colour measurement field protocol is explained in detail and repeatability of colour measurement was high. The yellow breast plumage from a total of 62 blue tits pairs was measured by using a portable spectrophotometer (CM-2600d, Minolta), covering the range of wavelengths between 360 and 740 nm. From the raw spectral reflectance data, individual carotenoid chroma was calculated as $(R_{700}-R_{450}) / R_{700}$. This is the relative difference in reflectance between the wavelengths of minimum (700 nm) and maximum (450 nm) absorbance of the two main carotenoids in blue tit plumage (lutein and zeaxanthin).

Statistical analysis

To investigate MHC-based pairing among mates, two analyses were performed. First, the relationship of MHC diversity between social pairs was investigated by Spearman correlation test. Next, the MHC allele similarity (D) between social pairs was calculated as twice the number of sharing alleles divided by the sum of the number of alleles in males and females individuals: $D = 2Fab / (Fa + Fb)$ (Wetton *et al.*, 1987). A score equal to one would indicate total allele MHC allele similarity, and a value equal to zero would indicate total MHC allele dissimilarity. Once MHC allele similarity was estimated, it was compared between social

pairs and random simulated pairs. To do that, the mean value of MHC allele similarity of social pairs was compared with the mean value of a random pairing distribution obtained by 10000 bootstraps. Ninety-five per cent confidence interval was generated from the random distribution. If MHC allele similarity from random pairs was contained inside the 95 % confidence interval, then there is statistically non-significant difference between the MHC allele similarity from social pairs and those obtained from the random distribution.

Finally, we investigated the relationship between MHC diversity and carotenoid chroma of individuals and then the relationship between the MHC diversity of one member of the social pair and the carotenoid chroma of the other member. These latter analyses were studied by applying a Spearman correlation test.

Analyses were performed in STATISTICA v.10 (StatSoft, 2011) or in R (R-project, 2012).

Results

MHC-I diversity and specific MHC alleles

A total of 56 blue tit pairs were included in the analyses. A maximum number of five MHC-I alleles were detected per individual (mean ± SD, 3.2 ± 0.95, range = 1-5). Thus, there was enough variation to investigate the role of MHC genes in host mate choice. MHC-I alleles corresponded to the previously detected alleles Paca UA*104 (JF742767), Paca UA*108 (JF742771), Paca UA*114 (JF742777) and Paca UA*117 (JF742780) (Schut *et al.*, 2011). Paca UA*236, Paca UA*249 and Paca UA*274 are new alleles and no sequencing was performed after RSCA screening. Two alleles Paca UA*236 and Paca UA*274 were found in very low frequency and were excluded from MHC-similarity analysis.

MHC-based mate choice

MHC diversity was positively associated among social pairs (Spearman Rank Test: R = 0.302, P = 0.017), (Fig. 1). MHC allele similarity between pairs (D) was 0.21. When this value was compared to the MHC allele similarity from a distribution generated at random there was

no significant difference (P = 0.33) (Fig. 2). This result supports a MHC similarity based assortative pairing.

MHC diversity and plumage chroma of blue tits pairs

No association was found neither between male nor between female MHC diversity and their carotenoid chroma (Spearman Rank Test: R = 0.006, P = 0.96 and R = -0.04, P = 0.75, respectively). That was true even after controlling by age (males: adults, R = 0.04, P = 0.81; juveniles, R = -0.02, P = 0.89; females: adults, R = -0.17, P = 0.36; juveniles, R = -0.04, P = 0.75). Also no association was found between female MHC diversity and male carotenoid chroma (Spearman Rank Test: R = 0.02, P = 0.90). However males with the higher MHC diversity were paired with females with the lower carotenoid chroma (Spearman Rank Test: R = -0.34, P < 0.01) (Fig. 3).

Discussion

In this study we investigated MHC-based pairing in a blue tit population from central Spain. Individuals were paired accordingly to MHC-I diversity, thus females mated with males with similar diversity. A similar result is observed in house sparrows, where females mate with males sharing similar alleles (Bonneaud *et al.*, 2006). During pair formation, females avoid dissimilar mates, pointing out a MHC-similar mate choice. In our study it was observed some MHC dissimilarity but it was not different compared to a random mating scenario, thus our results did not support MHC-dissimilarity mate choice in the studied population.

One hypothetical explanation for the pattern observed is that females would gain mating with similar males to avoid disrupting adaptation to local parasites (Bonneaud *et al.*, 2006; Loiseau *et al.*, 2009). In any population, it would be advantageous for the host to maintain a diverse pool of resistant alleles to cope with on-going parasite infections. By mating with dissimilar individuals, females could reduce the chances for their offspring to inherit their resistant alleles. In house sparrows, a selection accordingly to its own MHC diversity is observed, with low diverse females selecting higher diverse males (Griggio *et al.*, 2011) and this

kind of selection was suggested as a mechanism for females to either increase or optimize offspring MHC diversity.

In vertebrates, MHC-based mate choice has been suggested as a way for females to maximize/optimize offspring fitness (Milinski, 2006; Setchell & Huchard, 2010). In great reed warblers, the surviving offspring tend to have a high MHC allelic diversity (Hansson *et al.*, 2004) and, in house sparrows, offspring's MHC diversity depends on parent's MHC diversity (Bonneaud *et al.*, 2006). Therefore it seems, at least for these studies, a correlation between parent and offspring's MHC diversity. MHC diversity has been related to higher chances on parasite detection because it increases MHC molecules repertory. However the benefits of being diverse in the MHC could be due to higher chances of having a specific resistant allele. On the other hand, the relationship between heterozygote advantage and offspring fitness is not without controversy (see Kempenaers, 2007) thus an optimal rather than a higher number of MHC alleles would be advantageous as well (Reusch *et al.*, 2001; Wegner *et al.*, 2003). In this situation, females would select males to optimise MHC heterozygosity of their offspring. More support for the optimal hypothesis has been observed in the same species, with females discriminating among males genotype and females mated with males sharing similar alleles (Bonneaud *et al.*, 2006).

In passerines when MHC diversity between mates is similar, they have an increase success in extra-pair mating (Freeman-Gallant *et al.*, 2003; Richardson *et al.*, 2005). In blue tits, extra-pair copulation is relate to female body condition and genetic quality of the male, with heavy females searching for extra pair fertilizations when their social mate has low genetic quality (Dreiss *et al.*, 2008). Lean females however search for extra pair fertilizations when pair to similar genetic quality male and male extra-pair young fledge earlier than their siblings (Schlicht *et al.*, 2012). In our study population extra-pair copulation is quite elevated (Badás *et al.* personal communication), so it is likely that the pattern observed changed when extra-pair mating was taking into account.

As the colour of the males is a signal of good health status, females would gain by choosing a mate based on expression of plumage coloration (Jamieson, 2007), although a good

quality female would be also determinant on breeding success since females feed offspring as well. Breast yellowness is positively associated to parental investment and with the ability to collect carotenoid-rich food items and female breeding success (Senar *et al.*, 2002; Garcia-Navas *et al.*, 2012). Blood parasites affects blue tit breast colour (del Cerro *et al.*, 2010) and is a general observation that plumage colour is a signal of health status in males (Scott, 2005) but also in females (Midamegbe *et al.*, 2011; Holveck *et al.*, 2012), thus by choosing a showy mate the chances of disease transmission or death due to parasitic infections decrease (Scott, 2005). If there is compromise to express a yellow breast in more parasitized individuals, then individuals should discern among mates accordingly to breast colour. In this sense, no association was found between MHC diversity and plumage colour in blue tits, so breast colour seems not reflect MHC diversity. If low colour females are mated to higher diverse males it could indicate that they are probably following a cue different to yellowness, for example, ultraviolet crown colour (Hunt *et al.*, 1999; Doutrelant *et al.*, 2008).

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Figure 1. Association between male and female MHC-I allele diversity. Circles are proportional to the number of pairs.

Figure 2. Mean value of MHC-I allele similarity (D) between social pairs compared to a random pairing distribution. The mean value of social pairs is represented by a vertical line. Dashed lines represent 95 % confidence interval of mean value of social pairs.

Figure 3. Association between male MHC-I diversity and female carotenoid-based chroma.

Figure 1

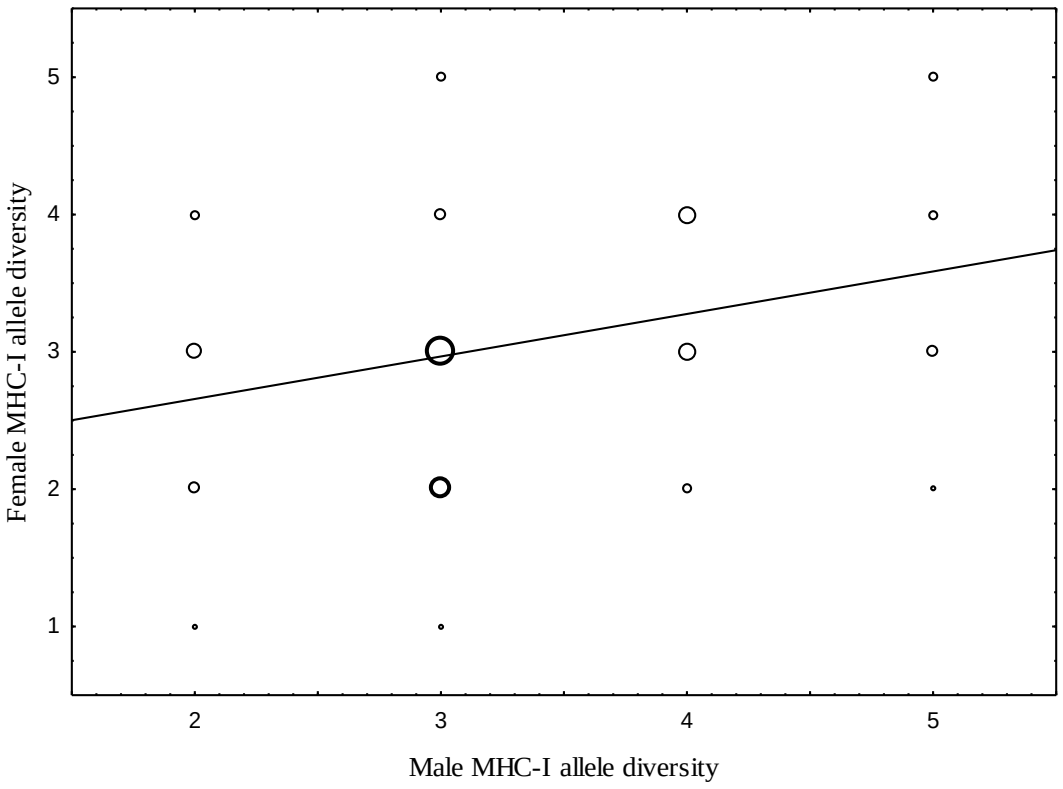

Female MHC-I allele diversity
5
4
3
2
1
2
3
4
5
Male MHC-I allele diversity

Figure 2

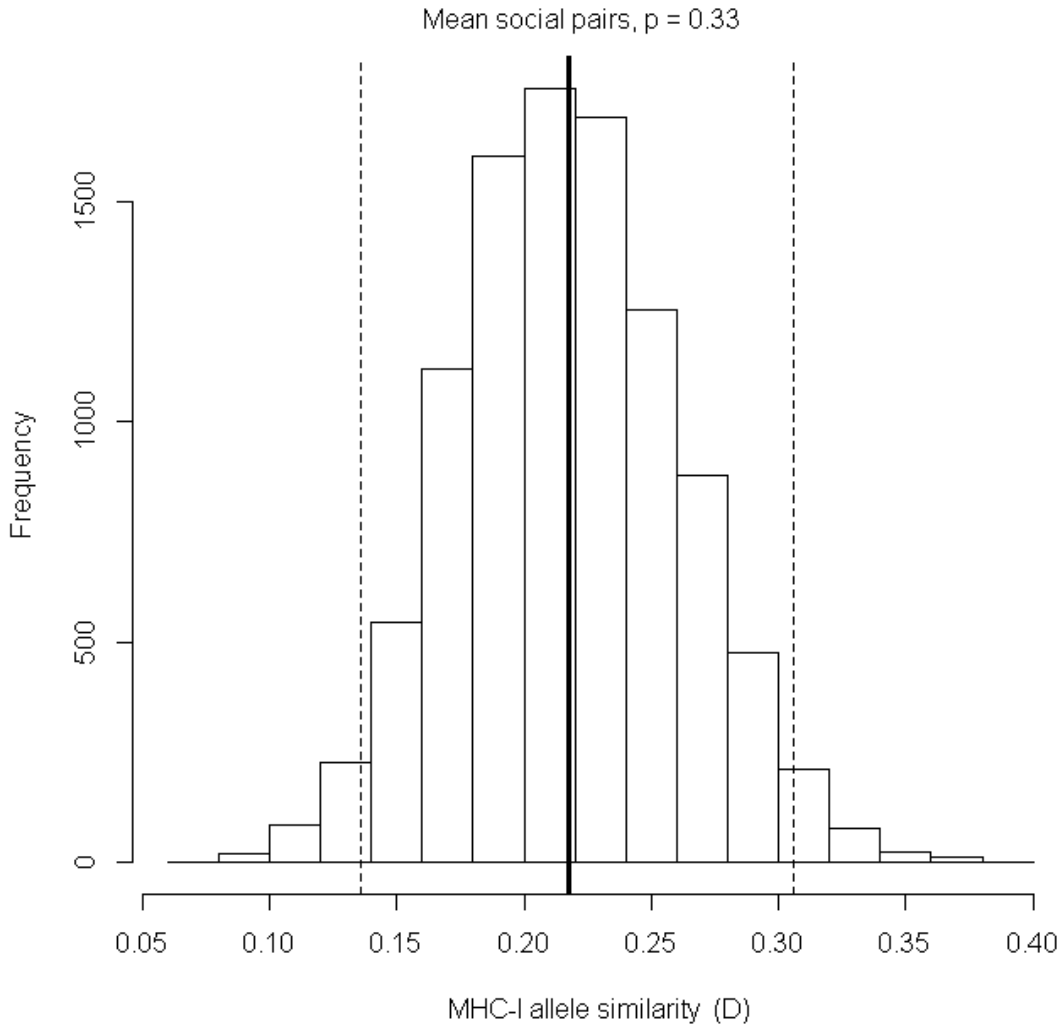
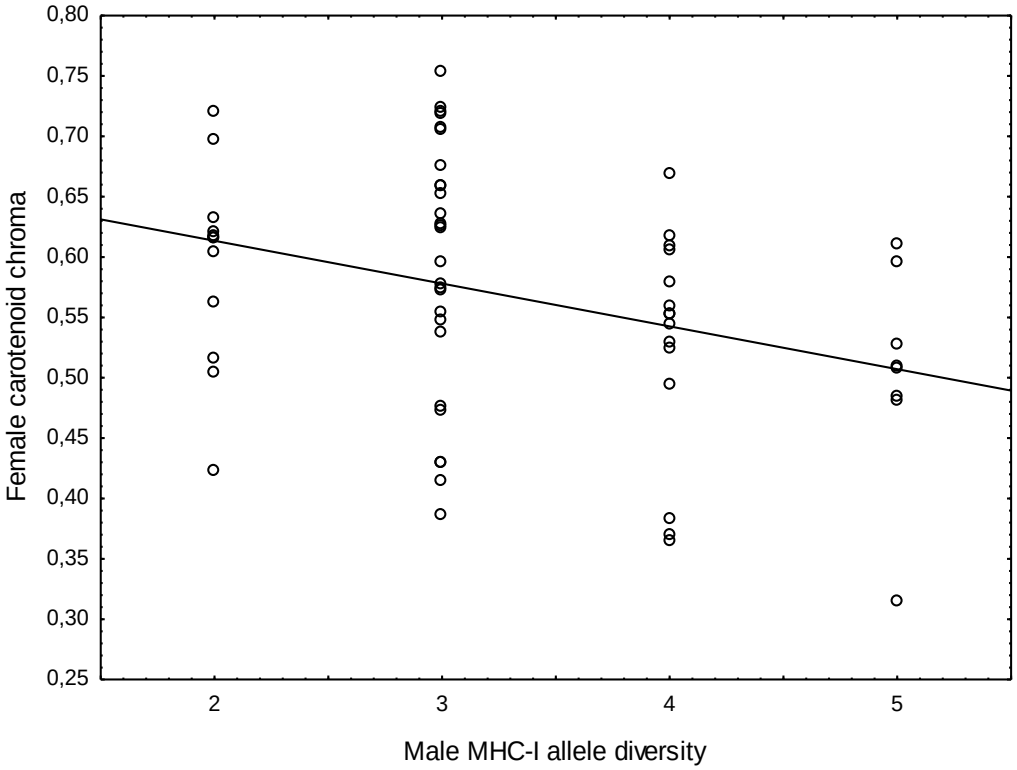

Mean social pairs, p = 0.33
Frequency
0
500
1000
1500
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
MHC-I allele similarity (D)

Figure 3


0,80
0,75
0,70
0,65
0,60
0,55
0,50
0,45
0,40
0,35
0,30
0,25
Female carotenoid chroma
2
3
4
5
Male MHC-I allele diversity

Capítulo 4

Diversidad del MHC de clase II en el herrerillo común: estudio preliminar

En este trabajo hemos caracterizado parcialmente el complejo principal de histocompatibilidad (MHC) de clase II B en el herrerillo común (*Cyanistes caeruleus*). La diversidad alélica del MHC fue estudiada en un total de 22 individuos de tres localidades europeas: España, Holanda y Suecia. Los genes de MHC fueron investigados por métodos basados en la PCR y polimorfismos de longitud de fragmentos de restricción (RFLP). Un total de 13 secuencias correspondientes al exón 2 fueron obtenidas independientemente a partir de ADN y/o ARN, confirmando así la transcripción y la probable funcionalidad de los genes. Nueve de 13 alelos se encontraron en más de un país, y dos alelos se encontraron en todos los países. Además se detectó selección positiva en la región de reconocimiento del péptido (PBR). Se detectó un máximo de tres alelos por secuenciación y el patrón de RFLP consintió entre 4 y 7 fragmentos, indicando un mínimo de 2-4 loci por individuo. En el análisis filogenético se observó que las secuencias obtenidas divergen de secuencias de otras aves paseriformes.

MHC class II B diversity in Blue tits: a preliminary study

Juan Rivero-de Aguilar, Elske Schut, Santiago Merino, Javier Martínez, Jan Komdeur, Helena Westerdahl

In this study we partly characterize Major Histocompatibility Complex (MHC) class II B in the blue tit (*Cyanistes caeruleus*). A total of 22 individuals from three different European locations: Spain, The Netherlands and Sweden were screened for MHC allelic diversity. The MHC genes were investigated using both PCR based methods and unamplified genomic DNA with restriction fragment length polymorphism (RFLP) and southern blots. A total of 13 different exon 2 sequences were obtained independently from DNA and/or RNA, thus confirming gene transcription and likely functionality of the genes. Nine out of 13 alleles were found in more than one country, and two alleles appeared in all countries. Positive selection was detected in the region coding for the peptide binding region (PBR). A maximum of three alleles per individual was detected by sequencing and the RFLP pattern consisted of 4-7 fragments, indicating a minimum number of 2-4 loci per individual. A phylogenetic analysis, demonstrated that the blue tit sequences are divergent compared to sequences from other passerines resembling a different MHC lineage than those possessed by the most passerines studied to date.

Introduction

The major histocompatibility complex (MHC) is a group of genes critical to immune function in vertebrates (Doherty and Zinkernagel, 1975; Klein, 1986). Due to its central role against infections from pathogens, the study of MHC genes has been the subject of many ecological and evolutionary studies (Sommer, 2005; Spurgin and Richardson, 2010), especially those studying host-parasite interactions (Westerdahl, 2007; Dionne, 2009). The characterization of MHC genes is constrained by its multigene nature, a result from gene duplications during evolutionary time and a turnover of new and old genes, the so-called birth-and-death model (Klein and Figueroa, 1986; Nei et al., 1997; Edwards and Hedrick, 1998; Nei and Rooney, 2005). Thus, vertebrates have its own particularities on MHC evolution, with orthologous and paralogous genes maintained mainly by natural selection from parasites but also by sexual selection (Hughes and Yeager, 1998). As a consequence, some MHC alleles have a long persistence time, even exceeding the species evolutionary time (i.e. trans-species polymorphism) (Klein and O'Huigin, 1994).

