INTERRELACIONES ENTRE HOSPEDADORES, VECTORES Y PARÁSITOS SANGUÍNEOS EN POBLACIONES DE AVES SILVESTRES.

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Interrelaciones entre hospedadores, vectores y parásitos sanguíneos en poblaciones de aves silvestres.

Memoria presentada por el Licenciado Josué Martínez de la Puente para optar al 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
Josué Martínez	Santiago Merino	Fco. Javier Martínez

A mis padres,

a Isabel

Índice

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Me fue concedida una beca de postgrado I3P-CSIC y una beca predoctoral de la estación biológica de El Ventorrillo-CSIC para la realización de esta tesis. Diferentes proyectos financiados por el Ministerio de Educación y Ciencia permitieron realizar estas investigaciones.

Las hojas comenzaban a amarillear cuando por aquel año de 2003 todo cambiaría para siempre. En respuesta a un correo me personé allí. Un señor de gesto serio, aún un desconocido, bajó al mostrador de entrada del MNCN. Casi sin verle el rostro, cerré los ojos. Aquí empezaría este sueño.

De pronto los volví a abrir. Ya no estábamos en el mismo sitio. Ahora, nos engullía un pequeño despacho en una esquina del museo, un lugar ocupado también por otras dos personas. Aquellos dos, una joven de la que recordaba su cara por algún fugaz cruce en la universidad y un joven de melena color fuego siempre sonriente. Es difícil hacer una retrospectiva del paso de estos años sin que una sonrisa furtiva irrumpa en mi rostro, y es que no puede ser de otro modo, cuando los mejores años de mi vida se han ido forjando a la par que el contenido de estas páginas. Al tiempo, casi como sin darme cuenta, estaba en otro despacho del museo, allí estaba un señor de voz grave sentado detrás de una gran mesa. "El señor de los pingüinos" pensé, ya que aún recordaba su cara de una charla en la facultad. Las visitas a ese despacho se hicieron frecuentes, donde, pegado a la tele, siempre me acompañaban otras personas que a la postre se convertirían también en piezas esenciales de todo esto. A la izquierda estaba Judith, una joven voluntariosa, inteligente, magnífica persona siempre presta a ayudarme con una sonrisa, con su forma de ser admirable y de la que aprendí y aprendo más de lo que ella jamás pudiera imaginar. Al otro lado, Elisa, cualquiera que la conozca podrá definirla en su pensamiento, porque ella es la prueba de que hay buenas personas en el mundo, listas, trabajadoras y admirables. Detrás estaba Juan, al que cualquiera puede admirar como científico, pero del que, una vez se le conozca, debe admirársele como persona; su conocimiento sobre la ecología, la evolución y su afán por compartirlo con cualquiera, estudiante o profesor, lo convierten en un lujo del que he podido disfrutar. Pero tras esos días, quizás meses, retorné a aquella esquina del museo del inicio de la historia. Las hojas del calendario iban pasando y con ellas, los frotis. En ese tiempo, empezaban a hacerse más frecuentes mis consultas a aquel muchacho de melena rojiza. Simplemente

genial, no se puede definir de otro modo a una persona como Gustavo. Los romanos aportaron grandes eventos en la historia de Segovia, y de manera similar, ese romano fue vital en mi historia particular. Incansable compañero, científico ejemplar, siempre dispuesto a ayudarme y aconsejarme, del que, como en los casos anteriores, me llevo un gran pedazo para siempre. Y así, la vida fue trascurriendo hasta que llego la primavera, por aquel entonces los pájaros empezarían a reproducirse. Esa reproducción supuso la invitación para participar en la temporada de campo, algo que ahora sé que cambió mi vida. Por aquel entonces, aún faltaban algunos exámenes por hacer, pero estos pasaron y finalmente, me incorporé de manera definitiva en el grupo. Con ello, cobrarían vida en esta historia aquellos que serían mis maestros en las labores del "pipeteo". Primero, una mujer, Sonia, más que una compañera, una voluntariosa maestra, una sonrisa que anticipa a una mejor persona, de incansable paciencia, genial. La otra persona es mi codirector, Javier, más lejos del museo, pero siempre pendiente y dispuesto. Profesor incansable, que arrastra en sus genes la crítica a la vida, pero que siempre está dispuesto a ayudarme, a discutir y a enseñarme. Esas sensacionales conversaciones en las que me despertó un afán por la autocrítica y por la duda, abriéndome los ojos siempre a otras maneras de pensar. No obstante la vida continuaba y no sin sus relativas dificultades y alegrías. En esos primeros momentos todo era diferente, las peticiones de becas, cuando eran posibles, siempre eran denegadas. La paciencia incansable y el constante apoyo de aquellos hombres que aceptaron incondicionalmente ser mis directores se convertía en ese hálito fundamental para continuar. Pero ese apoyo no estaba solo, venía acompañado de la incansable labor de los dos grandes pilares de mi vida. Por un lado, Pedro y María del Carmen o María del Carmen y Pedro y es que, como puede leerse en esa fachada de mi ciudad, tanto monta-monta tanto. Ellos, mis padres, son las personas que me han dado todo lo que soy, porque sus valores son los míos. Ellos pusieron la semilla que despertaría mi admiración por la naturaleza y mi amor por mi lugar de origen. Ellos me han enseñado el valor de la sencillez, el amor incondicional de unos padres por su hijo. Ellos me dieron la razón por la que un abrazo, un beso o una mirada tienen tanto mensaje oculto y es que sin ellos, nada, realmente nada, habría sido posible. El otro pilar fundamental de esta historia es Isabel, ya que en mi, nada es completo hasta que no está ella. Cuando una persona te mira de ese modo no puedes más que rogar que al mundo que se detenga, para siempre. Ella es la libertad que me condena y la condena que me libera. La razón paciente de cada amanecer. No obstante, a pesar de que todos ellos son piezas fundamentales en mi vida y en esta historia, si me limitase a ellos sería

tremendamente incompleta esta narración. Por eso hay que incorporar nuevos protagonistas, Antonia y Manolo, porque aunque ellos no estaban ya conmigo cuando estas letras se imprimían, todo lo que aprendí de ellos permanecía inmutable. A pesar de que la familia no se elije, existen ocasiones en las que uno encuentra personas excepcionales y se siente orgulloso de los que le rodean. Lejos de ser los únicos, ellos son uno de los ejemplos más significativos. Ellos eran la demostración de que el amor debe ser incondicional, su recuerdo ocupa gran parte de mi interior y hace que siempre permanezcan con vida. Dedicar esa mirada, esa sonrisa a una persona, cuando sólo puedes alimentarla con el último hálito de una vida, es el mejor regalo que se puede conceder. No obstante, nuevas pequeñas joyas aún me acompañan, logrando dar luz a este libro en blanco y negro. Gracias María, por ser tan brillante y dejarme disfrutar de ello.

Pero retornando a la historia, los días pasaban y con ellos aparecían nuevos protagonistas. Así, son muchos los compañeros, doctores y doctorandos, del museo, de El Ventorrillo y de otros lugares, que han hecho que estos fuesen sitios agradables donde estar y que los vínculos allí surgidos pudieran ir más lejos de lo meramente profesional. Sería injusto nombrar a algunos, cuando todos han sido tan importantes, porque las salidas del museo siempre son tremendamente gratificantes con ellos. Al tiempo, nuevos compañeros empezaban a ocupar los sitios dejados por los otros que se marchaban. Así aparecieron Juan primero, luego Sara y Rafa y finalmente Sonia. La suerte de narrar una historia como esta es que en ella hay una enorme diversidad de personajes. Así Juan, es esa magnífica persona del que todo el mundo debería tener el privilegio de disfrutar de su compañía. Transparente, paciente, amable, divertido y brillante compañero. Sara, esa persona enigmática que de pronto empezó a sentarse en la mesa de al lado, excelente compañera, trabajadora, inteligente, ese tipo de persona que te va conociendo poco a poco y que se deja conocer al mismo ritmo. Rafa, el hombre nacido de la diversidad, del que me llevo su sonrisa, su improvisación, su disposición a la ayuda y su magia personal. Y Sonia y Rodrigro, esas personas que apenas he podido conocer pero con las que también espero aprender durante este tiempo futuro. Y así, en este transcurrir, poco a poco, los compañeros que se hacían amigos y los amigos que se hacían más amigos todavía. Y es que un ecólogo no se puede considerar tal hasta que es consciente de sus interacciones con otros seres. En ciertos casos estas interacciones no siempre son positivas, pero afortunadamente yo he tenido la mejor de las suertes posibles. Natanael, José, Otones, Iñaki, Marta, Ricardo, Tamara,

Miriam, Kike, Rubén, Ana María, Fátima y todos aquellos compañeros y amigos que dentro y fuera del museo han hecho de esto un placer aún mayor. Muchos de ellos ya no están donde les conocí, pero el recuerdo perdura mucho más allá que estas páginas. Si el valor de las personas viene dado por el valor de sus amigos, siempre tendré un valor gracias a ellos. Pero, quizás más que nada por eso de que de vez en cuando uno encuentra una perla entre su tesoro, debo destacar aquí también mis grandes compañeros de discusiones a lo largo de estos años, Pedro y Óscar. Unas maravillosas personas que serán unos científicos de primer nivel, que sin conocerse entre sí comparten un interés inusitado por la ciencia, por el rigor de la misma y por la evolución. Dos grandes personas, dos grandes amigos.

Pero cualquiera que haya leído esta historia debe darse cuenta de que falta alguien. Ese personaje del principio de la historia. Aquel que hizo tangible lo que en aquel día era únicamente posible en el reino de Morfeo. Santi ha sido esa persona. Él, con ardua tarea, me enseñó a andar y a crecer en el camino de la ciencia, fue ese sustento que permitió que ese camino pedregoso se convirtiese en un sendero transitable. No encuentro palabras adecuadas para describir el día a día con él, porque independientemente de las que pudiera utilizar no bastarían para hacer justicia de lo que siempre me dio. Es una satisfacción invaluable que una persona, un científico, un director como él entre en tu vida. Una persona dispuesta a ofrecer siempre su apoyo incondicional, presto a enfrentarse a las dificultades que sean necesarias en pro de enseñar sus principios y defender sus razones; enseñándome en todo momento a valorar cada día más y más el fruto de nuestro trabajo. El maestro que ocupa una silla de la misma altura que el alumno, un científico con mayúsculas que hace de aquellos a los que dirige y enseña muchísimo más que unos alumnos o compañeros, una persona que siempre me enseñó, de primera mano, su amor por la ciencia y la evolución. Alguien a quien sin duda pediría de nuevo que fuese mi director si despertase y esto no hubiera sido más que un sueño aún por cumplir. Porque trabajar con un científico y una persona de su valía es un lujo del que todos querrían disfrutar. Gracias Santi por creer en mí y apoyarme desde siempre. Gracias, por hacer de este sueño, una realidad.

Introducción

La ecología es la ciencia que estudia la interacción de los organismos entre si y de estos con su entorno y, por tanto, aborda el estudio de relaciones simbióticas como el mutualismo, el comensalismo, la depredación o el parasitismo. Esta ciencia, como no podía ser de otro modo dentro del entorno biológico en el que se enmarca, debe entenderse bajo un contexto evolutivo, situándose como una pieza angular dentro de la biología evolutiva (Moreno 2008). A lo largo de los últimos tiempos, el estudio del parasitismo bajo un prisma ecológico está centrando cada vez más la atención de los investigadores (Poulin 1998), convirtiéndose en una línea de investigación elemental en la rama del conocimiento en la que se encuadra esta tesis, la ecología evolutiva.

El parasitismo se podría definir como una asociación interespecífica entre organismos, parásito y hospedador, en la que el primero, generalmente de menor tamaño y único beneficiado, presenta adaptaciones para vivir en, o sobre, organismos hospedadores de los que depende metabólicamente y con los que realiza un intercambio mutuo de sustancias (Cheng 1978, Møller 1997, Poulin 1998). Según esta definición, podrían catalogarse como parásitos multitud de organismos, desde virus y bacterias hasta metazoos, ampliando así la definición clásica de parásito generalmente limitada a organismos eucariotas. Por lo tanto, dada la considerable diversidad de organismos parásitos, no es de extrañar que el parasitismo se encuentre tan ampliamente extendido en la naturaleza, en la que prácticamente ningún organismo, al menos en alguna de sus fases vitales, está libre de contactar con algún parásito. De hecho, la diversidad de organismos parásitos es tan amplia que se estima que más de la mitad de los seres vivos lo son (Price 1980), pudiéndose encontrar ejemplos en la mayoría de los grupos taxonómicos conocidos (Maquardt *et al.* 2000). Ademas, los organismos parásitos presentes en ciertos ecosistemas pueden generar una biomasa tan importante o más que

la de algunos grupos de vertebrados (Kuris *et al.* 2008). De este modo, no es de extrañar que los parásitos "puedan afectar la relativa abundancia de diferentes especies a semejanza de los más importantes depredadores, lo que justificaría la inclusión del parasitismo como una fuerza biótica capaz de determinar la biodiversidad de las comunidades" (Poulin 1999). Así, aunque el nivel de virulencia de los patógenos pudiera variar en función de diferentes factores ligados a los parásitos o a los hospedadores (Merino 2002), tales como la tasa y mecanismos de transmisión y el nivel de infección de la población hospedadora, es indudable que el impacto que los parásitos generan les otorga una especial relevancia como reguladores de la evolución de sus hospedadores (Hamilton y Zuk 1982, Atkinson y van Ripper 1991, Møller 1997, Merino 2002, Navas *et al.* 2007). En esta simbiosis, los parásitos, principalmente debido a su reducido tamaño y la corta duración de sus ciclos vitales con respecto a los de los hospedadores, se posicionan con cierta ventaja en la carrera coevolutiva de armamentos.

En este contexto, diferentes estudios han profundizado sobre el papel que ejercen los parásitos como fuerza selectiva en sus hospedadores. Referente a este tema se ha propuesto, por ejemplo, que la reproducción sexual podría ser un mecanismo por el cual los organismos hospedadores pudieran incrementar su variabilidad genética y, con ello, mejorar sus mecanismos de defensa frente a sus patógenos (Merino 2002). De manera similar, diferentes estudios presentan al parasitismo como una fuerza selectiva fundamental para explicar el mantenimiento de ciertos rasgos que, a priori, suponen un coste importante a nivel fisiológico para los organismos. De este modo, si la presencia de ciertas patologías, como la talasemia en humanos, supone una ventaja frente a la infección por parásitos, sería esperable que estas características genéticas se fijasen en la población hospedadora a pesar de los importantes costes asociados al mantenimiento de dicho carácter (Flint *et al.* 1986). En este mismo sentido, en un estudio desarrollado recientemente con un modelo constituido por el nematodo *Caenorhabditis elegans* y la bacteria patógena *Pseudomonas aeruginosa*, Navas y colaboradores (2007) demostraron que únicamente los nemátodos mutantes que presentaban resistencia a la infección por esa bacteria eran capaces de incrementar su tamaño poblacional en presencia de la misma. No obstante, al comparar la cepa original con la mutante se demostró que la resistencia al patógeno estaba asociada con una alteración fisiológica deleterea en la tasa de respiración.

Del mismo modo, los parásitos también han sido señalados como uno de los principales protagonistas en la evolución de las especies por sus efectos sobre la

selección sexual. A lo largo de los últimos años, gracias principalmente a trabajos como el de Hamilton y Zuk (1982), los biólogos interesados en los mecanismos de evolución de la selección sexual, en especial de las aves, empezaron a incluir a los parásitos en sus estudios, lo que supuso un punto de inflexión en el desarrollo de esta disciplina científica. Así, los avances en este campo han permitido conocer que, como señala Møller (1994), existen diferentes mecanismos por los que los parásitos podrían ser mediadores en la selección sexual de sus hospedadores. En este sentido, algunas de las informaciones más importantes que ofrecerían las señales sexuales podrían ser el nivel de infección o la inmunocompetencia del emisor. Por ejemplo, la carga de parásitos podría condicionar a un individuo a la hora de seleccionar a su pareja, ya que, los individuos más parasitados podrían tener una capacidad parental más reducida, podrían incrementar la probabilidad de contagio de los parásitos o podrían carecer de genes de resistencia frente a ellos (Møller 1994). De este modo, el papel de los parásitos podría ser entendido bajo el contexto de la hipótesis del handicap, por la que los organismos que muestren caracteres sexuales secundarios más notables sufrirían unos mayores costes en términos fisiológicos debido al papel inmunosupresor de las hormonas reguladoras de la expresión de tales caracteres (Folstad y Karter 1992). De esta manera, la inmunosupresión podría traducirse en una mayor vulnerabilidad al ataque de los patógenos, de forma que sólo los individuos de buena calidad serían capaces de soportar el desarrollo de estos caracteres sin sufrir altas cargas parasitarias. En apoyo de esta hipótesis, modificaciones del nivel de testosterona en aves han demostrado que esta hormona incrementa tanto la intensidad de los caracteres sexuales secundarios de las aves como la susceptibilidad a ser parasitados (Mougeot *et al.* 2004). No obstante, ese incremento en la intensidad de infección podría deberse no sólo a la inmunosupresión asociada al tratamiento, sino a causas comportamentales de los hospedadores que aumentasen su probabilidad de contacto con los parásitos. En este sentido, un reciente estudio desarrollado por Mougeot y colaboradores (2005) puso de manifiesto que, aún bloqueando el efecto comportamental de la testosterona, las aves sufrían un incremento en la carga parasitaria asociado al incremento en los niveles de esta hormona, mostrando apoyo a la hipótesis del handicap de la inmunocompetencia (Folstad y Karter 1992).

Por otro lado, debido a los costes que los parásitos ejercen en sus hospedadores, el estudio del parasitismo se presenta también como una herramienta fundamental para comprender el efecto de éstos en el mantenimiento de sus poblaciones hospedadoras. En este sentido, los parásitos cobran un especial interés en la biología de la conservación.

Así, las infecciones por parásitos sanguíneos se presentan como una de las posibles causas de la pérdida de biodiversidad en sistemas insulares como Hawai (Warner 1968), situando a las especies patógenas como una de las causas más importante de la pérdida de endemismos. Por lo tanto, los controles sanitarios se hacen especialmente relevantes en planes de introducción de especies, con el fin de evitar la posible introducción de agentes patógenos en estas áreas (Cunningham 1996). No obstante, la posibilidad de que los individuos introducidos en nuevos lugares estén libres de parásitos, o sometidos a una mínima carga parasitaria, podría ser la causa por la que ciertas especies introducidas puedan alcanzar elevados crecimientos demográficos llegando a constituir plagas (Torchin *et al.* 2003). Por otro lado, también es importante señalar que la enorme pérdida de biodiversidad de vertebrados que estamos sufriendo hace que se estén perdiendo también una considerable diversidad de organismos parásitos asociada a la perdida de sus hospedadores (Koh *et al.* 2004). Bajo este principio de coextinción se sitúan como especies especialmente susceptibles aquellos parásitos más especialistas y aquellos que requieren la intervención de múltiples especies hospedadoras para completar su ciclo vital. La perdida del hospedador, en el primer caso, o la pérdida de algún hospedador intermediario, en el segundo, supondría la imposibilidad para completar el ciclo vital del parásito acarreando su extinción.

Del mismo modo, en este marco de cambio global en el que nos encontramos, el estudio de la ecología del parasitismo se presenta también como una estrategia fundamental con la que monitorizar la dinámica de las relaciones entre parásitos y hospedadores. En este contexto, los cambios ambientales, en cuanto al uso del terreno o los factores meteorológicos, se presentan como mecanismos mediadores fundamentales en la dinámica de transmisión de enfermedades parasitarias (Patz *et al.* 2000). De este modo, pequeños cambios en las condiciones ambientales, por ejemplo, incrementos de las temperaturas mínimas, pueden tener graves consecuencias aumentando la tasa de transmisión de enfermedades como la malaria y, por tanto, aumentando considerablemente la población expuesta a la infección.

En resumen, a lo largo de los últimos años multitud de investigadores han abordado el estudio de diferentes aspectos de la interacción entre los parásitos y los hospedadores siendo un elemento de interés común en áreas del conocimiento tan dispares como la microbiología, entomología, ornitología, medicina, veterinaria o ecología. En cada una de ellas se han abordado diferentes aspectos sobre la interacción entre parásitos y hospedadores, aunque en la mayoría de los casos el principal interés se

ha centrado en desenmascarar los efectos de los parásitos sobre sus hospedadores y en desarrollar todas aquellas estrategias posibles para combatirlos. En la mayoría de estos casos las investigaciones se han llevado a cabo con modelos de estudio en cautividad. Sin embargo, los estudios realizados sobre este tema, en condiciones naturales, encaminados a abordar los aspectos de la interacción entre parásitos, vectores y hospedadores son considerablemente más escasos. Esta escasez puede estar propiciada por las mayores dificultades inherentes a este tipo de estudio en condiciones naturales o a que éstos requieren de la combinación de conocimientos propios de diferentes especialidades. En este sentido, en numerosas ocasiones las mayores dificultades encontradas por los investigadores para realizar sus estudios en la naturaleza radican en la imposibilidad para manipular adecuadamente los modelos animales y en la dificultad para alcanzar tamaños muestrales lo suficientemente elevados con los que poner a prueba sus hipótesis. Por este motivo, cada vez más investigadores abordan el estudio de la relación parásito/hospedador utilizando como modelos diferentes especies de aves silvestres. Los principales motivos para su utilización radican en que muchas de estas especies son susceptibles de sufrir la presión de una enorme diversidad de parásitos, tanto ectoparásitos como endoparásitos, y su estudio, bajo condiciones naturales, permite alcanzar tamaños muestrales lo suficientemente elevados. Por lo tanto, en los estudios desarrollados en esta tesis también se utilizaron algunos de estos modelos con aves silvestres como hospedador vertebrado.

Todo estudio que pretenda ampliar el conocimiento sobre las relaciones entre parásitos y hospedadores en la naturaleza debe comenzar necesariamente por conocer los principales actores intervinientes en la interrelación bajo estudio. El uso de técnicas moleculares está siendo una herramienta eficaz para encuadrar taxonomicamente a los parásitos, especialmente en el caso de protozoos donde la ausencia de conocimientos sobre los ciclos vitales concretos de muchas especies ha llevado a cometer graves errores de determinacion. Por eso, uno de los primeros pasos que nos vimos obligados a dar fue el de profundizar en el conocimiento de las poblaciones parasitarias que infectaban a nuestras aves-modelo. Este primer paso nos condujo a la identificación correcta de uno de los parásitos sanguíneos que infectan nuestra población y, también, a la determinación de las especies de mosquitos que los atacan. Posteriormente, el objetivo de nuestro trabajo se centró en el estudio de las relaciones interespecíficas que mantienen los parásitos sanguíneos con sus hospedadores vertebrados o invertebrados. Las estrategias reproductivas de los parásitos sanguíneos y las potenciales

contraestrategias por parte de sus hospedadores, así como los efectos originados por los parásitos a largo plazo sobre el valor adaptativo de los hospedadores vertebrados, han sido las principales áreas abordadas en este estudio. A continuación, presentaremos someramente los problemas relacionados con cada uno de los componentes de las interrelaciones objeto de estudio: parásitos hemáticos, vectores y hospedador vertebrado.

Los parásitos sanguíneos: el caso de *Haemoproteus*

Los haemosporidios (Sporozoa: Haemosporida) son un grupo de protistas heteroxenos que requieren de la intervención de insectos dípteros hematófagos como vectores (Valkiūnas 2005). Este grupo engloba diferentes familias de parásitos como Plasmodiidae, Haemoproteidae, Leucocytozoidae y Garniidae que afectan a multitud de especies de vertebrados incluyendo anfibios, reptiles, aves y mamíferos. Estos parásitos se encuentran ampliamente distribuidos en la naturaleza, donde como en el caso de las aves, pueden llegar a infectar al 70% de las especies examinadas (Atkinson y van

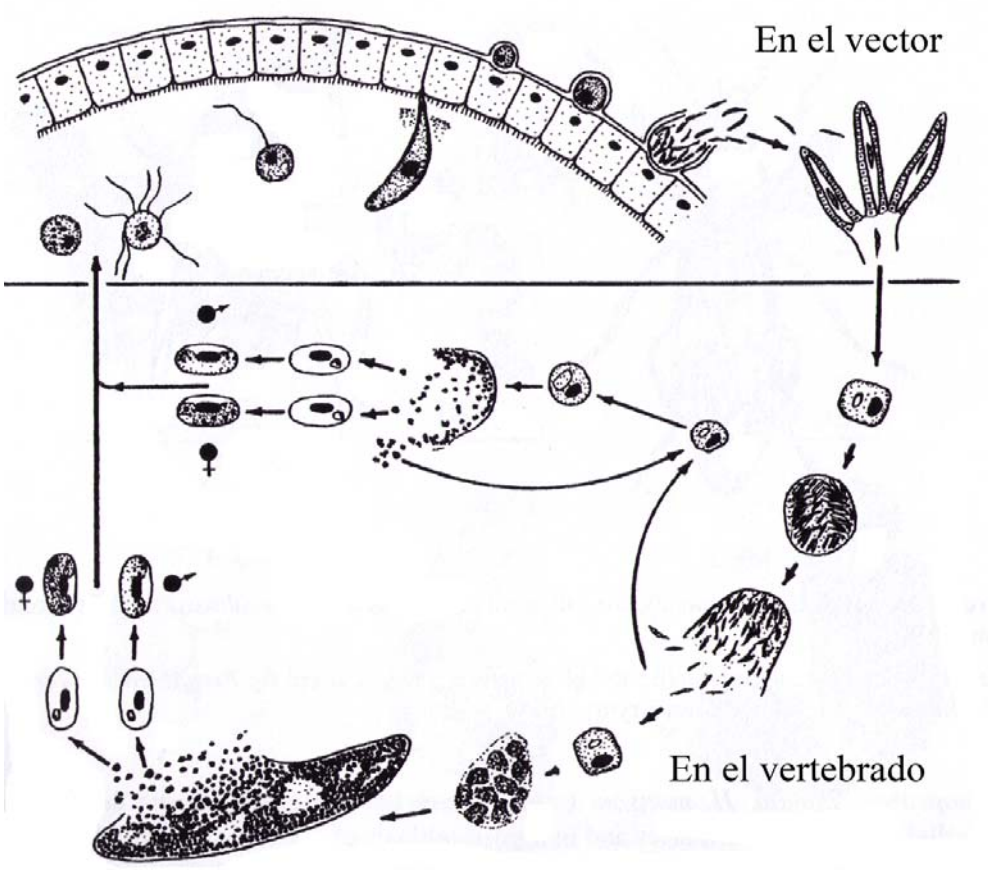

Figura 1. Representación del ciclo vital de *Haemoproteus mansoni* durante su fase en el insecto vector y en el hospedador vertebrado. Modificado de Valkiūnas 2005.

Ripper 1991). Además, desde el punto de vista sanitario, la importancia de estos parásitos es bien conocida por el elevado número de muertes que ocasionan en humanos (WHO 1994).