The MHC genes constitute the most polymorphic genes among vertebrates (Piertney and Oliver, 2006) and its study has been challenging, especially in non-model organisms (Babik, 2010). In passerines, the study of this genetic region is also complicated, due to the existence of concerted evolution among genes (Edwards et al., 1999; Hess and Edwards, 2002). Different molecular methods has been used to characterize MHC genes in birds, including PCR and non-PCR based methods (Babik, 2010). These studies involved the analysis of whole genetic region or partial regions, including complete or incomplete introns/exons. In this manner, several orders of passerine and non-passerine species have been studied to date (see Bollmer et al., 2010; Li et al., 2011; Miller et al., 2011). The first species with a completely sequenced MHC was a non-passerine, the chicken (*Gallus gallus*) and its MHC appeared simple and compact (Kaufman et al., 1999). Since then more species have been studied and the general conclusion is that the genetic organization of the passerines MHC seems to be more complex than that of non-passerines (Balakrishnan et al., 2010; Ekblom et al., 2011). This finding is supported by the derived phylogenetic position of passerines from non-passerines, where a minimal MHC seems

to be the ancestral condition for birds, at least for class II genes (Hughes et al., 2008; Balakrishnan et al., 2010). Although a ratite species was observed to have at least five MHC class II loci (Miller et al., 2011). Thus, passerines have a higher number of genes, larger class I and II genes (longer introns) and also non-functional genes (pseudogenes) (Edwards et al., 1998; Kaufman et al., 1999; Miller and Lambert, 2004a; Westerdahl et al., 2004b; Westerdahl, 2007; Bollmer et al., 2010). Both MHC class I and II genes has been studied in birds involving host-parasites implications. Having an elevated/optimal number of alleles (heterozygote advantage) and/or rare advantageous alleles (negative frequency-dependent selection) would be favoured by natural selection for parasite detection and elimination (Bodmer, 1972; Doherty and Zinkernagel, 1975; Wegner et al., 2003).

Different groups of MHC genes have been detected in different species. In the galliform birds there are two similar but independent MHC complexes, both with class I and class II genes (Briles et al., 1993; Strand et al., 2007). The B-complex has high polymorphic and expressed genes and has been associated to disease resistance (Kaufman, 2000) and the Y- complex (MHC-Y), a separate group of genes, less polymorphic and expressed genes to a lower extent. The Y-complex has been suggested to be involved in the innate immunity (Miller et al., 2004) and controversial associations among the Y-complex and Marek's disease have been reported (Wakenell et al., 1996; Vallejo et al., 1997). In some passerines low polymorphic genes seems reminiscent from the MHC-Y complex (Edwards et al., 2000; Gasper et al., 2001; Bonneaud et al., 2004; Jarvi et al., 2004), therefore a phylogenetic approach have been employed to classify groups of genes (Edwards et al., 1999).

In this study, we investigate MHC class II B genes in the blue tit (*Cyanistes caeruleus*) for the first time. The blue tit is established as a model species in different ecology studies where parasite prevalence, ecological factors and their effects on their host have been studied in depth (Hurtrez-Boussèz et al., 1997; Tripet and Richner, 1997; Fargallo and Merino, 1999; Merilä and Andersson, 1999; Wiles et al., 2000; Merino et al., 2006; Martínez-de la Puente et al., 2010). The blue tit MHC class I genes have recently been studied (Schut et al., 2011; Wutzler et al., 2012), therefore a preliminary characterization of the MHC class II B would be

determinant for a latter in depth molecular characterization, since it gives an idea of the complexity of the system (Wittzell et al., 1994; Babik, 2010). We used sequencing and restriction methods to investigate the exon 2 that codes from the most variable peptide binding region of the MHC class II molecule.

Material and methods

Study species

The blue tit is a small insectivorous passerine (Family Paridae) that breeds in the west Palearctic from Mediterranean to boreal zones (Cramp and Perrins, 1998). Most of the geographic range (75%) of this species is located within Europe (BirdLife, 2012). Blue tits willingly use nest-boxes for reproduction when provided. In the present study individuals sampled came from three different European locations where individuals breed in nest-boxes: Spain (Valsaín, 40º53' N, 4º01'W), Sweden (Revinge, 55º41'N, 13º26'E) and The Netherlands (The Vosbergen estate, 53º08'N, 06º35'E). The number of individuals sampled and their location of origin are detailed in Table 1 Supporting information.

PCR and sequencing

DNA exon 2 sequences

To get a measure of MHC diversity we investigated the exon 2 that code for the β_1 chain. The β_1 chain encodes part of the peptide binding region (PBR) of the MHC class II molecule. To that end whole blood samples (50-100 µl per bird) were obtained from the brachial vein and collected with a capillary tube in the field from 18 individuals (see Table 1 Supporting information). Blood was immediately stored in a cool box and later preserved either frozen at -80ºC, or in 99% ethanol, until molecular analysis. Genomic DNA was isolated either by standard phenol-chloroform extraction methods or by using the UltraClean DNA BloodSpin kit (MO BIO laboratories, Inc., California). Genomic exon 2 sequences were amplified by polymerase chain reaction (PCR) using standard procedures with AmpliTaq DNA Polymerase

PCR kit (Applied Biosystems, California). A single PCR reaction included 25 ng of genomic DNA, 0.5 μM of each of the primer 2ZFfw1 and 2ZFrv1 (Balakrishnan et al., 2010), 10 x PCR buffer, 0.5 μM dNTP, 2.0 mM MgCl and 1.0 unit of *Taq* polymerase in a final volume of 20 μl. The reaction was run in a thermal cycler Gene Amp PCR System 9600 (Perkin Elmer) at 94ºC for 2 min, 35 cycles of (94ºC for 30s, 60ºC for 30s and 72ºC for 30s), and 72ºC for 10 min. We previously tested how general the primers were in amplifying genomic DNA from several bird species including the blue tit. Also the primers has been utilized satisfactorily in other passerines (van Rensburg et al., 2012). The blue tit PCR product was checked on an ethidium bromide-stained 2% agarose gel for bands of the appropriate size. Because individuals are likely to be heterozygous and the possibility of amplifying more than one allele at any locus exists, amplicons were cloned in the vector pCR2.1 with the TOPO TA Cloning kit (Invitrogen, California) according to manufacturers' protocol. Clones with inserts were selected from colonies and diluted in 150 μl dd H_20 and heated to 95ºC for 3 min. Up to 20 clones of each individual were used for sequencing. To do this 1 μl of the clone dilution was amplified in a 20 μl PCR using 1 μM of cloning kit primers (M13 forward and M13 reverse), 1x PCR buffer, 0.125 mM dNTP, 2.0 mM MgCl, and 1.0 unit of *Taq* polymerase. The amplification consisted of 35 cycles at 94ºC, 60ºC, and 72ºC, each step for 30s. Positive PCR products were purified through precipitation in NH_4Ac via centrifugation and used as template in dye terminator sequencing reactions with Big Dye Terminator (Applied Biosystems, California). PCR conditions consisted of 25 cycles at 96ºC for 10s, 55ºC for 5s and 60ºC for 4 min. After precipitation in NaAc, sequences were run on an ABI PRISM Genetic Analyser 3730 (Applied Biosystems, California). Obtained sequences were read in one sequencing reaction. All sequences are the result of cloning events so each sequence corresponds to a clone. Only sequences that were found in at least two independent PCRs were regarded as verified sequences (Westerdahl et al., 2004b). Independent PCRs were performed either from the same individual or different ones. Unique sequences were also found but were considered non-verified. Non-verified sequences could be true sequences but also false sequences due to PCR mistakes. Primers were developed inside exon 2, therefore in this study we refer to "alleles" but

they do not encompass the entire length of exon 2. Also we expect not to detect all the possible alleles in the blue tit. Verified sequences were deposited in the GenBank (accession numbers JF775361 - JF775373).

cDNA exon 2 sequences

The purpose of this analysis was to obtain cDNA exon 2 of MHC class II B sequences from RNA and thus study transcription. Blood samples (100 µl per bird) were obtained from 7 individuals again by wing vein puncture (see Table 1 Supporting information). Blood from individuals C, D and E was used in both DNA and RNA analysis. Blood was preserved in 500 µl of Trizol-LS with the addition of 100 µl of K_2EDTA following Miller and Lambert (2003). Samples were stored at 4ºC for later RNA extraction. Total RNA was extracted as in Strandh et al. (2011). cDNA was obtained by RT-PCR using the two-step PCR reaction using the Retroscript kit according to protocol (Ambion, Applied Biosystems) again with the 2ZFfw1 and 2ZFrv1 primers. cDNA obtained was then used as a template on a standard PCR, where 2 µl of cDNA template was amplified with the same PCR reagents used for DNA. PCR conditions were 94ºC for 2 min, 35 cycles of (94ºC, 60ºC, and 72ºC, each step for 30s) and 72ºC for 5 min. PCR products were cloned and sequenced as in DNA methods described above. Again verified and non-verified sequences were found. Sequences were deposited in GenBank (accession numbers JF766222 - JF766234).

Restriction fragment length polymorphism

Another 17 individuals from two blue tit Swedish families were used for the RFLP analysis. These individuals were only used in this analysis and not in previous DNA or RNA analysis. With this analysis we wanted to obtain a rough description of MHC class II B genetic diversity. RFLP is based on restriction enzymes and the number of bands in each individual corresponds to the approximate number of MHC class II B alleles. To do this we performed a restriction cleavage with PvuII on 10 µg of genomic DNA following the methods described in

Westerdahl et al. (1999). The enzyme was previously tested to confirm its suitability. One of the verified cDNA sequences was used as a probe.

Data analyses

After manual alignment in BioEdit (Hall, 1999), DNA and RNA sequences were confirmed as exon 2 of MHC class II B using a BLAST search with NCBI GenBank. To measure sequence polymorphism the nucleotide diversity (π) and the number of segregating sites (S) were calculated from all alleles in DnaSP (see Librado and Rozas, 2009). These values were also calculated for other passerine sequences obtained from GenBank (Table 1). The same nucleotide length was used to calculate π and S for all the species. Next a Bayesian phylogeny was performed to study evolutionary relationships among the blue tit and other passerines. For this analyses we searched for all passerines exon 2 of MHC class II B sequences available from GenBank together with sequences corresponding to the BLB and YLB region from the chicken (*Gallus gallus*), the Eurasian black grouse (*Tetrao tetrix*) and the common mallard (*Anas platyrinchos*). From passerines functional and non-functional sequences were included. All the sequences were aligned by using MAFFT alignment implemented in Jalview (Waterhouse et al., 2009). Gblocks (Talavera and Castresana, 2007) selected for the most informative nucleotide positions under the less stringent option. Redundant sequences were discarded by using the redundancy removal option in Jalview. With this option sequences with a similarity above 90% were deleted from the alignment. jModeltest 0.0.1 (Posada, 2008) under AICc selected GTR as the suitable substitution model. The phylogeny was inferred in MrBayes v3.2 (Ronquist and Huelsenbeck, 2003) with 80 x 10^6 generations. The convergence of the parameter values sampled from the chains was checked by using the potential scale reduction factor (PSRF) once the standard deviation of split frequencies was below 0.01.

Subsequently, we also looked for signs of positive selection in the PBR. The non-synonymous (d_N) and synonymous (d_S) substitution ratio $\omega = d_N/d_S$ provide a measure of selection pressure at the amino acid level (Nei and Kumar, 2000; Yang and Nielsen, 2002). Neutral genes are supposed to have a $\omega = 1$ whereas genes under positive selection have $\omega > 1$.

We estimated ω ratio by calculating the average values of synonymous and non-synonymous substitutions per site by the Nei-Gojobori and maximum likelihood methods. In the first method the PBR nucleotide positions were previously defined based on the MHC structure determined by crystallography (Brown et al., 1993). In this method the Jukes Cantor correction was used (Nei and Gojobori, 1986) and a Z-test was performed for both, codons corresponding to PBR and non-PBR. Analyses were performed in MEGA 4.0 (Kumar et al., 2008). The second method calculates the positions under selection without any *a priori* information. This analysis was performed in CODEML program included in PAML 4 package (Yang, 2007). We tested the models M1a (nearly neutral), M2a (positive selection), M7 (beta) and M8 (beta & ω) of codon substitutions allowing the ω ratio to vary among sites. In the analysis a likelihood ratio test of positive selection was performed comparing model M1a against M2a and M7 against M8. P-values were calculated with a Chi–squared test.

Results

MHC diversity

Overall we obtained 217 verified sequences corresponding to blue tit exon 2 of MHC class II B from three different European locations (Table 2). 96 sequences were obtained from DNA and 121 from RNA. All sequences were searched in GenBank by using BLAST and they were similar to exon 2 of MHC class II B with a maximum identity of 82%. Sequences were 159 bp long (without primers) and covered 60% of exon 2 length which comprises 267 bp. 13 different alleles were verified obtained from DNA and RNA (Fig. 1 Supporting information). When these sequences were translated to amino acids, 12 amino acid sequences were obtained (Fig. 1). A total of 40 non-verified sequences were obtained, 38 from DNA and 2 from RNA (Table 2). No stop codon or shift in the reading frame was found in any sequence suggesting absence of pseudogenes in the samples. The maximum number of alleles found in an individual was four (individual S, Table 2), indicating the existence of at least two loci. Two individuals (Q and W) had three transcribed alleles (RNA), suggesting that both loci are expressed. Nine

out of 13 alleles were found in more than one country. Alleles Cyca-DAB*3 and Cyca-DAB*9 appeared in all countries.

The RFLP pattern consisted of 4-7 fragments per individual in the length range of 1-9 kb, each band corresponding to approximately one allele (Fig. 2). The intensity of the RFLP fragments were variable, either because their similarity to the probe differed or because RFLP fragments of certain lengths were more numerous than others (Westerdahl, 2003). Nearly all individuals had unique RFLP patterns except for the individuals F1 and 4 that shared the same bands, a likely result when comparing individuals from the same family. These results suggest a minimum number of four loci. The RFLP bands may correspond to both coding and non-coding genes, so it is possible that we are observing bands corresponding to pseudogenes that we do not amplify with our primers. On the other hand, one hybridising fragment could represent two genes with the same electrophoresis migration distance. Nucleotide diversity results from the blue tit and other passerines are presented in Table 1.

Phylogenetic analysis of class II B sequences

The Bayesian phylogeny on passerine MHC class II B sequences is presented in Fig. 3 (collapsed tree) and Fig. 2 Supporting information (extended tree). As expected, there were two well supported clades in the tree separating passerines from non-passerines. Inside passerines there was a well supported cluster (posterior probability of 97) with the majority of the passerine sequences (139 sequences out of 155). Inside this cluster there were well supported groups but also several sequences not resolved forming a polytomy. The sequences in this group included both non-functional alleles and alleles well assigned to be involved in peptide presentation. Interestingly, all the blue tit sequences appeared outside this major group and they formed a monophyletic cluster (posterior probability of 100) together with other passerine sequences. These sequences corresponded to the Dupont's lark (*Chersophilus duponti*) GU390279, the collared flycatcher (*Ficedula albicollis*) GU390277, woodchat shrike (*Lanius senator*) GU390285 and common raven (*Corvus corax*) GU390282, corresponding to sequences obtained with degenerated primers (Canal et al., 2010). Among these sequences non-functional

sequences were intermingled as well, i.e red-winged blackbird (*Agelaius phoeniceus*) AF030990, bluethroat (*Luscinia svecica*) FJ409236, FJ409241), slaty spinetail (*Synallaxis brachyura*) (AB531785, AB531793), blue-black grassquit (*Volatinia jacarina*) AB531794 and little greenbul (*Andropadus virens*) AY437894.

Selection analysis on exon 2

Putative PBR codons were inferred from HLA and then we did ω ratio analysis for the PBR and non-PBR, respectively, to detect selection. For the PBR, non-synonymous substitutions exceeded those of synonymous substitutions ((± SE) $d_N = 0.283 \pm 0.093$, $d_S = 0.134 \pm 0.062$, $\omega = 2.11$), hence there is a tendency for positive selection although this was not significantly different from 1 (Z = 1.566; P = 0.060). For the non-PBR, non-synonymous substitutions were similar to the synonymous substitutions ($d_N = 0.076 \pm 0.026$, $d_S = 0.078 \pm 0.033$, $\omega = 0.97$) and not significantly different from 1 (Z = -0.050; P = 1). For all positions (PBR and non-PBR together), non-synonymous substitutions exceeded those of synonymous substitutions ($d_N = 0.123 \pm 0.029$, $d_S = 0.090 \pm 0.028$, $\omega = 1.37$), also not significantly different from 1 (Z = 0.016, P = 0.181).

Maximun likelihood methods in CODEML found evidence of positive selection in the exon 2. Models involving selection (M2a and M8) fits the data significantly better than their respective neutral models (M1a and M7). Bayes empirical Bayes (BEB) found the amino acid sites 8, 31 and 42 to be under positive selection ($\omega > 1$). These positions were selected by both models M2a and M8. Position 8 fitted with that designated by Brown et al. (1993) to be a PBR position, whereas position 42 was one amino acid next to a designed PBR position. Position 31 coincided with a non-PBR position.

Discussion

MHC diversity

In this study we have examined the exon 2 of MHC class II B genes in the blue tit for the first time. We found a maximum of three expressed alleles per individual detected by

sequencing and a maximum of seven alleles detected by RFLP, suggesting at least two to four loci in the blue tit. We are cautious about the results observed and we propose them conservative and preliminary, since primers were designed over zebra finch sequences MHC class II sequences and are likely not amplifying all possible alleles. We checked this possibility adding new sequences to the dataset from recently sequenced species and several sequences were not detected, therefore blue tit MHC may be underestimated. Also, the values found for nucleotide diversity should be taken with caution, since the fact that non-verified sequences were amplified adverts that nucleotide diversity could increase if these sequences become corroborated. Taken this into account, we compared our results with that found in other birds. The total number of MHC class II B loci described in other passerines ranged from three (house sparrow and the red-winged blackbird) to 20 (common yellowthroat) and the number of transcribed loci ranged from three (house sparrow and the scrub jay) to eight (common yellowthroat) (see Bollmer et al., 2010). And the nucleotide diversity for exon 2 genes ranged from 0.007 (green bull) to 0.19 (house sparrow) (Bonneaud et al., 2004; Aguilar et al., 2006). When the blue tit sequence diversity was compared with some other passerines after controlling for the sequence length, a similar polymorphism was found (see Table 1). In the blue tit we found a value of $\pi = 0.10$, characteristic of polymorphic genes, although groups of sequences with low polymorphism have been found in some passerines (Edwards et al., 2000; Gasper et al., 2001; Bonneaud et al., 2004; Jarvi et al., 2004). But the values found in these cases are so low that the lack of polymorphism is evident (i.e the low π value found in the greenbul). Likewise, similar values of blue tit π have been found in non-passerines, i.e. in the chicken (0.107) or the black grouse (0.113) (Strand et al., 2007). Thus blue tit MHC diversity is similar to other passerines but resembling less diverse MHC genes. A low MHC class I genetic diversity in the blue tit has been reported by Schut et al. (2011) and Wutzler et al. (2012).

Several different hypotheses could explain the MHC diverse observed in the blue tit. A reduced MHC genetic diversity could be due to the population bottleneck the blue tits suffered during the last ice age (Kvist et al., 2004). In vertebrates a lack of MHC variability has been attributed to bottleneck events (Babik et al., 2009; Becker et al., 2009) and some bird species

with low MHC diversity have been reported to have passed a population bottleneck (Richardson and Westerdahl, 2003; Miller and Lambert, 2004b; Zhang et al., 2006; Bollmer et al., 2007). On the other hand, bird species affected by a greater diversity of parasites, either in time or space, should be selected to maintain/develop a more diverse MHC. Geographical variation in pathogen antigens leads to differential selection by same pathogens in different areas (Jeffery and Bangham, 2000) therefore a low diversity of the MHC could be explained by local adaptation to parasites (Klein, 1991; Westerdahl et al., 2005; Bonneaud et al., 2006). In this respect, we can expect that migratory birds exposed to a higher diversity of parasites (Møller and Erritzøe, 1998; Westerdahl et al., 2004a) could evolve a more diverse MHC than non migratory birds. The blue tit is considered a resident bird in Europe, except some migratory individuals from northern locations and some altitudinal movements produced during winter (Cramp and Perrins, 1998). Thus, when considering the passerine species included in our study there are some birds that fits with this prediction (migratory birds with higher diversity), but is not consistent among others. It will be necessary to increase the number of species in a comparative analysis to look for the potential effect of migratory behaviour on MHC genes diversity. Finally it will be important to identify the composition of parasite lineages present at any blue tit population and which MHC alleles are conferring resistance (Westerdahl et al., 2005; Martínez-de la Puente et al., 2011). In such a way we found some blue tit alleles shared between locations, a pattern consistent with gene flow among blue tit populations across Europe (Taberlet et al., 1992; Kvist et al., 2004).

MHC diversity is critical on antigen detection and affects host response to face pathogens, but reports in this respect are ambiguous. The heterozygote advantage predicts that having more MHC alleles increase the chance on antigen recognition and the negative frequency-dependent selection stands that specific MHC alleles are advantageous against parasite lineages adapted to common MHC alleles. Therefore different situations are expected to be found among species. Some vertebrate species with low MHC diversity are particularly susceptible to diseases (Mainguy et al., 2007) but also some species are not, as is reflected by their large population size or recent expansions (Babik et al., 2009; Radwan et al., 2010). In

birds, the chicken "minimal essential" MHC genes confers strong protection against Marek's disease, but a huge mortality is produced among susceptible individuals. Some vertebrates with reduced numbers of MHC genes showed highly divergent alleles, which might allow for the recognition of a wider range of pathogens (Sommer, 2005). Also variations in the level of polymorphism has been found to differ among bird species, thus some species with a unique class II B loci had more alleles than a species with more loci (Hughes et al., 2008). Thus the expression of a single gene could result in differences in resistance and susceptibility to infectious pathogens in individuals with different MHC haplotypes (Hepkema et al., 1993; Kaufman, 2000).

Blue tit phylogenetic analyses

MHC genes can be defined evolutionarily by clustering with respect to other known MHC genes in a phylogenetic context (Hess and Edwards, 2002). However, in the phylogeny based on bird MHC class II exon 2 the blue tit sequences were not clearly related to other well characterized sequences nor to non-functional ones. Instead they appeared to be forming a monophyletic basal group not clearly related to any sequences or group of sequences. Well characterized class II B sequences from other passerines grouped into a separate clade (group A in Fig. 3) and not close to blue tit sequences. Inside the group A several polytomies and low supported groups of sequences were observed. Similarly to our results, Balakrishnan et al. (2010) found a class II B zebra finch lineage situated at the base of other passerine class II sequences in a phylogeny performed with the same primers. They suggest that there is a novel locus that has not previously sequenced in birds, but it is unknown whether it is expressed or polymorphic. The phylogeny in Balakrishnan et al. (2010) was performed concatenating together exon 2 and 3 whereas in ours only the exon 2 was used. Phylogenies based on the exon 2 are less congruent than those based on exon 3 or both exon 2 and 3 combined (Hughes and Yeager, 1998; Miller et al., 2011) because exon 2 based trees may reflect selection rather than a duplication history (Reusch and Langefors, 2005). In this manner it is interesting that we found a similar basal lineage separated from the rest of passerines even though our phylogeny was

based only on exon 2. In the tree none of the sequences from other passerines mixed together with the blue tit sequences, therefore the blue tit sequences could be divergent with respect to those of other passerines and represent a different cluster of genes. This mean that closely related species have not had enough time to diverge and sequences remain similar and/or concerted evolution is acting then alleles are sometimes intermingled on trees (Vincek et al., 1997; Richardson and Westerdahl, 2003; Jarvi et al., 2004). Therefore it will be necessary to include data from species closely related to the blue tit (to date not available) in the phylogeny to confirm this possibility. Also blue tit genes could be paralogous with respect to the other passerines included in the analyses and the dissimilarity among genes is then because they did not descend from the same ancestral gene. On the other hand selective pressures from pathogens shared between two bird species could be counteracting genetic differences between species (Westerdahl, 2007) and as a consequence they would group together in the phylogeny.

Selection on exon 2

A method to confirm the effect of different selection pressures on the blue tit MHC is to look for signs of selection on the PBR. In the analysis in MEGA, where PBR positions where *a priori* assigned, we found that for the PBR d_N is higher than d_S (indicative of positive selection) and the difference was nearly significant (P=0.06). On the other hand, the analysis in CODEML found several amino acid positions under positive selection. Thus, there seems to be some positive selection acting in the PBR of blue tit exon 2 but not very strong. Accordingly we cannot reject the possibility that we are misidentifying PBR positions since they are estimated from results of a crystallographic study of human MHC class II B (Brown et al., 1993) and, therefore, they are not necessarily the same positions as in the avian MHC class II B. In this respect, in other avian studies where PBR positions were estimated following Brown et al. (1993), positive selection was detected. Also a lack of strong selection on the PBR could be due to the fact that we are including several non homologous loci in the analyses. In this study we did not assign sequences to a locus due to the similarity among sequences, therefore the ω ratio could be affected. In addition, we have only data of the PBR and non-PBR positions of one part

of exon 2 and the estimation of the ω may vary when the entire exon is amplified. The observation of trans-species polymorphism can be used to infer positive selection because it retains alleles for a long time (Bernatchez and Landry, 2003). Contrary to other species trans-species polymorphism was not observed in the blue tit. Selection has been found at different temporal scales, thus selection in the distant past can be detected as an excess of non-synonymous substitutions. But selection in the recent past or in the current generation can be detected considering other factors (e.g. measuring deviations from Hardy-Weinberg or finding correlations between disease resistance and MHC-allele or genotype (see Wegner et al., 2003). It will be necessary to incorporate these variables to correctly assess the effect of selection in the blue tit and to rule out the possibility that the soft selection could be signalling a change from a functional to a non-functional gene (Hughes and Nei, 1989).

Conclusions

With this study we have obtained a preliminary overview of the MHC class II B genes in the blue tit, characterizing exon 2 partially from several individuals originating in three European locations. Our primers were developed based on transcript sequences obtained from zebra finch RNA and they are supposed to amplify expressed class II B genes involved in peptide presentation. Primers even designed for the zebra finch are not very restricted to this species and could be considered general to apply in the blue tit. Using the same-species probes has revealed different diversity in MHC complexity among songbirds (Edwards et al., 1999). The combined results from sequencing and RFLP can be extremely valuable at the initial stages on MHC research in non-model vertebrates, until a new molecular method like next-generation sequencing based method could be developed (Babik, 2010). New molecular tools are proving that some variability is not measured by traditional molecular methods (Oomen et al., 2013), but to date no whole genome sequencing has been performed on this species. Although the methodology employed in this study is now increasingly being substituted by next generation sequencing is still possible to preliminarily characterize MHC in avian species or to reveal functional variation across populations (Whittaker et al., 2012). Now, more species are being

studied by applying new molecular tools and it promise a fascinating advance in MHC study. This is the first step towards a better understanding of the MHC class II B genes in the blue tit.

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Table 1. Sequence diversity of exon 2 of MHC class II B in the blue tit and other passerines. Sp = species. N = number of sequences, S = number of polymorphic sites, π = nucleotide diversity. SE = π standard deviation. Blue tit (Cyca, *Cyanistes caeruleus*), great reed warbler (Acar, *Acrocephalus arundinaceus*), red-winged blackbird (Agph, *Agelaius phoeniceus*), little greenbul (Anvi, *Andropadus virens*), Florida scrub jay (Apco, *Aphelocoma coerulescens*), house finch (Came, *Carpodacus mexicanus*), medium ground-finch (Gefo, *Geospiza fortis*), common yellow throat (Getr, *Geothlypis trichas*), Hawai´i Amakihi (Hevi, *Hemignathus virens*), house sparrow (Pado, *Passer domesticus*), savannah sparrow (Pasa, *Passerculus sandwichensis*), New Zealand robin (Peau, *Petroica australis*).

Sp	N	S	π	SE
Agph	10	76	0.20	0.01
Pado	12	82	0.19	0.02
Apco	10	67	0.17	0.02
Anvi	13	68	0.16	0.01
Came	6	53	0.16	0.02
Pasa	3	40	0.16	0.07
Getr	15	66	0.15	0.01
Acar	7	56	0.15	0.02
Hevi	11	44	0.14	0.01
Gefo	18	57	0.13	0.01
Peau	8	45	0.12	0.01
Cyca	13	47	0.10	0.01

Table 2. Number of verified and non-verified exon 2 sequences found per individual. Numbers without brackets = sequences obtained from DNA. Numbers in brackets = cDNA sequences obtained from RNA. Alleles = total number of verified alleles in an individual.

Sampling site	The Netherlands										Spain							Sweden					Total
Individual	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	W	
Verified sequences																							
Cyca-DAB*1	-	-	3 (7)	-	-	-	-	-	-	-	-	-	-	-	(6)		(8)	-	-	-	-	-	3 (21)
Cyca-DAB*2	-	-	-	-	8 (11)		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8 (11)
Cyca-DAB*3	-	2	-	-	-	-	-	-	-	-	-	-	-	-	(11)	-	-	3	-	-	-	-	5(11)
Cyca-DAB*4	-	-	-	-	-	-	-	1	-	-		6	-	-	-	(12)	-	-	-	-	-	-	7 (12)
Cyca-DAB*5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	2	-	-	(6)	4 (6)
Cyca-DAB*6	-	-	-	-	-	-	-	-		-	8			1	-	-	-	4	6	-	-	(5)	19 (5)
Cyca-DAB*7	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	1	-	1	(7)	3 (7)
Cyca-DAB*8	-	-	-	-	3 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3 (4)
Cyca-DAB*9	-	-	-	3 (3)	-	2	4	-		4			1	-	-	-	-	-	1	1	-	-	16 (3)
Cyca-DAB*10	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-		(3)	-	-	-	-	-	3 (3)
Cyca-DAB*11	6	-	-	5 (17)	-	-	-	-	-	-	-	-	-	-	-	(7)	-	-	-	-	-	-	11 (24)
Cyca-DAB*12	-	-	9 (7)	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	10 (7)
Cyca-DAB*13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(7)			4	-	-	4 (7)
Non-verified sequences																							
Sequence 1	-	-	-	-	-	-	-	-	-	-	-	-	19	-	-	-	-	-	-	-	-	-	19
Sequence 2	-	-	-	(2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(2)
Sequence 3	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
Sequence 4	-	-	-	-	-	-	-	-	-	-	-	-	-	9	-	-	-	-	-	-	-	-	9
Sequence 5	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	2
Sequence 6	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	2
Sequence 7	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	2
Sequence 8	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2

Figure legends

Figure 1:

Amino acid sequences translated from blue tit exon 2 cDNA sequences and other passerines. Identity with allele Cyca-DAB*1 is indicated by *dots. Asterisks* are the codons corresponding to the PBR. Anvi = *Andropadus virens* (AY437902), Acar = *Acrocephalus arundinaceus* (AJ404372), Came = *Carpodacus mexicanus* (U23969), Pado = *Passer domesticus* (AY518173), Tagu = *Taeniopygia guttata* (XM002193356).

Figure 2:

RFLP patterns with the restriction enzyme Pvu II and the blue tit MHC class II exon 2 probe, from 17 blue tit individuals corresponding to two Swedish families. M= mother F= father, numbers are the offspring. Family 1 = individuals from F1 to 6. Family 2 = individuals from F2 to 13. There are 4-7 fragments per individual in the size range 1-9 kb.

Figure 3:

Bayesian phylogeny of exon 2 of MHC class II B sequences (based on 159bp) from blue tit and other passerine and non-passerine species. Numbers on branches = the posterior probabilities values. * = Non-functional MHC class II. Codes following species name = GenBank accession numbers. Collapsed group A included: *Acrocephalus arundinaceus* (AJ404371, AJ404373, AJ404375, AJ404377, U24405, U24406, U24408), *Agelaius phoeniceus* (AF030987, AF030989, AF030994, U23970, U23971), *Andropadus virens* (AY437889, AY437890, AY437891, AY437892, AY437893, AY437900, AY437901, AY437904, AY437907, AY437908, AY437911, AY437912, AY437913), *Aphelocoma coerulescens* (U23958, U23959, U23961, U23962, U23963, U23966, U23972, U24401), *Atlapetes rufinucha* (AB531732, AB531795), *Cactospiza pallida* (AB531504, AB531518, AB531520), *Camarhynchus parvulus* (AB531822), *Carpodacus mexicanus* (AF241547, HQ203000), *Catamenia inornata* (AB531796), *Certhidea olivacea* (AB531513), *Coryphospingus cucullatus* (AB531639,

AB531729, AB531741, AB531744, AB531800, AB531802), *Dendroica adelaidae* (AB531663), *Erithacus rubecula* (GU390284*), Erythrura gouldiae* (EF535335, EF535337, EF535338, EF535342, EF535348, EF535448), *Euphonia musica* (AB531807), *Ficedula albicollis* (HQ678313, HQ678323, HQ678328, HQ678338, HQ678340, HQ678492), *Ficedula hypoleuca* (GU390232, GU390233, GU390235, GU390236, GU390238, GU390241, GU390248), *Geothlypis trichas* (GQ247570, GQ247571, GQ247574, GQ247575, GQ247588, GQ247597, GQ247612, GQ247617), *Lanius senator* (GU390287), *Lonchura striata* (L42334, L42335), *Loxigilla noctis* (AB531642), *Luscinia luscinia* (FJ529849, FJ529850, FJ529851, FJ529852, FJ529854, FJ529856, FJ529857), *Luscinia svecica* (FJ529861, FJ529863, FJ529870, FJ529871, FJ529876, FJ529878, FJ529883, GQ403040), *Passer domesticus* (AY518171, AY518172, AY518173, AY518176, AY518178, AY518179, AY518180, AY518181), *Passerculus sandwichensis* (AF420008), *Petroica australis* (AY428561, AY428562, AY428563, AY428564, AY730420), *Petroica traversi* (AY258333, AY258335), *Pheucticus aureoventris* (AB531787), *Phylloscopus collybita* (GU390293), *Poephila acuticauda* (EF535365, EF535366, EF535382, EF535416, EF535472, EF535493), *Ramphocelus carbo* (AB531656, AB531742), *Sicalis flaveola* (AB531517, AB531809), *Sporophila nigricollis* (AB531631, AB531812), *Sturnella bellicosa* (AB531813), *Tiaris bicolor* (AB531814), *Tiaris obscura*,(AB531577, AB531633, AB531634, AB531698), *Volatinia jacarina* (AB531640, AB531743, AB531819, AB531820) and *Zonotrichia capensis* (AB531665). Collapsed group B included: *Coturnix japonica* (AB181862), *Gallus gallus* (U91532, AJ248572), *Meleagris gallopavo* (AJ616892, AM233872), *Numida meleagris* (DQ885563), *Pavo cristatus* (AY928098), *Tetrao tetrix* (EF174544), *Phasianus colchicus* (AJ224344, AJ224346, AJ224347, AJ224349).

Fig. 1

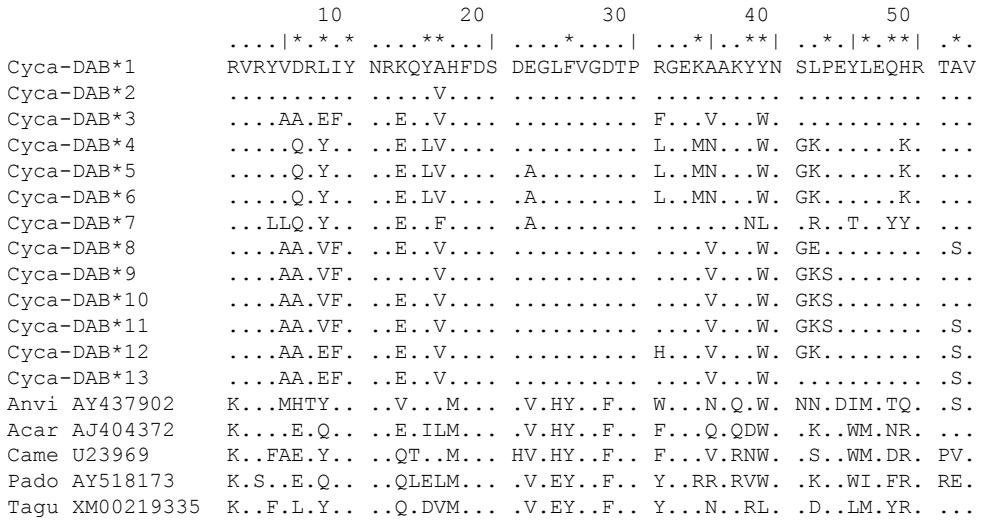

10 20 30 40 50
....|*.*.* ....**...| ....*....| ...*|..**| ..*.|*.**| .*.
Cyca-DAB*1 RVRYVDRLIY NRKQYAHFDS DEGLFVGDTP RGEKAAKYYN SLPEYLEQHR TAV
Cyca-DAB*2 ....V.... ....
Cyca-DAB*3 ....AA.EF. ..E..V.... ..... F...V...W. ....
Cyca-DAB*4 .....Q.Y.. ..E.LV.... ..... L..MN...W. GK......K. ...
Cyca-DAB*5 .....Q.Y.. ..E.LV.... .A..... L..MN...W. GK......K. ...
Cyca-DAB*6 .....Q.Y.. ..E.LV.... .A..... L..MN...W. GK......K. ...
Cyca-DAB*7 ...LLQ.Y.. ..E..F.... .A..... ....NL. .R..T..YY. ...
Cyca-DAB*8 ....AA.VF. ..E..V.... ....V...W. GE..... .S.
Cyca-DAB*9 ....AA.VF. .....V.... ....V...W. GKS..... ...
Cyca-DAB*10 ....AA.VF. ..E..V.... ....V...W. GKS..... ...
Cyca-DAB*11 ....AA.VF. ..E..V.... ....V...W. GKS..... .S.
Cyca-DAB*12 ....AA.EF. ..E..V.... ..... H...V...W. GK..... .S.
Cyca-DAB*13 ....AA.EF. ..E..V.... ....V...W. ..... .S.
Anvi AY437902 K...MHTY.. ..V...M... .V.HY..F.. W...N.Q.W. NN.DIM.TQ. .S.
Acar AJ404372 K....E.Q.. ..E.ILM... .V.HY..F.. F...Q.QDW. .K..WM.NR. ...
Came U23969 K..FAE.Y.. ..QT..M... HV.HY..F.. F...V.RNW. .S..WM.DR. PV.
Pado AY518173 K.S..E.Q.. ..QLELM... .V.EY..F.. Y..RR.RVW. .K..WI.FR. RE.
Tagu XM00219335 K..F.L.Y.. ..Q.DVM... .V.EY..F.. Y...N..RL. .D..LM.YR. ...