El ciclo vital de estas especies incluye fases de reproducción sexual y asexual (Fig. 1). De manera general, el parásito infecta al hospedador vertebrado en forma de esporozoito mediante la picadura de un insecto vector. Posteriormente, el parásito desarrolla una fase de reproducción asexual en los tejidos internos del hospedador, donde los merozoitos resultantes invadirán los eritrocitos (glóbulos rojos) del torrente circulatorio del hospedador. Allí, los parásitos continuarán desarrollándose en forma de gametocitos (precursores de gametos) (Fig. 2) o merontes (esquizontes). Cuando el insecto que actúa como vector consume la sangre de su hospedador ingiere los gametocitos parásitos, formas que posteriormente se desarrollarán en el tubo digestivo del vector en microgametos (machos) y macrogametos (hembra) dando lugar a la reproducción sexual del parásito. La unión de estos gametos formará el cigoto, que se diferenciará en un oocineto y, tras penetrar el tubo digestivo del vector, se dividirá mediante reproducción asexual (esporogonia) dando lugar a los esporozoitos. Estas últimas fases representan las formas infectivas del parásito que migrarán a las glándulas salivares del vector para introducirse en un nuevo hospedador vertebrado con la picadura del insecto. Aunque este pudiera considerarse el ciclo vital de los haemosporidios a grandes rasgos, existen marcadas diferencias entre los distintos géneros que engloba. Así, en el caso de *Plasmodium*, los eritrocitos del hospedador son susceptibles a ser parasitados por esquizontes y gametocitos, mientras que en otros géneros como *Haemoproteus* y *Leucocytozoon*, sólo los gametocitos infectan los eritrocitos del hospedador. Además, existen diferencias en cuanto a las especies de insectos que son susceptibles de transmitir cada una de estas especies de parásitos sanguíneos. Los insectos del género *Culicoides* (Ceratopogonidae) y los hipobóscidos (Hippobosciidae) son los principales vectores de *Haemoproteus*, los culícidos (Culicidae) de *Plasmodium* y los simúlidos (Simuliidae) de *Leucocytozoon* (para más detalles ver Atkinson y van Ripper 1991, Valkiūnas 2005). Debido a las diferencias encontradas en el ciclo vital y los vectores involucrados en la transmisión de los diferentes géneros de haemosporidios, algunos autores sugieren restringir el término malaria exclusivamente a aquellas enfermedades fruto de infecciones producidas por parásitos del género *Plasmodium,* excluyendo del término las infecciones producidas por los géneros *Haemoproteus* y *Leucocytozoon* (Valkiūnas *et al.* 2005). No obstante,

existe una marcada controversia en cuanto al empleo de esta terminología (Pérez-Tris *et al.* 2005), en parte debido al estrecho parentesco filogenético entre estos géneros, lo que apoyaría la inclusión de todos ellos como parásitos de la malaria aviar. En general, los estudios realizados hasta la fecha sobre la taxonomía de estos parásitos sanguíneos consideraban especialmente detalles sobre la abundancia y morfología del parásito en extensiones sanguíneas procedentes de muestras obtenidas de alguno de sus hospedadores vertebrados. De este modo, las características morfológicas, y otros detalles como la especie hospedadora en la que se encontraba el parásito, eran los principales argumentos en la descripción de especies nuevas de parásitos (Valkiūnas 2005). La razón de esto último es, al menos en parte, la asumida especificidad de las especies parásitas para infectar hospedadores estrechamente emparentados, al menos, a nivel de familia (Bennett *et al.* 1992; Atkinson y van Riper 1991; Valkiūnas 2005). Sin embargo, gracias a la incorporación de las técnicas moleculares y al análisis filogenético de las secuencias de ADN, se ha comprobado que el grado de especialización de los parásitos sanguíneos puede ser, al menos en ciertas ocasiones, menor de lo esperado (Ricklefs y Fallon 2002, Waldenström *et al.* 2002, Merino *et al.* 2008).

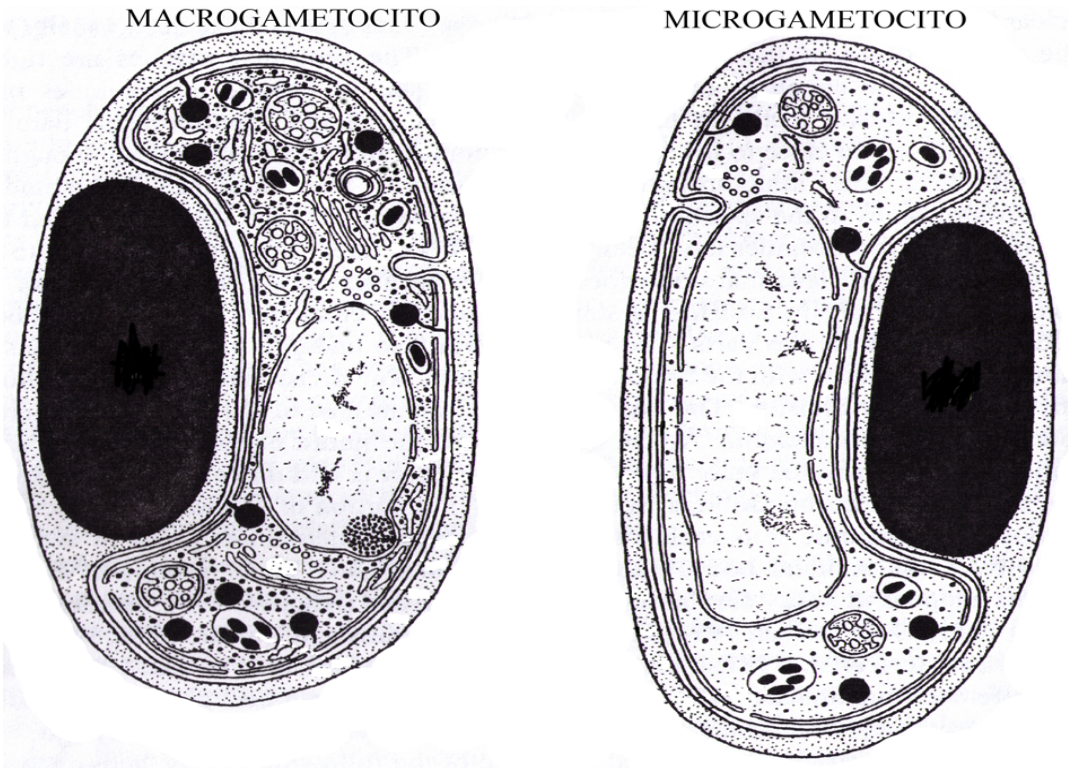

Figura 2. Representación de un macrogametocito y microgametocito maduro de un haemosporidio aviar infectando un eritrocito. En negro destaca el núcleo de la célula hospedadora. Modificado de Valkiūnas 2005.

Teniendo en cuenta las especies parásitas determinadas morfológicamente en cada especie hospedadora, la inclusión de estas nuevas técnicas moleculares está permitiendo obtener una mayor diversidad genética de líneas parásitas de lo esperado (Bensch *et al.* 2000, 2004) e incluso la descripción de especies crípticas (Pérez-Tris *et al.* 2007). No obstante, a pesar de las ventajas que suponen estas técnicas moleculares, también se discute la necesidad de conservar el uso de las técnicas tradicionales basadas en la observación directa de extensiones sanguíneas en los estudios de ecología del parasitismo, especialmente en los casos de infecciones parasitarias mixtas (Pérez-Tris y Bensch 2005, Valkiūnas *et al.* 2006). En este sentido, cada día existe un mayor consenso sobre la necesidad de compatibilizar el uso de las herramientas tradicionales y moleculares en los estudios de ecología del parasitismo. Este hecho es especialmente relevante en los estudios taxonómicos, ya que como sugiere Valkiūnas *et al.* (2007), los linajes genéticos de los parásitos podrían ser asignados a sus morfoespecies. De esta forma, se incrementaría la información disponible tanto para los biólogos evolutivos estudiosos de las relaciones filogenéticas de estos organismos, como para los parasitólogos tradicionales que investigan los ciclos vitales de los parásitos (ver también Valkiūnas *et al.* 2008b). Por ello, aunque ciertos estudios taxonómicos realizados sobre los parásitos de la malaria aviar, basados en metodologías clásicas, parecen reflejar cierta robustez (Martinsen *et al.* 2006), en el caso de otros parásitos sanguíneos, el mayor conocimiento de la historia vital de las especies puede traducirse en una profunda reestructuración de la taxonomía establecida (Desser 1980). De este modo, los estudios taxonómicos recientes de especies parásitas de aves y otros organismos silvestres aprovechan las ventajas obtenidas de la aplicación de ambas técnicas, tradicionales y moleculares, apoyando la necesidad de realizar estudios conjuntos incorporando el poder discriminatorio de ambas metodologías (Valkiūnas *et al.* 2007, 2008b, Merino *et al.* 2009). Al respecto, en el **capítulo 1** de esta tesis analizamos la morfología y características genéticas de un parásito sanguíneo intracelular presente en el herrerillo común.

En cualquier caso, como puede deducirse del ciclo vital general de los haemosporidios (Fig. 1), la probabilidad de contacto (de fecundación) entre macrogametos y microgametos, en el tubo digestivo del vector, es otro de los aspectos determinantes del éxito de transmisión del parásito. En este sentido y de acuerdo con Schall (2000), la cantidad de parásitos circulantes en el torrente sanguíneo del hospedador vertebrado se sitúa como un factor clave en la transmisión parasitaria,

produciéndose un incremento en la probabilidad de encuentro entre los gametos cuanto mayor sea la intensidad de infección del hospedador vertebrado. De este modo, un incremento en la abundancia de gametocitos en la sangre del hospedador podría considerarse como una estrategia ventajosa para este tipo de parásitos si con ello incrementan la probabilidad de transmisión, a pesar de comprometer la supervivencia de su hospedador (Ewald 1994). No obstante, bajo determinadas circunstancias, como por ejemplo en intensidades de infección bajas, los parásitos podrían disponen de otros mecanismos con los que incrementasen la probabilidad de encuentro entre los gametos en el insecto vector (Jovani 2002, Merino *et al.* 2004). En este sentido, recientemente se propuso la hipótesis de la "infección doble de gametocitos" como un medio por el que el parásito podría facilitar el encuentro entre macrogametos y microgametos en el vector gracias a la infección en la misma célula sanguínea hospedadora (eritrocito) por gametocitos de ambos sexos (Jovani 2002, ver **Capítulo 4**). De este modo, con la ingesta de un solo eritrocito, el vector podría ingerir al menos un macrogametocito y un microgametocito, lo que previsiblemente facilitaría, por una mayor proximidad, el encuentro entre gametos en el tubo digestivo del vector. No obstante, de acuerdo con Jovani (2002), para que las invasiones múltiples jueguen un papel relevante en la transmisión parasitaria deberían encontrarse de forma natural en los hospedadores y ser viables. En su apoyo, la ocurrencia de invasiones múltiples de eritrocitos ha sido constatada en diferentes especies de parásitos sanguíneos, tales como *Plasmodium*, *Haemoproteus* y *Leucocytozoon*, infectando multitud de especies de aves, reptiles y mamíferos, incluyendo a seres humanos (Wang 1970, Ahmed y Mohammed 1978, Lainson y Naiff 1998, Jovani *et al.* 2004, Valkiūnas 2005). A pesar de ello, para que este mecanismo suponga una ventaja adaptativa para los parásitos, las invasiones múltiples deberían además cumplir otras premisas (**Capítulo 4**). Estas premisas son: (1) que las invasiones múltiples deberían aumentar a medida que disminuya la intensidad de infección, cuando la probabilidad de transmisión del parásito se reduce y (2) que las invasiones múltiples deberían estar formadas predominantemente por gametocitos macho y hembra maduros. En el **capítulo 4** se profundiza en la comprobación de estas posibilidades.

Alternativamente y de acuerdo con varios estudios realizados recientemente en laboratorio, existe la posibilidad de que el sistema inmune del hospedador pudiera estar involucrado en la ocurrencia de las invasiones múltiples de eritrocitos. En un primer estudio desarrollado por Miller *et al.* (1984), los investigadores observaron que la

presencia de un anticuerpo monoclonal frente a *Plasmodium knowlesi* producía una reducción del número total de células infectadas y un incremento en el número de invasiones múltiples. Posteriormente y en apoyo del trabajo previo de Miller *et al.* (1984), Ramasamy y colaboradores (1999) encontraron, en un estudio *in vitro*, un incremento en el número de invasiones múltiples de eritrocitos cuando se añadía al medio un anticuerpo específico frente a *P. falciparum*. Además, la presencia de inmunoglobulinas procedentes de hospedadores que previamente habían sido infectados con *Plasmodium*, producía un incremento mayor en la frecuencia de aparición de estas invasiones múltiples comparado con la que producían las inmunoglobulinas procedentes de hospedadores no inmunizados (Ramasamy *et al.* 1999). Aunque ninguno de estos autores pudo probar un mecanismo claro por el que los anticuerpos podrían incrementar la presencia de invasiones múltiples, ellos propusieron que varios merozoitos unidos por anticuerpos podrían ser capaces de parasitar la célula hospedadora de manera conjunta, o bien de forma separada, si existiese una liberación previa de los anticuerpos en las proximidades del eritrocito (Fig. 3). En cualquier caso, si los hospedadores fueran capaces de incrementar la ocurrencia de invasiones múltiples de eritrocitos podrían reducir la cantidad total de células sanguíneas infectadas y, de esta forma, contribuir al éxito de transmisión del propio parásito. En el **capítulo 5** de esta tesis se aborda el estudio del sistema inmunitario del hospedador como mediador de la ocurrencia de invasiones múltiples de eritrocitos y en el **capítulo 6** se revisan todas las hipótesis

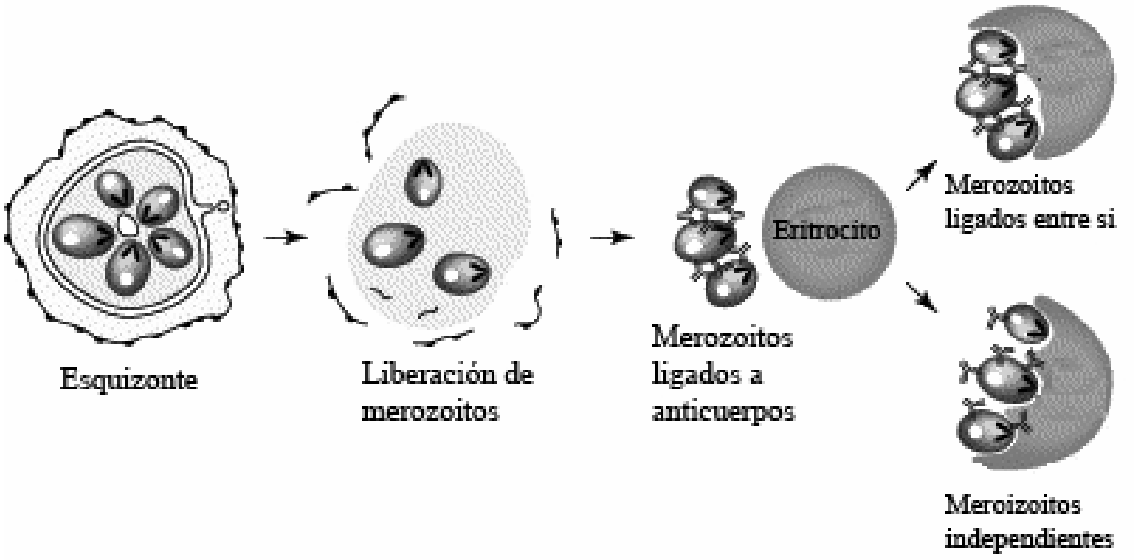

Figura 3. Representación de las hipótesis propuestas para explicar el efecto de los anticuerpos favoreciendo las invasiones múltiples de glóbulos rojos. Modificado de Ramasamy *et al.* 2001.

planteadas hasta el momento sobre la existencia de este fenómeno en la naturaleza.

Los vectores

Para que una especie parásita infecte una especie hospedadora tiene que cumplirse dos requisitos. En primer lugar, que el parásito pueda contactar con el hospedador y que, posteriormente, pueda asentarse en el mismo (Combes 2001). En este sentido, la imposibilidad de muchos parásitos para transmitirse por sus propios medios entre hospedadores hace que éstos requieran la intervención de otro organismo que actúe como vector. Por esta razón, el concepto general de vector podría hacer referencia al organismo capaz de transmitir un agente parásito entre hospedadores (Marquard *et al.* 2000). En la naturaleza existe una enorme diversidad de vectores, los cuales podrían dividirse en dos grupos principales, los vectores mecánicos (transmisores) y los biológicos. Los primeros son meros transportadores de los parásitos y los segundos son aquellos en los que el parásito desarrolla alguna parte de su ciclo vital haciendo del vector uno de sus hospedadores. Se han descrito numerosas especies como potenciales vectores de diferentes patógenos, siendo el grupo de los dípteros el más importante, ya que, presenta una multitud de especies implicadas en la transmisión de enfermedades tan importantes como la malaria, la fiebre amarilla o la lengua azul (Lehane 2005). En conjunto, estos vectores, y las enfermedades que transmiten, suponen un foco de investigación global en diferentes áreas por su importancia en los ecosistemas y por las numerosas pérdidas personales y materiales que producen en todo el mundo (WHO 1994, Lehane 2005, Ratnayake *et al.* 2006).

En general, cada especie de parásito se asocia con un número restringido de vectores (Lehane 2005, Hellgren *et al.* 2008) y las diferentes especies de vectores presentan una especificidad diferencial en cuanto a sus especies hospedadoras (Malmqvist *et al.* 2004). En este contexto, una alta especificidad en la selección de hospedadores por parte de los vectores, alimentándose predominantemente o exclusivamente sobre ciertas especies, podría restringir el contacto entre las especies de parásitos sanguíneos y sus hospedadores (Hellgren *et al.* 2008). No obstante, también existe la posibilidad de que los vectores sean capaces de consumir sangre de diferentes especies hospedadoras infectándose con diversas líneas de parásitos (Gager *et al.* 2008) lo que podría facilitar, al menos en parte, el salto de líneas parásitas a nuevas especies hospedadoras. Esta especificidad diferencial de los vectores con respecto a sus hospedadores podría deberse, al menos en parte, a diferencias en su capacidad para

detectar los atrayentes emitidos por ellos. Este hecho, puede ser debido a que los vectores presentan una diferente cantidad o sensibilidad de receptores frente a estos atrayentes (Braverman y Hulley 1979 y referencias allí citadas) o a la presencia, o ausencia, de adaptaciones morfológicas que les permitan fijarse de una manera efectiva a su fuente de alimento (Crosskey 1990). En este sentido, se hace necesario el estudio de las especies de vectores que parasitan a las aves en la naturaleza, prestando especial atención a aquellos factores que determinan su interacción con los hospedadores, ya que en último término, éstos estarían condicionando, al menos en parte, la transmisión de los parásitos sanguíneos entre las diferentes especies de hospedadores. Aunque diferentes taxones de insectos tienen importancia en la transmisión de estas enfermedades, es especialmente destacable el caso del suborden Nematocera que incluye familias como Simuliidae, Ceratopogonidae y Culicidae, considerados, en conjunto, el grupo más importante de insectos que actúan como vectores.

Los simúlidos (Díptera: Simuliidae) son un grupo de insectos con una amplia distribución, generalmente de color negro y con longitudes que suelen oscilar entre 1 y 5 milímetros. Por otro lado, dentro de la familia Ceratopogonidae destaca la importancia de los insectos del género *Culicoides* que engloba una diversidad de más de 1200

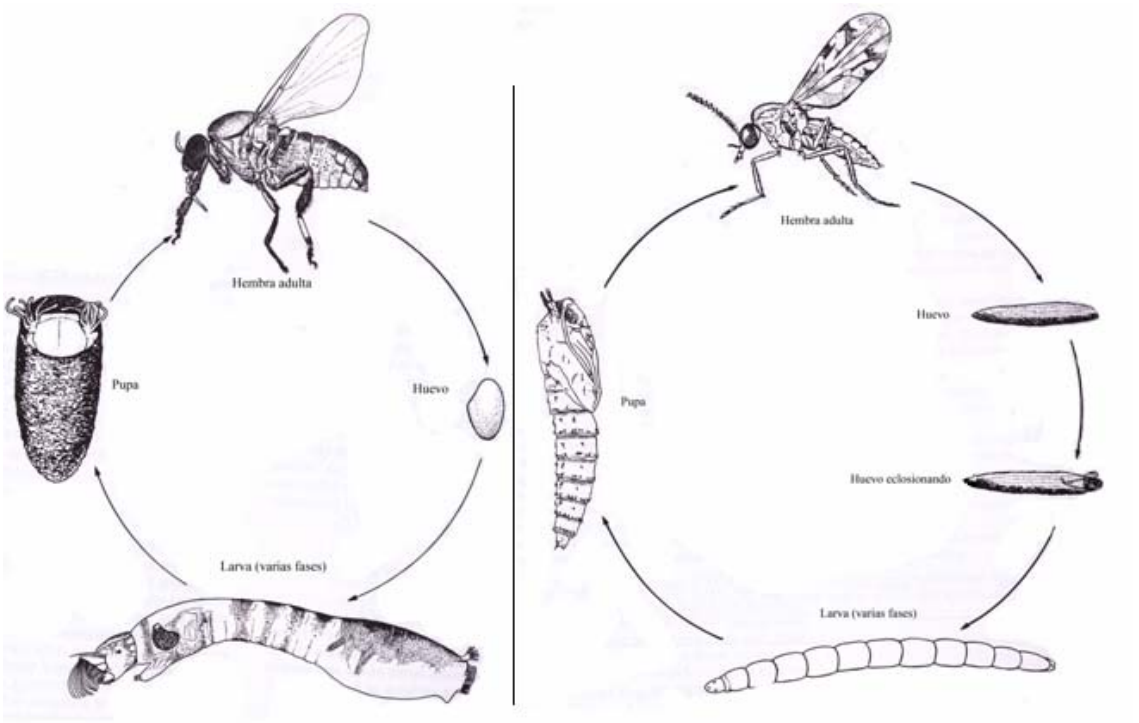

Figura 4. Esquema general del ciclo vital de los simúlidos (izquierda) y *Culicoides* (derecha). En ambos casos se alternan fases en forma de adulto reproductor, huevo, larvas y pupa. El número de fases larvales varía entre ambos grupos. Modificado de Marquardt *et al.* 2000.

especies (Beckenbach y Borkent 2003) ocupando la mayoría de las tierras emergidas del planeta (Kettle 1995, Marquardt *et al.* 2000). El ciclo vital de ambos grupos comprende las diferentes fases de una metamorfosis completa, es decir, forma de huevo, larvas y pupa, siendo de esta última de la que emergerá el adulto (Fig. 4). En términos generales, simúlidos y *Culicoides* presentan una serie de claras diferencias con respecto a su ciclo vital (Lehane 2005). Por ejemplo, una importante diferencia ecológica es con respecto a los requerimientos hídricos para realizar sus puestas, ya que los simúlidos prefieren aguas claras y bien oxigenadas de cursos corrientes en rápidos o cascadas, mientras que las especies del género *Culicoides* realizan sus puestas en terrenos fangosos. Además, mientras que los simúlidos son considerados de actividad predominantemente diurna, los representantes del género *Culicoides* muestran un marcado patrón de actividad nocturno. Por otro lado, los *Culicoides* y simúlidos adultos de ambos sexos, consumen soluciones azucaradas de origen vegetal como fuente de alimento. Además, las hembras adultas, con la excepción de unas pocas especies, requieren de una toma de sangre para el desarrollo de sus huevos como fuente proteica de alimento previa a la puesta. Es en este momento cuando las hembras son susceptibles de adquirir los parásitos sanguíneos presentes en el hospedador vertebrado. Si, posteriormente, se produjese el desarrollo y evolución morfológica del hemoparásito, estos insectos se convertirán en vectores de la parasitosis en cuestión. La alternancia entre los ciclos de reproducción (emparejamiento y puesta de huevos) y los periodos de ingesta de sangre se prolonga a lo largo de toda la vida de estos insectos.

Por su parte, como en el caso de los hospedadores vertebrados, los vectores también sufren unos importantes costes asociados a la infección por parásitos sanguíneos. En este sentido, los parásitos, en su tránsito a través del epitelio intestinal de los insectos, producen severas alteraciones en las células del tubo digestivo y alteran la flora bacteriana comensal necesaria para completar la dieta hematófaga. En conjunto, estos, y otros factores, hacen que los insectos desarrollen respuestas inmunológicas frente a la infección por parásitos sanguíneos (Dimopoulos *et al.* 1998, Lehane *et al.* 2004). De este modo, los costes asociados a la infección y al mantenimiento de la respuesta inmunológica son la causa, al menos en parte, del incremento en la mortalidad y el descenso en la fecundidad observada en los insectos infectados (Ferguson *et al.* 2003; Valkiūnas y Iezhova 2004). Así, nos encontraríamos ante un escenario en el que los vectores pretenderían evitar los costes asociados a la infección, lo que podría disminuir la eficacia biológica de los parásitos. Mientras, los parásitos intentarían

incrementar su probabilidad de transmisión por medio de un aumento en la probabilidad de que los vectores consuman sangre de aquellos hospedadores infectados. Por ello, la posibilidad de que los insectos hematófagos pudieran evitar consumir sangre de hospedadores infectados podría suponer una estrategia asociada al comportamiento eficaz para evitar los costes de la infección (ver Tomás *et al.* 2008b). No obstante, este no parece ser el caso en humanos, donde se observa un incremento en la probabilidad de ser picado por mosquitos cuando los hospedadores están infectados por parásitos de la malaria (Lacroix *et al.* 2005). El papel de los parásitos sanguíneos incrementando la atracción de vectores hacia sus hospedadores parece estar ligada a la patología de dicha infección, en la que los hospedadores estarían sufriendo procesos febriles y un incremento en su tasa metabólica basal, lo que les haría más conspicuos a sus vectores. En este sentido, multitud de sustancias olorosas o derivados del metabolismo emitidos por los hospedadores, tales como el dióxido de carbono, el ácido láctico o el octenol, son estimulantes de ciertos receptores de los insectos (Bhasin *et al.* 2000, Grant y Kline 2003) y, por lo tanto, sustancias frente a las que los vectores son capaces de responder (Blackwell *et al.* 1996, Gibson y Torr 1999, Mordue 2003, Mands *et al.* 2004). De este modo, todos aquellos factores que afecten la emisión de estas sustancias atrayentes, tales como el estado de infección u otros como el tamaño del hospedador o su actividad metabólica, se sitúan como factores clave en la relación entre insectos hematófagos y sus hospedadores. Los **capítulos 2 y 3** de esta tesis profundizarán sobre este aspecto.