Fig. 2

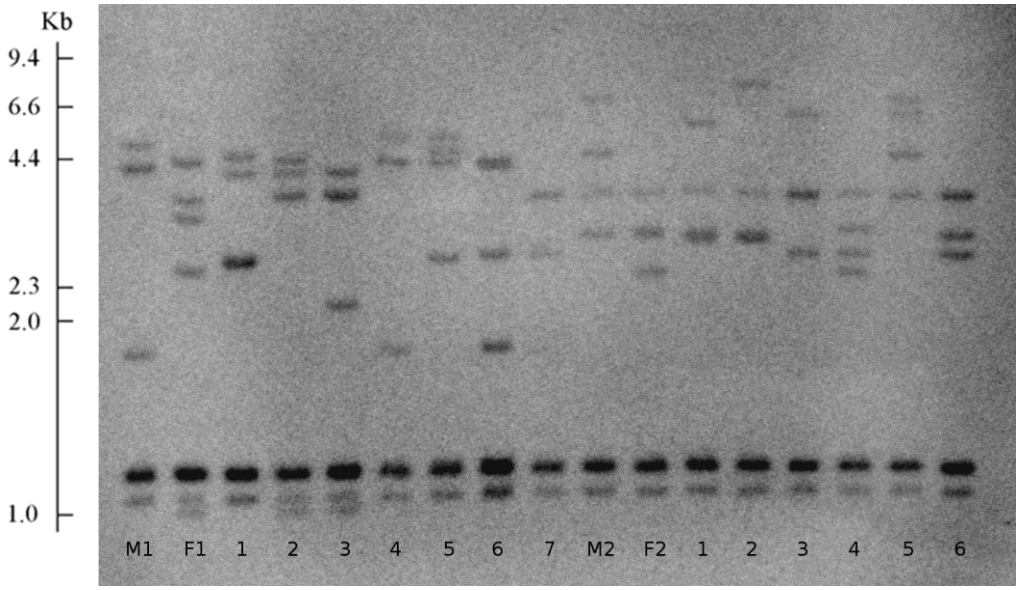
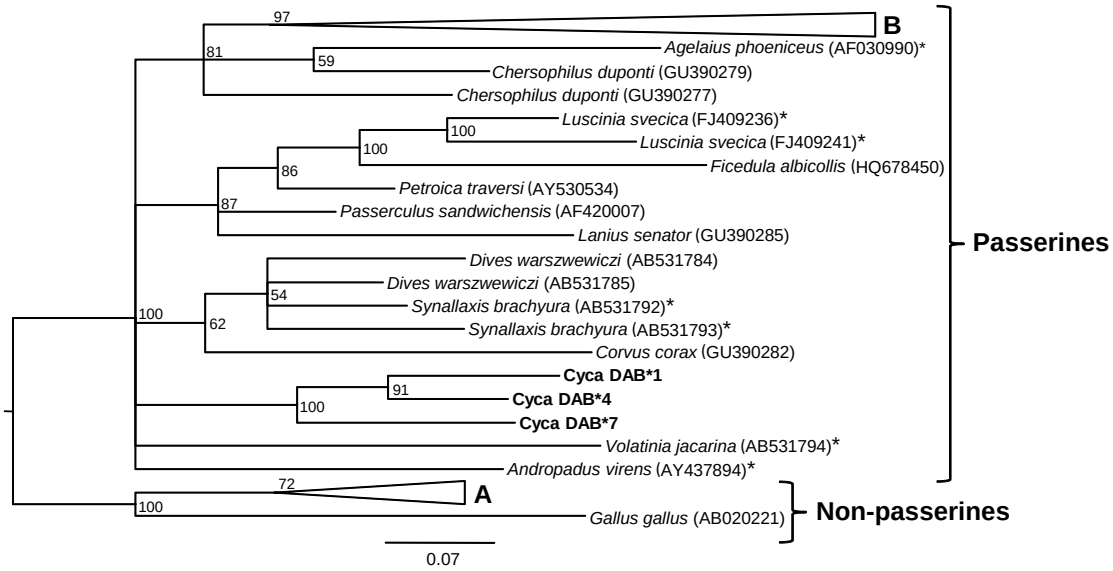


Fig. 3


97
B
81
59
Agelaius phoeniceus (AF030990)*
Chersophilus duponti (GU390279)
Chersophilus duponti (GU390277)
Luscinia svecica (FJ409236)*
100
Luscinia svecica (FJ409241)*
100
Ficedula albicollis (HQ678450)
86
Petroica traversi (AY530534)
87
Passerculus sandwichensis (AF420007)
Lanius senator (GU390285)
Dives warszwewiczi (AB531784)
Dives warszwewiczi (AB531785)
54
Synallaxis brachyura (AB531792)*
100
62
Synallaxis brachyura (AB531793)*
Corvus corax (GU390282)
Cyca DAB*1
91
Cyca DAB*4
100
Cyca DAB*7
Volatinia jacarina (AB531794)*
Andropadus virens (AY437894)*
Passerines
72
A
100
Gallus gallus (AB020221)
Non-passerines
0.07

Supporting information

Table 1. Sample sites, number of individuals sampled and kind of analysis. + = the individual was used for the analysis. - = the individual was not used for the analysis.

Sampling site	The Netherlands										Spain							Sweden				
Individuals	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	W
DNA Analysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	-
RNA Analysis	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	+

Figure legends

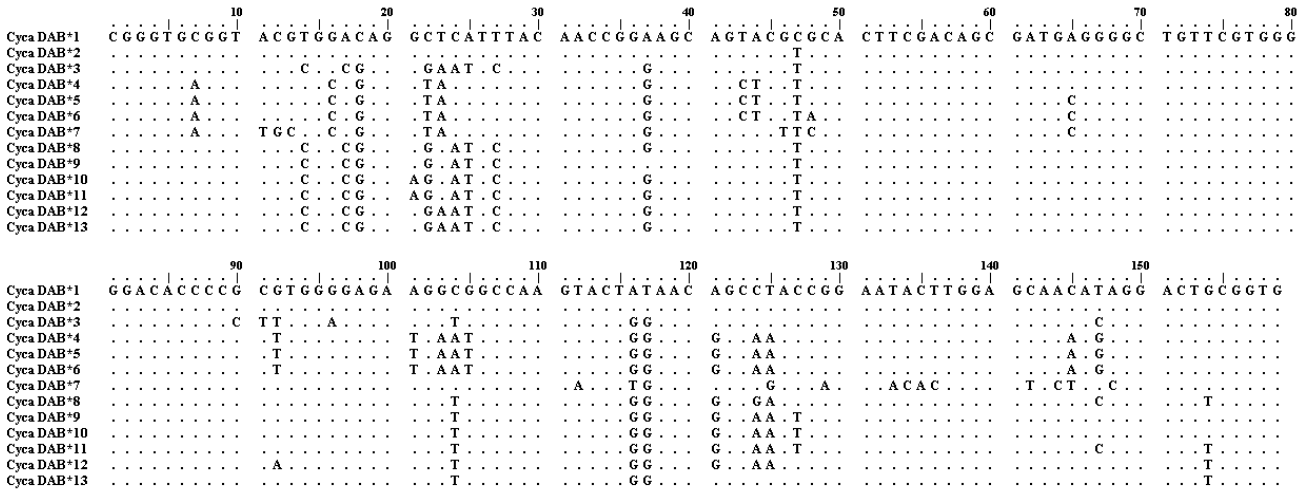
Fig. 1:

Total MHC class II B blue tit alleles obtained from all DNA and cDNA sequences (amplified with the degenerated primers 2ZFfw1 and 2ZFrv1). Identity with allele Cyca-DAB*1 is indicated by *dots.*

Fig. 2:

Bayesian phylogeny from the blue tit and other passerines with non-collapsed nodes. Decimal numbers on branches = posterior probability. MHC class II B blue tit alleles are indicated by an arrow.

Fig. 1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Cyca DAB*1 CGGGTGCGGT ACGTGGACAG GCTCATTTAC AACCGGAAGC AGTACGCGCA CTTCGACAGC GATGAGGGGC TGTTCGTGGG
Cyca DAB*2
Cyca DAB*3
Cyca DAB*4
Cyca DAB*5
Cyca DAB*6
Cyca DAB*7
Cyca DAB*8
Cyca DAB*9
Cyca DAB*10
Cyca DAB*11
Cyca DAB*12
Cyca DAB*13
90 100 110 120 130 140 150
Cyca DAB*1 GGACACCCCG CGTGGGGAGA AGGCGGCCAA GTACTATAAC AGCCTACCGG AATACTTGGA GCAACATAGG ACTGCGGTG
Cyca DAB*2
Cyca DAB*3
Cyca DAB*4
Cyca DAB*5
Cyca DAB*6
Cyca DAB*7
Cyca DAB*8
Cyca DAB*9
Cyca DAB*10
Cyca DAB*11
Cyca DAB*12
Cyca DAB*13

Fig.2


GU39023
HQ67832
HQ67833
HQ67834
GU39028
HQ67831
FJ52987
GQ40304
HQ67832
FJ52985
FJ52986
FJ52986
FJ52987
GU39023
FJ52985
GU39023
GU39023
GU39023
FJ52987
GU39024
GU39024
FJ52985
FJ52988
FJ52984
AJ40437
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EF53538
EF53549
EF53536
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AB53169
AY51817
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AB53181
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AB53182
AF42000
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AB53173
AB53163
AB53164
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AB53152
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GQ24761
HQ20300
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U2440
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Cyca
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Cyca
AB53179
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AJ22434
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DQ88556
AJ24857
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AY92809
AB02022
0.07

English abstract

Introduction

Diseases are considerate selective forces that make up the populations structure affecting survival of the individuals and affecting their fitness. The Major Histocompatibility Complex or MHC is central in the immune response, and these genes are thought to be of important adaptive significance, particularly for resistance to parasites and pathogens.

Antigen detection depends on the MHC structure and function. Individual MHC alleles may be especially effective at presenting antigens from a particular infection, and, hence, in combating specific pathogens in birds. Associations between MHC alleles and resistance/susceptibility to parasites have been found in vertebrates. Therefore, it is of interest to characterize the genetic components of host immunity and to elucidate the way in which they face the infectious diseases.

In addition, numerous observational and experimental studies have shown that female birds prefer males with the brightest and most intense plumage colours. One of the potential selective pressures proposed to explain the evolution of female mate choice is that plumage colour may be signaling health status and parasite resistance; thus, females would acquire selective advantages by mating with colourful males.

Objectives and results

I. Major histocompatibility complex (MHC) genes are central for the adaptive immune response against parasites. Several resistance/susceptibility associations between specific MHC alleles and parasites have been found in vertebrates, including birds, both from captivity and wild populations. Here, we investigate such associations in a natural population of a passerine bird, the blue tit *Cyanistes caeruleu*s, in central Spain. A diverse community of blood parasites including *Haemoproteus* and *Leucocytozoon* species adversely affects this bird population. MHC-I genes were analysed to search for associations among MHC diversity and/or specific MHC alleles, and parasite prevalence and intensity of infection. The statistical analyses revealed that individuals with elevated MHC diversity or carrying specific alleles (Paca UA*104 or Paca UA*108) had higher intensity of infection by *Leucocytozoon* but no significant relationship was detected with *Leucocytozoon* prevalence. Paradoxically, the group of individuals recruited one year later had higher allelic diversity and presented the allele Paca UA*104 in higher frequency, indicating that the association with *Leucocytozoon*

had not deleterious consequences. In addition, the males or young birds with Paca UA*104 or Paca UA*108 alleles were more prone to suffer from high intensity of infection. No significant associations with prevalence or intensity of infection were observed for *Haemoproteus*. In conclusion, (i) the relationship between MHC-I and susceptibility to *Leucocytozoon* would be a side effect of the allele selection conducted by an unknown virulent pathogen and (ii) other factors such as sex and age would play an important role in determining pathogenicity.

II. Carotenoids are molecules that birds are not able to synthesize and therefore, must be acquired through their diet. These pigments, besides their function giving red and yellow colours when deposited in feathers, seem to act as immune-stimulators and antioxidants in the organism. Hence, only the healthiest individuals would be able to express carotenoid-based ornaments to a larger extent without compromising their physiological functions. Various studies have reported that birds infected by parasites are paler than those uninfected, but, to our knowledge, none of them assessed the possible effect of multiple infections by blood parasites on plumage colour. By comparing the yellow colour in the breast plumage of blue tits, *Cyanistes caeruleus*, between birds infected by different number of blood parasite genera, we found that those birds infected by more than one genus were paler than those parasitized just by one. In addition, we have examined the potential role of carotenoid-based plumage colour of blue tits as a long-term indicator of other parameters of health status, such as body condition and immunoglobulin and HSPs (heat shock proteins) levels. Our results indicate that brightly coloured birds had lower HSP70 levels than pale birds, but we did not find any significant association between colour and body condition or immunoglobulin levels. Overall, these results support the role of carotenoid-based colours as indicators of health status in blue tits.

III. Major histocompatibility complex (MHC) genes encode proteins involved on parasite antigen recognition. MHC diversity and specific MHC alleles has been related to both resistance and susceptibility to parasite infections. Therefore, MHC-based mate choice could act as a sexual mechanism to increase/optimize offspring fitness. In this study we investigate a blue tit population in central Spain to search for associations between MHC-I and mate choice. We also investigate breast yellow plumage colour as a signal of infection status affecting mate choice. To that end, we screened MHC-I alleles corresponding to the peptide binding region (PBR). We found assortative mating

according to MHC-I diversity. Females were paired with males that had similar MHC-I diversity. In addition, the apparent MHC compatibility-based mate choice pattern was not statistically different from a random pairing sample. These results point out to the occurrence of a mating system where individuals select pairs similar to each other in MHC-I genes. This finding is in accordance with host/parasite systems where parents invest in parental care and both members of the pair evaluate the quality of its mate. Furthermore, the less colourful females where paired to the more MHC diverse males, suggesting a female searching for high quality males.

IV. In this study we partly characterize Major Histocompatibility Complex (MHC) class II B in the blue tit (*Cyanistes caeruleus*). A total of 22 individuals from three different European locations: Spain, The Netherlands and Sweden were screened for MHC allelic diversity. The MHC genes were investigated using both PCR based methods and unamplified genomic DNA with restriction fragment length polymorphism (RFLP) and southern blots. A total of 13 different exon 2 sequences were obtained independently from DNA and/or RNA, thus confirming gene transcription and likely functionality of the genes. Nine out of 13 alleles were found in more than one country, and two alleles appeared in all countries. Positive selection was detected in the region coding for the peptide binding region (PBR). A maximum of three alleles per individual was detected by sequencing and the RFLP pattern consisted of 4-7 fragments, indicating a minimum number of 2-4 loci per individual. A phylogenetic analysis, demonstrated that the blue tit sequences are divergent compared to sequences from other passerines resembling a different MHC lineage than those possessed by the most passerines studied to date.

Conclusions

- Individuals with high MHC diversity or specific MHC alleles had a higher intensity of infection by *Leucocytozoon*.

- The high prevalence and the low intensity of infection suggest that the parasite does not exert an excessive selective pressure on the blue tit population.

- Birds parasitized by a single genus of blood parasite had higher carotenoid chroma and thus showed a more colourful plumage than birds parasitized by more than one parasite.

- Individuals were paired accordingly to MHC-I diversity, thus females mated with males with similar diversity.

- The results did not support MHC-dissimilarity mate choice in the studied population.

- A maximum of at least two to four MHC-II loci was detected in the blue tit.

- The blue tit MHC-II diversity is similar to other passerines but resembling less diverse MHC genes.

Publicaciones originales según formato de revista científica

Se presentan copias de todos aquellos artículos que han sido publicados en diferentes revistas científicas. El formato en que se presentan corresponde al formato de edición de cada una de estas publicaciones.

Oecologia
DOI 10.1007/s00442-009-1510-y

PHYSIOLOGICAL ECOLOGY - ORIGINAL PAPER

Carotenoid-based plumage colouration is associated with blood parasite richness and stress protein levels in blue tits (*Cyanistes caeruleus*)

Sara del Cerro · Santiago Merino · Josué Martínez-de la Puente · Elisa Lobato · Rafael Ruiz-de-Castañeda · Juan Rivero-de Aguilar · Javier Martínez · Judith Morales · Gustavo Tomás · Juan Moreno

Received: 30 December 2008 / Accepted: 30 October 2009

Abstract Carotenoids are molecules that birds are not able to synthesize and therefore, must be acquired through their diet. These pigments, besides their function of giving birds red and yellow colouration when deposited in feathers, seem to act as immune-stimulators and antioxidants in the organism. Hence, only the healthiest individuals would be able to express carotenoid-based ornaments to a larger extent without compromising the physiological functions of carotenoids. Various studies have reported that birds infected by parasites are paler than those uninfected, but, to our knowledge, none of them has assessed the possible effect of multiple infections by blood parasites on plumage colour. By comparing the yellow colour in the breast plumage of blue tits, *Cyanistes caeruleus*, between birds infected by different numbers of blood parasite genera, we found that those birds infected by more than one genus were paler than those parasitized just by one. In addition, we examined the potential role of carotenoid-based plumage colour of blue tits as a long-term indicator of other parameters of health status, such as body condition and immunoglobulin and heat shock protein (HSP) levels. Our results indicate that more brightly coloured birds had lower HSP70 levels than paler birds, but we did not find any significant association between colour and body condition or immunoglobulin levels. In addition, we found a positive significant association between *Haemoproteus* density of infection and HSP60 levels. Overall, these results support the role of carotenoid-based colours as indicators of health status in blue tits and show detrimental effects of parasitism on this character.

Keywords Bird · Health status · Heat shock proteins · Multiple infections · Yellow breast

Communicated by Heli Siitari.

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Introduction

Numerous observational and experimental studies have shown that female birds prefer males with the brightest and most intense plumage colours (e.g. Zuk et al. 1990; Hill 1991; MacDougall and Montgomerie 2003), although the proximate reasons for this choice are not fully understood. One of the potential selective pressures proposed to explain the evolution of female mate choice is that plumage colour may signal health status and parasite resistance (Hamilton and Zuk 1982); thus, females would acquire selective advantages by mating with colourful males. Several hypotheses have been developed in this respect. The influential

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hypothesis of Hamilton and Zuk (1982) posits that females choosing a brightly coloured male may gain benefits for their nestlings in the form of heritable resistance to parasites. Also, females may avoid parasite transmission to themselves and their nestlings (Clayton 1990) or they may benefit from a higher parental effort performed by a healthy male (Read 1990). Despite the role of colourful plumage having been studied especially in males, it is known that females also show conspicuous plumages in several species. Traditionally, it has been assumed that this might be due to a genetic correlation with male ornamentation, thereby suggesting that female colouration was not functional (Lande 1980). However, males may suffer costs associated with reproduction and a way to reduce these costs would be choosing a showy female if showiness signals her good quality (Amundsen 2000). Indeed, evidence is accumulating on the adaptive function of female ornamentation (see Kraaijeveld et al. 2007 for a review).

Carotenoid-based colours are very common among birds (Goodwin 1984). It has been proposed that the honesty of carotenoid-based ornaments as quality signals is based on the multiple functions of carotenoids in the organism. Besides being deposited in feathers and thus giving yellow and red colours to birds' plumages, they seem to participate as immune-stimulators and antioxidants in the organism (Lozano 1994; Møller et al. 2000). Among their functions we can cite their role as scavengers of free radicals and immunosuppressive peroxides (e.g. Chew 1993; Surai and Speake 1998; von Schantz et al. 1999; Møller et al. 2000; but see Costantini and Møller 2008), as well as enhancers of the production of lymphocytes, the phagocytic ability of neutrophils and macrophages, and of tumour immunity (Møller et al. 2000; Surai et al. 2001). In addition, carotenoids are pigments that vertebrates cannot synthesize de novo, so they must be obtained through the diet (Hill 1992; Olson and Owens 1998). Hence, according to Hamilton and Zuk (1982), only individuals of high quality (i.e. those with higher resistance to parasites and/or higher foraging capacity) would be capable of being intensely coloured without compromising the quantity of carotenoids allocated to other physiological functions.

The negative effects of blood parasites on plumage colours have been reported in previous studies (Weatherhead 1990; Sundberg 1995; Merilä et al. 1999; Hõrak et al. 2001), those birds heavily infected by parasites being normally less pigmented than lightly infected ones, and on the whole, parasitized birds being paler than unparasitized ones. However, these studies on the effects of blood parasites on plumage colour are based on only one parasite species, whereas, to our knowledge, there is no study examining the possible effect of joint infections by multiple blood parasites on carotenoid-based plumage colour. Such studies may contribute to a better understanding of the role of carotenoid-based plumage colourations as quality signals, since birds in the wild are normally infected by different parasite species simultaneously (Merino et al. 2000; Valkiūnas et al. 2003). Furthermore, it has been shown that hosts can suffer more severe harmful effects, like anaemia (Graham et al. 2005), loss of body mass (Evans and Otter 1998; Graham et al. 2005; Marzal et al. 2008), production of a less saturated carotenoid-based colouration (Brawner et al. 2000) and reduction of survival (Evans and Otter 1998; Davidar and Morton 2006; Arriero and Møller 2008; Marzal et al. 2008) due to multiple simultaneous infections, if compared to single ones.

The aim of this study was to evaluate the role of a carotenoid-based trait, the yellow breast plumage colouration of blue tits (*Cyanistes caeruleus*), as a long-term indicator of health status in a population commonly infected by several species of blood parasites (Merino et al. 2000). The blue tit is a small (11 g) insectivorous passerine, very common in Europe. It is a slightly sexually dichromatic species, males being more intensely coloured than females (Cramp 1998). In blue tit males, yellow carotenoid-based colouration has been shown to indicate parental investment (Senar et al. 2002), while in females it reflects their reproductive capacity (Doutrelant et al. 2008). Moreover, there is assortative mating by this trait (Hidalgo-García 2006; Ferns and Hinsley 2008), which suggests that yellow plumage colouration might be sexually selected in both sexes. The health status variables studied here were parasite richness (number of parasite genera birds are infected with), *Haemoproteus* infection intensity, body condition index and the levels of total immunoglobulins and stress proteins (heat shock proteins; HSPs). Immunoglobulins play an important role in the specific humoral immune response of vertebrates, being responsible for antigen recognition (Roitt et al. 1996). Measures of humoral immune response are becoming widely used in many ecological studies (Norris and Evans 2000; Martínez-de la Puente et al. 2007a; Tomás et al. 2007), and fitness costs of immune defence against parasites and trade-offs in the face of limited resources have been reported (Deerenberg et al. 1997; Moreno et al. 1999; Soler et al. 2002; Merino et al. 2006; Moreno et al. 2008). HSPs are molecules that maintain cellular homeostasis by responding to a wide array of stressors, like heat (Gehring and Wehner 1995), toxins (Mariño et al. 1999), oxidant compounds (Martínez et al. 1999a), cold (Martínez et al. 2001) and parasites (Merino et al. 1998; Martínez et al. 1999b; Tomás et al. 2005). Likewise, studies on the ecological and evolutionary role of the HSP-mediated stress response have been published in recent years (see Sørensen et al. 2003 for a review).

We hypothesize that if the yellow colour is a signal of phenotypic quality, more intensely coloured birds should be lightly infected by *Haemoproteus* and they should harbour

fewer blood parasite genera. Also, colourful birds would be in better body condition and would show higher levels of immunoglobulins (immunocompetence) and lower stress protein levels.

Materials and methods

The study was carried out during the 2004 and 2007 breeding seasons in a Pyrenean oak (*Quercus pyrenaica*) forest located in Valsaín, central Spain (Segovia province, 40º53′N, 4º01′W, 1,200 m a.s.l.), where a population of blue tits breeds in wooden nest boxes. This population has been studied since 1991 (e.g. Merino et al. 2000; Martínez-de la Puente et al. 2006). Every year, nest boxes are periodically inspected to determine the laying date (day 1 = 1 April), clutch size, hatching date and fledging success.

After removing cases of birds recaptured in 2007 (five birds) to avoid pseudoreplication, we kept samples from 166 birds (85 females: 30 yearlings and 55 adults, and 81 males: 34 yearlings and 47 adults) in 2004 and 58 birds (29 females: 9 yearlings and 20 adults and 29 males: 14 yearlings and 15 adults) in 2007.

In both study years, adult birds were trapped in nest boxes twice. In the first capture (when nestlings were 3 days old) they were ringed if necessary, weighed with an electronic balance to the nearest 0.1 g and sampled for blood (see below). In addition, 0.1 mg of an antimalarial drug, primaquine, diluted in 0.1 ml of saline solution or the same volume of saline solution was injected subcutaneously into the abdominal region for another experimental purpose (see Martínez-de la Puente et al. 2006, 2007b; Tomás et al. 2008). During the second capture (10 days later), tarsus length was measured with a digital calliper to the nearest 0.01 mm. Birds were sexed and classified as yearling or older (≥2 years) according to plumage characteristics as described by Svensson (1992). Breast yellow plumage colour was objectively measured using a portable spectrophotometer (CM-2600d, Minolta), that covers the range of wavelengths between 360 and 740 nm. In order to prevent the incidence of ambient light the spectrophotometer measuring mask was placed perpendicularly against one flank of the breast feather surface to take colour measures while holding the bird horizontally. From the raw spectral reflectance data, we computed the "carotenoid chroma" $(R_{700}-R_{450})/R_{700}$ for each individual (Andersson and Prager 2005). This is the relative difference in reflectance between the wavelengths of minimum (700 nm) and maximum (450 nm) absorbance of the two main carotenoids in blue tit plumage (lutein and zeaxanthin). This measure has the advantage of being a strong correlate of perceived chroma and also the best spectrometric estimate of actual carotenoid concentration (Andersson and Prager 2005). During 2004, two consecutive colour measurements were taken from some individuals and the values of carotenoid chroma from these measurements were averaged to be used in the final analyses. As our methods of colour estimates had reasonably low measurement error to sufficiently describe inter-individual differences in carotenoid chroma [repeatability (Lessells and Boag 1987) of two consecutive measurements $R_i = 0.66$, $F_{13,14} = 4.83$; $P < 0.01$], just one spectral measurement was taken from each individual in 2007. As in other studies based on the use of spectrophotometers not including UV reflectance (Figuerola et al. 1999; Senar et al. 2002; Hidalgo-García 2006; Ferns and Hinsley 2008), we face a potential problem since blue tit visual perception extends into the UV range of the spectrum (Hunt et al. 1998; Örnborg 2002). However, our main aim is to explore whether carotenoid chroma as experienced by human observers is associated with some health parameters of birds. Therefore, objective measurements of chroma in the human-visible part of the spectrum appear adequate for our purposes.

For the molecular detection of blood parasites (*Haemoproteus*, *Leucocytozoon*, *Plasmodium*, *Lankesterella* and *Trypanosoma*), a drop of blood from the brachial vein of birds captured in 2004 was stored at −20°C until processed, whereas samples of 2007 were stored in FTA cards (Whatman, UK). DNA was extracted using a commercial kit (UltraClean DNA BloodSpin kit; MO BIO Laboratories, Calif.) or, in the case of FTA cards, by applying the protocol described in Merino et al. (2008). In both cases we immediately amplified the cytochrome B gene using the primers described in Table 1. Polymerase chain reactions (PCRs) used 25-µl reaction volumes containing 20 ng template DNA, 50 mM KCl, 10 mM TRIS–HCl, 1.5 $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, Calif.). The reactions were cycled under the following conditions using a thermal cycler (MasterCycler Personal, Eppendorf): 94°C for 10 min (polymerase activation), 40 cycles at 95°C for 40 s, annealing temperature for 1 min (see Table 1), 72°C for 1 min, and a final extension at 72°C for 10 min. In some cases there was not enough blood to detect the presence of all parasite genera in samples from 2004; therefore, we used data obtained from microscopical observations of blood smears in these cases. Blood smears were prepared immediately upon extraction and were air-dried, fixed in absolute ethanol and stained with Giemsa stain for 45 min. Half of a smear was scanned under an optical microscope at 200× to search for large parasites such as *Trypanosoma* and *Leucocytozoon*, whereas small intra-erythrocytic parasites, such as *Haemoproteus, Lankesterella* and *Plasmodium* were detected in the other half of the smear at 1,000× with the oil immersion objective (see Merino et al. 1997). In spite of the different efficiency of

Table 1 Sequences and annealing temperatures of the primers

Primer	Annealing	Parasite	Sequence (5′→3′)
LDLd	58	*Leucocytozoon*	CAT TCY ACW GGT GCA TCT TT
LDRd	58	*Leucocytozoon*	CTG GAT GWG ATA ATG GWG CA
PLAS-F	60	*Plasmodium*	GTA ACA GCT TTT ATG GGT TAC
4292Rw[a]	60	*Plasmodium*	TGG AAC AAT ATG TAR AGG AGT
HML	58	*Haemoproteus*	GCT ACT GGT GCT ACA TTT GT
HMR	58	*Haemoproteus*	CCT AAA GGA TTA GAG CTA CC
S-755[b]	60	*Trypanosoma*	CTA CGA ACC CTT TAA CAG CA
S-823[b]	60	*Trypanosoma*	CGA AYA ACT GCY CTA TCA GC
Hep900F	58	*Lankesterella*	GTC AGA GGT GAA ATT CTT AGA TTT G
Hep1615R	58	*Lankesterella*	AAA GGG CAG GGA CGT AAT C

[a] Primer previously published by Beadell et al. (2004)

[b] Primers previously published by Maslov et al. (1996)

molecular and microscopic techniques in detection of blood parasites (higher for molecular methods in all cases: 47.5 vs. 32.3% for *Trypanosoma*; 89.3 vs. 63.5% for *Leucocytozoon*; 85.6 vs. 72.5% for *Haemoproteus*; 47.7 vs. 32.9% for *Lankesterella* and 69.9 vs. 2% for *Plasmodium* according to molecular and microscopic detection, respectively), we included data from microscopy when necessary to increase sample size. However, as this increased the possibility of including false negatives, we also conducted the analyses including only data from molecular detection. We assigned birds to the following groups depending on their blood parasite richness, i.e. the number of blood parasite genera they were infected with: (1) one blood parasite genus, (2) two blood parasite genera, (3) three blood parasite genera, and (4) four or five blood parasite genera (see Results).

The intensity of infection by *Haemoproteus* was quantified as the number of parasites per 2,000 erythrocytes in blood smears (Merino et al. 1997). However, in some cases the parasite was detected by molecular methods and not by microscopy. In order to include these cases in the analysis on intensity of infection the variable was transformed into categories according to its distribution: (1) 0–10 parasites/2,000 erythrocytes; (2) 11–20 parasites/2,000 erythrocytes; (3) 21–40 parasites/2,000 erythrocytes; (4) 41–60 parasites/2,000 erythrocytes; and (5) 61–140 parasites/2,000 erythrocytes. Infections by other parasites showed low intensities and were not quantified.

Only in 2004, part of the blood extracted was centrifuged (2,000 *g*, 5 min) with a portable centrifuge (1,201–220 V; Labnet, Woodbridge, N.J.) which separated the cellular and plasma fractions. The cellular fraction was used to determine the levels of two different HSPs, HSP60 and HSP70, through Western blot following the protocol described in Tomás et al. (2004). The plasmatic fraction was used to determine immunoglobulin levels through a direct enzyme-linked immunosorbent assay (ELISA), using a polyclonal rabbit antichicken IgG conjugated with peroxidase (Sigma A-9046; Sigma, St Louis, Mo.). For details on the methodology see Martínez et al. (2003). Body condition index was calculated as mass/tarsus length during both years.

Treatment with primaquine reduces the intensity of infection by *Haemoproteus* and the prevalence of infection by *Leucocytozoon* in blue tits (Merino et al. 2000). To avoid any confounding effect of this treatment (e.g. on blood parasite infection), data from the first capture were used in the analyses, which was available for all variables except for plumage colour and tarsus length (obtained in the second capture). It should also be noted that in 2003 and 2006, i.e. the two breeding seasons previous to those included in the present study (when birds moulted the new plumage colour was measured the following breeding season), no bird was treated with primaquine. This precludes that any relationship found between blood parasitaemias and plumage colour of birds can be associated with previous primaquine treatments.

To study the relationship of the different variables (categorical variable: blood parasite richness; continuous variables: body condition index, levels of immunoglobulin, HSP60 and HSP70) with plumage colour (carotenoid chroma), general regression models (Statistica 2001; StatSoft) were used. Year, date of measurement, sex, age and treatment were controlled for. For simplicity only two-way interactions between categorical variables were included. Models were obtained by a backward elimination procedure. In addition, we studied the variation of the intensity of infection by *Haemoproteus* when infecting a host alone or together with other blood parasite genera and its association with carotenoid chroma and other health parameters using a general linear model, controlling for year, sex and age of birds. All the variables showed a normal distribution except date of measurement which was logarithmically transformed to satisfy assumptions of normality. Differences in sample sizes reflect missing values due to, for instance, inability to obtain enough blood from all birds.

Results

The prevalence of blood parasites was considerable, *Haemoproteus* being the most frequent blood parasite (84% in 2004 and 81% in 2007), followed by *Leucocytozoon* (87% in 2004 and 57% in 2007). Other blood parasites were also present but with lower prevalences: *Trypanosoma* (29% in 2004 and 19% in 2007), *Lankesterella* (45% in 2004 and 38% in 2007) and *Plasmodium* (65% in 2004 and 36% in 2007). The number of birds in each category of blood parasite richness was: (1) 21 birds (10 in 2004 and 11 in 2007); (2) 57 birds (33 in 2004 and 24 in 2007); (3) 74 birds (60 in 2004 and 14 in 2007); (4) 69 birds (49 in 2004 and 7 in 2007 with four different parasite genera and 12 in 2004 and 1 in 2007 infected by five parasite genera). Only one bird of 2007 was unparasitized and was not included in the analyses.

The model obtained when relating carotenoid chroma to blood parasite richness and body condition is presented in Table 2. Multiple blood parasite infections were more likely to be present in paler birds (those with lower carotenoid chroma) as compared to those parasitized by just one parasite genus. Post hoc comparisons showed that carotenoid chroma was drastically lower in birds infected by more than one blood parasite [Fig. 1; Fisher least significant difference (LSD): $P < 0.05$], but did not differ between birds infected with two, three or four genera. Additionally, carotenoid chroma of birds was significantly different between years, birds of 2007 being more colourful than birds of 2004. Moreover, we found an effect of year × blood parasite richness on carotenoid chroma. Post hoc comparisons of birds infected by the same number of blood parasite genera in different years indicated that carotenoid chroma of birds infected by two or three blood parasite genera in 2004 was lower than carotenoid chroma of birds infected by the same number of blood parasite genera in 2007 (Fig. 2; Fisher LSD: $P < 0.01$). In addition, in 2004, birds infected by one blood parasite genus had significantly higher carotenoid chroma values than birds infected by two or three blood parasite genera (Fig. 2; Fisher LSD: $P < 0.05$), whereas in 2007, birds infected by one blood parasite genus were slightly more intensely coloured than birds infected by four or five blood parasite genera (Fig. 2; Fisher LSD: $P = 0.05$) but not than birds infected by two or three blood parasite genera (Fig. 2; Fisher LSD: $P > 0.05$). On the other hand, we did not find a significant effect of date of measurement, sex or age on carotenoid chroma ($P > 0.05$). However, we found an effect of the treatment and sex × age and sex × treatment interactions on plumage colour. Young females were significantly paler than adult females and young males but not than adult males, whereas untreated males were significantly more colourful than females and treated males and overall, untreated birds had higher levels of carotenoid chroma. Finally, carotenoid chroma was not significantly related to body condition or to any of the other two-way interactions ($P > 0.05$). The final

Table 2 Model obtained when exploring the relationship of carotenoid chroma of blue tit breast plumage in 2004 and 2007 with parasite richness and body condition

Effect	*df*	*F*	*P*
Year	1,205	7.132	0.008
Treatment	1,205	5.494	0.020
Blood parasite richness	3,205	4.984	0.002
Sex × age	1,205	5.374	0.021
Sex × treatment	1,205	7.295	0.007
Year × blood parasite richness	3,205	3.142	0.026

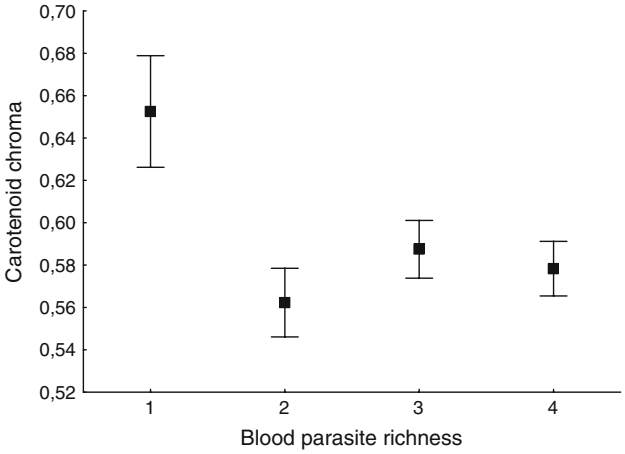

Fig. 1 Carotenoid chroma variation in relation to blood parasite richness. *Bars* indicate SE. *1* One blood parasite genus, *2* two blood parasite genera, *3* three blood parasite genera, *4* four or five blood parasite genera

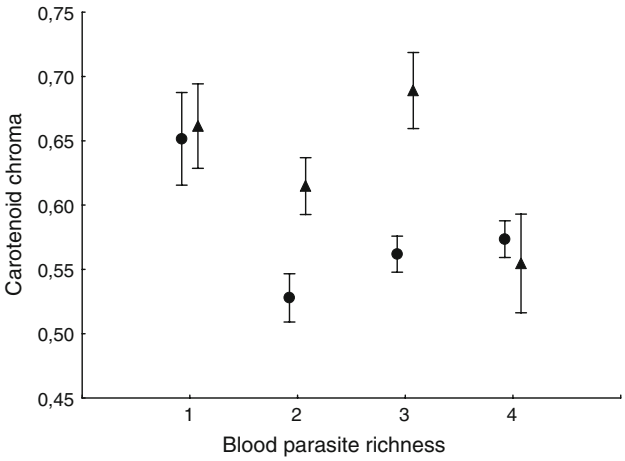

Fig. 2 Carotenoid chroma variation in relation to year × blood parasite richness interaction. *Circles* Data from 2004, *triangles* data from 2007. *Bars* indicate SE

model explained 18% of the variation in carotenoid chroma ($F_{10,205} = 5.56$, $P < 0.001$). We attained similar conclusions when analysing data based only on PCR (year, $F_{1,156} = 9.16$, $P < 0.01$; blood parasite richness, $F_{3,156} = 2.82$, $P < 0.05$; age × treatment, $F_{1,156} = 5.26$, $P < 0.05$; sex × treatment, $F_{1,156} = 5.78$, $P < 0.05$; rest of variables and interactions, $P > 0.05$). However, in this case post hoc comparisons indicated that carotenoid chroma was higher in birds infected by one blood parasite genus than in those infected by two or four genera ($P < 0.05$) but it did not differ between birds infected by one genus and three blood parasite genera ($P > 0.05$). Post hoc comparisons of the interaction age × treatment indicated that untreated adult birds were more colourful than medicated adult birds ($P < 0.01$).

Carotenoid chroma was not significantly related to immunoglobulin levels ($P > 0.05$), although higher levels of HSP70 in blood were significantly associated with a paler colour (lower carotenoid chroma) in birds (Table 3; Fig. 3). In contrast, we did not find a significant relationship between HSP60 levels and carotenoid chroma ($P > 0.05$). This model retained the effect of treatment, blood parasite richness, sex × age and sex × treatment and explained 12% of variation in carotenoid chroma ($F_{7,139} = 3.98$, $P < 0.001$). The model based only on PCR data retained the significant relationship between carotenoid chroma and HSP70 ($F_{1,99} = 7.01$; $P < 0.01$) and the effect of the interaction sex × treatment ($F_{1,99} = 4.08$, $P < 0.05$), whereas the association of chroma with the rest of the variables was not significant (all $P > 0.05$).

Finally, the intensity of infection by *Haemoproteus* in our blue tit population did not differ significantly between different categories of parasite richness ($F_{3,175} = 0.71$, $P > 0.05$) and it was not significantly associated with carotenoid chroma ($F_{1,175} = 0.03$, $P > 0.05$), body condition ($F_{1,175} = 0.20$, $P > 0.05$), immunoglobulin levels ($F_{1,119} = 0.24$, $P > 0.05$) or HSP70 levels ($F_{1,119} = 0.05$, $P > 0.05$). However, we found a positive significant association with HSP60 levels ($F_{1,119} = 10.00$, $P < 0.01$; Fig. 4). In addition, *Haemoproteus* infection intensity was higher in 2007 ($F_{1,175} = 7.08$, $P < 0.01$) and in young birds ($F_{1,175} = 25.61$, $P < 0.001$).