Por otro lado, la interacción entre insectos y hospedadores también depende de diferentes factores abióticos, tales como la temperatura o la precipitación, ya que estas variables afectan de manera significativa el desarrollo, supervivencia y capacidad de vuelo de los insectos hematófagos (ver, por ejemplo, McCreadie *et al.* 1985, Shipp *et al.* 1988, Martin *et al.* 1994, Bishop *et al*. 1996, Tun-Lin *et al.* 2000, Su y Mulla 2001). Se ha constatado que cuanto mayor sea la independencia de los ectoparásitos de sus hospedadores mayor será la susceptibilidad de estos insectos a los condicionantes ambientales. De este modo, sería esperable una gran influencia de estos factores sobre los insectos voladores hematófagos, los cuales sólo entran en contacto con sus hospedadores durante cortos periodos de tiempo. Incidiendo en este aspecto, la mayoría de los estudios que han comprobado los efectos ambientales sobre la abundancia general de insectos hematófagos, o su capacidad para localizar a sus hospedadores, se han venido realizando mediante la captura de los insectos con trampas de luz o mediante el muestreo directo sobre la fauna que parasitan. En este último caso, los insectos

presentes sobre la superficie de los animales, generalmente, domésticos o sometidos a cautiverio son recogidos por aspirado. Por el contrario, el efecto de estas variables ambientales sobre la interacción entre insectos hematófagos y hospedadores silvestres es poco conocido, especialmente en el caso de las aves, donde las dificultades para capturar los vectores que entran en contacto con ellas son elevadas. Basándonos en los escasos estudios sobre este tema, la temperatura y previsiblemente la velocidad del viento, podrían ser los factores más relevantes que afectan la abundancia de vectores en los nidos de las aves (Smith *et al.* 1998) y la variabilidad interanual en estos factores pudiera ser, al menos en parte, la responsable de la variabilidad en la abundancia de estos vectores encontrada en los nidos de aves silvestres en diferentes años (Tomás *et al.* 2008a). En el **capítulo 3** se profundiza sobre el efecto de los factores ambientales en la abundancia de insectos voladores hematófagos en los nidos de aves paseriformes silvestres.

Las aves silvestres como hospedadores

Los parásitos producen considerables costes directos e indirectos en sus hospedadores, debido a los recursos que drenan de éstos y mediante los costes asociados a la respuesta inmunitaria que los hospedadores desencadenan en su lucha contra los parásitos. De este modo, los parásitos se sitúan como importantes moduladores del tamaño poblacional de los hospedadores (Anderson y May 1978, May y Anderson 1978, Hudson y Dobson 1997, Tompkins y Begon 1999), ejerciendo sobre ellos una enorme presión selectiva por diferentes vías que les afectarán, en último caso, en términos de supervivencia y éxito reproductor (ver revisiones de Lehmann 1993, Tompkins y Begon 1999). Al respecto, es sabido que los parásitos de aves pueden disminuir el éxito reproductor de sus hospedadores por varios medios. Por un lado, los parásitos son capaces de ejercer efectos adversos directamente sobre la descendencia, ya sea en términos físicos como fisiológicos, reduciendo la probabilidad de supervivencia de los polluelos. De este modo, una reducción experimental de la carga parasitaria de los nidos en los que se desarrollan los polluelos permite mejorar su estado de salud y aumentar su probabilidad de supervivencia (Møller 1990, Lehmann 1993, Merino y Potti 1998, Tomás *et al.* 2008). Por otro lado, también es posible que los parásitos disminuyan el éxito reproductor de sus hospedadores debido al compromiso entre la asignación de recursos a la respuesta inmunitaria y al esfuerzo reproductor (Sheldon y Verhulst 1996). Este hecho podría conducir a una reducción del esfuerzo parental en

aquellos individuos que sufren los costes de la infección. En este sentido, la reducción experimental de la intensidad de infección, mediante la administración de un tratamiento antiparasitario, permitiría a los individuos adultos reproductores incrementar los recursos asignados al cuidado parental (Tomás *et al.* 2007). Los efectos de este incremento en el cuidado parental podrían notarse mediante el aumento del éxito reproductor, ya sea por el incremento del tamaño de puesta (Marzal *et al.* 2005) o por el número de volantones (Merino *et al.* 2000).

Por otro lado, existen multitud de estudios mostrando también claras evidencias a favor del impacto de los parásitos en la supervivencia de las aves silvestres. Las conclusiones más sólidas provienen de los estudios realizados experimentalmente, pues como sugieren Hudson y Dobson (1997), la mejor metodología para comprobar los efectos parasitarios sobre la supervivencia de las aves consiste en el seguimiento de los individuos después de la administración de un tratamiento antiparasitario. Así, diferentes estudios alterando experimentalmente la intensidad de infección por ectoparásitos demostraron la importancia de estos organismos en la supervivencia de sus hospedadores (Lehmann 1993, Brown *et al.* 1995). No obstante, posiblemente debido a la mayor dificultad para modificar la carga de los endoparásitos en animales silvestres, en especial en el caso de las aves, existe un reducido número de estudios que han comprobado de una manera experimental el impacto de los endoparásitos sobre la supervivencia de estos organismos. En este sentido, en los trabajos desarrollados sobre este tema, empleando como modelo el lagópodo escocés (*Lagopus lagopus*) y el eider común (*Somateria mollissima*), se han descrito incrementos en la probabilidad de supervivencia de estas especies cuando fueron sometidas a un tratamiento experimental que reducía su intensidad de infección por parásitos intestinales (Hudson y Dobson 1991, Hanssen *et al.* 2003). Estos estudios evidencian el papel esencial que juegan estos organismos parásitos en la supervivencia y dinámica poblacional de las aves. Sin embargo, este papel nunca ha sido comprobado experimentalmente en estudios realizados en condiciones naturales con aves silvestres infectadas por parásitos sanguíneos. Desde hace años, se viene comprobando el efecto deletéreo que ejercen los parásitos sanguíneos sobre las aves aunque en la mayoría de los casos estos registros provienen de estudios realizados en laboratorio con animales de experimentación (Atkinson y van Ripper 1991). La escasez de registros de aves silvestres muertas, o que sufren graves patologías debidas a altas intensidades de infección (Atkinson y van Ripper 1991, Valkiūnas 2005), dificultaba en gran medida la posibilidad de extrapolar

las evidencias recabadas sobre el efecto de los parásitos a las condiciones naturales. Este hecho, propiciaba la idea de que la infección por ciertas especies de parásitos sanguíneos como *Haemoproteus* pudieran ser relativamente benignas (Bennett *et al.* 1993). No obstante, como había sugerido Stone y colaboradores (1971), es posible que en un primer momento los investigadores infravaloraran los casos de muerte inducida por parásitos, como los de la malaria en aves, al atribuir los síntomas, en especial los neurológicos, a otras causas como la intoxicación por pesticidas. En este sentido, diferentes autores han sugirido la importancia de los parásitos sanguíneos reduciendo la probabilidad de supervivencia de las aves bajo condiciones naturales (Richner *et al.* 1995; Nordling *et al.* 1998; pero ver Stjernman *et al.* 2004). Además, más recientemente, Marzal y colaboradores (2008) encontraron que la probabilidad de supervivencia en el avión común (*Delichon urbica*) se veía reducida cuando las aves estaban infectadas por alguna línea genética de parásitos sanguíneos de los géneros *Haemoproteus* y *Plasmodium.* Estas evidencias han sido nuevamente apoyadas en estudios comparativos que reflejaban una menor supervivencia en aquellas especies de aves que presentaban una mayor prevalencia de infección (Møller y Nielsen 2007, Arriero y Møller 2008).

En este sentido, asumiendo los costes que los parásitos infringen en sus hospedadores, es esperable que éstos desarrollen diferentes mecanismos con los que evitar o mitigar el impacto asociado a dichas infecciones. Aunque los hospedadores utilizan diferentes estrategias, incluidas las comportamentales, para evitar el contacto de los parásitos o reducir los efectos que estos les infringen (Bucher 1988, Clayton y Wolfe 1993, Merino y Potti 1995), el sistema inmune es la vía de defensa más compleja y eficaz contra ellos. Este sistema presenta una considerable diversidad de mecanismos efectores que podrían dividirse en dos grupos, el componente innato y el adquirido. Las respuestas que median cada uno de estos componentes presentan marcadas diferencias. En el primer caso, la respuesta es de carácter inespecífico mientras que en el segundo, las respuestas son específicas y generan resistencia frente al patógeno. Además, los mecanismos efectores desencadenados durante una respuesta específica pueden estar principalmente vinculados a la participación de las celulas del sistema inmunitario (respuesta celular) o a la producción de moléculas solubles proteicas (inmunidad humoral). Entre los componentes de la respuesta humoral destaca el papel de las inmunoglobulinas, moléculas producidas y secretadas por los linfocitos B que reconocen específicamente los antígenos presentes en los agentes patógenos.

A lo largo de los últimos años, diferentes autores han desarrollado metodologías para determinar el nivel de estas proteínas plasmáticas (p.ej. Gustafsson *et al.* 1994, Hasselquist *et al.* 1999, Johnsen y Zuk 1999, Martínez *et al.* 2003). Una de ellas, la medida de inmunoglobulinas totales (Martínez *et al.* 2003), permite conocer la capacidad inmune de las aves silvestres con la ventaja de requerir la toma de una pequeña muestra sanguínea. No obstante, como ocurre con otras medidas del estado inmunitario de los organismos silvestres, aún existe controversia sobre su interpretación (Sheldon y Verhulst 1996, Norris y Evans 2000), ya que un alto nivel de inmunoglobulinas puede ser entendido como reflejo de una alta inmunocompetencia o, al contrario, como reflejo de una relativa susceptibilidad a una infección. Por otro lado, los hospedadores también son capaces de hacer frente a organismos patógenos, y a los efectos que producen, mediante otros mecanismos diferentes del sistema inmune. Al respecto, las proteínas de estrés (HSPs de sus siglas en inglés, *heat shock proteins*) se presentan como unas moléculas altamente conservadas a lo largo de la evolución y esenciales en el mantenimiento de la homeostasis celular ante situaciones de estrés. Estas proteínas participan en la síntesis, ensamblaje y transporte de proteínas y, también, en la degradación y eliminación de proteínas desnaturalizadas (Morimoto 1991, Sørensen *et al.* 2003). Originalmente estas proteínas fueron descubiertas en organismos expuestos a temperaturas elevadas, de ahí el nombre de HSPs, aunque también son inducidas ante otros factores estresantes incluyendo el estrés nutricional, estrés oxidativo, senescencia, radiación o, incluso, la infección por parásitos (Merino *et al.* 1998, 2002, Moreno *et al.* 2002, Sørensen *et al.* 2003). Por lo tanto, los hospedadores son capaces de desencadenar diferentes respuestas frente a los parásitos, incluyendo la respuesta inmunitaria y la respuesta al estrés. En este marco, los organismos experimentan un compromiso entre los beneficios derivados de reducir el impacto de las infecciones y los costes de mantener las respuestas defensivas. Uno de los costes sería el condicionamiento de la eficacia de una de las respuestas en función de los recursos asignados a otra. Este reparto de recursos incluso podría afectar funciones vitales tales como el esfuerzo reproductor. En este sentido, existen estudios demostrando como un cambio en los niveles de proteínas de estrés puede comprometer la inmunocompetencia en las aves (Merino *et al.* 2006, Morales *et al.* 2006), lo que a su vez podría tener importantes repercusiones en la supervivencia de estas especies (Møller y Saino 2004) debido a la mayor depredación sufrida por aquellos organismos inmunosuprimidos (Møller y Erritzøe 2000). A pesar de los costes asociados a la

síntesis de estas proteínas, el incremento de los niveles de HSPs durante ciertos periodos de tiempo podría otorgarles múltiples ventajas al mantener la homeostasis celular (Sørensen *et al.* 2003) y otras funciones vitales como la respuesta inmune mediada por el sistema mayor de histocompatibilidad (Srivastava 2002). Además, un incremento en los niveles de HSPs puede incrementar la probabilidad de supervivencia de los organismos (Tatar *et al.* 1997, Sørensen y Loeschcke 2004). Por lo tanto, en el **capítulo 7** de esta tesis abordamos de manera conjunta los efectos del parasitismo, la inmunocompetencia (nivel de inmunoglobulinas plasmáticas totales) y los niveles de estrés fisiológico (nivel de proteínas de estrés) sobre la supervivencia de las aves bajo condiciones naturales, aportando la primera prueba experimental de los efectos de una reducción de la infección por parásitos sanguíneos sobre la supervivencia en aves silvestres.

Zona de estudio y hospedadores vertebrados

El área de estudio se localiza en un bosque caducifolio situado en los Montes de Valsaín (40° 53′ 74N, 4°01′ O, 1200 m.s.n.m.). Estos montes se localizan en la vertiente noroeste de la Sierra de Guadarrama (Sistema Central), enclavados dentro del término municipal de San Ildefonso, en la provincia de Segovia, y tienen en la actualidad una extensión aproximada de 10.700 Ha amparados bajo ciertas figuras de protección legal. La propiedad de este territorio forestal pertenece al Organismo Autónomo Parques Nacionales y engloba tres tipos de bosques principales, encinar, pinar y melojar (Tornero Gómez 2005). En la zona en la que se desarrolló este estudio, el estrato arbóreo está compuesto principalmente por roble melojo (*Quercus pyrenaica*) aunque también es posible encontrar ejemplares aislados de pino silvestre (*Pinus silvestris*) y fresno (*Fraxinus angustifolia*). Por su parte, el estrato arbustivo esta constituido principalmente por jaras (*Cistus laurifolius*) aunque también existen manchas de zarzas (*Rubus* sp.) y rosales silvestres (*Rosa* sp.). Este bosque se encuentra situado en el piso bioclimático supramediterráneo con un ombroclima subhúmedo con temperaturas medias anuales que oscilan entre los 8 y los 12 ºC (Izco 1984).

Anualmente, se realizó el seguimiento de la población de aves que ocupan alrededor de 300-350 nidales colocados en el área de estudio. Cada año se realizó el seguimiento de su reproducción y, al final de cada una de estas estaciones reproductoras, se procedió a la limpieza de los nidales retirando los nidos que habían sido construidos por las aves. A lo largo de los años de estudio, las especies que

utilizaron estos nidales fueron principalmente el herrerillo común (*Cyanistes caeruleus*), el papamoscas cerrojillo (*Ficedula hypoleuca*), el carbonero común (*Parus major*) y el trepador azul (*Sitta europaea*), si bien estas dos últimas especies fueron mucho menos abundantes. Aunque en algunos capítulos se emplearon otras especies como modelo de estudio (**capítulo 3**), la principal especie utilizada fue el herrerillo común (*Cyanistes caeruleus,* anteriormente *Parus caeruleus*).

Figura 5. A la derecha, investigador realizando las tareas de campo en la zona de estudio. A la izquierda, ejemplar adulto de herrerillo común portando una oruga en su pico antes de su entrada en el nido. Imágenes cedidas por Santiago Merino y Ángel M. Sánchez, respectivamente.

El herrerillo común *Cyanistes caeruleus* L. es un pequeño ave paseriforme de entre 10-12 gramos de peso que anida en los bosques caducifolios y mixtos de la región paleártica occidental. A esta especie se la considera sedentaria en la mayoría de su área de distribución (Cramp y Perrins 1998) aunque, en las poblaciones más norteñas, se considera que puede realizar algunos movimientos dispersivos. Las parejas de esta especie se adaptan fácilmente a criar en cajas nido en el área de estudio. Las hembras construyen los nidos utilizando diferentes materiales de origen animal y vegetal, principalmente pelo y musgo, realizando posteriormente una única puesta de entre 4 y 14 huevos con un promedio de 9.1 huevos (Fargallo 1997). Durante la fase de crecimiento de los polluelos en los nidos, ambos progenitores se encargan de suministrar el alimento a los polluelos hasta que abandonan el nido. Este acontecimiento ocurre a una edad que oscila entre los 17 y los 20 días desde la fecha de eclosión. De media, un total de 7.8 polluelos consiguen volar de cada nido (Fargallo 1997). Los

ejemplares adultos de esta especie presentan un hermoso y colorido plumaje. En el centro de la cabeza destacan el color azul, en la frente y las mejillas el color blanco y a la altura del ojo atraviesa una banda negra que cruza desde el pico a la parte posterior de la cabeza. El dorso presenta una coloración verdosa, con las alas y la parte superior de la cola de color azul intenso. Además, presenta una coloración ventral amarilla con una marcada banda negra sobre el pecho (Fig. 5). Aunque los machos presentan un color ligeramente más vistoso y un tamaño algo mayor, no se considera que esta especie presente dimorfismo sexual notable.

Objetivos

- **Capítulo 1:** Determinar y clasificar taxonómicamente, en base a caracteres morfológicos y genéticos, una especie de parásito hemático intracelular que infecta al herrerillo común.

- **Capítulo 2:** Comprobar los efectos de la reducción experimental de la carga de parásitos sanguíneos en aves adultas y de la fumigación de sus nidales sobre la abundancia de vectores del género *Culicoides* en nidos de herrerillo común.

- **Capítulo 3:** Comprobar el papel de los factores ambientales, tales como la precipitación, la temperatura y la velocidad del viento, como determinantes de la abundancia de insectos vectores hematófagos en nidos de diferentes especies de aves silvestres.

- **Capítulo 4:** Estudiar si la ocurrencia de invasiones parasitarias múltiples, en eritrocitos de aves, es un fenómeno favorecido por los propios parásitos sanguíneos como un mecanismo para incrementar su éxito de transmisión.

- **Capítulo 5:** Estudiar el papel del sistema inmune del hospedador sobre la frecuencia de aparición de invasiones parasitarias múltiples en eritrocitos de aves silvestres.

- **Capítulo 6:** Revisar las hipótesis propuestas sobre la ocurrencia de invasiones múltiples de eritrocitos por parásitos de la malaria o parásitos emparentados.

- **Capítulo 7:** Estudiar los efectos de la infección por parásitos sanguíneos, sobre el sistema inmunitario y las proteínas de estrés, en la supervivencia interanual de aves silvestres.

Resultados principales y discusión de los capítulos

Capítulo 1:

En este capítulo se estudió las características morfológicas y genéticas de una especie de parásito que infecta los linfocitos del herrerillo común en la fase de gamonte. Sobre la base de sus características morfológicas, esta especie presenta considerables similitudes con las especies del género *Hepatozoon*. Sin embargo, los análisis del tamaño de las células parásitas en extensiones sanguíneas mostraron que este parásito presenta un tamaño ligeramente inferior, aunque estadísticamente significativo, al presentado por *Hepatozoon parus*, especie descrita originalmente por Bennett y Peirce (1989) en el carbonero común (*Parus major*) y característica de la familia Paridae (Tabla 1). Además, la comparación de un fragmento amplificado de 1484 pares de bases del gen ribosomal 18S, basada en el método BLAST, permitió comprobar que *Lankesterella minima* es la especie que mayor valor de identidad muestra con la especie indeterminada (93%). La posición taxonómica de esta especie parásita fue determinada mediante análisis filogenético basado en el fragmento del gen ribosomal 18S, revelando un mayor parentesco con *L. minima* que con *H. parus*. En conclusión, este estudio refleja que la descripción de especies de estos grupos, basada exclusivamente en los caracteres morfológicos de alguna de sus fases vitales y en la especificidad de hospedador, no permite la correcta asignación de estas especies a un grupo taxonómico concreto y, por tanto, no deben utilizarse exclusivamente en la descripción de estas especies.

	Parásito estudiado	*H. parus*
Longitud	9.0 (1.0)	10.4 (1.2)
Anchura	3.4 (0.7)	4.0 (0.6)
Área	27.2 (5.7)	34.0 (7.0)
Núcleo del parásito		
Longitud	3.3 (0.9)	4.0 (0.8)
Anchura	2.5 (0.7)	2.2 (0.9)
Área	8.4 (3.4)	8.0 (2.8)
Vacuola del parásito		
Diámetro	2.3 (0.3)	2.1 (0.4)
Área	4.7 (1.3)	3.1 (1.1)

Tabla 1. Comparación de las medidas (media ± desviación estándar) en micrómetros de los parámetros morfológicos del parásito estudiado y de *Hepatozoon parus* (Bennett y Peirce 1989).

Capítulo 2:

En este estudio se identificaron a nivel específico los vectores del género *Culicoides* capturados en nidales de herrerillo común. Por orden de abundancia, las

especies identificadas fueron *C. kibunensis*, *C. festivipennis*, *C. segnis*, *C. truncorum*, *C. pictipennis* y *C. circumscriptus*. Del total de insectos muestreados, únicamente dos individuos fueron machos. El hecho de que la inmensa mayoría de los insectos capturados, en el interior de los nidales, fuesen hembras concuerda con la circunstancia de que sean los ejemplares de este sexo los únicos que desarrollan una dieta hematófaga. Además, este dato no apoya la posibilidad de que los machos ocupen territorios próximos a los hospedadores con el fin de conseguir cópulas (Yuval 2006).

El tamaño de nidada se relacionó significativa y positivamente con la abundancia de *Culicoides*, posiblemente debido a que un mayor número de polluelos son capaces de producir más atrayentes de insectos. Este resultado apoyaría otros trabajos previos que encontraban una asociación entre la carga de vectores y el tamaño del hospedador (Dow *et al.* 1957, Anderson y DeFoliart 1961, Tomás *et al.* 2008a pero ver Rätti *et al.* 2006). Con respecto al tratamiento experimental, se encontró una mayor abundancia de *C. festivipennis* en nidos control ocupados por parejas medicadas, resultado apoyado por un trabajo previo realizado en el mismo área de estudio (Tomás *et al.* 2008b). Este hecho podría deberse al desarrollo de un mecanismo en los vectores para consumir sangre de hospedadores poco infectados, reduciendo así la posibilidad infección por parásitos sanguíneos. Alternativamente, un aumento de la actividad física de las aves medicadas podría facilitar su detección por los insectos. Por otra parte, la abundancia de hembras de *Culicoides*, con una toma reciente de sangre, se redujo significativamente en nidos fumigados a pesar de que la fumigación con insecticida no redujo la abundancia total de *Culicoides* en los nidales de herrerillo común, como previamente había sido descrito (Tomás *et al.* 2008a). Sin embargo, este tratamiento sí redujo las picaduras de estos insectos en los polluelos (Fig. 6) y, por lo tanto, los costes asociados al consumo de sangre por parte de estos vectores, bien en términos de pérdida de sangre o bien de transmisión de parásitos sanguíneos.

En conclusión, la intensidad de infección, la utilización de sustancias con propiedades insecticidas y el tamaño de nidada son factores clave que pueden determinar la abundancia de insectos vectores en los nidos de las aves silvestres.

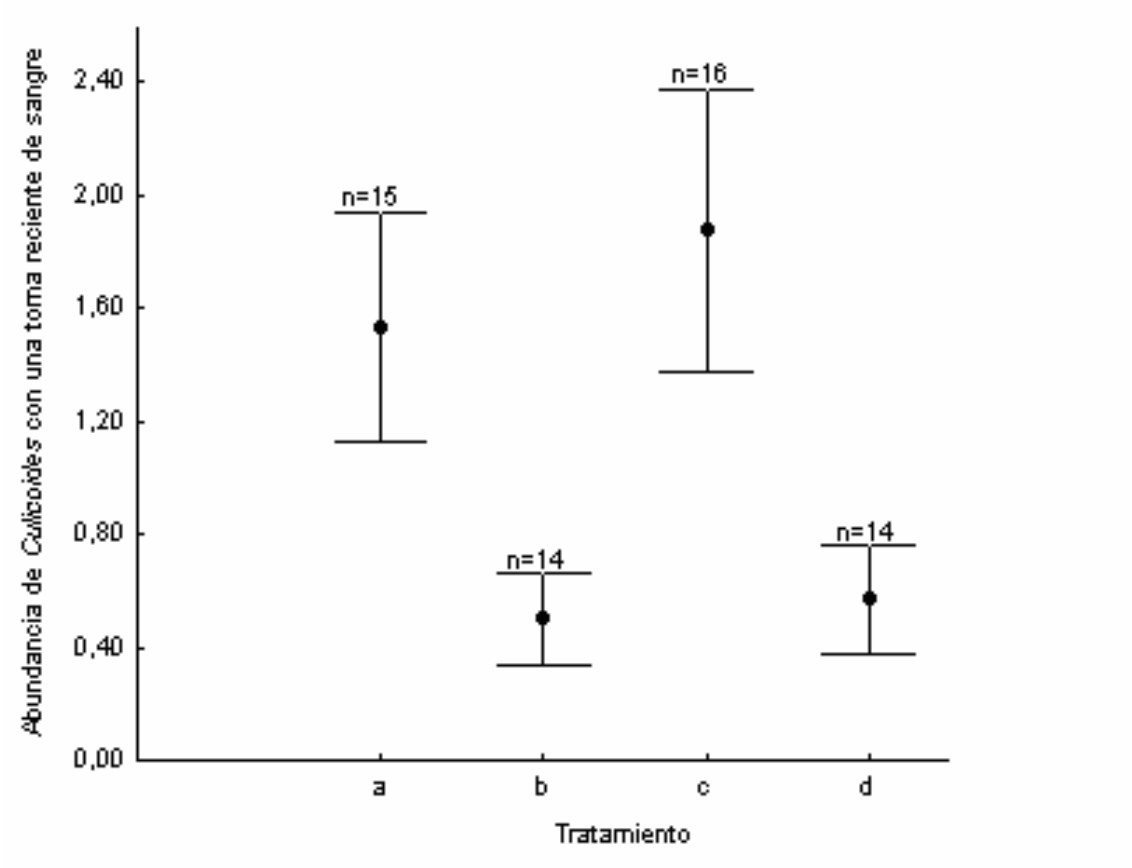

Figura 6. Abundancia media (± error estándar) de *Culicoides* con una toma reciente de sangre en su abdomen en nidales de parejas medicadas ocupando nidos control (a), parejas control ocupando nidos fumigados (b), parejas control ocupando nidos control (c) y parejas medicadas ocupando nidos fumigados (d).

Capítulo 3:

En este capítulo se estudió el efecto de diferentes factores bióticos (tamaño de la nidada y especie hospedadora) y abióticos (temperatura, precipitación y velocidad del viento) sobre la abundancia de simúlidos y *Culicoides* en nidos de tres especies de aves que anidan en la misma población, el herrerillo común, el papamoscas cerrojillo y el carbonero común.

Se observó que la abundancia de simúlidos disminuyó con el avance de la estación reproductora mientras que la abundancia de *Culicoides* aumentó, lo que posiblemente sea debido a las diferencias de requerimientos hídricos que presentan ambos grupos para realizar sus puestas (Lehane 2005). Por otra parte, apoyando los resultados del capítulo anterior, la abundancia de ambos vectores mostró relaciones significativas y positivas con el tamaño de nidada. Además, se encontró una mayor abundancia de *Culicoides* en nidales de papamoscas cerrojillo que en nidos de páridos (Fig. 7), sugiriendo que factores ligados a cada una de las especies, tales como el

tamaño o la fecha de puesta, el tipo de nido o el color de las aves, podrían afectar a la abundancia de los insectos que parasitan a cada especie.