Table 3 Model obtained when exploring the association of levels of immunoglobulins and stress proteins with carotenoid chroma of blue tit breast plumage in 2004. *HSP* Heat shock protein

Effect	*df*	*F*	*P*
Treatment	1,139	3.935	0.049
Blood parasite richness	3,139	3.243	0.024
HSP70 levels	1,139	5.354	0.022
Sex × age	1,139	3.273	0.041
Sex × treatment	1,139	4.608	0.034

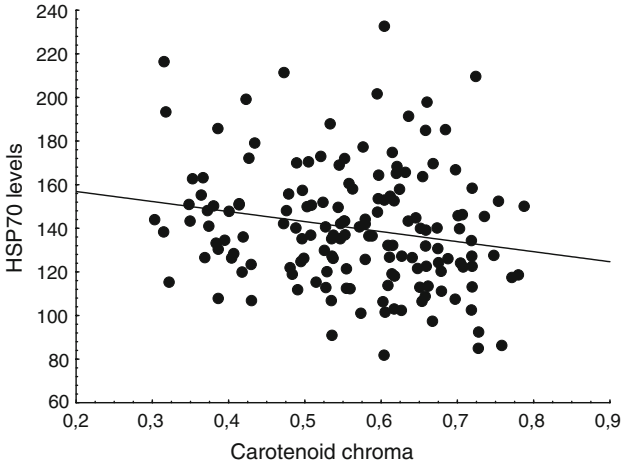

Fig. 3 Relationship between heat shock protein (*HSP*) 70 levels and carotenoid chroma of breast plumage colour of blue tits in 2004

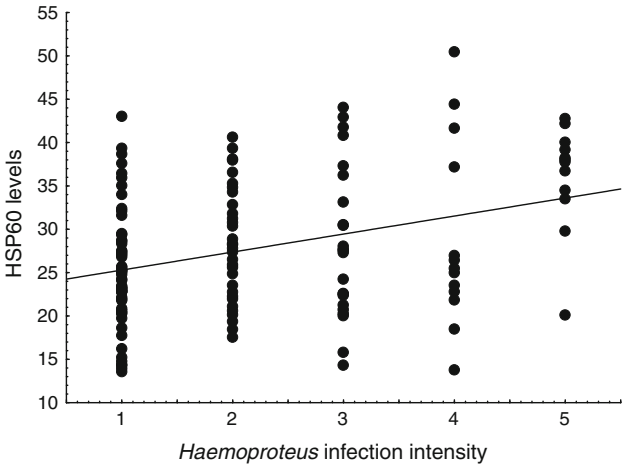

Fig. 4 Relationship between HSP60 levels and *Haemoproteus* infection intensity of blue tits in 2004. Intensity of infection was transformed to include data of infections only detected by molecular methods (see text)

Discussion

The most novel result of this study is the relationship of multiple blood parasite infections with carotenoid chroma. Birds parasitized by a single genus of blood parasite had higher carotenoid chroma and thus showed a more colourful plumage than birds parasitized by more than one parasite genus. In spite of the temporal separation between the moult and the reproductive season (when we took colour measurements), the relationship between parasite richness and carotenoid chroma could be expected. Birds being able to develop a conspicuous plumage during the moult could show a better health status in the next spring, especially because most of these blood parasite infections are chronic with birds suffering relapses while breeding (Valkiūnas

2005). Birds in poor health would be less efficient in acquiring carotenoid-rich food (Hill 1991, 1992; Senar et al. 2002) and consequently would exhibit a paler plumage. A previous experiment in the same study population showed that females medicated with primaquine did not vary in body condition during the breeding season, whereas body condition was reduced in control females during the same period, suggesting that blood parasites have negative effects on female condition (Merino et al. 2000). In addition, Tomás et al. (2007) showed that females treated with high doses of the antimalarial drug were able to increase their reproductive effort in comparison to control females and those treated with low doses of the drug. These results support the existence of harmful effects induced by parasitism, parasitized birds being in poor condition and therefore showing a paler colour.

Furthermore, according to the hypotheses that deposition of carotenoids into feathers is traded-off with their participation in physiological functions as immune-stimulators and antioxidants (Lozano 1994; Møller et al. 2000), parasitized birds would have less carotenoids available for plumage pigmentation. An alternative non-excluding mechanism would be that carotenoid-based traits are signalling levels of other non-pigment antioxidants that might protect carotenoids from free radical attacks and make them available for sexual advertisements (Hartley and Kennedy 2004). Additionally, apart from the passive "protection mechanism" (Hartley and Kennedy 2004), the allocation of colourless antioxidants to sexual signalling may promote an active mechanism to increase the amount of pigments (Pérez et al. 2008). Another hypothesis suggests a trade-off between lipids being used for energy generation or for absorption and/or transportation of carotenoids (Fitze et al. 2007). Whatever the mechanism implied, it appears that carotenoid-based plumage colouration signals health status in our blue tit population, as multiple infections negatively affect the showiness of the yellow breast plumage.

Negative effects of multiple infections by blood parasites in birds have been also found in other studies (Davidar and Morton 2006; Arriero and Møller 2008; Marzal et al. 2008). This could be due to additive effects of infection by different blood parasite genera. Another possibility is that infection by a second genus could induce competition for resources between different parasites (Frank 1996) or activation of the immune response (Read and Taylor 2000), which would induce parasites to become more virulent than if they infected hosts separately (Frank 1996). It is assumed that parasites which grow more rapidly inflict more damage on their hosts and are competitively superior to less harmful parasites (see de Roode et al. 2005). Thus, if parasite competition is occurring, higher *Haemoproteus* infection intensity would be expected in birds infected by several blood parasites. In fact, we can expect that the intensity of *Haemoproteus* increases with the number of parasite genera infecting the same bird. However, there were not significant differences in the intensity of infection by *Haemoproteus* among birds infected by different numbers of parasite genera in our blue tit population. Therefore, competition between *Haemoproteus* and other parasites apparently does not exist or at least it is not reflected in an increase in the intensity of *Haemoproteus* infection. Nevertheless, further studies to explore the interactions between parasites inside a common host and their effects are needed.

On the other hand, we found an inter-annual variation in the effect of multiple infections on carotenoid chroma. In 2007, birds parasitized by two and three blood parasite genera were significantly more colourful than birds infected by two and three blood parasite genera in 2004. Carotenoids must be acquired through the diet (Olson and Owens 1998), caterpillars being the major source of these pigments for blue tits (Slagsvold and Lifjeld 1985). Thus, a year may be "favourable" for birds because there is a higher abundance of food (greater carotenoid availability) and/or a lower abundance of parasites, and consequently, more carotenoids would be available to be deposited into birds' feathers. Hence, it was not surprising to find inter-annual variation in the carotenoid chroma of birds, those of 2007 being more colourful than those of 2004. In this respect, the prevalence of blood parasites in 2007 was lower than in 2004 although *Haemoproteus* infection intensity was higher in 2007. Likewise, Hõrak et al. (2000) showed that great tit nestlings which grew up in a "bad" year had paler yellow breasts than nestlings which grew up in a "good" year. Different environmental conditions can also be found between habitats, explaining a large part of the variation in the colour of birds from habitats of different quality. Blue tits inhabiting structurally complex forests obtain more caterpillars (Ferns and Hinsley 2008), and consequently present higher chroma values than blue tits from poor quality forests (Arriero and Fargallo 2006). Our results suggest that birds of 2007 infected by two or three blood parasite genera had higher carotenoid availability for pigmentation in late 2006 (when they moulted their plumage) than birds of 2004 infected by two or three blood parasite genera in late 2003. However, birds infected by four or five blood parasite genera presented similar values of carotenoid chroma in both years, suggesting that highly parasitized birds are the palest birds independently of favourability of environmental conditions. Our main results were still significant when we only analysed parasite data based on PCR, supporting the association between carotenoid chroma and blood parasite richness and HSP70 levels. However, we can not rule out completely the possibility that differences between results using the expanded (PCR plus microscopic data) or the reduced sample (only PCR data) were due to the presence of false negatives and not only to reduction in sample size.

In addition, we did not find any relationship between *Haemoproteus* infection intensity and carotenoid-based colour of the breast, suggesting that blood parasite richness per se and not *Haemoproteus* intensity influenced carotenoid-based colour in our population. Whereas Merilä et al. (1999) found a negative relationship between *Haemoproteus* infection intensity and plumage yellowness of male greenfinches, *Carduelis chloris*, other authors did not find this relationship (Seutin 1994; Dufva and Allander 1995). These contradictory results may be due to the use of different methods to quantify haematozoan infections, as suggested by Clayton (1991), as well as differences in the colour variables used. On the other hand, we found a positive relationship between HSP60 levels and *Haemoproteus*. This is not surprising because in an experiment consisting of a reduction in the intensity of infection by *Haemoproteus* and the prevalence of infection by *Leucocytozoon* in female blue tits, Tomás et al. (2005) found that control females had a higher final level of HSP60 than medicated ones. Also, other studies on birds suggested a role of this protein in responses to parasitism (Merino et al. 1998; Merino et al. 2002; Arriero et al. 2008). Parasites may cause increases in HSP60 by at least three different, non mutually exclusive mechanisms: it could be a response to the fever occasioned by the infection, i.e. HSP60 could be increased in response to a heat shock (Garbe 1992); the increase in HSP60 could be due to a pathogen-induced necrosis (Moseley 2000); and secretion/excretion products from parasites could induce protein expression (Martínez et al. 1999b). Our result adds support to the hypothesis that parasites are the stressors that cause the increase in HSP60 values in blue tits. However, a relationship does not seem to exist between parasitism and HSP70 levels (Merino et al. 2002; Tomás et al. 2005), suggesting that this protein responds in a different manner, or in response to different stressors, than HSP60. More studies are needed to clarify these associations.

Sex and age differences of carotenoid-based colouration of blue tits have been reported in some populations (Figuerola et al. 1999; Hidalgo-García 2006) but not in others (Ferns and Hinsley 2008). In this study, adult birds of both sexes showed similar values of carotenoid chroma, whereas young males were significantly more colourful than young females. Additionally, we did not find age differences in the colour of males but we did in the colour of females, young females being paler than adult females. Therefore, it seems that blue tits are more or less dimorphic in the yellow plumage of the breast depending on the population considered. This may be due to the partial dependence of carotenoid-based plumage colour on environmental factors (Hadfield and Owens 2006) such as habitat and population density.

We found a relationship of primaquine treatment with carotenoid chroma, which was especially strong in males. Untreated birds showed significantly higher values of carotenoid chroma than treated birds. We treated birds with primaquine months after the deposition of carotenoids in feathers during the moult, so we did not expect a direct effect of this drug on birds' colours. It could be that this was a spurious effect, i.e. the colour of untreated birds was more intense prior to treatment; however, another possibility could be that higher provisioning rates of treated birds (Tomás et al. 2007) induced a higher abrasion of birds' plumages due to rubbing against nest box entrance or meant less time was devoted to feather maintenance activities resulting in paler colours (McGraw and Hill 2004). Studies taking into account colouration before treatment are needed to clarify the effect of primaquine on carotenoid-based plumage colour.

Since the carotenoid chroma of plumage reflected to a certain degree the blood parasite richness within a host, we could also expect it to reflect body condition, and immunoglobulin and HSP levels. Although Senar et al. (2003) found that carotenoid-based plumage colour of the breast of the great tit *Parus major* was correlated with the nutritional condition, as estimated by the rate of tail growth (see also Senar et al. 2008), and several authors have reported positive correlations between carotenoid-based colours and immune function (Dufva and Allander 1995; McGraw and Ardia 2003; Alonso-Álvarez et al. 2004), we could not find any association between carotenoid chroma and immunoglobulin levels or body condition. However, birds with lower carotenoid concentrations in feathers had higher HSP70 levels. These proteins prevent cellular homeostasis alteration and are involved in many different functions like protein synthesis, folding and transport, as well as in degradation of misfolded, non-functional proteins (Morimoto 1991). These proteins are implicated in responses to a wide array of stressors including blood parasites (Merino et al. 1998; Tomás et al. 2005). As previously reported (Merino et al. 2002; Tomás et al. 2005), we did not find *Haemoproteus* infection intensity significantly associated with levels of HSP70. However, the relationship between HSP70 and plumage colour may be in part related to an infection-associated stress. The fact that blood parasite infections are chronic and maintained in birds for several months and even years with periods of relapses (Valkiūnas 2005) may help to understand the existence of these associations between long-term stress indicators such as HSPs levels and plumage colours. On the other hand, this association may be due to the function of carotenoids as antioxidants in the organism (Lozano 1994; Møller et al. 2000) since HSPs respond to oxidant compounds as well (Martínez et al. 1999a). Therefore, more colourful birds would have more carotenoids available for this antioxidant function, fewer oxidant compounds and consequently lower HSP70 levels. As discussed above, HSP70 seems to respond in a different manner, or in response to different stressors, than HSP60.

Overall, based on the full data set, carotenoid-based plumage colouration seems to be a long-term indicator of health status in this population of blue tits. This study reveals negative effects of multiple infections by blood parasites on carotenoid-based plumage colour. Further experiments are needed to better understand the effects of multiple parasite infections on hosts and the mechanisms underlying carotenoid-based colouration.

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MHC class II B diversity in blue tits: a preliminary study

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Cyanistes caeruleus, major histocompatibility complex, *Parus caeruleus*, passerine.

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Abstract

In this study, we partly characterize major histocompatibility complex (MHC) class II B in the blue tit (*Cyanistes caeruleus*). A total of 22 individuals from three different European locations: Spain, The Netherlands, and Sweden were screened for MHC allelic diversity. The MHC genes were investigated using both PCR-based methods and unamplified genomic DNA with restriction fragment length polymorphism (RFLP) and southern blots. A total of 13 different exon 2 sequences were obtained independently from DNA and/or RNA, thus confirming gene transcription and likely functionality of the genes. Nine out of 13 alleles were found in more than one country, and two alleles appeared in all countries. Positive selection was detected in the region coding for the peptide binding region (PBR). A maximum of three alleles per individual was detected by sequencing and the RFLP pattern consisted of 4–7 fragments, indicating a minimum number of 2–4 loci per individual. A phylogenetic analysis, demonstrated that the blue tit sequences are divergent compared to sequences from other passerines resembling a different MHC lineage than those possessed by most passerines studied to date.

Introduction

The major histocompatibility complex (MHC) is a group of genes critical to immune function in vertebrates (Doherty and Zinkernagel 1975; Klein 1986). Due to its central role against infections from pathogens, the study of MHC genes has been the subject of many ecological and evolutionary studies (Sommer 2005; Spurgin and Richardson 2010), especially those studying host-parasite interactions (Westerdahl 2007; Dionne 2009). The characterization of MHC genes is constrained by its multigene nature, a result from gene duplications during evolutionary time and a turnover of new and old genes, the so-called birth-and-death model (Klein and Figueroa 1986; Nei et al. 1997; Edwards and Hedrick 1998; Nei and Rooney 2005). Thus, vertebrates have their own particularities on MHC evolution, with orthologous and paralogous genes not only maintained mainly by natural selection from parasites but also by sexual selection (Hughes and Yeager 1998). As a consequence, some MHC alleles have a long persistence time, even exceeding the species evolutionary time (i.e., trans-species polymorphism) (Klein and O'Huigin 1994).

The MHC genes constitute the most polymorphic genes among vertebrates (Piertney and Oliver 2006) and its study has been challenging, especially in non-model organisms (Babik 2010). In passerines, the study of this genetic region is also complicated, due to the existence of concerted evolution among genes (Edwards et al. 1999; Hess and Edwards 2002). Different molecular methods have been used to characterize MHC genes in birds, including PCR and non-PCR-based methods (Babik 2010). These studies involved the analysis of whole genetic region or partial regions, including complete or incomplete introns/exons. In this manner, several orders of passerine and non-passerine species have been studied to date (see Bollmer et al. 2010; Li et al. 2011; Miller et al. 2011). The first species with a completely sequenced MHC was a non-passerine, the chicken (*Gallus gallus*) and its MHC appeared simple and compact (Kaufman et al. 1999).

Since then more species have been studied and the general conclusion is that the genetic organization of the passerines MHC seems to be more complex than that of non-passerines (Balakrishnan et al. 2010; Ekblom et al. 2011). This finding is supported by the derived phylogenetic position of passerines from non-passerines, where a minimal MHC seems to be the ancestral condition for birds, at least for class II genes (Hughes et al. 2008; Balakrishnan et al. 2010). Although a ratite species was observed to have at least five MHC class II loci (Miller et al. 2011). Thus, passerines have a higher number of genes, larger class I and II genes (longer introns) and also nonfunctional genes (pseudogenes) (Edwards et al. 1998; Kaufman et al. 1999; Miller and Lambert 2004a; Westerdahl et al. 2004b; Westerdahl 2007; Bollmer et al. 2010). Both MHC class I and II genes have been studied in birds involving host-parasites implications. Having an elevated/optimal number of alleles (heterozygote advantage) and/or rare advantageous alleles (negative frequency-dependent selection) would be favored by natural selection for parasite detection and elimination (Bodmer 1972; Doherty and Zinkernagel 1975; Wegner et al. 2003).

Different groups of MHC genes have been detected in different species. In the galliform birds there are two similar, but independent MHC complexes, both with class I and class II genes (Briles et al. 1993; Strand et al. 2007). The B-complex has high polymorphic and expressed genes and has been associated with disease resistance (Kaufman 2000) and the Y-complex (MHC-Y), a separate group of genes, less polymorphic, and expressed genes to a lower extent. The Y-complex has been suggested to be involved in the innate immunity (Miller et al. 2004) and controversial associations among the Y-complex and Marek's disease have been reported (Wakenell et al. 1996; Vallejo et al. 1997). In some passerines, low polymorphic genes seem reminiscent from the MHC-Y-complex (Edwards et al. 2000; Gasper et al. 2001; Bonneaud et al. 2004; Jarvi et al. 2004), therefore a phylogenetic approach has been employed to classify groups of genes (Edwards et al. 1999).

In this study, we investigate MHC class II B genes in the blue tit (*Cyanistes caeruleus*) for the first time. The blue tit is established as a model species in different ecology studies where parasite prevalence, ecological factors, and their effects on their host have been studied in depth (Hurtrez-Boussèz et al. 1997; Tripet and Richner 1997; Fargallo and Merino 1999; Merilä and Andersson 1999; Wiles et al. 2000; Merino et al. 2006; Martínez-de la Puente et al. 2010). The blue tit MHC class I genes have recently been studied (Schut et al. 2011; Wutzler et al. 2012), therefore a preliminary characterization of the MHC class II B would be a determinant for a later in-depth molecular characterization, as it gives an idea of the complexity of the system (Wittzell et al. 1994; Babik 2010). We used sequencing and restriction methods to investigate the exon 2 that codes from the most variable peptide-binding region (PBR) of the MHC class II molecule.

Materials and Methods

Study species

The blue tit is a small insectivorous passerine (Family Paridae) that breeds in the west Palearctic from Mediterranean to boreal zones (Cramp and Perrins 1998). Most of the geographic range (75%) of this species is located within Europe (BirdLife 2012). Blue tits willingly use nest boxes for reproduction when provided. In this study, individuals sampled came from three different European locations where individuals breed in nest boxes: Spain (Valsaín, 40°53′N, 4°01′W), Sweden (Revinge, 55°41′N, 13°26′E) and The Netherlands (The Vosbergen estate, 53°08′N, 06°35′E). The number of individuals sampled and their location of origin are detailed in Table S1.

PCR and sequencing

DNA exon 2 sequences

To get a measure of MHC diversity we investigated the exon 2 that code for the β_1 chain. The β_1 chain encodes part of the PBR of the MHC class II molecule. To that end, whole blood samples (50–100 μL per bird) were obtained from the brachial vein and collected with a capillary tube in the field from 18 individuals (see Table S1). Blood was immediately stored in a cool box and later preserved either frozen at −80°C or in 99% ethanol, until molecular analysis. Genomic DNA was isolated either by standard phenol-chloroform extraction methods or by using the UltraClean DNA BloodSpin kit (MO BIO laboratories, Inc., CA). Genomic exon 2 sequences were amplified by polymerase chain reaction (PCR) using standard procedures with AmpliTaq DNA Polymerase PCR kit (Applied Biosystems, CA). A single PCR reaction included 25 ng of genomic DNA, 0.5 μmol/L of each of the primer 2ZFfw1 and 2ZFrv1 (Balakrishnan et al. 2010), 10× PCR buffer, 0.5 μmol/L dNTP, 2.0 mmol/L MgCl, and 1.0 unit of *Taq* polymerase in a final volume of 20 μL. The reaction was run in a thermal cycler Gene Amp PCR System 9600 (Perkin Elmer, Foster City, CA) at 94°C for 2 min, 35 cycles of (94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec), and at 72°C for 10 min. We previously tested how general the primers were in amplifying genomic DNA from several bird species including the blue tit and also whether the primers had been utilized satisfactorily in other passerines (van Rensburg et al. 2012). The blue tit PCR product was checked on an

ethidium–bromide-stained 2% agarose gel for bands of the appropriate size. Because individuals are likely to be heterozygous and the possibility of amplifying more than one allele at any locus exists, amplicons were cloned in the vector pCR2.1 with the TOPO TA Cloning kit (Invitrogen, CA) according to manufacturers' protocol. Clones with inserts were selected from colonies and diluted in 150 μL dd H_20 and heated to 95°C for 3 min. Up to 20 clones of each individual were used for sequencing. To do this, 1 μL of the clone dilution was amplified in a 20 μL PCR using 1 μmol/L of cloning kit primers (M13 forward and M13 reverse), 1× PCR buffer, 0.125 mmol/L dNTP, 2.0 mmol/L MgCl, and 1.0 unit of *Taq* polymerase. The amplification consisted of 35 cycles at 94°C, 60°C, and 72°C, each step for 30 sec. Positive PCR products were purified through precipitation in NH_4Ac via centrifugation and used as template in dye terminator sequencing reactions with Big Dye Terminator (Applied Biosystems). PCR conditions consisted of 25 cycles at 96°C for 10 sec, 55°C for 5 sec, and 60°C for 4 min. After precipitation in NaAc, sequences were run on an ABI PRISM Genetic Analyzer 3730 (Applied Biosystems). Obtained sequences were read in one sequencing reaction. All sequences are the result of cloning events so each sequence corresponds to a clone. Only sequences that were found in at least two independent PCRs were regarded as verified sequences (Westerdahl et al. 2004b). Independent PCRs were performed either from the same individual or different ones. Unique sequences were also found, but were considered non verified. Non-verified sequences could not only be true sequences but also false sequences due to PCR mistakes. Primers were developed inside exon 2, therefore in this study we refer to "alleles" but they do not encompass the entire length of exon 2. In addition, we do not expect to detect all the possible alleles in the blue tit. Verified sequences were deposited in the GenBank (accession numbers JF775361 - JF775373).

cDNA exon 2 sequences

The purpose of this analysis was to obtain cDNA exon 2 of MHC class II B sequences from RNA and thus study transcription. Blood samples (100 μL per bird) were obtained from seven individuals again by wing vein puncture (see Table S1). Blood from individuals C, D, and E was used in both DNA and RNA analysis. Blood was preserved in 500 μL of Trizol-LS with the addition of 100 μL of K_2EDTA following Miller and Lambert (2003). Samples were stored at 4°C for later RNA extraction. Total RNA was extracted as in Strandh et al. (2011). cDNA was obtained by RT-PCR using the two-step PCR reaction using the Retroscript kit according to protocol (Ambion, Applied Biosystems) again with the 2ZFfw1 and 2ZFrv1 primers. cDNA obtained was then used as a template on a standard PCR, where 2 μL of cDNA template was amplified with the same PCR reagents used for DNA. PCR conditions were 94°C for 2 min, 35 cycles of (94°C, 60°C, and 72°C, each step for 30 sec) and 72°C for 5 min. PCR products were cloned and sequenced as in DNA methods described above. Again verified and non-verified sequences were found. Sequences were deposited in GenBank (accession numbers JF766222 - JF766234).

Restriction fragment length polymorphism

Another 17 individuals from two blue tit Swedish families were used for the restriction fragment length polymorphism (RFLP) analysis. These individuals were only used in this analysis and not in previous DNA or RNA analysis. With this analysis we wanted to obtain a rough description of MHC class II B genetic diversity. RFLP is based on restriction enzymes and the number of bands in each individual corresponds to the approximate number of MHC class II B alleles. To do this, we performed a restriction cleavage with PvuII on 10 μg of genomic DNA following the methods described in Westerdahl et al. (1999). The enzyme was previously tested to confirm its suitability. One of the verified cDNA sequences was used as a probe.

Data analyses

After manual alignment in BioEdit (Hall 1999), DNA and RNA sequences were confirmed as exon 2 of MHC class II B using a BLAST search with NCBI GenBank. To measure sequence polymorphism the nucleotide diversity (π) and the number of segregating sites (S) were calculated from all alleles in DnaSP (see Librado and Rozas 2009). These values were also calculated for other passerine sequences obtained from GenBank (Table 1). The same nucleotide length was used to calculate π and S for all the species. Next a Bayesian phylogeny was performed to study evolutionary relationships among the blue tit and other passerines. For this analyses, we searched for all passerines exon 2 of MHC class II B sequences available from GenBank together with sequences corresponding to the BLB and YLB region from the chicken (*Gallus gallus*), the Eurasian black grouse (*Tetrao tetrix*), and the common mallard (*Anas platyrinchos*). From passerines functional and non-functional sequences were included. All the sequences were aligned by using MAFFT alignment implemented in Jalview (Waterhouse et al. 2009). Gblocks (Talavera and Castresana 2007) selected for the most informative nucleotide positions under the less stringent option. Redundant sequences were discarded by using the redundancy removal option in Jalview. With this option sequences with a similarity above 90% were deleted from

Table 1. Sequence diversity of exon 2 of MHC class II B in the blue tit and other passerines.

Sp	*N*	*S*	π	SE
Agph	10	76	0.20	0.01
Pado	12	82	0.19	0.02
Apco	10	67	0.17	0.02
Anvi	13	68	0.16	0.01
Came	6	53	0.16	0.02
Pasa	3	40	0.16	0.07
Getr	15	66	0.15	0.01
Acar	7	56	0.15	0.02
Hevi	11	44	0.14	0.01
Gefo	18	57	0.13	0.01
Peau	8	45	0.12	0.01
Cyca	13	47	0.10	0.01

Sp, species; *N*, number of sequences; S, number of polymorphic sites; π, nucleotide diversity. SE, π standard deviation. Blue tit (Cyca, *Cyanistes caeruleus*), great reed warbler (Acar, *Acrocephalus arundinaceus*), red-winged blackbird (Agph, *Agelaius phoeniceus*), little greenbul (Anvi, *Andropadus virens*), Florida scrub jay (Apco, *Aphelocoma coerulescens*), house finch (Came, *Carpodacus mexicanus*), medium ground-finch (Gefo, *Geospiza fortis*), common yellow throat (Getr, *Geothlypis trichas*), Hawai'i Amakihi (Hevi, *Hemignathus virens*), house sparrow (Pado, *Passer domesticus*), savannah sparrow (Pasa, *Passerculus sandwichensis*), New Zealand robin (Peau, *Petroica australis*).

the alignment. jModeltest 0.0.1 (Posada 2008) under corrected Akaike information criteria (AiCc) selected generalised time-reversible (GTR) as the suitable substitution model. The phylogeny was inferred in MrBayes v3.2 (Ronquist and Huelsenbeck 2003) with 80×10^6 generations. The convergence of the parameter values sampled from the chains was checked by using the potential scale reduction factor (PSRF) once the standard deviation of split frequencies was below 0.01.

Subsequently, we also looked for signs of positive selection in the PBR. The non-synonymous (d_N) and synonymous (d_S) substitution ratio $\omega = d_N/d_S$ provide a measure of selection pressure at the amino acid level (Nei and Kumar 2000; Yang and Nielsen 2002). Neutral genes are supposed to have a $\omega = 1$ whereas genes under positive selection have $\omega > 1$. We estimated ω ratio by calculating the average values of synonymous and non-synonymous substitutions per site by the Nei-Gojobori and maximum likelihood methods. In the first method, the PBR nucleotide positions were previously defined based on the MHC structure determined by crystallography (Brown et al. 1993). In this method, the Jukes Cantor correction was used (Nei and Gojobori 1986) and a *Z*-test was performed for both, codons corresponding to PBR and non-PBR. Analyses were performed in MEGA 4.0 (Kumar et al. 2008). The second method calculates the positions under selection without any *a priori* information. This analysis was performed in CODEML program included in PAML 4 package (Yang 2007). We tested the models M1a (nearly neutral), M2a (positive selection), M7 (beta) and M8 (β and ω) of codon substitutions allowing the ω ratio to vary among sites. In the analysis, a likelihood ratio test of positive selection was performed comparing model M1a against M2a and M7 against M8. *P*-values were calculated with a chi-squared test.

Results

MHC diversity

Overall, we obtained 217 verified sequences corresponding to blue tit exon 2 of MHC class II B from three different European locations (Table 2). A total of 96 sequences were obtained from DNA and 121 from RNA. All sequences were searched in GenBank by using BLAST and they were similar to exon 2 of MHC class II B with a maximum identity of 82%. Sequences were 159 bp long (without primers) and covered 60% of exon 2 length, which comprises 267 bp. Thirteen different alleles were verified obtained from DNA and RNA (Fig. S1). When these sequences were translated to amino acids, 12 amino acid sequences were obtained (Fig. 1). A total of 40 non-verified sequences were obtained, 38 from DNA and 2 from RNA (Table 2). No stop codon or shift in the reading frame was found in any sequence suggesting absence of pseudogenes in the samples. The maximum number of alleles found in an individual was four (individual S, Table 2), indicating the existence of at least two loci. Two individuals (Q and W) had three transcribed alleles (RNA), suggesting that both loci are expressed. Nine out of 13 alleles were found in more than one country. Alleles Cyca-DAB*3 and Cyca-DAB*9 appeared in all countries.

The RFLP pattern consisted of 4–7 fragments per individual in the length range of 1–9 kb, each band corresponding to approximately one allele (Fig. 2). The intensity of the RFLP fragments were variable, either because their similarity to the probe differed or because RFLP fragments of certain lengths were more numerous than others (Westerdahl 2003). Nearly all individuals had unique RFLP patterns except for the individuals F1 and 4 that shared the same bands, a likely result when comparing individuals from the same family. These results suggest a minimum number of four loci. The RFLP bands may correspond to both coding and non-coding genes, so it is possible that we are observing bands corresponding to pseudogenes that we do not amplify with our primers. On the other hand, one hybridizing fragment could represent two genes with the same electrophoresis migration distance. Nucleotide diversity results from the blue tit and other passerines are presented in Table 1.

Table 2. Number of verified and non-verified exon 2 sequences found per individual.

Sampling site	The Netherlands										Spain							Sweden					
Individual	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	W	Total
Verified sequences																							
Cyca-DAB*1	–	–	3 (7)	–	–	–	–	–	–	–	–	–	–	–	(6)		(8)	–	–	–	–	–	3 (21)
Cyca-DAB*2	–	–	–	–	8 (11)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	8 (11)
Cyca-DAB*3	–	2	–	–	–	–	–	–	–	–	–	–	–	–	(11)	–	–	3	–	–	–	–	5 (11)
Cyca-DAB*4	–	–	–	–	–	–	–	1	–	–	–	6	–	–	–	(12)	–	–	–	–	–	–	7 (12)
Cyca-DAB*5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	2	–	–	(6)	4 (6)
Cyca-DAB*6	–	–	–	–	–	–	–	–	–	–	8			1	–	–	–	4	6	–	–	(5)	19 (5)
Cyca-DAB*7	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	1	–	1	(7)	3 (7)
Cyca-DAB*8	–	–	–	–	3 (4)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3 (4)
Cyca-DAB*9	–	–	–	3 (3)	–	2	4	–		4			1	–	–	–	–	–	1	1	–	–	16 (3)
Cyca-DAB*10	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	(3)	–	–	–	–	–	3 (3)
Cyca-DAB*11	6	–	–	5 (17)	–	–	–	–	–	–	–	–	–	–	–	(7)	–	–	–	–	–	–	11 (24)
Cyca-DAB*12	–	–	9 (7)	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	10 (7)
Cyca-DAB*13	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	(7)			4	–	–	4 (7)
Non-verified sequences																							
Sequence 1	–	–	–	–	–	–	–	–	–	–	–	–	19	–	–	–	–	–	–	–	–	–	19
Sequence 2	–	–	–	(2)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	(2)
Sequence 3	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2
Sequence 4	–	–	–	–	–	–	–	–	–	–	–	–	–	9	–	–	–	–	–	–	–	–	9
Sequence 5	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	2
Sequence 6	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	2
Sequence 7	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	2
Sequence 8	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2

Numbers without brackets, sequences obtained from DNA. Numbers in brackets, cDNA sequences obtained from RNA. Alleles: total number of verified alleles in an individual.

```
                        10         20         30         40         50
                   ....|*.*.* ....**...| ....*....| ...*|..**| ..*.|*.**| .*.
Cyca-DAB*1         RVRYVDRLIY NRKQYAHFDS DEGLFVGDTP RGEKAAKYYN SLPEYLEQHR TAV
Cyca-DAB*2         .......... .....V.... .......... .......... .......... ...
Cyca-DAB*3         ....AA.EF. ..E..V.... .......... F...V...W. .......... ...
Cyca-DAB*4         .....Q.Y.. ..E.LV.... .......... L..MN...W. GK......K. ...
Cyca-DAB*5         .....Q.Y.. ..E.LV.... .A........ L..MN...W. GK......K. ...
Cyca-DAB*6         .....Q.Y.. ..E.LV.... .A........ L..MN...W. GK......K. ...
Cyca-DAB*7         ...LLQ.Y.. ..E..F.... .A........ .......NL. .R..T..YY. ...
Cyca-DAB*8         ....AA.VF. ..E..V.... .......... ....V...W. GE........ .S.
Cyca-DAB*9         ....AA.VF. .....V.... .......... ....V...W. GKS....... ...
Cyca-DAB*10        ....AA.VF. ..E..V.... .......... ....V...W. GKS....... ...
Cyca-DAB*11        ....AA.VF. ..E..V.... .......... ....V...W. GKS....... .S.
Cyca-DAB*12        ....AA.EF. ..E..V.... .......... H...V...W. GK........ .S.
Cyca-DAB*13        ....AA.EF. ..E..V.... .......... ....V...W. .......... .S.
Anvi AY437902      K...MHTY.. ..V...M... .V.HY..F.. W...N.Q.W. NN.DIM.TQ. .S.
Acar AJ404372      K....E.Q.. ..E.ILM... .V.HY..F.. F...Q.QDW. .K..WM.NR. ...
Came U23969        K..FAE.Y.. ..QT..M... HV.HY..F.. F...V.RNW. .S..WM.DR. PV.
Pado AY518173      K.S..E.Q.. ..QLELM... .V.EY..F.. Y..RR.RVW. .K..WI.FR. RE.
Tagu XM00219335    K..F.L.Y.. ..Q.DVM... .V.EY..F.. Y...N..RL. .D..LM.YR. ...
```

Figure 1. Amino acid sequences translated from blue tit exon 2 cDNA sequences and other passerines. Identity with allele Cyca-DAB*1 is indicated by *dots*. *Asterisks* are the codons corresponding to the PBR. Anvi, *Andropadus virens* (AY437902); Acar, *Acrocephalus arundinaceus* (AJ404372); Came, *Carpodacus mexicanus* (U23969); Pado, *Passer domesticus* (AY518173); Tagu, *Taeniopygia guttata* (XM002193356).

Phylogenetic analysis of class II B sequences

The Bayesian phylogeny on passerine MHC class II B sequences is presented in Figure 3 (collapsed tree) and Figure S2 (extended tree). As expected, there were two well supported clades in the tree separating passerines from non-passerines. Inside passerines there was a well supported cluster (posterior probability of 97) with the majority of the passerine sequences (139 sequences out of 155). Inside this cluster there were well supported groups but also several sequences not resolved forming a polytomy. The sequences in this group included both

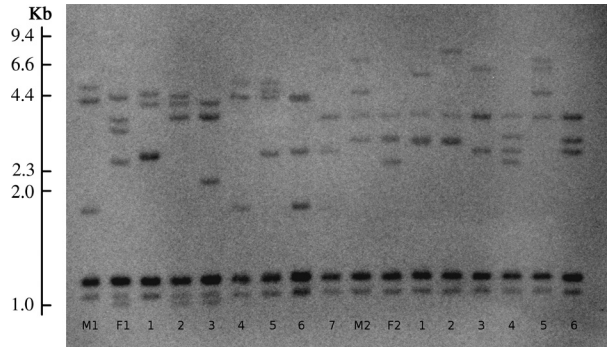

Figure 2. RFLP patterns with the restriction enzyme Pvu II and the blue tit MHC class II exon 2 probe, from 17 blue tit individuals corresponding to two Swedish families. M, mother; F, father; numbers are the offspring. Family 1, individuals from F1 to 6. Family 2, individuals from F2 to 13. There are 4–7 fragments per individual in the size range 1–9 kb.

non-functional alleles and alleles well assigned to be involved in peptide presentation. Interestingly, all the blue tit sequences appeared outside this major group and they formed a monophyletic cluster (posterior probability of 100) together with other passerine sequences. These sequences corresponded to the Dupont's lark (*Chersophilus duponti*) GU390279, the collared flycatcher (*Ficedula albicollis*) GU390277, woodchat shrike (*Lanius senator*) GU390285, and common raven (*Corvus corax*) GU390282, corresponding to sequences obtained with degenerated primers (Canal et al. 2010). Among these sequences non-functional sequences were intermingled as well, that is, red-winged blackbird (*Agelaius phoeniceus*) AF030990, bluethroat (*Luscinia svecica*) FJ409236, FJ409241, slaty spinetail (*Synallaxis brachyura*) AB531785, AB531793, blue-black grassquit (*Volatinia jacarina*) AB531794, and little greenbul (*Andropadus virens*) AY437894.

Selection analysis on exon 2

Putative PBR codons were inferred from HLA and then we did ω ratio analysis for the PBR and non-PBR, respectively, to detect selection. For the PBR, non-synonymous substitutions exceeded those of synonymous substitutions ($[\bar{X} \pm SE]$ $d_N = 0.283 \pm 0.093$, $d_S = 0.134 \pm 0.062$, $\omega = 2.11$), hence there is a tendency for positive selection although this was not significantly different from 1 ($Z = 1.566$; $P = 0.060$). For the non-PBR, non-synonymous substitutions were similar to the synonymous substitutions ($d_N = 0.076 \pm 0.026$, $d_S = 0.078 \pm 0.033$, $\omega = 0.97$) and not significantly different from 1 ($Z = -0.050$; $P = 1$). For all positions (PBR and non-PBR together), non-synonymous substitutions exceeded those of synonymous substitutions ($d_N = 0.123 \pm 0.029$, $d_S = 0.090 \pm 0.028$, $\omega = 1.37$), also not significantly different from 1 ($Z = 0.016$, $P = 0.181$).

Maximun likelihood methods in CODEML found evidence of positive selection in the exon 2. Models involving selection (M2a and M8) fits the data significantly better than their respective neutral models (M1a and M7). Bayes empirical Bayes (BEB) found the amino acid sites 8, 31, and 42 to be under positive selection ($\omega > 1$). These positions were selected by both models M2a and M8. Position 8 fitted with that designated by Brown et al. (1993) to be a PBR position, whereas position 42 was one amino acid next to a designed PBR position. Position 31 coincided with a non-PBR position.