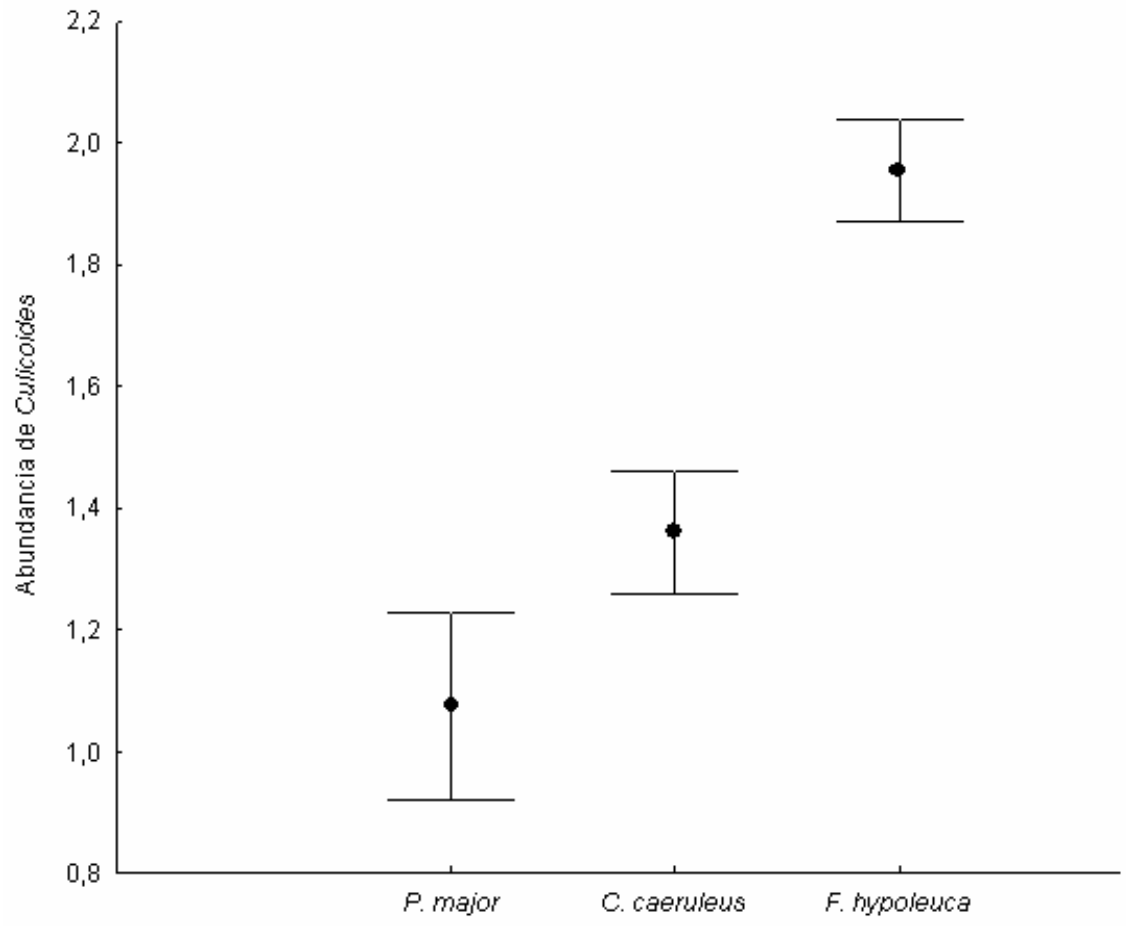

Figura 7. Abundancia media (± error estándar) de *Culicoides* capturados en nidales de tres especies de aves, el carbonero común *P.major*, el herrerillo común *C. caeruleus* y el papamoscas cerrojillo *F. hypoleuca*.

Por otro lado, se encontró una asociación significativa y negativa entre la abundancia de simúlidos y la temperatura mínima medida durante el periodo de captura de los insectos. Además, la velocidad del viento medido a primera hora de la mañana redujo significativamente la abundancia de simúlidos y *Culicoides* en los nidales. Por el contrario, la abundancia de simúlidos se asoció positivamente con la velocidad del viento medido a las 18:00 horas. Estos resultados apoyarían otros estudios previos que han encontrado relaciones significativas de diferentes factores ambientales sobre la abundancia de insectos en la naturaleza (Martin *et al.* 1994, Smith *et al.* 1998), evidenciando que estos factores pueden tener efectos diferentes en función del grupo de vectores estudiados.

En conclusión, la abundancia de insectos voladores hematófagos, en nidales de aves silvestres, está determinada por factores ligados a la especie hospedadora como el tamaño de nidada y por variables abióticas ligadas a la meteorología.

Capítulo 4:

La ocurrencia de las invasiones múltiples de eritrocitos por gametocitos de *Haemoproteus* puede ser un mecanismo adaptativo para incrementar la probabilidad de transmisión del parásito al facilitar el encuentro de los microgametos y macrogametos en el insecto que actúa de vector. Los resultados obtenidos en este capítulo evidencian que la intensidad de infección es el factor que mejor explica la ocurrencia del fenómeno, encontrándose una asociación positiva y significativa entre la intensidad de infección por el parásito *Haemoproteus majoris* y el número de invasiones múltiples de eritrocitos. Esto fue cierto antes y después de la administración de un tratamiento que redujo la intensidad de infección por este parásito. Por su parte, el tratamiento de medicación no tuvo un efecto significativo en el número de invasiones múltiples. Estos resultados apoyarían a los encontrados en otros estudios, en los que se muestra el nivel de parasitemia como principal responsable en la ocurrencia de invasiones múltiples (Wang 1970, Jovani y Sol 2005). Además, en contra de la hipótesis estudiada, encontramos que la mayoría de los gametocitos que formaban invasiones múltiples no alcanzaban la madurez y, cuando lo hacían, las invasiones múltiples estaban formadas predominantemente por gametocitos del mismo sexo. Estos resultados son una primera evidencia clara en contra de la posibilidad de que los parásitos de la malaria u otros parásitos emparentados pudieran favorecer la ocurrencia de invasiones múltiples en la naturaleza para aumentar su éxito de transmisión. Por tanto, este estudio apoyaría la hipótesis de que la ocurrencia de invasiones múltiples en infecciones por malaria, y en otras parasitosis emparentadas, es un mecanismo debido a altas intensidades de infección aunque otros factores relacionados con el hospedador pudieran jugar también un papel importante en la frecuencia de aparación de este fenómeno.

Capítulo 5:

En este capítulo pusimos a prueba la hipótesis que sitúa al sistema inmune del hospedador como mecanismo mediador de la ocurrencia de invasiones parasitarias múltiples en eritrocitos. Para ello, determinamos la frecuencia de aparición de invasiones múltiples, en machos y hembras de herrerillo común, en relación con el nivel

de inmunoglobulinas totales en sangre. La medicación con primaquina disminuyó significativamente la intensidad de infección en las hembras pero no en los machos. Además, se observó que las hembras presentaron mayores niveles de inmunoglobulinas que los machos. En ambos casos, la intensidad de infección se correlacionó con la presencia de invasiones múltiples. Además, en las hembras pero no en los machos, la ocurrencia de invasiones múltiples se relacionó positivamente con el nivel de inmunoglobulinas. Estos resultados fueron concordantes en dos momentos diferentes de la reproducción, cuando los polluelos tenían 3 y 13 días de edad. En el caso de los machos, la medicación favoreció la ocurrencia de invasiones múltiples, si bien, otras variables como el tamaño de nidada podrían estar afectando esta última relación. En conjunto, estos resultados suponen la primera evidencia en condiciones naturales del papel del sistema inmune del hospedador favoreciendo la ocurrencia de invasiones múltiples. Además, estos resultados corroboran los obtenidos previamente en trabajos de laboratorio (Miller *et al.* 1984, Ramasamy *et al.* 1999) y mantienen abierta la posibilidad de que las invasiones múltiples pudieran, en parte, estar causadas por los hospedadores con el objetivo de reducir los costes de la infección y, posiblemente, dificultar el éxito de transmisión de los parásitos.

Capítulo 6:

Se realizó una revisión exhaustiva de los estudios publicados hasta el momento sobre la ocurrencia de invasiones múltiples en infecciones producidas por parásitos de la malaria y otras parasitosis emparentadas. Se han postulado tres posibles causas para explicar la presencia de dos o más formas morfológicas parasitarias en la misma célula hospedadora: (i) por un mecanismo asociado a la actividad del parásito, (ii) debido a la actividad del hospedador o bien (iii) por un mecanismo azaroso asociado a altas intensidades de infección (Fig. 8). En ciertas ocasiones, los estudios realizados sobre la ocurrencia de este fenómeno han reflejado su estrecha relación con altas intensidades de infección (Wang 1970, Jovani y Sol 2005). No obstante, aunque se necesita investigar más sobre estos aspectos, otros estudios han presentado claras evidencias en favor del papel del hospedador como favorecedor de la ocurrencia de las invasiones múltiples (Miller *et al.* 1984, Ramasamy *et al.* 1999, **capítulo 5**). Estas evidencias entran en conflicto con las que soportan la actividad del propio parásito como hipótesis alternativa para explicar este evento (Jovani 2002, **capítulo 4**).

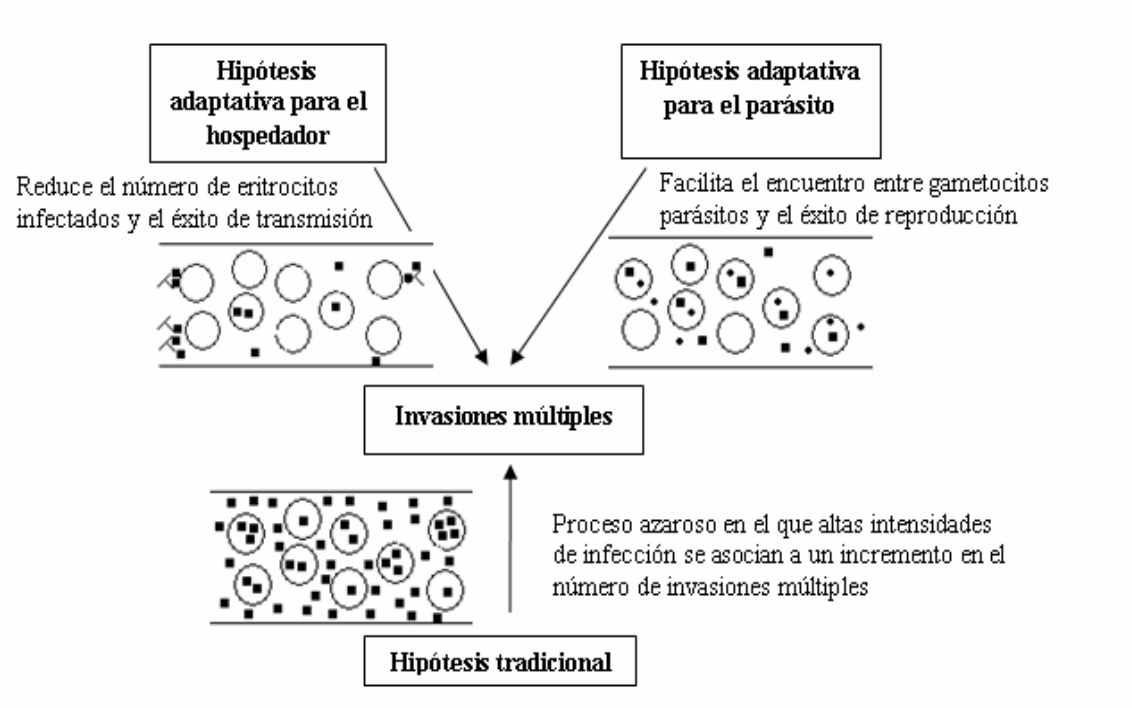

Figura 8. Esquema de las hipótesis propuestas sobre la ocurrencia de invasiones múltiples de eritrocitos por parásitos de la malaria y otros parásitos emparentados.

Capítulo 7:

En este estudio se investigó el efecto de la administración de un fármaco antimalárico en la probabilidad de supervivencia del herrerillo común mediante técnicas de captura-recaptura. La administración de una dosis subcurativa de este medicamento redujo la intensidad de infección por *Haemoproteus majoris* en las hembras pero no en los machos. Además, los análisis estadísticos mostraron que la interacción entre la medicación y el sexo fue significativa, de modo que las hembras medicadas tuvieron una mayor probabilidad de supervivencia que las hembras control (Fig. 9). Sin embargo, no se encontró un efecto significativo del tratamiento sobre la supervivencia de los machos. Del mismo modo, cuando se incluyeron en los análisis el cambio en los niveles de inmunoglobulinas totales y de proteínas de estrés (HSPs), presentes en el torrente circulatorio de las aves adultas cuando la edad de los polluelos era de 3 y 13 días, se observó un efecto significativo sobre la probabilidad de supervivencia. Los factores que mejor explicaron este resultado fueron el cambio de los niveles de HSPs y la interacción entre el sexo y el tratamiento. En conjunto, estos resultados demuestran por primera vez de forma experimental, utilizando un tratamiento de medicación, el impacto de los parásitos sanguíneos sobre la supervivencia de las aves silvestres y apoyan los

obtenidos en otros trabajos previos (Richner *et al.* 1995; Nordling *et al.* 1998, Møller y Nielsen 2007, Arriero y Møller 2008, Marzal *et al.* 2008). Adicionalmente, este estudio presenta la primera evidencia sobre el papel de las proteínas de estrés como favorecedoras de la supervivencia de las aves silvestres, como había sido previamente demostrado en otros organismos (Tatar *et al.* 1997, Sørensen y Loeschcke 2004).

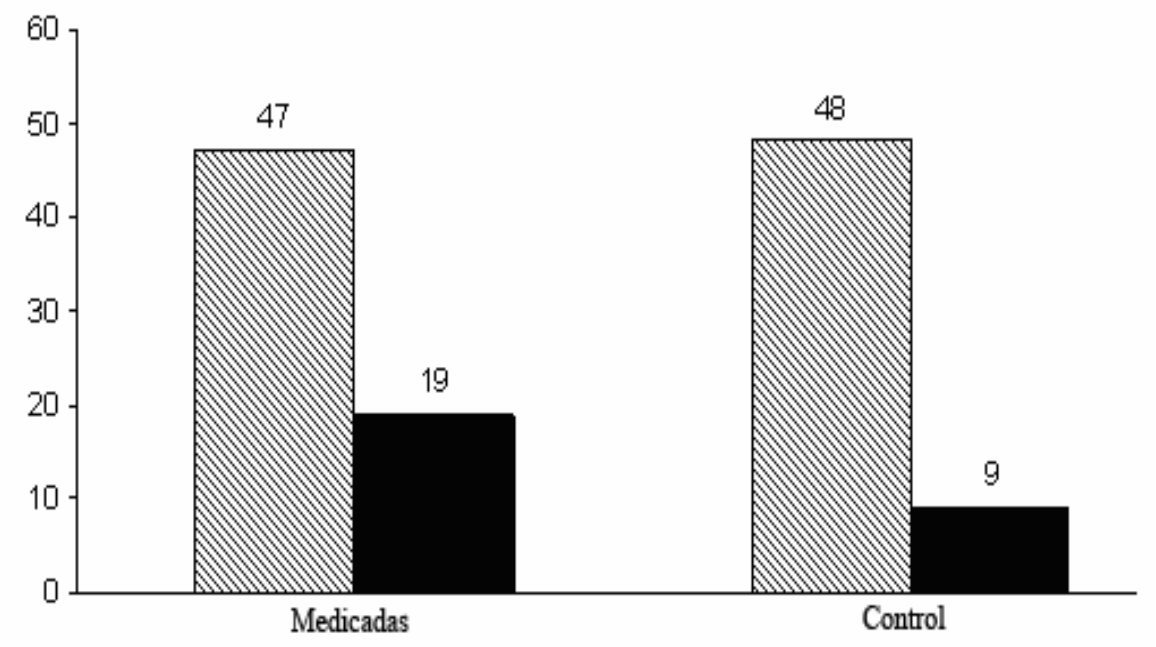

Figura 9. Hembras medicadas y control durante el tratamiento experimental en el año 2004. Las columnas barreadas muestran las no supervivientes y las columnas en negro son aquellas que si sobrevivieron hasta el año siguiente.

Discusión integradora

El parasitismo representa una entramada red de relaciones complejas en la que están involucrados una enorme diversidad de organismos, en nuestro caso, parásitos sanguíneos, vectores (dípteros) y hospedadores (aves).

El estudio de la ecología del parasitismo en condiciones naturales no siempre es sencillo debido principalmente a dos grandes aspectos, la enorme diversidad de organismos que pueden interactuar y la multitud de factores que pueden afectar estas relaciones. Uno de los principales problemas que frecuentemente se encuentran los estudiosos de la ecología del parasitismo en animales silvestres es el escaso conocimiento que se tiene sobre los ciclos vitales y la taxonomía de las especies parásitas. Esto supone un problema de base que obliga a los investigadores a realizar estudios taxonómicos, o de identificación de los organismos parásitos, como el que se presenta en el **capítulo 1**. En este sentido, y a pesar del enorme conocimiento disponible sobre las especies parasitas presentes en las aves, los estudios moleculares revelan la existencia de una diversidad parasitaria considerablemente mayor que la recabada empleando únicamente técnicas de microscopía y criterios de especificidad de hospedadores, es decir, mediante los estudios taxonómicos clásicos. Por ello, es necesario la inclusión de nuevas herramientas de análisis molecular, basadas en la amplificación del material genético del parásito, que faciliten la determinación y el encuadre taxonómico de estos organismos (Valkiūnas e*t al.* 2007, 2008b, Merino *et al.* 2009). Estos dos aspectos son extremadamente complejos de llevar a cabo usando únicamente las herramientas tradicionales, ya que en muchas ocasiones únicamente se dispone de muestras de alguna de las fases vitales del parásito (Smith 1996). De esta forma, la incorporación de los métodos geneticos esta permitiendo la reorganizacion de algunos grupos establecidos de parásitos. Además, recientemente Valkiūnas y colaboradores (2008a) llamaron la atención de los investigadores sobre la necesidad de aglutinar el conocimiento de estas dos grandes escuelas taxonómicas, tradicionales y moleculares. Ello permitiría asignar las líneas genéticas de los parásitos recientemente descritas a sus morfoespecies correspondientes. Sin embargo, esta lúcida recomendación es difícil llevarla a cabo debido a la falta de investigadores conocedores de las bases para la identificación taxonómica mediante microscopía de estos organismos.

Por otro lado, otra de las dificultades que frecuentemente se encuentran los investigadores que tratan las interrelaciones entre parásitos y hospedadores radica en la correcta identificación de los mediadores de esta relación, los insectos que actúan como vectores. De manera general, los estudiosos de los parásitos que infectan a las aves silvestres poseen unos amplios conocimientos sobre la identificación de los hospedadores y de sus endoparásitos, pero la enorme diversidad de vectores que existen en la naturaleza dificulta la posibilidad de que estos investigadores engloben también conocimientos sobre la taxonomía de los insectos. En este contexto, en el **capítulo 2 y 3** se identificó y cuantificó la abundancia de los diferentes vectores que afectan a las aves silvestres en sus nidos, lo que puso de manifiesto la considerable diversidad de vectores presentes en el área de estudio, potencialmente importantes en la transmisión de diferentes parásitos como *Leucocytozoon* y *Haemoproteus*. La correcta identificación de las especies de vectores, que afectan a las aves en sus nidos, se hace fundamental para esclarecer en un futuro aquellos potencialmente relevantes en la transmisión de los parásitos y para esclarecer aspectos referidos a su ciclo vital.

De este modo, una vez identificados los organismos involucrados en el ciclo epidemiológico de los parásitos sanguíneos, es decir, los insectos que actúan como vectores y los hospedadores vertebrados, los estudios dentro de un marco ecológico se encaminarían ha dilucidar la influencia que poseen cada uno de ellos en el ciclo en cuestión. Ello conduce, por ejemplo, a comprobar si las distintas especies de vectores se ven atraídas a los nidos por distintos factores o si los diferentes tratamientos experimentales afectan del mismo modo a las distintas especies de insectos. En este sentido, en los **capítulos 2 y 3** se estudiaron los diferentes efectos de los factores bióticos o ambientales en la localización de los hospedadores vertebrados por parte de los insectos hematófagos. Hasta el momento, existe un profundo desconocimiento sobre las especies de vectores que afectan a las aves silvestres y sobre los mecanismos que median esta interacción, principalmente debido a las enormes dificultades para muestrear y cuantificar los vectores que afectan a las aves en sus nidos. A pesar de la existencia de numerosos estudios sobre estos temas, las conclusiones obtenidas deben ser tomadas con cautela dado que, en la mayoría de los casos, se utilizaron modelos mantenidos en cautiverio (pero ver también Smith *et al.* 1998 como modelo de estudio en aves silvestres). En conjunto, estos trabajos, y otros asociados al tema, ponen de manifiesto que los vectores deben utilizar diferentes receptores que les permitirían responder frente a estímulos visuales, térmicos o químicos emitidos por sus

hospedadores (Gibson y Torr 1999, Lehane 2005). Del mismo modo, los factores ambientales son también potencialmente influyentes en la relación entre los ectoparásitos y las aves en sus nidos (Merino y Potti 1996, Smith *et al.* 1998, Dawson *et al.* 2005). Esta hipótesis tendría una especial relevancia con los insectos voladores, ya que al ser éstos más independientes de sus hospedadores vertebrados también están más afectados por los factores ambientales que otros insectos hematófagos.

En este marco en el que nos encontramos, donde diferentes organismos interactúan entre si y diferentes factores parecen mediar esta relación, se presenta una interesante oportunidad para comprobar las diferentes estrategias y contraestrategias con las que cada uno de estos organismos intentarían maximizar los beneficios y minimizar los costes frutos de esta interacción. En este sentido, dado que los vectores poseen diferentes mecanismos con los que detectar las sustancias emitidas por sus hospedadores, todos aquellos factores que incrementen la emisión de estos atrayentes, como por ejemplo la infección por parásitos sanguíneos, podrían facilitar la localización de los hospedadores por parte de los vectores (Lacroix *et al.* 2005). No obstante, teniendo en cuenta los costes que los parásitos infringen a los vectores, si los insectos pudiesen detectar el estado de infección del hospedador por alguna vía, sería esperable que estos consumieran sangre preferentemente de los hospedadores menos infectados (Tomás *et al.* 2008b). Esta hipótesis estaría reforzada por algunos resultados mostrados en el **capítulo 2**, donde se encontró una mayor abundancia de *Culicoides festivipennis* en nidales ocupados por aves que habían sido medicadas frente a parásitos sanguíneos.

Por otro lado, asumiendo los costes infringidos por los parásitos sobre sus hospedadores vertebrados, podría predecirse que éstos deberían también minimizar la probabilidad de ser infectados por parásitos empleando todas aquellas estrategias que les permitan paliar el impacto producido por las infecciones. En este sentido, se han descrito multitud de mecanismos por los que los hospedadores podrían mitigar los costes de ser parasitados, bien evitando el contacto con dichos parásitos (Merino y Potti 1995) o bien intentando controlar sus niveles de infección mediante mecanismos fisiológicos y/o comportamentales, tales como el consumo de sustancias exógenas (Clayton y Wolfe 1993) o el uso de sustancias con propiedades insecticidas (Bucher 1988, Lafuma *et al.* 2001, Shutler y Campbell 2007, pero ver Mennerat *et al.* 2008). Al respecto, merece la pena destacar que las aves emplean ciertas sustancias con capacidad insecticida en la construcción de los nidos aunque los resultados sobre su eficacia en condiciones naturales no son concluyentes, máxime en el caso de los insectos

hematófagos voladores, los cuales presentan una independencia de los nidos de las aves considerablemente mayor que otros ectoparásitos como pulgas, ácaros o larvas hematófagas de moscas. Los resultados presentados en el **capítulo 2** arrojan algo de luz sobre este tema, ya que la utilización de sustancias insecticidas en los nidos, si bien no afecta significativamente a la abundancia total de vectores en el interior de los nidales, sí podría reducir la cantidad de insectos que logran alimentarse satisfactoriamente de las aves. De esta forma, las aves podrían disminuir los costes asociados a la pérdida de sangre y reducir la probabilidad de ser infectadas por los parásitos que transmiten.

Por otro lado, los parásitos sanguíneos, debido a que requieren la intervención de varios organismos para completar su ciclo vital, deberían maximizar su probabilidad de transmisión a través de todos aquellos hospedadores a los que infectan. Los estudios realizados hasta el momento sobre los parásitos de la malaria aviar, incluyendo diferentes especies de *Plasmodium* y otros parásitos emparentados, han demostrado que la intensidad de infección y la proporción de sexos son factores clave en el éxito de transmisión de estos parásitos desde el hospedador vertebrado al vector (Schall 2000, Merino *et al.* 2004). En otros estudios se ha sugerido que las invasiones parasitarias múltiples de eritrocitos podrían representan otra estrategia para incrementar la probabilidad de encuentro entre macrogametos y microgametos en el vector y, por lo tanto, para incrementar el éxito de reproducción del parásito en el mismo (Jovani 2002). Sin embargo, los resultados mostrados en **capítulo 4** permitirían rechazar esta hipótesis de partida. Ademas, de los datos presentados en el **capítulo 5** se desprende la posible implicación de un nuevo factor en la ocurrencia de las invasiones parasitarias múltiples. En concreto, se presenta la primera evidencia en hospedadores silvestres de un mecanismo por el cual las aves podrían reducir la cantidad total de eritrocitos infectados por parásitos sanguíneos mediante la inclusión de varias células parásitas dentro de la misma célula hospedadora. Aunque, no existe consenso en cuanto a los mecanismos que pudieran mediar la ocurrencia de este fenómeno, se postula que los anticuerpos bivalentes del hospedador pudieran estar involucrados en la formación de invasiones múltiples de eritrocitos por diferentes vías (Miller *et al.* 1984, Ramasamy *et al.* 1999). Estos anticuerpos podrían provocar una reducción del número total de eritrocitos infectados con infecciones simples, lo que a la postre podría reducir los costes de la infección y comprometer el éxito de transmisión del parásito (ver **capítulos 5 y 6**).

Finalmente, una vez conocidos algunos de los mecanismos que subyacen a la interacción entre los parásitos y sus hospedadores, unas de las cuestiones clave es

esclarecer cómo los parásitos son capaces de afectar la evolución y el tamaño poblacional de sus hospedadores, un foco de investigación fundamental en los estudios sobre la ecología del parasitismo. A lo largo de los últimos años se ha comprobado que las reducciones experimentales en la intensidad de infección, por parásitos sanguíneos, incrementa el éxito reproductor en aves silvestres (Merino *et al.* 2000, Marzal *et al.* 2005). Complementando estos resultados previos, los datos presentados en el **capítulo 7** sobre el efecto de la administración de un tratamiento antiparasitario, demuestran que estos parásitos también son capaces de afectar la supervivencia de las aves silvestres. La modificación experimental de la carga de parásitos sanguíneos no siempre es sencilla, lo que parece contribuir a las escasas evidencias experimentales presentadas sobre el impacto de la intensidad de parasitación sobre la supervivencia de las aves silvestres. Por ello, y hasta donde llega nuestros conocimientos, el efecto de los parásitos sobre sus hospedadores se ha realizado con modelos infectados con parásitos intestinales (Hudson y Dobson 1991, Hanssen *et al.* 2003). En el caso de los parásitos sanguíneos, diferentes estudios correlacionales y comparativos (p. ej. Møller y Nielsen 2007, Arriero y Møller 2008, Marzal *et al.* 2008) han prestado apoyo al impacto de estos organismos sobre la supervivencia de las aves. Por su parte, otros estudios en los que se modifica el esfuerzo reproductor de las aves con el objetivo de afectar la carga parasitaria de las mismas han presentado también ciertas evidencias adicionales sobre el efecto negativo de los parásitos incrementando la mortalidad de las aves (Richner *et al.* 1995; Nordling *et al.* 1998, pero ver Stjernman *et al.* 2004). No obstante, es esencial una modificación experimental directa la carga parasitaria para discernir con total seguridad si el efecto sobre la supervivencia se debe al cambio en la carga parasitaria o a otras posibles tratamientos que medien la modificación de la intensidad de infección, como pudiera ser el esfuerzo reproductor (ver Stjernman *et al.* 2004). El efecto del tratamiento antiparasitario, que genera una reducción de la intensidad de infección, podría incrementar la supervivencia de las aves mediante una reducción de los recursos destinados al mantenimiento de la respuesta inmune (Tomás *et al.* 2007) y del gasto metabólico basal (Martínez *et al.* 2004). Estos efectos podría redundar, de manera conjunta, en una disminución de la pérdida de peso corporal (Merino *et al.* 2000, Tomás *et al.* 2005) que permitiera a las aves, en último término, reducir la probabilidad de ser depredadas (Hudson *et al*. 1992). Por otro lado, diferentes factores podrían mediar la relación entre los hospedadores y sus parásitos. Uno de ellos, el sexo, se presenta como un factor que determina la intensidad de infección que sufren los hospedadores y los

mecanismos que éstos emplean en su lucha contra los parásitos (Zuk y McKean 1996, Møller *et al.* 1998, Klein 2004). Estos mecanismos de lucha pueden tener un origen ecológico y/o fisiológico y podrían contribuir a la aparición de una susceptibilidad diferencial a las infecciónes parasitarias en función del sexo de los individuos (Zuk y McKean 1996, Ferrari *et al.* 2007). Del mismo modo, el sexo se presenta también como un factor clave mediando, al menos en parte, la eficacia de los fármacos en los organismos (Gordi *et al.* 2002, Klein 2004, Pinsonneault y Sadée 2004), lo que podría variar los efectos producidos al ser administrados. De acuerdo con ello, en el **capítulo 7** se observa que factores ligados al sexo median la eficacia diferencial del tratamiento antiparasitario, lo que a la postre podría influir en la supervivencia de las aves. Además, de acuerdo con otros resultados obtenidos en este mismo capítulo, podemos concluir que si bien la respuesta mediada por proteínas de estrés puede ser costosa en términos fisiológicos (Merino *et al.* 2006, Morales *et al.* 2006), su implicación en diferentes funciones vitales, como el mantenimiento de la homeostasis celular (Sørensen *et al.* 2003) y su implicación en diferentes aspectos de la respuesta inmune (Srivastava 2002), podría suponer una ventaja adaptativa para los organismos contribuyendo finalmente al aumento de su probabilidad de supervivencia (Tatar *et al.* 1997, Sørensen y Loeschcke 2004).

Conclusiones

- En estudios taxonómicos de parásitos sanguíneos de aves silvestres es necesario utilizar conjuntamente técnicas moleculares y microscópicas.

- El uso de sustancias insecticidas en los nidos reduce el consumo de sangre de los vectores aunque no su atracción. Esto puede beneficiar a las aves hospedadoras al reducir la pérdida de sangre y la probabilidad de infección por parásitos.

- Ciertos factores ambientales, como la temperatura mínima y la velocidad del viento, conjuntamente con algunos factores bióticos, como la intensidad de parasitación, la especie hospedadora y el tamaño de nidada, son variables determinantes de la abundancia de vectores que parasitan a las aves en sus nidos. Por lo tanto, deben tenerse en cuenta en estudios ecológicos sobre las interacciones de la tríada vector-ave-parásito.

- La ocurrencia de invasiones múltiples de eritrocitos por parásitos del género *Haemoproteus* en aves silvestres no es un mecanismo adaptativo del parásito para favorecer su éxito de transmisión.

- La ocurrencia de invasiones múltiples se asocia con un mayor nivel de inmunoglobulinas plasmáticas en las hembras pero no en los machos del herrerillo común, apuntando a que estas invasiones se deban a la existencia de un mecanismo adaptativo de defensa del hospedador.

- Las invasiones múltiples de eritrocitos deben considerarse mayoritariamente como fruto de altas intensidades de infección y como un mecanismo defensivo mediado por el sistema inmune del hospedador.

- El sexo, la reducción de la intensidad parasitaria y el incremento a lo largo de la estación reproductora de los niveles de proteínas de estrés determinan la probabilidad interanual de supervivencia en el herrerillo común.

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CAPÍTULO 1

La caracterización molecular del gen del 18S rADN de un *Hepatozoon* aviar revela que está estrechamente relacionado con *Lankesterella*.

Como una parte de un estudio intensivo de las infecciones por parásitos sanguíneos en una población del paseriforme herrerillo común (*Cyanistes caeruleus*, Paridae), detectamos una especie parásita que, basado en la similitud morfológica, fue identificado inicialmente como *Hepatozoon parus*, la única especie de este género parásito descrita en aves de esta familia. No obstante, medidas morfológicas demostraron que *H. parus* es ligeramente más largo que el parásito encontrado en nuestra población. Una caracterización molecular de la especie parásita se realizó mediante la amplificación del gen 18S rAND, usando cebadores ("primers") descritos previamente para amplificar *Hepatozoon* sp. en pitones acuáticas. Otros cebadores fueron desarrollados en base a la nueva secuencia obtenida. El fragmento de 1.484 pares de bases amplificado reveló que, de acuerdo con los análisis comparados usando BLAST, la especie parásita de nuestra población de aves está más estrechamente emparentada con *Lankesterella minima* que con las especies de *Hepatozoon* de otros vertebrados (93% de identidad). Además, análisis filogenéticos usando el los procedimientos de parsimonia y Kimura relacionaron inequívocamente la especie parásita detectada mediante PCR con *L. minima*. Los valores de "bootstrap" obtenidos fueron 97% y 100% respectivamente. Estos resultados implican que la especie parásita es una especie de lankesterélido en vez de un *Hepatozoon*.

Molecular characterization of the 18S rDNA gene of an avian *Hepatozoon* reveals that it is closely related to *Laesterella*.

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As a part of intensive study of blood parasite infections in a population of the passerine bird blue tit (*Cyanistes caeruleus*, Paridae), we detected a parasite species that, based on its morphological similarity, was tentatively identified as *Hepatozoon parus,* the only species of this parasite genus described from birds of this family. However, morphological measurements show that *H. parus* is slightly larger than the parasite detected in our population. A molecular characterization of the parasite species was conducted by amplification of the 18S rDNA gene, using primers that were reported previously to amplify in *Hepatozoon* sp. of water pythons. Additional primers were developed based on the new sequence obtained. The 1484 bp fragment amplified reveals that the parasite from our bird population is more closely related to *Lankesterella minima* than to *Hepatozoon* species from other vertebrates according to analysis using the BLAST comparison method (93% identity). In addition, phylogenetic analyses using parsimony and Kimura procedures unequivocally related the parasite species detected by PCR with *L. minima*. The bootstrap values obtained were 97% and 100%, respectively. These results imply that this parasite is a species of a lankesterellid instead of *Hepatozoon*.

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Introduction

Species of *Hepatozoon* represents a group of apicomplexan blood parasites described from terrestrial vertebrates and hematophagous invertebrates (Smith 1996). Gamonts, the blood stage of the parasite in their vertebrate hosts, usually parasitize leukocytes in mammals and birds. Several *Hepatozoon* species have been described from birds in the last years (see review in Bennett *et al.* 1992). These descriptions are mostly based in morphometric characteristics of the gamonts, as well as in the supposed host family specificity (Bennett *et al.* 1992). Most of the infections in birds by these parasites are light and, in general, are overlooked by researchers or assigned to a number of genera as *Haemogregarina* sp. or *Atoxoplasma* sp. Therefore, some confusion exists with respect to the taxonomic position of these parasites. Smith (1996), in his detailed review of *Hepatozoon*, recommended to include all the infections by haemogregarines from birds within *Hepatozoon*, until more data on the life cycle of these parasites allow for their placement into a correct genus. Some reports of species of *Haemogregarina*, *Lankesterella*, and even *Atoxoplasma* from birds are also known and several authors have tried to rearrange these parasites into one of those genera, as some synonymy apparently exists (Levine 1982, Desser 1993).

The taxonomy of avian *Lankesterella* spp. has been also debated during years, as these parasites have been various included as species of *Haemogregarina*, *Toxoplasma*, *Atoxoplasma*, and *Lankesterella* (Box 1971). More recently, some studies have situated *Lankesterella* spp. infections of birds as species of *Isospora* (Box 1975, Desser 1980). Levine (1982) related all this controversy to the fact that several different genera of protozoa have stages in blood cells that look alike, and that they cannot be assigned to their proper genera without knowing their life cycles. He also accepted the opinion expressed by Baker *et al.* (1972), that birds have 2 types of blood-inhabiting coccidians that are difficult to separate based on morphological ground. One group (probably not homogeneous) was referred to as atoxoplasms and the other consisted of hemogregarines. Levine (1982) resurrected *Atoxoplasma* and differentiated it from *Lankesterella*. He based his proposal on the observation that the latter presents both merozoites and sporozoites in blood cells, and transmission is accomplished by ingestion of sporozoite-infected mites (thus, *Atoxoplasma* spp. are homoxenous and *Lankesterella* spp. heteroxenous).

The potential confusion between species of *Lankesterella* and *Atoxoplasma* emerged again when Upton *et al.* (2001) pointed out that the observation by Lainson

(1959) of both gametes and polyzoic oocysts in extraintestinal tissues of infected sparrows clearly differentiated these species from those transferred by Box (1975) from *Atoxoplasma* to *Isospora*. Therefore, Lainson (1959) was probably working on a species within Lankesterellidae. More recently, *Atoxoplasma* has been proposed to be a synonym of *Isospora* based in morphological and molecular data (Barta *et al.* 2005). Based on these references, one can easily understand the conclusion of Desser (1993): "The taxonomy of haemogregarines is a mess". He also predicted that when additional information on life histories became available, many of the species of hemogregarines will be invalidated or transferred from one genus to another. In conclusion, blood stages of several parasites from 2 different groups are, therefore, easily confused: (1) hemogregarines, which consist of adeleid (suborder Adeleorina) blood parasites that have circulating gamonts within blood cells and use ectoparasitic hematophagous invertebrates as biological vectors in which sporogony occurs, i.e. *Hepatozoon* spp., and (2) coccidia from suborder Eimeriorina, which consist of circulating zoites (either sporozoites or merozoites). Sporozoites in blood cells usually belong to 1 of the 2 genera within the Lankesterellidae (either *Schellackia* or *Lankesterella* spp.), that use ectoparasitic hematophagous invertebrates as vectors. Merozoites in the circulation are usually associated with avian isosporan parasites in the Eimeriidae (*Isospora* spp. or, historically, *Atoxoplasma* spp.). These latter parasites are actually typical intestinal coccidia that have circulating extraintestinal stages. They are homoxenous and infected birds shed oocysts in the feces.

Although morphological confusion is still possible among blood stages of several of these parasites infecting birds and their life cycles are still mostly unknown, the use of molecular tools may help to place these hematozoans within their correct genera. Here, we present the results of a study carrying out the first molecular characterization of a supposed avian *Hepatozoon*, which place this parasite close to a *Lankesterella* species.

Material and methods

Blood smears were collected from reproductive blue tits (*Cyanistes caeruleus*) in a population breeding in nest-boxes near the locality of Valsaín, Segovia, central Spain (40º53'N, 4º01'W) during the spring of 2004. Birds were captured and a blood sample was obtained from the brachial vein. A drop of blood was immediately smeared, air-dried, and later fixed with 95% ethanol. The rest of the blood was stored inside a plastic

tube in a cool-box and later frozen at –80 C until analyzed. Blood smears were stained with Giemsa (1/10 v/v) for 45 min. Smears were checked microscopically in search of blood parasites and positive slides for the putative *Hepatozoon* species were selected for the study. Slides are deposited at the collection of Museo Nacional de Ciencias Naturales, Madrid, Spain (accession numbers: MNCN 35.02/24, MNCN 35.02/25 and 35.02/26, blood smears from *Cyanistes caeruleus).*

Parasite morphometric measurements were made with the aid of image analyzer software (Scion Image, Frederick, Maryland) from pictures of parasites taken under 100X oil immersion objective in an Olympus BX41 optic microscope. Length, width, and area of the parasite and parasite nucleus, along with the diameter and area of the paranuclear refractile body, were measured for each parasite.

Genomic DNA was obtained from blood of infected *Cyanistes caeruleus* using the UltraClean DNA BloodSpin kit (MO BIO Laboratories, Inc., Solana Beach, California). Initially, partial amplification of 18S rDNA gene was accomplished by PCR using the primers HepF300 and Hep900 (Table 1), as described previously by Ujvari *et al.* (2004) in their study of *Hepatozoon* sp. in water pythons. PCR reactions consisted of 25 µl reaction volumes containing 20 ng template DNA, 50 mM KCl, 10mM Tris-HCl, 1.5 MgCl2, 0.2 mM of each dNTP, 1 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, 60 C for 1 min, 72 C for 1 min, and a final extension at 72 C for 10 min. Once a partial sequence of the small ribosomal subunit of the putative *Hepatoozon* sp. was obtained, it was used for designing new primers destined to amplify most of the small ribosomal subunit gene. These new primers designed by us were called Hep50F/Hep631R, Hep623F/Hep1615R, and Hep800F/Hep1615R and are described in Table 1. PCR reactions and thermal profile were identical to those described above except for annealing, 56 C for Hep50F/Hep631R, and 58 C for Hep623F/Hep1615R and Hep800F/Hep1615R.

The DNA fragments obtained after PCR assays were recovered from agarose gels and subjected to direct sequencing. DNA fragments obtained in at least 2 separate amplifications were sequenced using an ABI 3130 (Applied Biosystems) automated sequencer.

DNA sequences were aligned using the CLUSTALW program (Thompson *et al.* 1994). The BIOEDIT program (Hall, 1999) was used to edit the sequences. The

TREECONW (Van de Peer and De Wachter, 1993) and MEGA3.1 (Kumar e*t al.* 2004) software packages were used in phylogram construction/drawing. Computer programs were set at their default parameters in all analyses. Phylogenetic analyses were performed using the parsimony method and the Kimura model. Tree consistency was estimated by bootstrap analysis with 100 replications.

Table 1. List of primers used in this study.

Primers	Sequences (5'→3')
HepF300	GTT TCT GAC CTA TCA GCT TTC GAC G
Hep900	CAA ATC TAA GAA TTT CAC CTC TGA C
Hep623F	GGA TTT CTG CCG TGA TCG TC
Hep631R	CGT CGG ACG ATC ACG G
Hep50F	GAA ACT GCG AAT GGC TCA TT
Hep800F	GTC AGA GGT GAA ATT CTT AGA TTT G
Hep1615R	AAA GGG CAG GGA CGT AAT C

Results

Overall, 59 blue tit smears (31.2% of 189 samples) were found infected by the parasite (Fig. 1). Morphometric measurements differed significantly from those reported by Bennett and Peirce (1989) for *Hepatozoon parus,* the only species within this genus described for birds of the Paridae (Bennett and Peirce 1989, Peirce 2005, see Table 2).

Table 2. Comparison of the morphometric parameters of the Lankesterellid species with *Hepatozoon parus*. Numbers in parenthesis show standard deviation. N, number of infected cells examined. All measurements are given in micrometers. Paranuclear refractile body (PRB) of the Lankesterellid is compared to parasite vacuole of *H. parus*. Parasite measurements were statistically compared between both species by *t*-test for independent samples. *= P<0.001, †= *P*<0.0001, ns= no significant differences. ‡Data from Bennett and Peirce (1989).

	Avian lankesterellid (N = 81)	*H. parus*‡ (N = 215)
Parasite		
Length†	9.0 (1.0)	10.4 (1.2)
Width†	3.4 (0.7)	4.0 (0.6)
Area†	27.2 (5.7)	34.0 (7.0)
Parasite nucleus		
Width*	2.5 (0.7)	2.2 (0.9)
Area ^{ns}	8.4 (3.4)	8.0 (2.8)
Vacuole		
Diameter*	2.3 (0.3)	2.1 (0.4)
Area†	4.7 (1.3)	3.1 (1.1)

On the other hand, a fragment of 1484 bp of the small ribosomal subunit was sequenced from 1 hemoprotozoan isolate. Using the BLAST comparison method, the species with the highest sequence identity (93%) was *Lankesterella minima*. To assess genetic polymorphism in the 18S gene, new amplifications of 700 bp were performed in other 6 isolates using Hep800F/Hep1615R primers. ClustalW alignment of the 7 sequences showed that all isolates were identical. Phylogenetic analyses using parsimony method and Kimura model unequivocally related the parasites detected by PCR with *Lankesterella minima* (Figs. 2, 3). The bootstrap values obtained were 97% and 100%, respectively. The measurements of this parasite indicate that it is substantially smaller than *Hepatozoon parus* (Table 2). Molecular characterization of the 18s rDNA gene clearly indicates that the parasite is closely related to a *Lankesterella* species (see above).

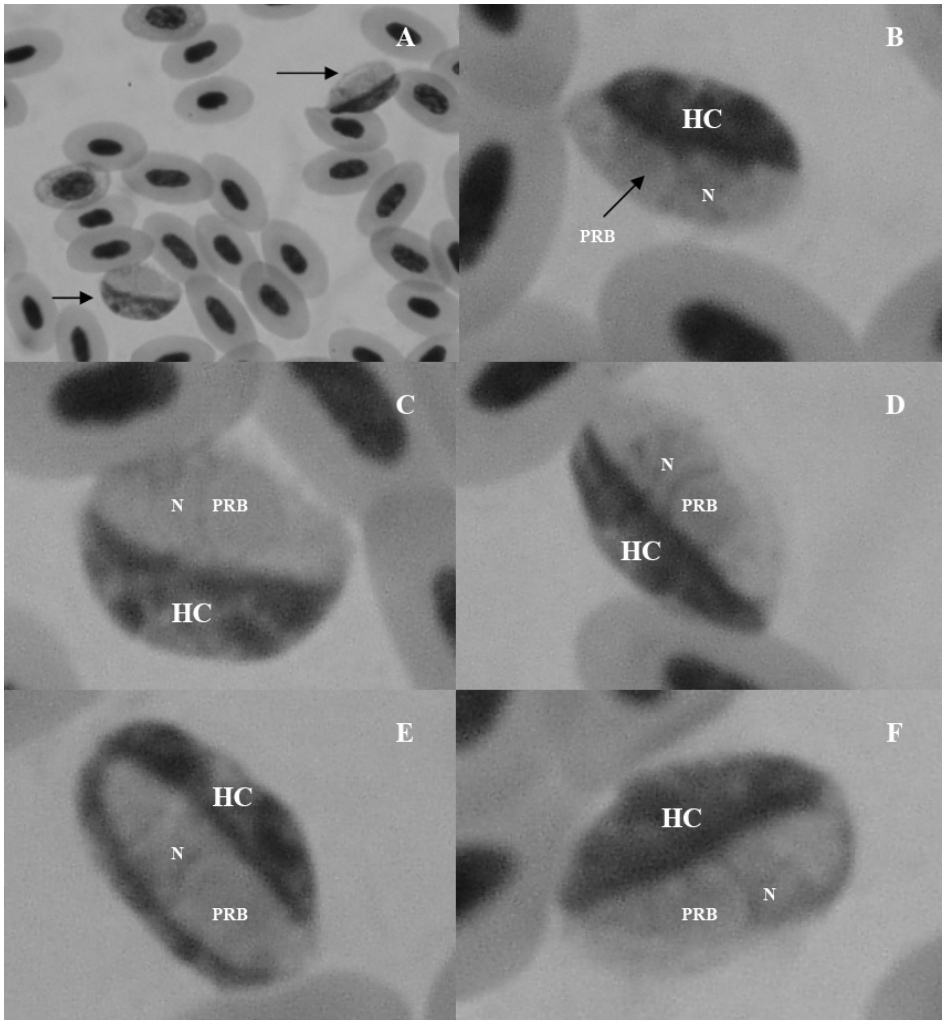

Figure 1. Microphotographs of blood smears showing lymphocytes infected with the Lankesterellid (A-F). Peripheral blood from blue tits (*Cyanistes caeruleus*) were employed to prepare the smears. HC: Host cell; N: Parasite nucleus; PRB: Paranuclear refractile body.

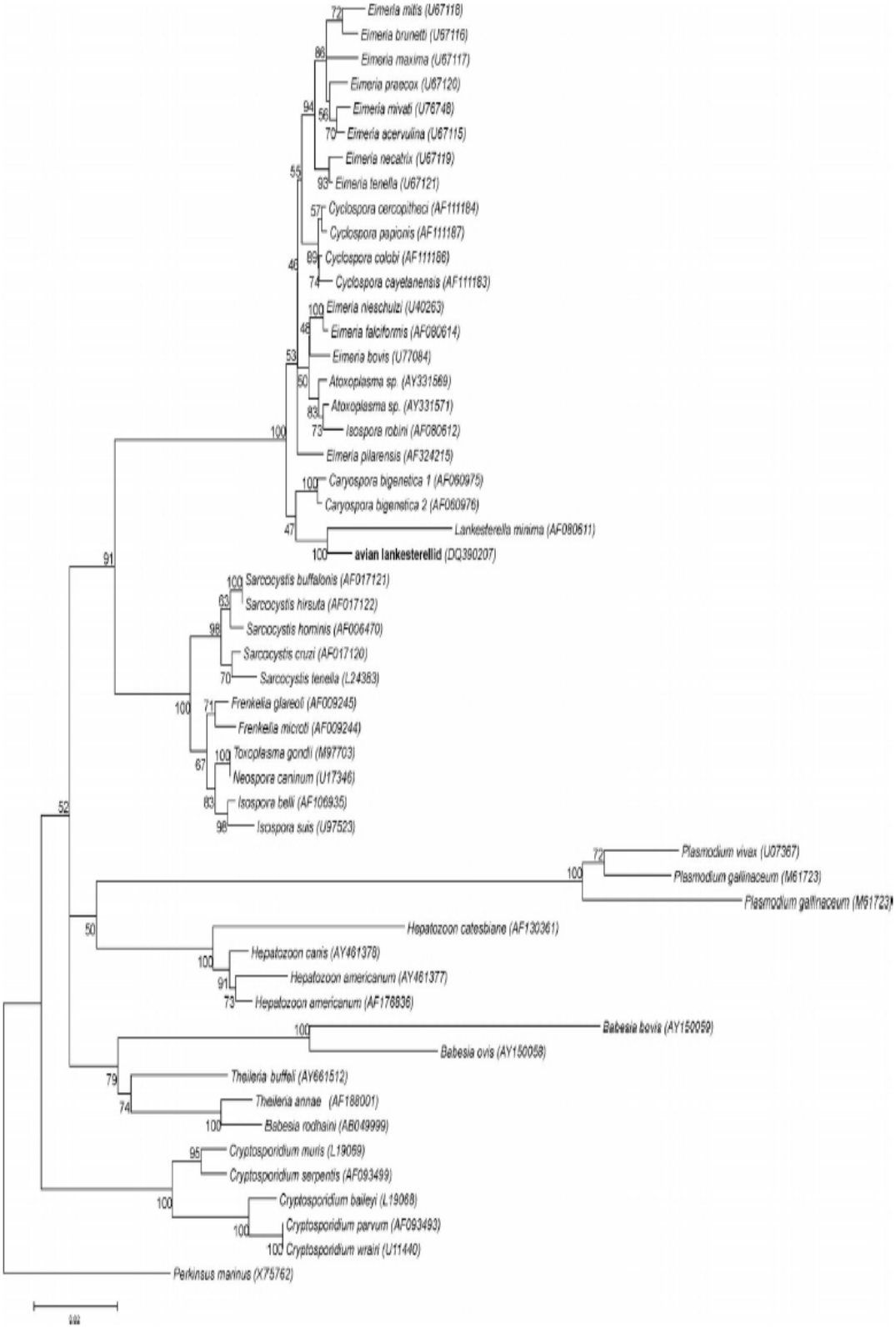

Figure 2. Phylogenetic tree obtained with the MEGA3 software package using the model of Kimura. Bootstrap values (with 100 replications) are shown at the corresponding nodes. Figures in brackets after the species name indicate the GenBank accession number of the isolate. The avian Lankesterellid species appears in bold.

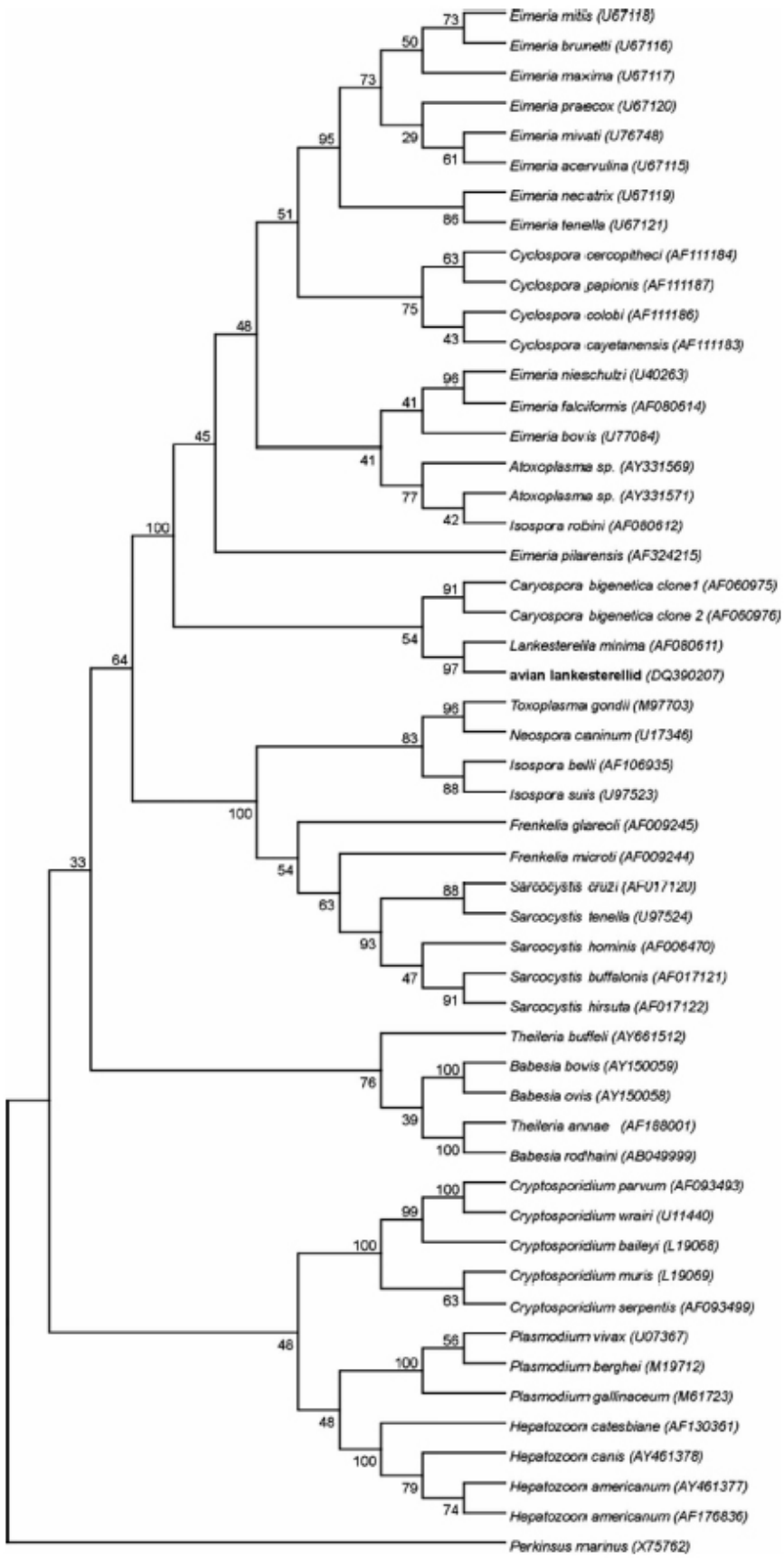

Figure 3. Phylogenetic tree obtained with the MEGA3 software package using maximum parsimony method. Bootstrap values (with 100 replications) are shown at the corresponding nodes. Figures in brackets after the species name indicate the GenBank accession number of the isolate. The avian Lankesterellid species appears in bold.