Discussion

MHC diversity

In this study, we have examined the exon 2 of MHC class II B genes in the blue tit for the first time. We found a maximum of three expressed alleles per individual detected by sequencing and a maximum of seven alleles detected by RFLP, suggesting at least two to four loci in the blue tit. We are cautious about the results observed and we propose them conservative and preliminary, as primers were designed over zebra finch sequences MHC class II sequences and are likely not amplifying all possible alleles. We checked this possibility adding new sequences to the dataset from recently sequenced species and several sequences were not detected, therefore blue tit MHC may be underestimated. Also, the values found for nucleotide diversity should be taken with caution, as the fact that non-verified sequences were amplified adverts that nucleotide diversity could increase if these sequences become corroborated. Taken this into account, we compared our results with that found in other birds. The total number of MHC class II B loci described in other passerines ranged from three (house sparrow and the red-winged blackbird) to 20 (common yellowthroat) and the number of transcribed loci ranged from three (house sparrow and the scrub jay) to eight (common yellowthroat) (see Bollmer et al. 2010). And the nucleotide diversity for exon 2 genes ranged from 0.007 (green bull) to 0.19 (house sparrow) (Bonneaud et al. 2004; Aguilar et al. 2006). When the blue tit sequence diversity was compared with some other passerines after controlling for the sequence length, a similar polymorphism was found (see Table 1). In the blue tit we found a value of $\pi = 0.10$, characteristic of polymorphic genes, although groups of sequences with low polymorphism have been found in some passerines (Edwards et al. 2000; Gasper et al. 2001; Bonneaud et al. 2004; Jarvi et al. 2004). But the values found in these cases are so low that the lack of

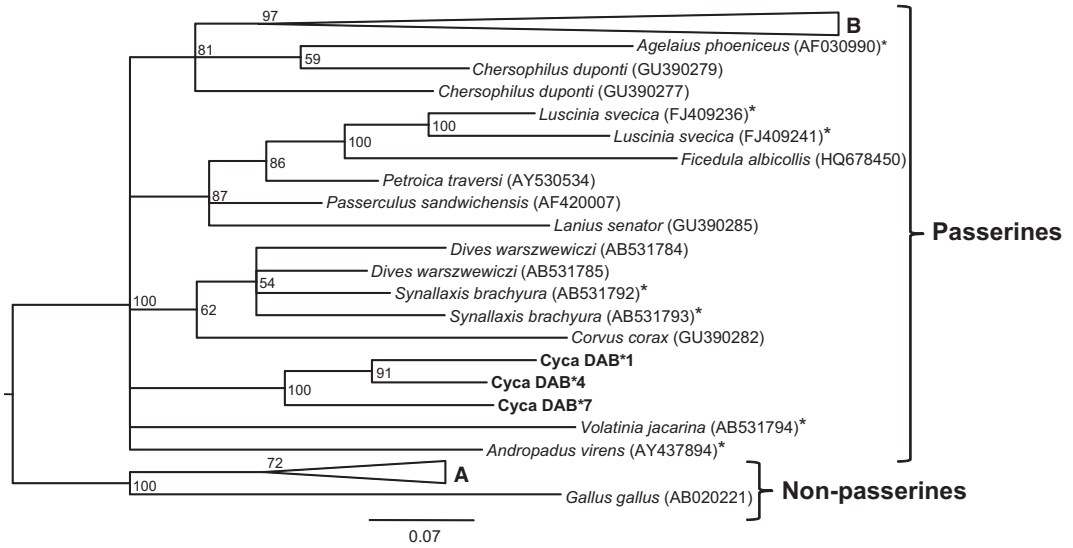

Figure 3. Bayesian phylogeny of exon 2 of MHC class II B sequences (based on 159 bp) from blue tit and other passerine and non-passerine species. Numbers on branches, the posterior probabilities values. *, Non-functional MHC class II. Codes following species name, GenBank accession numbers. Collapsed group A included the following: *Acrocephalus arundinaceus* (AJ404371, AJ404373, AJ404375, AJ404377, U24405, U24406, U24408), *Agelaius phoeniceus* (AF030987, AF030989, AF030994, U23970, U23971), *Andropadus virens* (AY437889, AY437890, AY437891, AY437892, AY437893, AY437900, AY437901, AY437904, AY437907, AY437908, AY437911, AY437912, AY437913), *Aphelocoma coerulescens* (U23958, U23959, U23961, U23962, U23963, U23966, U23972, U24401), *Atlapetes rufinucha* (AB531732, AB531795), *Cactospiza pallida* (AB531504, AB531518, AB531520), *Camarhynchus parvulus* (AB531822), *Carpodacus mexicanus* (AF241547, HQ203000), *Catamenia inornata* (AB531796), *Certhidea olivacea* (AB531513), *Coryphospingus cucullatus* (AB531639, AB531729, AB531741, AB531744, AB531800, AB531802), *Dendroica adelaidae* (AB531663), *Erithacus rubecula* (GU390284), *Erythrura gouldiae* (EF535335, EF535337, EF535338, EF535342, EF535348, EF535448), *Euphonia musica* (AB531807), *Ficedula albicollis* (HQ678313, HQ678323, HQ678328, HQ678338 HQ678340, HQ678492), *Ficedula hypoleuca* (GU390232, GU390233, GU390235, GU390236, GU390238, GU390241, GU390248), *Geothlypis trichas* (GQ247570, GQ247571, GQ247574, GQ247575, GQ247588, GQ247597, GQ247612, GQ247617), *Lanius senator* (GU390287), *Lonchura striata* (L42334, L42335), *Loxigilla noctis* (AB531642), *Luscinia luscinia* (FJ529849, FJ529850, FJ529851, FJ529852, FJ529854, FJ529856, FJ529857), *Luscinia svecica* (FJ529861, FJ529863, FJ529870, FJ529871, FJ529876, FJ529878, FJ529883, GQ403040), *Passer domesticus* (AY518171, AY518172, AY518173, AY518176, AY518178, AY518179, AY518180, AY518181), *Passerculus sandwichensis* (AF420008), *Petroica australis* (AY428561, AY428562, AY428563, AY428564, AY730420), *Petroica traversi* (AY258333, AY258335), *Pheucticus aureoventris* (AB531787), *Phylloscopus collybita* (GU390293), *Poephila acuticauda* (EF535365, EF535366, EF535382, EF535416, EF535472, EF535493), *Ramphocelus carbo* (AB531656, AB531742), *Sicalis flaveola* (AB531517, AB531809), *Sporophila nigricollis* (AB531631, AB531812), *Sturnella bellicosa* (AB531813), *Tiaris bicolor* (AB531814), *Tiaris obscura*,(AB531577, AB531633, AB531634, AB531698), *Volatinia jacarina* (AB531640, AB531743, AB531819, AB531820) and *Zonotrichia capensis* (AB531665). Collapsed group B included: *Coturnix japonica* (AB181862), *Gallus gallus* (U91532, AJ248572), *Meleagris gallopavo* (AJ616892, AM233872), *Numida meleagris* (DQ885563), *Pavo cristatus* (AY928098), *Tetrao tetrix* (EF174544), *Phasianus colchicus* (AJ224344, AJ224346, AJ224347, AJ224349).

polymorphism is evident (i.e., the low π value found in the greenbul). Likewise, similar values of blue tit π have been found in non-passerines, that is, in the chicken (0.107) or the black grouse (0.113) (Strand et al. 2007). Thus, blue tit MHC diversity is similar to other passerines, but resembling less diverse MHC genes. A low MHC class I genetic diversity in the blue tit has been reported by Schut et al. (2011) and Wutzler et al. (2012).

Several different hypotheses could explain the MHC diverse observed in the blue tit. A reduced MHC genetic diversity could be due to the population bottleneck the blue tits suffered during the last ice age (Kvist et al. 2004). In vertebrates, a lack of MHC variability has been attributed to bottleneck events (Babik et al. 2009; Becker et al. 2009) and some bird species with low MHC diversity have been reported to have passed a population bottleneck (Richardson and Westerdahl 2003; Miller and Lambert 2004b; Zhang et al. 2006; Bollmer et al. 2007). On the other hand, bird species affected by a greater diversity of parasites, either in time or space, should be selected to maintain/develop a more diverse MHC. Geographical variation in pathogen antigens leads to differential selection by same pathogens in different areas (Jeffery and Bangham 2000), therefore, a low diversity of the MHC could be explained by local adaptation to parasites (Klein 1991; Westerdahl et al. 2005; Bonneaud et al. 2006). In this respect, we can expect that migratory birds exposed to a higher diversity of parasites (Møller and Erritzøe 1998; Westerdahl et al. 2004a) could evolve a more diverse MHC than nonmigratory birds. The blue tit

is considered a resident bird in Europe, except some migratory individuals from northern locations and some altitudinal movements produced during winter (Cramp and Perrins 1998). Thus, when considering the passerine species included in this study there are some birds that fits with this prediction (migratory birds with higher diversity), but is not consistent among others. It will be necessary to increase the number of species in a comparative analysis to look for the potential effect of migratory behavior on MHC genes diversity. Finally, it will be important to identify the composition of parasite lineages present in any blue tit population and which MHC alleles are conferring resistance (Westerdahl et al. 2005; Martínez-de la Puente et al. 2011). In this way, we found some blue tit alleles shared between locations, a pattern consistent with gene flow among blue tit populations across Europe (Taberlet et al. 1992; Kvist et al. 2004).

MHC diversity is critical on antigen detection and affects host response to face pathogens, but reports in this respect are ambiguous. The heterozygote advantage predicts that having more MHC alleles increases the chance on antigen recognition and the negative frequency-dependent selection stands that specific MHC alleles are advantageous against parasite lineages adapted to common MHC alleles. Therefore, different situations are expected to be found among species. Some vertebrate species with low MHC diversity are particularly susceptible to diseases (Mainguy et al. 2007) although some species are not, as is reflected by their large population size or recent expansions (Babik et al. 2009; Radwan et al. 2010). In birds, the chicken "minimal essential" MHC genes confer strong protection against Marek's disease, but a huge mortality is produced among susceptible individuals. Some vertebrates, with reduced numbers of MHC genes showed highly divergent alleles, which might allow for the recognition of a wider range of pathogens (Sommer 2005). Also variations in the level of polymorphism have been found to differ among bird species, thus some species with a unique class II B loci had more alleles than a species with more loci (Hughes et al. 2008). Thus, the expression of a single gene could result in differences in resistance and susceptibility to infectious pathogens in individuals with different MHC haplotypes (Hepkema et al. 1993; Kaufman 2000).

Blue tit phylogenetic analyses

MHC genes can be defined evolutionarily by clustering with respect to other known MHC genes in a phylogenetic context (Hess and Edwards 2002). However, in the phylogeny based on bird MHC class II exon 2 the blue tit sequences were not clearly related to other well characterized sequences nor to non-functional ones. Instead they appeared to form a monophyletic basal group not clearly related to any sequences or group of sequences. Well characterized class II B sequences from other passerines grouped into a separate clade (group A in Fig. 3) and not close to blue tit sequences. Inside group A, several polytomies and low supported groups of sequences were observed. Similar to our results, Balakrishnan et al. (2010) found a class II B zebra finch lineage situated at the base of other passerine class II sequences in a phylogeny performed with the same primers. They suggest that there is a novel locus that has not previously sequenced in birds, but it is not known whether it is expressed or polymorphic. The phylogeny in Balakrishnan et al. (2010) was performed concatenating together exon 2 and 3 whereas in this study only the exon 2 was used. Phylogenies based on the exon 2 are less congruent than those based on exon 3 or both exon 2 and 3 combined (Hughes and Yeager 1998; Miller et al. 2011) because exon 2-based trees may reflect selection rather than a duplication history (Reusch and Langefors 2005). In this manner, it is interesting that we found a similar basal lineage separated from the rest of passerines even though our phylogeny was based only on exon 2. In the tree none of the sequences from other passerines mixed together with the blue tit sequences, therefore, the blue tit sequences could be divergent with respect to those of other passerines and represent a different cluster of genes. This mean that closely related species have not had enough time to diverge and sequences remain similar and/or concerted evolution is acting then alleles are sometimes intermingled on trees (Vincek et al. 1997; Richardson and Westerdahl 2003; Jarvi et al. 2004). Therefore, it will be necessary to include data from species closely related to the blue tit (to date not available) in the phylogeny to confirm this possibility. Also, blue tit genes could be paralogous with respect to the other passerines included in the analyses and the dissimilarity among genes then is because they did not descend from the same ancestral gene. On the other hand, selective pressures from pathogens shared between two bird species could be counteracting genetic differences between species (Westerdahl 2007) and as a consequence they would group together in the phylogeny.

Selection on exon 2

A method to confirm the effect of different selection pressures on the blue tit MHC is to look for signs of selection on the PBR. In the analysis in MEGA, where PBR positions where a priori assigned, we found that for the PBR d_N is higher than d_S (indicative of positive selection) and the difference was nearly significant ($P = 0.06$). On the other hand, the analysis in CODEML found several amino acid positions under positive selection. Thus, there seems

to be some positive selection acting in the PBR of blue tit exon 2, but it is not very strong. Accordingly, we cannot reject the possibility that we are misidentifying PBR positions as they are estimated from results of a crystallographic study of human MHC class II B (Brown et al. 1993) and, therefore, they are not necessarily the same positions as in the avian MHC class II B. In this respect, in other avian studies where PBR positions were estimated following Brown et al. (1993), positive selection was detected. Also a lack of strong selection on the PBR could be due to the fact that we are including several non homologous loci in the analyses. In this study, we did not assign sequences to a locus due to the similarity among sequences, therefore, the ω ratio could be affected. In addition, we have only data of the PBR and non-PBR positions of one part of exon 2 and the estimation of the ω may vary when the entire exon is amplified. The observation of trans-species polymorphism can be used to infer positive selection because it retains alleles for a long time (Bernatchez and Landry 2003). Contrary to other species trans-species polymorphism was not observed in the blue tit. Selection has been found at different temporal scales, thus selection in the distant past can be detected as an excess of non-synonymous substitutions. However, selection in the recent past or in the current generation can be detected considering other factors (e.g., measuring deviations from Hardy–Weinberg or finding correlations between disease resistance and MHC-allele or genotype (see Wegner et al. 2003). It will be necessary to incorporate these variables to correctly assess the effect of selection in the blue tit and to rule out the possibility that the soft selection could be signalling a change from a functional to a non-functional gene (Hughes and Nei 1989).

Conclusions

With this study we have obtained a preliminary overview of the MHC class II B genes in the blue tit, characterizing exon 2 partially from several individuals originating in three European locations. Our primers were developed based on transcript sequences obtained from zebra finch RNA and they are supposed to amplify expressed class II B genes involved in peptide presentation. Primers even designed for the zebra finch are not very restricted to this species and could be considered general to be applied in the blue tit. Using the same-species probes has revealed different diversity in MHC complexity among songbirds (Edwards et al. 1999). The combined results from sequencing and RFLP can be extremely valuable at the initial stages on MHC research in non-model vertebrates, until a new molecular method like next-generation sequencing based method could be developed (Babik 2010). New molecular tools are proving that some variability is not measured by traditional molecular methods (Oomen et al. 2013), but to date, no whole genome sequencing has been performed on this species. Although the methodology employed in this study is now increasingly being substituted by next-generation sequencing is still possible to preliminarily characterize MHC in avian species or to reveal functional variation across populations (Whittaker et al. 2012). Now, more species are being studied by applying new molecular tools and this approach promises a fascinating advance in MHC study. This is the first step toward a better understanding of the MHC class II B genes in the blue tit.

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Conflict of interest

None declared.

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Supporting Information

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

Figure S1. Total MHC class II B blue tit alleles obtained from all DNA and cDNA sequences (amplified with the degenerated primers 2ZFfw1 and 2ZFrv1). Identity with allele Cyca-DAB*1 is indicated by *dots*.
Figure S2. Bayesian phylogeny from the blue tit and other passerines with non-collapsed nodes. Decimal numbers on branches, posterior probability. MHC class II B blue tit alleles are indicated by an arrow.
Table S1. Sample sites, number of individuals sampled and kind of analysis. +, the individual was used for the analysis. −, the individual was not used for the analysis.

Supporting information

Table 1. Sample sites, number of individuals sampled and kind of analysis. + = the individual was used for the analysis. - = the individual was not used for the analysis.

Sampling site	The Netherlands										Spain							Sweden				
Individuals	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	W
DNA Analysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	-
RNA Analysis	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	+

Figure legends

Fig. 1:

Total MHC class II B blue tit alleles obtained from all DNA and cDNA sequences (amplified with the degenerated primers 2ZFfw1 and 2ZFrv1). Identity with allele Cyca-DAB*1 is indicated by *dots.*

Fig. 2:

Bayesian phylogeny from the blue tit and other passerines with non-collapsed nodes. Decimal numbers on branches = posterior probability. MHC class II B blue tit alleles are indicated by an arrow.

Fig. 1

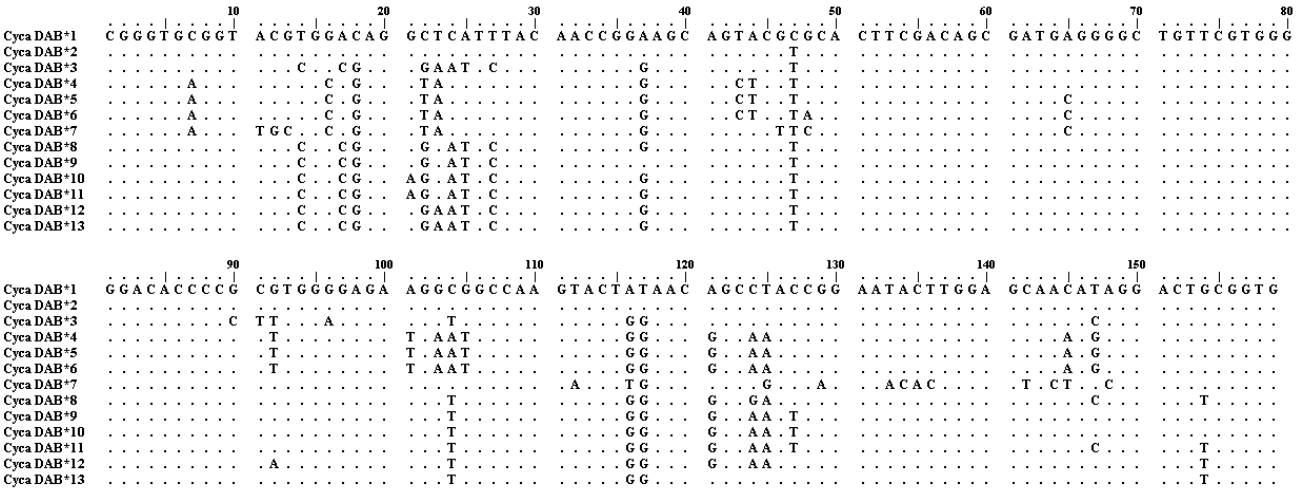
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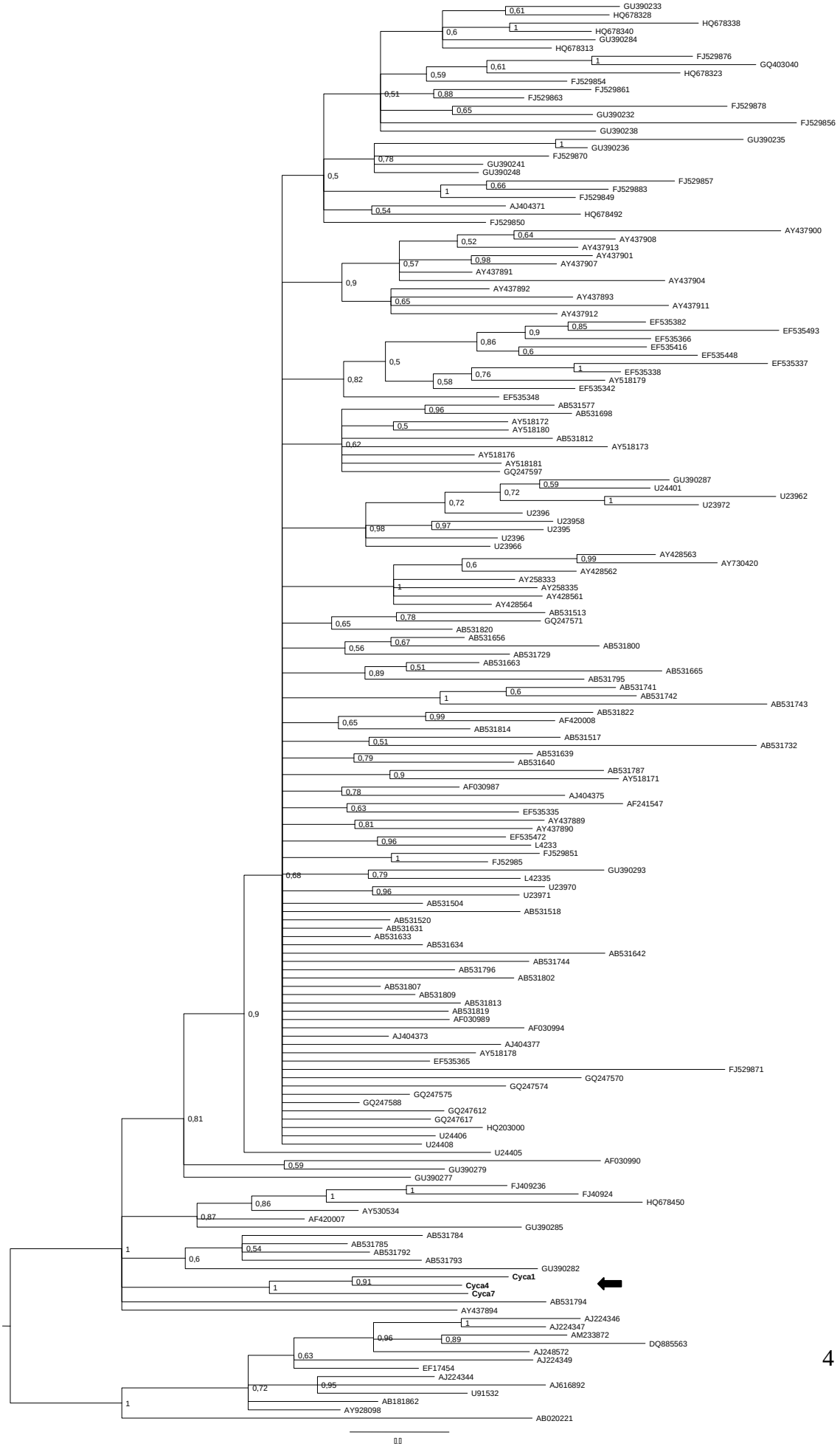
Fig.2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