Discussion

The lankesterellid species measured above was previously assigned to *Hepatozoon parus* (Fargallo and Merino 1999, Merino *et al.* 2000, and subsequent references by the same authors) due to its morphological similarity to that species (see Fig. 1; Bennett and Peirce 1989). In fact, only by careful measurement of the parasite can we conclude that highly significant size differences exist between both parasites. However, these differences are not enough to separate species of *Lankesterella* from *Hepatozoon* among the 15 avian species of *Hepatozoon* considered valid (Peirce 2005). There are even smaller species than the lankesterellid measured here (see Table 2; Bennett and Peirce 1989, Bennett *et al.* 1992). Description of *Hepatozoon* species of birds has been exclusively based in morphological measurements of parasites in vertebrate hosts, as the life cycles for all the species described is unknown. In addition, it has been assumed that species of *Hepatozoon* are host specific at the familial or sub-familial level (Bennett *et al.*, 1992). According to Smith (1996) and Peirce (2005), previously described avian species of *Hepatozoon* should be considered valid until new data on life cycles can be obtained. Avian *Hepatozoon* species have been described from parasites previously identified as *Atoxoplasma*, *Haemogregarina*, and *Lankesterella*. Smith (1996) claimed that the inconsistency of morphological characteristics of the parasites in the hemogregarine complex, especially in gamonts, and their low host specificity render their differentiation difficult. In addition, Desser (1993) pointed out that because of the similarity between some lankesterellid sporozoites and hemogregarine gamonts, certain species designated as *Lankesterella* are hemogregarines. Our data imply that the opposite may also be the case; some species considered hemogregarines are lankesterellids. Although, *Lankesterella* species are actually confined to amphibian and reptile hosts (Desser 1993) and the species of *Atoxoplasma* from birds have been transferred to *Isospora* (Barta *et al.* 2005, Schrenzel *et al.* 2005), it is possible that some species of hemogregarines in birds are better placed within the Lankesterellidae (see also Upton *et al.* 2001).

The difficulty of obtaining data on life cycles for several blood parasite species of birds is now partially overcome by the use of molecular and phylogenetic techniques. In fact, the use of DNA analyses represents a considerable advance in systematics of protozoan infections (Carreno *et al.*1999, Barta *et al.* 2001, Criado-Fornelio *et al.* 2003, 2004). Our phylogenetic analyses using the 1484 bp fragment amplified from avian *Hepatozoon* sp. led us to draw the following conclusions. First, the parasite from our

blue tit population is more closely related to *Lankesterella minima* than to *Hepatozoon* species from lower vertebrates or mammals. Second, phylogenetic analyses using parsimony method and Kimura model unequivocally related the parasite detected by PCR to the lankesterellids. At least some parasites considered as bird haemogregarines are likely lankesterellids, and can not be described as *Isospora* species based solely on bloodstream stages. The conclusion by Upton (2000) that "the genus Atoxoplasma Garnham, 1950 is probably a composite of avian isosporans and avian lankesterellids" is thus supported by our results.

Finally, based solely on rDNA sequence and the phylogenetic relationships inferred herein, the transfer or redefinition of avian *Hepatozoon* species are not warranted at this time, but this should be considered after additional *Hepatozoon* spp. sequences from birds become available.

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CAPÍTULO 2

Efectos de los tratamientos de medicación y fumigación sobre la composición de especies y la abundancia de *Culicoides* en nidos de aves: un estudio experimental en el herrerillo común (*Cyanistes caeruleus*).

Los mecanismos que afectan los patrones de distribución de vectores en sus hospedadores deben influir en la población y en la evolución de la dinámica de los vectores, los hospedadores y los parásitos que transmiten. Aquí estudiamos el papel de diferentes factores afectando la composición y abundancia de especies del género *Culicoides* encontrada en nidos de herrerillo común (*Cyanistes caeruleus*). Para ello, identificamos 1531 hembras y 2 machos de 7 especies diferentes de Culicoides, siendo *C. simulator* la especie más abundante seguida de *C. kibunensis*, *C. festivipennis*, *C. segnis*, *C. truncorum*, *C. pictipennis* y *C. circumscriptus*. Desarrollamos un experimento combinado de medicación y fumigación asignando los tratamientos de forma aleatoria entre los nidos, generando grupos de parejas medicadas y controles criando en nidos fumigados y controles. Las parejas medicadas fueron inyectadas con el fármaco antimalárico primaquina diluido en solución salina mientras que a las parejas control se les inyectó con solución salina. El tratamiento de fumigación se llevó a cabo usando una solución insecticida o agua en nidos tratados como fumigados o controles respectivamente. El tamaño de nidada fue el principal factor asociado con la abundancia de *Culicoides* probablemente debido a que un mayor número de polluelos deben producir mayores cantidades de atrayente de estos vectores. Además, las aves medicadas frente hemoparásitos criando en nidales no fumigados presentaron una mayor abundancia de *C. festivipennis* que el resto de grupos experimentales. También encontramos que el tratamiento de fumigación redujo la abundancia de *Culicoides* con una toma reciente de sangre en sus abdómenes tanto en los nidos con parejas medicadas como en las parejas control, indicando una reducción del éxito de consumo de sangre producido por el insecticida. Estos resultados representan una primera evidencia sobre el papel de diferentes factores en la determinación de la infracomunidad de *Culicoides* en nidos de aves silvestres.

Effects of medication and fumigation treatments on the species composition and abundance of *Culicoides* in avian nests

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Mechanisms affecting patterns of vector distribution among host individuals may influence the population and evolutionary dynamics of vectors, hosts and the parasites transmitted. We studied the role of different factors determining the species composition and abundance of *Culicoides* found in nests of blue tit (*Cyanistes caeruleus*). We identified 1531 females and 2 males of 7 different *Culicoides* species in nests, with *C. simulator* as the most abundant species, followed by *C. kibunensis*, *C. festivipennis*, *C. segnis*, *C. truncorum*, *C. pictipennis* and *C. circumscriptus*. We conducted a medication x fumigation experiment randomly assigning bird nests to different treatments, thereby generating groups of medicated and control pairs breeding in fumigated and control nests. Medicated pairs were injected with the antimalarial drug primaquine diluted in saline solution while control pairs were injected with saline solution. The fumigation treatment was carried out using insecticide solution or water for fumigated and control nests respectively. Brood size was the main factor associated with the abundance of biting midges probably because more nestlings may produce more quantities of vector attractants. In addition, birds medicated against hemoparasites breeding in not fumigated nests supported a higher abundance of *C. festivipennis* than the rests of the groups. Also, we found that the fumigation treatment reduced the abundance of engorged *Culicoides* in both medicated and control nests, thus indicating a reduction of feeding success produced by the insecticide. These results represent a first evidence for the role of different factors determining the *Culicoides* infracommunity in wild avian nests.

Parasitology, 2ª revisión.

Introduction

As indicated by Bush *et al.* (2001) "the study of parasitism from an ecological perspective is a fascinating exploration of organisms that make their living at the expense of others". Parasites are important organisms driving the evolution of their hosts (Navas *et al.* 2007), affecting them in terms of both reproductive success and survival (Hudson and Dobson 1991, Lehmann 1993, Merino *et al.* 2000). Of all parasite species known, blood sucking insects are especially interesting because they not only affect their hosts directly by draining their resources but also indirectly as vectors of multiple pathogens. In this context, the study of biting midges of the genus *Culicoides* Latreille (Diptera: Ceratopogonidae), a worldwide distributed genus, with about 1254 described species (Beckenbach and Borkent 2003), present in most terrestrial habitats (Kettle 1995, Marquardt *et al.* 2000), is of great importance not only because females are obligate blood feeders attacking an enormous diversity of vertebrates (Downes 1958, Kettle 1995, Marquardt *et al.* 2000), but also because biting midges are vectors of a large number of transmissible agents, some of them with economic and veterinary importance, including viruses (Braverman *et al.* 1996, Mellor *et al.* 2000) and other parasites such as protozoa and filarial worms (Fallis and Wood 1957, Atkinson *et al.* 1983, Shelley and Coscarón 2001, Garvin and Greiner 2003, Mullens *et al*. 2006).

Female biting midges, the only sex that requires blood meals, are infected by blood parasites when they obtain a blood meal from an infected host. With the exceptions of few non-biting species and autogenous species which require a blood meal only after laying their first egg batch, most *Culicoides* females need to obtain blood for their first ovarian development (Downes 1958). Many studies on *Culicoides* in the fields of medical and veterinarian sciences have been conducted to identify the mechanisms affecting their host selection processes and feeding patterns. However, in the wild, there is scant information about ecological relationships between *Culicoides* and their hosts, especially for the case of wild birds. The main reason for the scarcity of this kind of studies is probably the absence of an effective method of capture. Usually, biting midges are captured using different gadgets such as light traps, CO_2 traps placed close to the animals or directly vacuuming them from the animals' bodies (i.e. Bennett 1960, Braverman *et al.* 1976, Zimmerman and Turner 1983, Mushi *et al.*1999, Yu *et al.* 2000, Mullens *et al.* 2005). However these methods are difficult to use in avian nests, especially for the study of midges attacking bird species breeding in nests placed in cavities.

Arthropod–host interactions involve fascinating behavioral processes and chemosensory mechanisms and chemicals that allow vectors to express host-selection behaviours that results in non-random biting (Mukabana *et al.* 2002, Tomás *et al.* 2008b). Visual as well as antennal and maxillary receptors may be involved in host-location (Bowen 1991). *Culicoides* have receptors sensitive to a diversity of host derived products such as lactic acid, 1-octen-3-ol and CO_2 (Bhasin *et al.* 2000a, Grant and Kline 2003) which produce attractive effects (Blackwell *et al.* 1996, Gibson and Torr 1999, Mands *et al.* 2004, Marquardt *et al.* 2000, Mordue 2003). Also, the presence of volatile pheromones produced by parous midge females may attract other females, as reported by Blackwell *et al.* (1994) in their study on an autogenous species, the biting midge *C. impunctatus*. In addition, as may occur under natural conditions, hosts-derived volatile components may interact with parous female pheromones, either attracting or repelling females as a function of the relative doses of each chemical (Blackwell *et al.* 1996).

Also, host infection status may be a key factor affecting host location by vectors, because infection could affect host metabolism and therefore host-derived attractants (Torres-Estrada and Rodríguez 2003, Lacroix *et al.* 2005). In humans, individuals with high intensities of infection by malaria are more susceptible to the attack by vectors (Lacroix *et al.* 2005). However, this may not be the case for birds (Tomás *et al.* 2008b) where higher abundances of biting midges were found in nests of female blue tits with experimentally reduced intensities of infection by medication with an antimalarial drug, an effective method to reduce the intensity of infection by the *Culicoides* transmitted malaria-like *Haemoproteus* (Merino *et al.* 2000, Tomás *et al.* 2005, Martínez–de la Puente *et al.* 2007). Biting midges may prefer to feed on less infected birds because blood parasites may reduce their survival (Valkiūnas and Iezhova 2004). On the other hand, the infection status could also affect host susceptibility to vector attacks through other ways such as reducing host antimosquito behaviours (Torres-Estrada and Rodríguez 2003). It is known that hosts use a diversity of insect-repelling strategies to avoid the attack of biting midges including anti-insect behaviours (Edman *et al.* 1974, Mooring *et al.* 2003, Darbro and Harrington 2007) or the use of plants with insecticide properties (Bucher 1988, Clark 1991, Lafuma *et al.* 2001). Humans, due to the sanitary and economical importance of *Culicoides* (Mellor *et al.* 2000, Ratnayake *et al.* 2006) also use different insecticides to control midge populations. There is evidence of lower abundances of *Culicoides* in fumigated farms as compared to non-fumigated ones (Sarto

i Monteys and Saiz-Ardanaz 2003, also see Satta *et al.* 2004) that may reduce the costs associated with the activity of biting insects. In the case of birds, some species introduce in their nests plants with insect-repellent properties that could reduce the abundance of ectoparasites in avian nests (Bucher 1988, Clark 1991). Both naturally derived and synthesized components have been tested for their repellent effect on biting midges (Braverman and Chizon-Ginzburg 1997). In wild populations, birds may also benefit from the use of insecticides if they reduce biting midge densities. In the case of blue tits, it has been suggested that the use of green plants could be a mechanism of protection against parasites (Cowie and Hinsley 1988, Banbura *et al.* 1994, Petit *et al.* 2002). However, the effect of plant-derived repellents could be different among parasite species because there are both attraction and repellency effects of a particular compound among *Culicoides* species (Braverman *et al.* 1999). To reveal the potential effect of insecticides on *Culicoides* infracommunities in avian nests, studies in wild populations should be done. In this respect, we found in a previous study that the use of an insecticide treatment was not effective in reducing the abundance of *Culicoides* in blue tit nests, although a differential specific susceptibility of *Culicoides* species to the insecticide treatment could affect these results (Tomás *et al.* 2008a).

Understanding the interactions between biting midges and birds are especially interesting for the case of hole-nesting species because some of them predate on insect pests of gardens and forests. In order to study the factors affecting *Culicoides* host location in wild populations of hole-nesting birds we conducted an experimental study on blue tits (*Cyanistes caeruleus*). Our predictions are: i) higher abundances of female *Culicoides* will be found in those nests with larger broods given that more nestlings produce more host attractants (such as CO_2); ii) a reduction in the abundance of *Culicoides* is expected for those nests fumigated with insecticide compared to control nests; and iii) according to a previous study in blue tits (Tomás *et al.* 2008b), we expect a higher abundance of *Culicoides* in nests attended by breeding pairs medicated against malaria-like parasites. Under these premises, we studied the *Culicoides* species composition and abundance in blue tit *Cyanistes caeruleus* nests with special emphasis on parous and engorged *Culicoides* females, because parous females are potential hemoparasite vectors and engorged females have fed recently on a host.

Methods

This study was carried out in a population of blue tits *Cyanistes caeruleus* breeding in nest-boxes during the spring of 2005 in a Pyrenean Oak *Quercus pyrenaica* deciduous forest located in Valsaín (Segovia, 40° 53′ 74N, 4°01′ W, 1200 m a.s.l.). When nestlings were 3 days old, nests were randomly assigned to fumigation and medication treatments that generated medicated x fumigated nests (n=14), medication control x fumigated nests (n=14), medicated x fumigation control nests (n=15) and medication control x fumigation control nests (n=16). The medication consisted in a subcutaneous injection of 0.1 ml of the antimalarial drug primaquine (Sigma, St Louis, MO, USA) diluted in saline solution (concentration $1 mg \cdot ml^{-1}$) when nestlings were 3 days old. Control pairs were injected with the same volume of saline solution. Treatment with primaquine causes a reduction in the intensity of infection by blood parasites in the study population (Merino *et al.* 2000, Tomás *et al.* 2005, Martínez-de la Puente *et al.* 2007). The fumigation treatment was carried out at three different times (at the nestling ages of 3, 7 and 11 days) with an insecticide solution (Stockade ©, Fort Dodge Veterinaria, S.A., Vall de Bianya, Girona, Spain) comprising 0.5 % Permethrin and 1 % Piperonyl butoxide. Nestlings were extracted from nests prior to fumigation and left again in the nest immediately after treatment. This treatment has been previously used to reduce ectoparasite populations in nests without detection of any deleterious effect for nestlings (Tomás *et al.* 2007). The same methodology was employed in control nests using water instead of insecticide. During two days after the last fumigation, we captured *Culicoides* using the method described and tested by Tomás *et al.* (2008a). This method consisted in the placement inside the nest-boxes of plastic Petri dishes (8.5 cm diameter; 56.7 cm^2) layered with 0.5 ml of commercially available body gel-oil (Johnson's© baby chamomilla, Johnson and Johnson, Dusseldorf, Germany). This gel-oil is made up of paraffinum liquidum, hexyl laurate, ethylene/propylene/styrene copolymer, cyclopentasiloxane, butylene/ethylene/styrene copolymer, chamomilla recutita, bisabolol and perfume [FPT1353]. The comparison between medication control x fumigation and medication control x fumigation control groups was previously reported by Tomás *et al.* (2008a) in the context of a methodological study to determine the efficacy of such a sticky media to collect biting midges. On day 13 brood sizes for each nest were recorded and Petri dishes removed and stored in a freezer until their examination. In the laboratory, biting midges were removed from dishes using xilene and maintained in absolute ethanol until their identification. All *Culicoides* species were initially sorted on their wing pattern under an Olympus SZH stereomicroscope (10x to

64x magnification). However, given their minute size (usually no longer than 3 mm), for more accurate diagnosis, it was necessary to dissect many of the midges and make microscopic slide preparations of their body parts. For fixing them we used Tendeiro solution (distilled water: 35 ml; chloral hydrate: 40 g; glacial acetic acid: 18 ml; polyvinyl alcohol: 7 g). To identify them to specific level we used Kremer's (1966) and Delécolle's (1985) morphological keys. *Culicoides* were sexed and the parity of females determined as follows: nulliparous (those that have never fed on blood), parous (those showing a burgundy pigment in the subcutaneous cells of the abdomen indicating a previously digested blood meal; see Dyce 1969) or engorged females (those with a blood meal still not completely digested in their abdomen). We assume that engorged females fed blood on birds (nestlings or adults) from the nest-box were they were captured.

Total abundance of *Culicoides* and each specific abundance were logarithmically (log10) transformed to normalize distributions. General regression models (GRM) (Statistica version 6.0, StatSoft, Inc. 2001) applying the forward stepwise solution, were used to investigate the relationships between the total abundance of *Culicoides* and the abundance of each species, including in the model the treatment (4 groups) as a factor and brood size and phenology (a potential confounding variable estimated as hatching date of the brood) as covariables. Results were also confirmed using backward stepwise solutions. Residuals of the models were tested for normality. Variables reflecting total abundances included the total number of nulliparous, parous and engorged females per nest. In addition, when residuals of the models did not follow a normal distribution, nonparametric analyses were conducted. Simple correlations and Kruskal-Wallis tests were used to test for the effect of each brood size, seasonality and treatment on the abundance of total parous females and the abundance of total engorged females (both not normally distributed variables, even after log transformation). Analyses for *Culicoides* species were restricted to the three more abundant species, *C. simulator*, *C. kibunensis* and *C. festivipennis* (see Table 1).

Results

A total of 1531 female biting midges of 7 different species were captured in 57 nests. Only two males (one *C. kibunensis* and one *C. festivipennis*) were captured. In two additional nests we did not capture any biting midge (Table 1). In addition, 41 biting midges (2.6% of the total) could not be identified because of the absence of wings or

other anatomical structures. However, unidentified individuals were also considered in total abundances. In each nest, we captured an average of 26.64 (SD 39.06, range 0-208) biting midges from 3.14 (SD 1.50, range 0-6) different species.

Table 1. Female abundance and prevalence (in brackets) of each *Culicoides* species captured in blue tits nests during the breeding season of 2005. Female biting midges were identified as nulliparous, parous and engorged (see text for details).

Species	Nulliparous	Parous	Engorged	Total
C. simulator	751 (86.4)	76 (35.6)	44 (45.8)	871 (93.2)
C. kibunensis	322 (79.7)	42 (37.3)	9 (11.9)	373 (81.4)
C. festivipennis	121 (61.0)	60 (30.5)	5 (5.1)	186 (62.7)
C. segnis	59 (42.4)	9 (13.6)	7 (11.9)	75 (50.8)
C. truncorum	14 (13.6)	3 (5.1)	2 (3.4)	19 (20.3)
C. pictipennis	2 (3.4)	2 (3.4)	0 (0)	4 (6.8)
C. circumscriptus	3 (5.1)	0 (0)	0 (0)	3 (5.1)
Total	1272 (91.5)	192 (62.7)	67 (55.9)	1531

The abundance of total *Culicoides* females captured in avian nests was strongly and positively associated with brood size (Fig.1; model: adjusted R^2=0.11, p<0.01; brood size: $F_{1,57}$=7.83, p<0.01). The same positive significant association was found for the abundance of *Culicoides simulator* (model: adjusted R^2=0.13, p<0.003; brood size: $F_{1,57}$=9.72, p<0.003). We also found a significant positive association between the abundance of *C. kibunensis* and both brood size and phenology (model: adjusted R^2=0.18, p<0.002; brood size: $F_{1,56}$=12.37, p<0.001, phenology: $F_{1,56}$=8.19, p<0.01). In addition, we found a significant effect of treatment on the abundance of *C. festivipennis* females (model: adjusted R^2=0.14, p<0.01; treatment: $F_{3,55}$=4.27, p<0.01). As residuals of this model did not follow a normal distribution, we also tested for the effect of the treatment on the abundance of *C. festivipennis* using nonparametric statistics and obtaining the same conclusion (Fig. 2; Kruskal-Wallis test: $H_{3,59}$=11.12, p=0.01), that is the medicated x fumigation control nests had a higher abundance of *C. festivipennis* than the other groups.

We did not find any significant association between the abundance of parous females and brood size, phenology or treatment (all p>0.09). In addition, the abundance of engorged *Culicoides* females was significantly higher in nests with larger broods (n=59; r_s=0.33; p=0.01). Also, the abundance of engorged females was significantly associated with treatment (Fig. 3; $H_{3,59}$=8.41, p=0.04), with lower abundances of engorged females for fumigated nests (both medication control x fumigated and

medicated x fumigated) than the non-fumigated groups. The abundance of engorged females and phenology were not significantly associated (n=59; r_s=0.09; p=0.49).

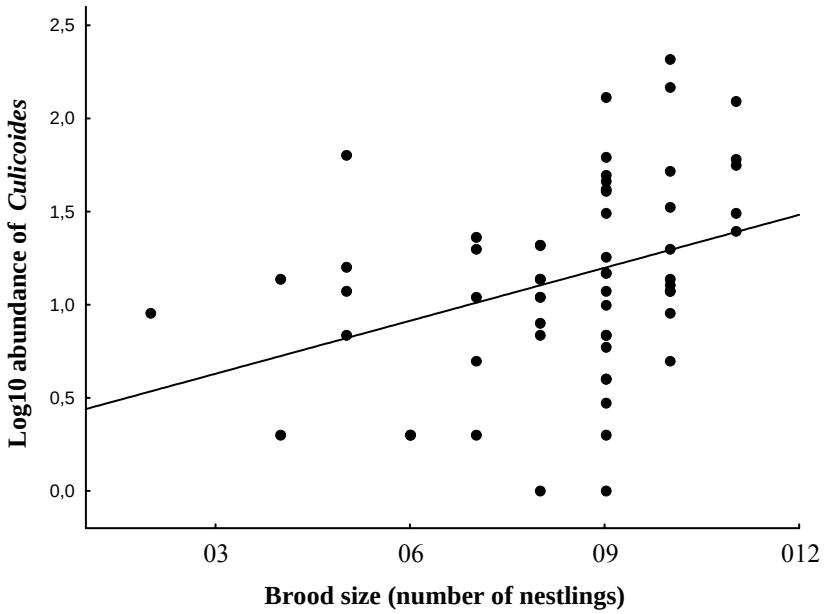

Figure 1. Relationship between the abundance of *Culicoides* and brood size in blue tit nests during the spring of 2005 (adjusted r^2 = 0.11, p<0.01).

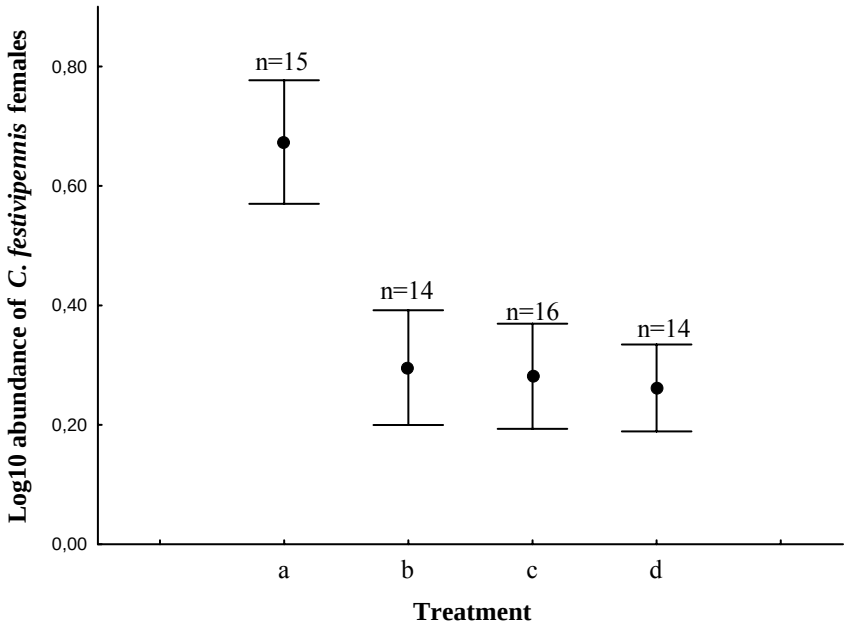

Figure 2. Abundance of *C. festivipennis* in each experimental group ($H_{3,59}$=11.12, p=0.01). Bird pairs were randomly assigned to fumigation and medication treatments that generated medicated x fumigation control nests (a), medication control x fumigated nests (b), medication control x fumigation control nests (c) and medicated x fumigated nests (d). Bars denote standard errors.

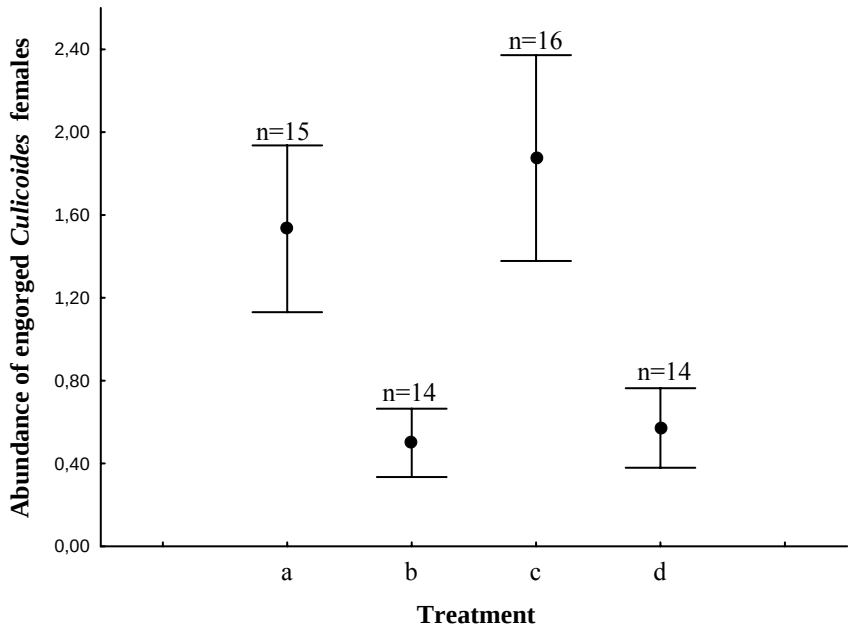

Figure 3. Abundance of engorged female *Culicoides* in each experimental group ($H_{3,59}$=8.41, p=0.04). Bird pairs were randomly assigned to fumigation and medication treatments that generated medicated x fumigation control nests (a), medication control x fumigated nests (b), medication control x fumigation control nests (c) and medicated x fumigated nests (d). Bars denote standard errors.

Discussion

Here we report the *Culicoides* infracommunity composition and examine different factors determining their abundance in wild blue tit nests. All the *Culicoides* species found in this study have been previously cited for the Iberian Peninsula (Delécolle 2002), and three of them, *Culicoides festivipennis*, *C. kibunensis* (quoted as *C. cubitalis*) and *C. truncorum* (quoted as *C. sylvarum*) have been previously captured on wild avian hosts (buzzards *Buteo buteo* nests; Votýpka *et al.* 2002, Podlipaev *et al.* 2004). We found that three *Culicoides* species had prevalences above the 60% and that a very low proportion of nests were free of biting midges. Because vector abundances may determine the prevalence of blood parasites in their hosts (Yu *et al.* 2000), our results are in accordance with a previous study in the same host population reporting a high prevalence of infection by hemoparasites (Merino *et al.* 2000). Also, the possibility that male midges occupied a position near the hosts to increase their probability of mating with females while or before they are feeding (Yuval 2006), is not supported by our results due to the fact that only two males were captured inside bird nest-boxes. In the case of these midge species, it is more likely that mating takes place out of nest-boxes over prominent landmarks such as trees or even people (Marquardt *et al.* 2000).

Many, if not all, biting insects have evolved a complex sensory system designed to detect and locate hosts with different receptors including chemo- and visual-receptors (Gibson and Torr 1999, Grant and Kline 2003). Blood sucking insects use host-derived odours as cues to detect their hosts (Gibson and Torr 1999, Mordue 2003). As shown in electrophysiological studies on several *Culicoides* species, these products are effective in stimulating biting midge receptors (Bhasin *et al.* 2000a, Grant and Kline 2003, Sollai *et al.* 2007), and their attractive effect on *Culicoides* species have been reported both when they are present on their own (Blackwell *et al.* 1996, Braverman *et al.* 2000 but see Bhasin *et al.* 2000b) or in interaction with other host products (such as CO_2) (Gibson and Torr 1999, Bhasin *et al.* 2000b, but see Braverman *et al.* 2000). For that reason, if more nestlings are capable of producing a higher amount of these products we could expect the pattern obtained here, with higher abundances of *Culicoides* in nests with larger broods. Accordingly, the abundance of *Culicoides* in avian nests increased with nestling age (a correlate of nestling size) (Tomás *et al.* 2008a). Overall, here we report the effect of brood size as a variable affecting the abundance of *Culicoides* in avian nests. In a previous study Tomás *et al.* (2008b) reported the effect of other variables (nest size, nestling condition, female infection status, the abundance of other ectoparasites and parental provisioning rates) also affecting the total abundance of *Culicoides* in avian nests however they did not find any significant effect of nestling mass (a correlate of brood size) on total *Culicoides* abundance. This difference between our study and Tomás *et al.* (2008b) may be due to differences in the experimental design between both studies. For example, we captured biting midges with Petri dishes during a period of two days while Tomás *et al.* (2008b) captured *Culicoides* using a piece of plastic tape during one day. In addition, a considerable lower number of *Culicoides* were captured during 2005 (1531 *Culicoides* females) than in 2004 (more than 2300 *Culicoides*) when the study by Tomás *et al.* (2008b) was carried out. Differences in the species composition of *Culicoides* between both studies may also affect results, but unfortunately this information in not available for the study by Tomás *et al.* (2008b). More experimental studies modifying brood size or the concentration of host attractants should be done in avian nests to reveal the actual importance of these cues to host detection by ornithophilic midges.

Also, our results represent an experimental evidence for the role of blood parasite infection on biting midge attraction and successful midge feeding in the wild. The higher intensity of infection by blood parasites in humans increases the attraction of

vectors (Lacroix *et al.* 2005). However, the higher abundance of *C. festivipennis* females found in medicated x fumigation control nests do not support the role of the medication treatment in reducing the vector attraction by birds. This medication treatment produces a significant reduction in the malaria-like *Haemoproteus* parasites, the more common blood parasite infecting blue tits (Merino *et al.* 2000, Tomás *et al.* 2005, Martínez-de la Puente *et al.* 2007), so that our results suggest that vectors are attracted to hosts with lower intensities of infection by parasites that also infect and damage biting midges (Desser and Yang 1973; Valkiūnas and Iezhova 2004). In support of that, in a previous study carried out in the same population during 2004 (Tomás *et al.* 2008b) authors found a higher abundance of biting midges in nests from medicated females.

On the other hand, although the total abundance of *Culicoides* was not affected by the fumigation treatment, we clearly found an effect of the insecticide on the abundance of engorged females, suggesting that the insecticide reduced the efficiency of blood feeding by midges. In previous studies where we used the same insecticide, a significant reduction in the abundance of other ectoparasites (fleas, mites and blowflies) was found (Tomás *et al.* 2007, Lobato *et al.* 2008), suggesting that the higher mobility of biting midges with respect to other nest ectoparasites (mites, fleas and blowflies) could explain the differential efficiency of the treatment between nest-dweller and flying ectoparasites. In addition, we can expect that birds using plants with insecticide properties similar to those of the insecticide used here have a reduced probability of infection by blood parasites transmitted by midges and suffer from a lower impact of vector blood feeding.

Finally, we found that phenology is an important abiotic factor affecting the abundance of some vector species probably due to its association with meteorological conditions. Many reports on the effects of both meteorological factors and seasonality on *Culicoides* biology in terms of development, adult survival, distribution, abundance and activity rates exist (Bishop *et al*. 1996, Gerry and Mullens 2000, Mellor *et al.* 2000, Wittmann *et al.* 2001, Garvin and Greiner 2003, Sarto i Monteys and Saiz-Ardanaz 2003, Lysyk and Danyk 2007). The relationship between the abundance of *C. kibunensis* and host phenology suggests that early dates in the host breeding season were less favourable for the development of this species. In sum, these results represent a first evidence for different factors determining the *Culicoides* infracommunity in wild avian nests.

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CAPÍTULO 3

¿Afectan las condiciones meteorológicas a la abundancia de insectos hematófagos en nidos de aves?

Estudiamos los efectos de factores meteorológicos (temperatura, precipitaciones y velocidad del viento) sobre la abundancia de simúlidos y *Culicoides* en nidos de tres especies de aves paseriformes, el herrerillo común *Cyanistes caeruleus*, el carbonero común *Parus major* y el papamoscas cerrojillo *Ficedula hypoleuca* criando en el mismo área. Para tal propósito, consideramos también los diferentes factores relacionados con los hospedadores (fecha de eclosión, tamaño de nidada y especie hospedadora). Encontramos que la abundancia de simúlidos fue afectada negativamente por la temperatura mínima. Además, la abundancia de simúlidos y *Culicoides* fue afectada negativamente por la velocidad del viento a las 7:00, aunque la abundancia de simúlidos se relacionó positivamente con la velocidad del viento a las 18:00. También encontramos una mayor abundancia de simúlidos y *Culicoides* en nidos con mayores tamaños de nidada criando más tarde en la estación reproductora. Además, encontramos una abundancia significativamente mayor de *Culicoides* en nidos de papamoscas cerrojillo que en nidos de páridos. Los resultados presentados en este estudio representan, hasta donde conocemos, la primera demostración de la importancia conjunta de los efectos ambientales y relativos a la especie hospedadora sobre la abundancia de insectos voladores vectores en especies silvestres que anidan en cavidades.

Does weather affect the abundance of biting flies in avian nests?

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We have studied the effects of weather (temperature, rainfall and wind speed) on the abundance of blackflies and biting midges in nests of three passerine avian species, blue tits *Cyanistes caeruleus*, great tits *Parus major* and pied flycatchers *Ficedula hypoleuca,* breeding in the same area. To that end, we have controlled for different host-related factors (hatching date, brood size and host species). Blackfly abundance was negatively affected by minimum temperature. In addition, the abundance of blackflies and biting midges was negatively affected by wind speed at 07:00, but the abundance of blackflies was positively associated to wind speed at 18:00. We also found a higher abundance of both blackflies and biting midges in nests with larger broods breeding later in the season. In addition, a significantly higher abundance of biting midges in pied flycatcher nests with respect to tit nests was found. These results represent, to our knowledge, the first reporting both environmental and host-related effects on the abundance of flying insect vectors in nests of wild hole-nesting birds.

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Introduction

Host-parasite interactions are crucial in the ecology of most organisms, and are receiving increasing attention in the last decades (Poulin 1998). Parasites are organisms of certain species with adaptations for living in or on individuals of other species or feed on them, thereby affecting their metabolic efficiency or their life history trade-offs (Møller 1997, Poulin 1998). Haematophagous insects not only consume host blood but serve also as vectors of a diversity of pathogens, and can affect different host life history traits including health status (Tomás et al. 2008b), survival (Fitch et al. 1946, Hunter et al. 1997, Smith et al. 1998) and reproductive success (Bukaciński and Bukaciński 2000).

Factors affecting host location and the density of host and parasite populations are important determinants of host-vector interactions. Host location is a complex behavioural task that enhances the probability of contact of parasites with their blood meal source (Sutcliffe 1986). This behaviour could be divided into three phases: (i) appetitive searching, (ii) activation and orientation, and (iii) attraction (Lehane 2005), although the definition of these phases could vary between authors (Kettle 1995). Host location is driven by a diversity of stimuli including visual and olfactory cues (Bradbury and Bennett 1974a, 1974b; Sutcliffe 1986, Bowen 1991). Many studies testing the attraction of flying insects to animals or host-derived attractants have been conducted under laboratory conditions and in the field (Bennett 1960, Fallis and Smith 1964, Bennett et al. 1972, Greiner et al. 1978, Blackwell et al. 1996, Bashin et al. 2000, Ojanen et al. 2002, Mullens et al. 2005). These studies suggest that in natural conditions, many host-derived products or the combination of several of them may act as attractants of parasites to their hosts. However, there is a paucity of information for wild populations, especially in the case of breeding birds, about the factors determining the associations between biting flies and their hosts.

In this respect, environmental factors may strongly affect host-parasite interactions in different ways because insects are heterothermic organisms that depend greatly on environmental variables to activate their metabolism and behaviour. A large literature on flying insects have reported the concomitant effects of temperature and many other weather variables such as rainfall or wind speed affecting on breeding, abundance, survival and activity of haematophagous species (Kettle 1969, McCreadie et al. 1985, Shipp et al. 1988, Martin et al. 1994, Bishop et al. 1996, Kettle et al. 1998, Mellor *et al*. 2000, Tun-Lin *et al*. 2000, Su and Mulla 2001, Cilek and Schaediger 2004,

Mullens *et al*. 2005), as well as on host-ectoparasite interactions (Merino and Potti 1996, Smith *et al*. 1998, Calvete *et al*. 2003, Hubálek *et al*. 2003). For example, the host-seeking activity of ticks, determined as the abundance of individuals captured on low vegetation, was affected by temperature and humidity (Hubálek *et al*. 2003). In red-legged partridges, the abundance of lice was positively associated with temperature and a climate-derived vegetation index (Calvete *et al*. 2003). In an inter-annual study of weather effects on nest-dwelling ectoparasites (mites, fleas and blowfly larvae), Merino and Potti (1996) found a lower abundance and prevalence of these ectoparasites in the coldest and wettest year. Other studies have also studied the effects of meteorological conditions on ectoparasite loads in birds (i. e. Moyer *et al*. 2002, Dawson *et al*. 2005, Carrillo *et al*. 2007). However, there is a scarcity of information about the effects of weather on the abundance or prevalence of flying ectoparasites in avian nests. Thus, Smith *et al*. (1998) reported a decrease in blackfly abundance in red-tailed hawk nests when ambient temperatures were below 14ºC. Also, variation in weather conditions may partly explain the variation in vector abundance found in blue tit nests in different breeding seasons (Tomás *et al*. 2008a).

Blackflies (Diptera: Simuliidae) are small, dark, stout-bodied and hump-backed insects with a wide geographic distribution (Kettle 1995, Bush *et al*. 2001, Lehane 2005). Species identification of this group is difficult and uses several adult, pupal and larval characters (Kettle 1995). In their early stages, blackflies are limited to fluvial ecosystems, breeding in running water. Most adult blackflies are essentially diurnal, many of them showing a bimodal behaviour pattern with maximum activity in the early morning and afternoon (Lehane 2005, McCreadie *et al*. 1985, Grillet *et al*. 2005). Although there are some reports of nocturnal blackfly activity, the level of activity is low (McCreadie *et al*. 1985) and there is no evidence of blackflies attacking birds after dark (Bennett 1960). On the other hand, biting midges (Diptera: Ceratopogonidae) are worldwide distributed insects grouped in the genus *Culicoides* that includes more than 1400 species (Mellor *et al*. 2000). With the exception of some species, most of the biting midges are crepuscular or nocturnal, showing the peak of activity during the evening and the first half of the night (Maquardt *et al*. 2000, Lehane 2005). This is the case especially during spring, summer and autumn when a pronounced burst of activity at sunset is found in different biting midge species (Kettle *et al*. 1998).

Adult blackflies and biting midges of both sexes feed on sugary solutions, but females also consume the blood necessary for developing their eggs (Downes 1958,

Kettle 1995). Both blackflies and biting midges have been reported as blood feeders on many animals, including humans, other mammals and birds (Downes 1958, Kuusela 1979, Kettle 1995, Marquardt *et al*. 2000, Malmqvist *et al*. 2004). These insects play an important role as vectors of a diversity of pathogens such as viruses (Mellor *et al.* 2000, Mead *et al*. 2004), filarial worms (Shelley and Coscarón 2001, Kutin *et al*. 2004) and many other parasites including malaria-like parasites (Fallis and Bennett 1958, Bennett 1961, Votýpka and Svobodova 2004, Mullens *et al*. 2006). The lack of an effective method to capture these vectors from bird species while nesting until recently (Tomás *et al*. 2008a), may be the main reason for the scarcity of ecological studies on flying ectoparasites of wild birds. In fact, biting midges are considered as one of the least known groups of haematophagous insects (Mellor *et al*. 2000). It should be mentioned that some of these parasites are pests for livestock (Kettle 1995), and therefore of economic (Ratnayake *et al*. 2006) and sanitary (Braverman *et al*. 1996) interest, and that they may affect the conservation of endangered species (Adler *et al*. 2007).

The aim of this study is to identify the concomitant effects of different weather variables on the abundance of blackflies and biting midges in nests of three coexisting wild hole-nesting avian species, the blue tit *Cyanistes caeruleus* (previously *Parus caeruleus*), the pied flycatcher *Ficedula hypoleuca* and the great tit *Parus major* after control for different biotic factors potentially affecting these relationships. These avian species differ in several aspects of their breeding phenology including laying date, brood size and the duration of the nestling period (see Cramp 1998).

Methods

Our study was carried out during the spring of 2007 in a coexisting population of great tits, blue tits and pied flycatchers breeding in nest-boxes in a Pyrenean oak *Quercus pyrenaica* forest located in Valsaín (Segovia, 40° 53′ 74N, 4°01′ W, 1200 m a.s.l.). Wooden nest-boxes were hanging from branches at about 4 m above the ground. Periodical visits in the course of the breeding season allowed us to record hatching date and brood size of each nest. To capture flying insects, we placed inside and close to the roof of each nest-box a plastic Petri dish (8.5 cm diameter; 56.7 cm^2) with a thinly spread layer of approximately 0.5 ml of commercially available body gel-oil (Johnson's© baby chamomilla, Johnson and Johnson, Dusseldorf, Germany), facing downwards and supported on a wire netting following the method described and tested by Tomás *et al*. (2008a). Petri dishes were placed in nest-boxes when nestlings had

attained two thirds of the nestling period (10 days for great and blue tits and 9 days for pied flycatcher nestlings) and they were maintained during a period of 3 days. Although daily changes of Petri dishes could provide data to detect daily variation in biting flies activity this possibility was discarded to reduce avian disturbance during reproduction that could produce nest desertion. When Petri dishes were removed from the nest-boxes, the number of nestlings in each nest was recorded. In the laboratory, we quantified the abundance of blackflies and biting midges collected in Petri dishes under a binocular lens (Motic K700; 46.5 x magnification). Here we only include control nests from different experiment carry out in our avian populations, with the exception of an experiment consisting in supply snail shells to some great and blue tits while others were maintained as controls during the egg laying period. This treatment will not be considered in subsequent analyses because there were no significant effects of this experiment on insect abundance (all $p>0.34$).

The Spanish National Meteorological Institute provided weather data from the nearest meteorological station of Segovia, approximately 9 km from the study area. This station was selected for offering the more complete records of weather variables among the closer stations to the area. Data from this meteorological station have been previously used to found clear effects of weather on several factors related to avian biology in the same study area (Sanz *et al*. 2003, Lobato *et al*. 2006). Daily meteorological data provided include maximum and minimum temperature, rainfall and wind speed. The latter variable was recorded at four different times of the day (00:00, 07:00, 13:00 and 18:00, local time GTM + 01:00). To estimate temperature and wind speed during the insect sampling period, we calculated the average of each maximum and minimum temperatures and wind speeds recorded at each recording time during the period of vector capture. Also, to estimate the effect of rainfall on vector abundance, we calculated a presence/absence index of precipitation during the sampling period of biting flies.

Statistical analyses were conducted by using general linear models (GLM) (Statistica version 6.0, StatSoft, Inc. 2001). To investigate the variables affecting the total abundance of blackflies and biting midges we included in the model the avian host species (3 groups), the presence/absence of rainfall and their interaction as factors, as well as hatching date (a variable reflecting avian reproductive phenology), brood size, average minimum temperature, average maximum temperature and average wind speed as covariables. In order to simplify and because blackflies are considered diurnal while

the majority of biting midges are crepuscular (see introduction for more details and references), we only included average wind speed recorded at 7:00, 13:00 and 18:00 in the model explaining abundance of blackflies and average wind speed recorded at 00:00, 7:00 and 18:00 in the model explaining abundance of biting midges. The total abundances of blackflies and biting midges were logarithmically (log10) transformed to normalize their distributions. Residuals of each model were tested for normality.

Results

A total of 86 nests (33 blue tit nests, 36 pied flycatcher nests and 17 great tit nests) were included in this study. As expected, hatching date differed between species ($F_{2,83}$=66.17, p<0.001) with great tits breeding earlier in the season followed by blue tits and pied flycatchers. In addition, avian species differed in brood size ($F_{2,83}$=71.72, p<0.001). As expected from the ornithological literature, post-hoc analyses revealed that brood size was lower in pied flycatcher than in blue tits (LSD test, p<0.001) and great tits (p<0.001) while no significant differences between blue and great tits were found (p=0.18).

Table 1. Prevalence (%), mean abundance (± SD) and range of infection intensity of blackflies and biting midges captured in nests of great tits (*Parus major*), blue tits (*Cyanistes caeruleus*) and pied flycatchers (*Ficedula hypoleuca*) during the breeding season of 2007. Prev.= Prevalence, Abund.=Abundance.

	Blackflies			**Biting midges**		
	Prev.	Abund.	Range	Prev.	Abund.	Range
Great tit	88	10.9 ± 10.7	0-38	100	30.2 ± 41.7	1-132
Blue tit	88	9.0 ± 11.3	0-43	94	48.2 ± 61.2	0-266
Pied flycatcher	56	2.1 ± 3.5	0-18	100	173.8 ± 274.1	5-1587

Overall, 556 blackflies were captured in nests (Table 1). Results from the model are shown in Table 2 (model: adjusted r^2=0.52, p<0.001). The abundance of blackflies in avian nests was significantly and negatively associated with the minimum temperature (Fig. 1) and wind speed at 07:00 (Fig. 2) but positively with wind speed at 18:00. In addition, we found positive associations between the abundance of blackflies and brood size and hatching date. The rest of the variables included in the model were not significantly associated with the abundance of blackflies.

Table 2. Results of a General Lineal Model relating the abundance of blackflies in avian nests with variables under study. Significant relationships at P<0.05 are marked in bold.

	D.f.	F	P
Maximum temperature	1,73	0.003	0.96
Minimum temperature	1,73	4.00	**0.049**
Wind speed at 07:00	1,73	8.35	**<0.01**
Wind speed at 13:00	1,73	0.09	0.77
Wind speed at 18:00	1,73	4.80	**0.03**
Hatching date	1,73	9.45	**<0.01**
Brood size	1,73	21.12	**<0.001**
Presence of rainfalls	1,73	2.06	0.16
Avian species	2,73	0.46	0.63
Avian species*rainfall interaction	2,73	1.78	0.18

Table 3. Results of a General Lineal Model relating the abundance of biting midges in avian nests with variables under study. Significant relationships at P<0.05 are marked in bold.

	D.f.	F	P
Maximum temperature	1,73	<0.001	0.98
Minimum temperature	1,73	0.05	0.82
Wind speed at 00:00	1,73	0.15	0.70
Wind speed at 07:00	1,73	4.16	**0.045**
Wind speed at 18:00	1,73	1.96	0.17
Hatching date	1,73	7.52	**<0.01**
Brood size	1,73	8.94	**<0.01**
Presence of rainfalls	1,73	3.18	0.08
Avian species	2,73	5.11	**<0.01**
Avian species*rainfall interaction	2,73	1.04	0.36

Overall, 8360 biting midges were captured (Table 1). Results from the model are shown in Table 3 (model: adjusted r^2=0.39, p<0.001). The abundance of biting midges was significant and negatively associated with wind speed at 07:00. In addition, the abundance of biting midges was significant and positively associated with hatching date

and brood size. Also, biting midges was significantly more abundant in pied flycatcher nests than in tit nests, while differences between nests of blue and great tits did not reach significance. Finally, the abundance of biting midges tended to be higher with the presence of rainfalls. The rest of the variables included in the model were not significantly associated with the abundance of biting midges.

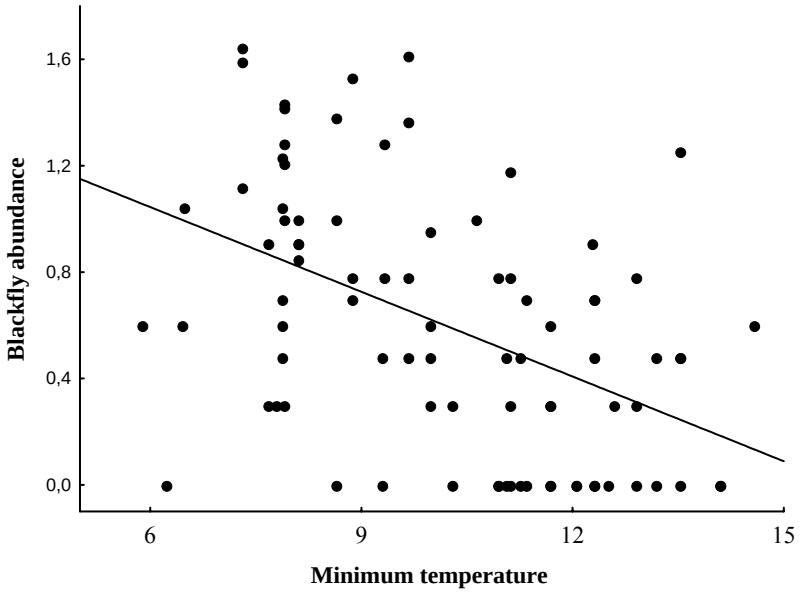
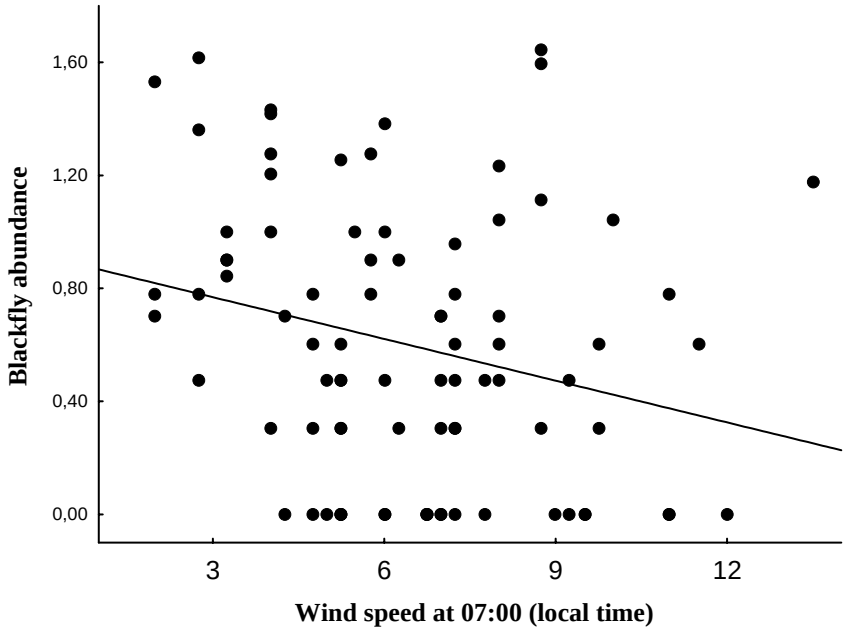


Figure 1. (up) Relationship between average minimum temperature and the abundance of blackflies (adj. r^2=0.22, p<0.001) in avian nests. The abundance of blackflies was log10 transformed. Temperature was measured in ºC. Regression line is shown.

Figure 2. (down) Relationship between the log10 transformed abundance of blackflies and average wind speed (Km/h) measured at 07:00 local time (adj. r^2=0.05, p=0.02). Regression line is shown.



Discussion

Here we present the first evidence of a strong effect of weather measured as minimum temperature and wind speed on the abundance of biting flies in avian nests. Environmental effects on flying insects may vary between insect species, in relation to their specific biological developmental requirements, breeding habitat preferences or their patterns of activity. The close association between weather variables makes it difficult to disentangle the potential differential effects of each weather variable on the abundance or activity of biting flies. However, studies in laboratory conditions found that the flight activity of blackflies varied in response to changes in either temperature or humidity conditions, while the other variable was kept constant (Shipp *et al*. 1998), suggesting that different combinations of humidity and temperature may affect the activity of these flies in the wild. In this respect, according to our results, the abundance of blackflies decreases with an increase in minimum temperatures suggesting that, in our study area, high minimum temperatures may negatively affect blackflies in their development or in host location activity. Also, if temperature and rainfall recorded during the capture period was partially related with conditions during previous days, it could be possible that the increase in the minimum temperature could indirectly affect the abundance of blackflies through its effect on the availability of breeding sites. That is, small streams in our study area usually dry up as temperature increases (authors pers. obs.) and it is known that blackflies need running water for breeding (Kettle 1995, Lehane 2005). In addition, the positive effect of rainfalls which may directly favour the availability of breeding sites is also partially supported by the marginally positive relationship found between the presence of rainfall and the abundance of biting midges.

In addition, we also found a clear negative effect of wind speed at 07:00 on the abundance of both blackflies and biting midges in avian nests. It is generally accepted that adult biting midges are nocturnal or crepuscular while the activity of adult blackflies is essentially diurnal, with a peak of activity early in the morning and ceasing with the onset of darkness (Lehane 2005, see more references in introduction). Our results agree with this pattern because higher wind speeds at these times may hinder biting flies from contacting hosts by reducing their flight capacity or affecting their capacity for host-location. Although the degree of the adverse effect of wind speed on insect abundance may vary between insect species (Edwards *et al*. 1987), it is generally accepted that catches of biting fly species are inversely related to wind speed with studies on different blackfly and biting midge species reporting the importance of wind

speed as an limiting factor affecting biting flies activity and biting habits (Davies 1957, Kettle 1969a, Kettle 1969b, Edwards *et al*. 1987, Fredeen and Mason 1991, Martin *et al*. 1994, Kettle *et al*. 1998, Maquardt *et al*. 2000). In addition, it was previously suggested by Smith *et al*. (1998) that the abundance of blackflies in avian nests may decrease as wind velocity increases, however in that study authors did not collect systematic data on wind velocity to test this assumption. For that reason, our results are, to our knowledge, the first describing negative effects of wind speed on the abundance of biting flies in avian nests. However, we also found a positive effect of wind speed at 18:00 on the abundance of blackflies in avian nests. The differential effect of wind measured at 07:00 and 18:00 on blackflies could be due to the fact that in the later time temperatures are usually hotter than early in the morning and wind could reduce the temperature favoring blackflies mobility. In fact, Berzina (1953) (see in Davies 1957) found that wind depressed the blackfly landing activity on man at unfavourable temperatures while stimulated it at optimum temperatures.

Also, our results support the role of biotic factors affecting biting flies host-seeking activity, especially those related to phenology, brood size and host species. The association found here between biting flies abundance and hatching date is in accordance with previous studies on biting flies and birds in the same area (Tomás *et al*. 2008b, Martínez-de la Puente submitted). Another study in central Spain, also found an increase in the abundance of blowfly pupae in pied flycatcher nests with the advancing season (Merino and Potti 1995) pointing out to an effect of weather conditions on development and/or activity of flying insects. In addition, we found a strong positive association between brood size and biting flies' abundance. Previously, Anderson and DeFoliart (1961) found that host size, more than other factors, explained the abundance of *Simulium rugglesi* being attracted to various bird species, and other studies lend support to the association between brood size of birds and mosquitoes and biting midges abundance (Dow *et al*. 1957, Tomás *et al.* 2008a, Martínez-de la Puente *et al*. submitted, but see Rätti *et al*. 2006). Moreover, we found a significant difference in the abundance of biting midges between tit and pied flycatcher nests. This fact also suggest that factors different from brood size such as a differential attractiveness of biting midges to bird coloration (see Bennett *et al*. 1972, Bradbury and Bennett 1974a, 1974b; but see Yezerinac and Weatherhead 1995) or simply the coincidence at the end of the season between the higher abundance of biting midges and pied flycatchers breeding period, could affect the differential abundance of these insects in avian nests.

To conclude, our results are especially relevant in a scenario of climate change that may contribute to the spread of several diseases, including those transmitted by haematophagous insects (Githeko *et al*. 2000). A better knowledge of determinants of biting fly densities is required given their role in favouring the evolution of virulence of several important diseases (Ewald 1994).

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CAPÍTULO 4

¿Son las infecciones múltiples de gametocitos en parásitos de la malaria una adaptación para asegurar su fertilidad?

Las infecciones múltiples, aquellas causadas por más de una parásito en el mismo eritrocito, podrían ser adaptativas para los parásitos de la malaria como un mecanismo para asegurar su fertilidad. Alternativamente, estas infecciones podrían ser una simple consecuencia de un proceso no adaptativo forzando a varios parásitos a competir por los recursos en una célula hospedadora. Aves hospedadoras infectadas con *Haemoproteus* fueron medicadas con primaquina o inyectadas con solución salina y en ellas se determinó la intensidad de infección así como el número, estado de maduración y sexo de las infecciones múltiples. De acuerdo a nuestros resultados, las infecciones múltiples dependen de la intensidad de infección, los gametocitos raramente alcanzan la madurez y cuando lo hacen, generalmente están formadas por gametocitos del mismo sexo. El papel de las infecciones múltiples como un mecanismo para asegurar la fertilidad de los parásitos no es apoyado por estos resultados.

Are multiple gametocyte infections in malarial parasites an adaptation to ensure fertility?

Josué Martínez de la Puente, Santiago Merino, Gustavo Tomás, Juan Moreno, Judith Morales, Elisa Lobato

Multiple infections, those by more than one parasite in the same erythrocyte, may be adaptive for the malarial parasite as a means to ensure fertility. Alternatively they may simply be the consequence of a non-adaptive process forcing several parasites to compete for resources in one host cell. Avian hosts infected with *Haemoproteus* were medicated with primaquine or injected with saline solution and the density of infection and number, maturity and sex of mature multiple infections counted. Multiple infections depend on density of infection, and maturity is attained rarely and usually by gametocytes of the same sex. The role of multiple infections for fertility insurance is not supported by these results.

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Introduction

Within the phylum Apicomplexa the haemospororins, or malarial parasites, produce sexual stages called gametocytes in their vertebrate host. Once in the dipteran vector, a macrogametocyte produces a single female gamete that must be fertilised by one of the several (up to eight) male gametes produced by each microgametocyte. Mated gametes tend to originate from a single blood meal within a few minutes from ingestion by the insect vector. For most species of these parasites the sex ratio in the blood sexual stage is easily identifiable in blood smears and several studies have pursued the adaptive significance of sex ratio (the proportion of male gametocytes) in malarial parasites when a single (or low numbers of) genotype is present in a host (Read *et al.* 1992, Paul *et al*. 1995, West *et al.* 2001, Read *et al.* 2002). Hamilton (1967) demonstrated that when a small number of females produce offspring that mate among themselves, a female skewed sex ratio is favoured by natural selection. This situation is called local mate competition (LMC) because the higher number of females reduces competition among brothers for mates (Taylor 1981). In the extreme, where all matings are between sibs, the prediction is that females produce enough sons to mate with all their daughters. Haemosporidians have life cycles that might lead to biased sex ratios through LMC; as males produce several gametes, a female skew in sex ratio may maximize successful transmission by optimizing zygote production (Ghiselin, 1974, Pickering 1980, Schall 1989, Read *et al.* 1992, Paul *et al*. 1995, West *et al.* 2001). However, less biased sex ratios may also be adaptive under several circumstances (Shutler and Read 1998, Paul, *et al.* 1999, 2000). For example, in cases of infections by several clones, those producing more male gametocytes will obtain more mates and thus a greater contribution to the next generation (Fisher 1930, Hamilton 1967, Read *et al.* 1992). Alternatively, under low gametocyte densities male gametes would have difficulty meeting female gametes and natural selection would favour a less female biased sex ratio to ensure mating of female gametes (Shutler and Read 1998, Paul *et al.* 1999, Paul *et al.* 2000, West *et al.* 2002, Gardner *et al.* 2003). The low probability of a male gamete encountering a female gamete can result from a low number of gametocytes in the vector´s bloodmeal, or reduced gamete mobility or viability due to immune responses. This "fertility insurance hypothesis" has received recent empirical support from an experimental study of variation on sex ratios in the avian malarial parasite *Haemoproteus*. A change from female-skewed towards male-skewed sex ratios

followed medication with the antimalarial drug primaquine, which produces a reduction in density of infection by the parasite (Merino *et al.* 2004).

Malarial parasites may produce multiple gametocyte infections (MGIs) where there is more than one parasite in the same erythrocyte (Fallis and Desser 1977, Jovani *et al.* 2004, Valkiūnas 2005). These infections have been reported in taxonomic studies of malarial parasites from several vertebrates and are usually considered as by-products of high asexual and sexual parasite densities of infection inducing the infection of the same cell by two parasites, which are thereby forced to compete for the food available for their development inside the cell (Valkiūnas 2005). However, Jovani (2002) has proposed that this multiple infection may also be a way to facilitate encounters between gametes and thus function as a fertility insurance mechanism. Jovani (2002) assumes two prerequisites for his hypothesis to work: 1) that male-female multiple infections naturally occur, and 2) that male-female multiple infections are viable. Both prerequisites are apparently fulfilled (Jovani 2002, Jovani *et al.* 2004) although viability of apparently mature multiple infections should be more thoroughly investigated. Although Jovani (2002) did not propose mixed multiple infections as an adaptive strategy by the parasite but as a beneficial by-product of a random process (Jovani personal communication), it is obvious that parasites being able to produce multiple infections by gametocytes of different sex may obtain a selective advantage when there exist difficulties for male gametes to reach female gametes. Thus, we propose that multiple infections by gametocytes of different sexes inside the same erythrocyte may be an adaptive strategy by malarial parasites to ensure fertility. In fact, this resembles adaptive syzygy (when a single male and a single female gametocyte pair together physically or in close proximity just prior to fertilization), and local mate competition in this case may therefore be not relevant (West *et al.* 2000).

The hypothesis of multiple infections as an adaptive strategy by the parasite to facilitate gamete encounters or of multiple infections as a non-adaptive by-product of high intensities of infection produce contrasting predictions which can easily be tested:

1) Under the adaptive hypothesis we expect an increase in the number of multiple infections as density (abundance of gametocytes in blood) decreases. This is because fertility insurance is especially needed in conditions of low density of gametocytes. However, under the non-adaptive hypothesis we can expect a higher number of multiple infections as density of infection increases, because it is more probable

that two parasites reach the same cell when many parasites are looking for cells to infect.

2) Multiple infections must be able to reach maturity by both parasites infecting the same cell in most cases under the adaptive hypothesis, but not under the alternative non-adaptive hypothesis where parasites may compete for resources, leading to most of them not reaching maturity.

3) Multiple infections may also be produced preferentially by parasites of different sexes if they are produced to ensure fertility, but this is not needed under the non-adaptive hypothesis (infection by parasites of the same sex may also be frequently found).

Gametocyte density in birds infected by *Haemoproteus* tends to show a maximum during host reproduction and to decrease towards the end of the reproductive season due to the effect of host immune responses (Atkinson and Van Riper 1991, Morales *et al*. 2004). In fact, if immune pressure reduces gametocyte or gamete survival or motility, a shift towards less biased sex ratios is predicted to favour fertility insurance (West *et al*. 2001). So we can also expect that reduction in gametocyte density due to immune responses may play a role in a potential adaptive response by the parasite producing more multiple gametocyte infections. Here we present data on the number of multiple infections, their maturity and sex in a wild population of breeding birds infected by the malarial parasite *Haemoproteus* and being subjected to experimental reduction of infection by medication with the antimalarial primaquine, in order to test these predictions. We compare the effects of experimental reductions and of natural reductions in gametocyte abundance due to immune responses on the number and composition of multiple gametocyte infections.

Material and methods

The study was conducted during the spring of 2004 using a blue tit (*Parus caeruleus*) population breeding in nest-boxes, in a Pyrenean oak (*Quercus pyrenaica*) forest in Valsain (Segovia, central Spain). Infection by malarial parasites in this population has been under study since 1994 (Fargallo and Merino 1999). Adult birds were randomly assigned to one of two treatments (medicated and control groups) when their nestlings were three days old. Birds were captured and injected subcutaneously with either 0.1 mg of the antimalarial primaquine (Sigma, St Louis, MO, USA) diluted in 0.1 ml of

saline solution (medicated group) or with the same volume of saline solution (control group). Primaquine has been previously used as a subcurative dose to reduce the density of infection by blood parasites in wild birds (Merino *et al.* 2000). Primaquine has been shown to have effects on gametocytes of several species of *Plasmodium* (Lopez-Antuñano 1999, WHO 2001). Apparently primaquine acts by binding and modifying the parasite´s DNA and disrupting parasite mitochondrial membranes (Lopez-Antuñano 1999, Baird and Rieckmann 2003). Based on these mechanisms of action, we did not expect a differential effect of medication on gametocytes of each sex. Before injection, a blood sample was obtained from the braquial vein (initial sample). Ten days later birds were recaptured and a second blood sample was obtained (final sample). Prepatence time for *Haemoproteus* is about 12-14 days as shown by the lack of infections on smears from nestlings of 13 days. For infections developed in internal organs, the time for release and maturation of new gametocytes should be lower. Therefore, ten days allows for the new production and release of gametocytes to blood and for maturation of current MGIs, while excluding any newly inoculated infections which can affect results after drug treatment.

Blood samples were immediately smeared and air-dried and later fixed in ethanol (96%) and stained with Giemsa (1/10 v/v) for 45 minutes. Each smear was examined for extra- and intra-erythrocytic haematozoa, following the procedure reported by Merino and Potti (1995) and Merino *et al.* (1997). We calculated the density of infection by *Haemoproteus majoris* as the number of parasites per 2000 erythrocytes scanned at 1000x magnification. The number of MGIs (two or three parasites infecting the same erythrocyte) was also counted per 2000 erythrocytes (Jovani 2004). All smears were checked by the same person (J.M-P). MGIs were considered mature when parasites were sufficiently developed to differentiate their sex (that is approximately when they reach at least 75% of their maximum size).

Samples were included in the analyses when found infected by the malarial parasite *Haemoproteus* in at least one of the two smears (initial or final sample). Host sex was not included in analyses as it did not influence the results. The intensities of parasite infection were normalized by log transformation (initial density K-S $d = 0.071$, $p > 0.20$; final density K-S $d=0.048$, $p > 0.20$).

Results

Overall, 115 adult blue tits were found infected (59 medicated and 56 controls). Before the primaquine injection (initial sample) no significant differences in gametocyte density were observed between individuals subjected subsequently to different treatments (t-test independent by groups; t=0.86; d.f.=113; p=0.39). The density of infection by *Haemoproteus* was significantly reduced for medicated birds (t-test for dependent samples: t=3.60; d.f.=58; p=0.001). There was also a reduction in gametocyte density for control birds but this was not significant (t=1.03; d.f.=55; p=0.31). Mean density (SE) was 26.88 (2.69) for initial infections (Controls= 23.05 (3.25); Medicated= 30.50 (4.22)) and 21.16 (2.48) for final infections (Controls= 21.62 (3.57); Medicated= 20.71 (3.48)).

The number of multiple infections detected and their maturity and sex are shown in Table 1. Only 3 multiple infections were triple and 109 were double. All triple and most of double infections were immature (Table 1). The number of mature MGIs is maintained for medicated and control hosts across the experiment (6 mature MGIs for medicated birds and 7 mature MGIs for controls). The number of MGIs was significantly and positively correlated with the density of the infection in both captures (Spearman Correlation; initial: r_s= 0.57; n= 115; p< 0.001; final: r_s= 0,70; n= 59; p< 0.001 for medicated and r_s= 0.48; n= 56; p <0.001 for controls). In addition, initial density of infection was significantly higher when MGIs were present as compared to infections where they were absent (t= 6.60; n= 115; p< 0.0001). This was also true for controls and medicated individuals for final samples (t= 3.47; n= 56; p= 0.001 and t= 7.05; n= 59; p< 0.001 respectively).

Table 1. Number of MGIs detected per 2000 erythrocytes with respect to their maturity and sex composition. Percentages are shown in parenthesis.

	Immature	Same sex	Male-female	N
Initial	45 (77.6)	13 (22.4)	0 (0)	32 (27.8)
Final	41 (75.9)	12 (22.2)	1 (1.9)	29 (25.2)
Total	86 (76.8)	25 (22.3)	1 (0.9)	61 (26.5)

On the other hand, we have not observed any relationship between treatment (medicated or control) and the presence or absence of MGIs for final samples (with Yates´ correction: χ^2_2=0.49; d.f.=1; p=0.49). The number of new MGIs found in final samples did not differ from the number of those lost for medicated or control hosts

(McNemar χ^2 <0.001; p>0.99 and χ^2 =0.45; p= 0.50 respectively). The number of MGIs counted on initial and final samples were significantly correlated (r_s= 0.32; n= 115; p< 0.001), although this circumstance was only maintained for the medicated group (r_s= 0.51; n= 59; p< 0.001) and not for controls (r_s= 0.079; n= 56; p= 0.56). The average MGI density and the ratio of MGIs to single infections for hosts with MGIs are shown in Table 2. Both measures indicate that MGIs are present in low densities/proportions as compared to single infections. Density and ratio did not vary between initial and final samples for medicated or control hosts (P>0.19, data not shown).

Table 2. Average MGI density (infections per 2000 erythrocytes) and ratio of MGI to single gametocyte infections for hosts with MGIs. S.E. is shown in parenthesis.

		Density MGIs	Ratio MGIs/single infections	N
Medicated	Initial	2.06 (0.41)	0.04 (0.01)	16
	Final	2.18 (1.74)	0.05 (0.03)	17
Control	Initial	1.56 (0.20)	0.08 (0.02)	16
	Final	1.42 (0.67)	0.05 (0.05)	12

Discussion

Our results do not support the adaptive hypothesis of multiple infections being a parasite strategy to ensure fertility. Infections by several merozoites in the same erythrocyte are relatively frequently encountered (26.5%), but they are dependent on a high density of infection and independent of a reduction due to both natural immunity and experimental medication. In his detailed review of avian malarias, Valkiūnas (2005) also reported that multiple infections are positively related to the density of infection.

Following the non-adaptive hypothesis we can expect a reduction in MGIs with reduction in density but an increase following the adaptive hypothesis. The higher reduction in density of infection produced in medicated hosts did not produce a higher number of MGIs as we could expect following the adaptive hypothesis. However, the number of new MGIs produced ten days after injection with primaquine did not differ from the number of MGIs lost in the same period, and therefore we can not support any of the hypotheses in this respect. In any case, the tight positive relationship between density of infection and MGIs is maintained for medicated hosts, and therefore the adaptive hypothesis is not supported. With respect to the second prediction, our data clearly support the non-adaptive hypothesis as immature MGIs represent more than 76% of the total number. Only infections by two parasites reach maturity (Valkiūnas

2005, this study). Although most multiple infections in our study are produced by two parasites, only a reduced number of MGIs reach maturity (23.2%). In addition, in only one case did we detect MGIs by parasites of both sexes and the number of mature MGIs did not change across the experiment with treatment. Therefore the third prediction of the adaptive hypothesis is also not supported.

The positive correlation between the initial and final number of MGIs only for medicated hosts may be related to the lower natural immune responses in medicated hosts (authors unpublished). In this case, it is possible that natural immunity in controls affects MGIs slightly more than in the case of medicated hosts, where density of infection is reduced mainly through medication and less through their own defences.

However, although not being a good mechanism to facilitate gamete encounters under low parasite density, multiple infections may be still of some help by ensuring fertility as they are present even in final samples and their number is significantly correlated with initial numbers for medicated individuals. However, for multiple infections being able to play a role in fertility insurance, they should develop to maturity before being ingested by the vector. Only if multiple infections produced by parasites of the same sex are viable, they may increase probabilities of fertilization by altering the sex ratio of blood parasites in the vector blood meal. Overall, these facts imply that multiple infections are not very effective in ensuring fertility in *Haemoproteus majoris* infections. Our results show that multiple infections are more probably produced by a random infection when intensities are high and that they probably do not play an important role in fertility insurance for malarial parasites. However, as most of MGIs apparently do not attain maturity, competition by resources inside erythrocyte may not be the cause of this failure. Double infections may be caused when two merozoites being antibody cross-linked contact and infect the cell at the same time (Ramasamy *et al.* 1999). That being the case, merozoites in double infections may be negatively affected by previous contact with antibodies. Moreover, gametocyte sex ratios in this species have been found to be close to 50:50 in similar conditions (Merino *et al*. 2004), so we can expect about 50 % of infections by gametocytes of the same sex and about 50% of male-female infections. However, these proportions are clearly not found in our sample (Table 1). One potential explanation for this fact is that antibodies were only able to cross-link merozoites of the same sex. In this case MGIs were not produced at random and the successful transmission of the parasite was indeed reduced in this way. The infection of the erythrocyte may allow parasites to avoid further immune responses

but not necessarily to increase successful transmission, as maturity by these infections is not always completed. In the case that multiple infections have more difficulties to attain maturity or that they are not viable, the double infections produced by bivalent antibodies may reduce successful transmission of the parasite. Mechanisms of reduction in transmission efficiency may finally render increases in virulence, whilst parasites try to maintain higher densities in hosts' blood. Alternatively, it is possible that the red blood cell type may determine which gametocyte sex is able to develop inside, thus producing preferentially MGIs of the same sex, or even that MGIs were produced close to schizonts producing, preferentially or exclusively, cells of one sex. On the other hand, it is possible that MGIs are important in species were red cells are likely to become limiting, for example when both asexual and sexual parasites utilize the red cells. Another possibility is that parasites do not have a facultative MGI response to their own population size, and might only be selected for in species where gametocyte density is always low enough to compromise fertilisation success. All these possibilities merit further investigations.

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CAPÍTULO 5

¿Puede el sistema inmune del hospedador provocar invasiones múltiples de eritrocitos *in vivo*? Efectos diferenciales de la medicación y del sexo del hospedador en un modelo de estudio silvestre relacionado con la malaria.

Las invasiones o infecciones múltiples (MIs), aquellas en las que más de un parásito infecta un mismo eritrocito, pueden ser el resultado de altas intensidades de infección o, por el contrario, deberse a factores relacionados de los parásitos o con el hospedador. De acuerdo con esta última posibilidad, hasta donde nosotros sabemos, sólo tres estudios sobre malaria desarrollados en el laboratorio sobre malaria han encontrado un incremento en la ocurrencia de invasiones múltiples en presencia de anticuerpos frente a antígenos parasitarios. En este contexto, comprobamos la posibilidad de que la ocurrencia de invasiones múltiples esté influenciada por el estado del sistema inmune del hospedador, usando como modelo de estudio el parásito emparentado con la malaria *Haemoproteus* infectando al herrerillo común (*Cyanistes caeruleus*). Las aves infectadas con *Haemoproteus* se medicaron con primaquina o con solución salina y se determinó su intensidad de infección y la presencia de invasiones múltiples. La medicación redujo significativamente la intensidad de infección por *Haemoproteus* en las hembras pero no en los machos. En el caso de las hembras, la presencia de invasiones múltiples estuvo positivamente asociada con la intensidad de infección y con el nivel de inmunoglobulinas en ambas capturas pero no encontramos asociación significativa entre el tratamiento y la presencia de invasiones múltiples. En los machos, la intensidad de infección pero no el nivel de inmunoglobulinas estuvo positivamente asociado con la presencia de invasiones múltiples. Además, los machos medicados presentaron más frecuentemente invasiones múltiples que los machos control. Estos resultados representan una primera evidencia en la naturaleza sobre la posible implicación del sistema inmune del hospedador produciendo invasiones múltiples.

Can the host immune system promote multiple invasions of erythrocytes *in vivo*? Differential effects of medication treatment and host sex in a wild malaria-like model.

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Multiple invasions (MIs) or infections, those by more than one parasite in the same erythrocyte, could be the result of parasite density or, alternatively, be due to parasite related factors or host related factors. According to the last possibility, to our knowledge, only three laboratory studies of malaria have found an increase in the occurrence of MIs when antibodies to parasite antigens were present. Therefore, we tested the possibility that MIs were influenced by host immune status, using as model the malaria-like parasite *Haemoproteus* infecting blue tits (*Cyanistes caeruleus*). Avian hosts infected with *Haemoproteus* were medicated with primaquine or injected with saline solution and the density of infection and the presence of MIs counted. Medication treatment reduced significantly the density of infection by *Haemoproteus* in females but not in males. For females, the presence of MIs was positively associated with both the density of infection and the immunoglobulin levels on each capture, but no association was found between the treatment and the presence of MIs. For males, the density of infection but not the immunoglobulin levels was positively associated with the presence of MIs. In addition, medicated males supported more MIs than controls. Our results represent the first line of evidence in the wild for a possible role of host immune system promoting MIs.

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Introduction

Traditionally, multiple invasions (MIs) of erythrocytes in malaria and malaria-like diseases have been considered as a by-product of higher parasite densities as shown by studies on infections in both human and non-humans (Wang 1970, Fallis and Desser 1977, Valkiūnas 2005, Martínez-de la Puente *et al.* 2006). However, in recent years, other host and parasite related factors have been also considered as determinants of the occurrence of MIs. For example, Jovani (2002) proposed that MIs composed by male and female gametocytes could be a random phenomenon that could increase the transmission success in malaria parasites. Under this assumption, a "parasite adaptive hypothesis" of MIs was proposed by Martínez-de la Puente *et al.* (2006), suggesting that parasites could promote MIs to increase their probability to be paired into the vector and thus to increase their possibilities of successful transmission.

Alternatively, MIs could be induced by host-related factors. Studies in the laboratory have found clear associations between host immune defences and the occurrence of MIs (Miller *et al.* 1984, Frazén *et al.* 1989, Ramasamy *et al.* 1999). Miller *et al.* (1984) found that the presence of monoclonal antibodies (mAb) to *Plasmodium knowlesi* reduced the total number of infected cells and increased the number of MIs. Later, Frazén *et al.* (1989) found an increase in both the number of single invasions and MIs by *P. falciparum* parasites in cultures when a mAb to an asparagine-rich protein of *P. falciparum* was added to the medium. More recently, Ramasamy *et al.* (1999) in an *in vitro* study also found an increase in the number of MIs when a specific antibody against *P. falciparum* was present. However, contrary to Frazén *et al.* (1989), Ramasamy *et al.* (1999) found that the increase in the number of MIs was not always accompanied by a change in the total number of infections. In addition, Ramasamy *et al.* (1999) also found that the presence of IgG at concentrations of 20-200 μg/ml from hosts previously infected with *Plasmodium* significantly increased the occurrence of MIs with respect to similar concentrations of IgG from pre-immunized animals, suggesting that the same process could also occur *in vivo* (Ramasamy *et al.* 1999).

According to a host adaptive hypothesis of MIs, the increase in the number of MIs by the effect of host immune system may benefit hosts if they reduce the number of infected cells and consequently the anaemia provoked by the rupture of infected erythrocytes. At the same time, due to the difficulties suffered by parasite gametocytes to reach maturity in MIs (Ahmed and Mohammed 1978, Inselburg 1983, Martínez-de la Puente *et al.* 2006), hosts could therefore affect the parasite transmission success

reducing the probability of infection or re-infections in their population as well as the number of parasite reservoirs.

The aim of this study is to investigate if the level of total immunoglobulins is involved in the occurrence of MIs in nature as has been proposed here according to previous studies in the laboratory.

Material and methods

This study was conducted during the spring of 2004 using a wild population of blue tits *Cyanistes caeruleus* breeding in nest-boxes in Valsaín, near Segovia, central Spain (40^o 53' N, 4^o 01' W). Adult birds were captured at two different stages of reproduction, first when attending nestlings of 3 days of age (initial capture) and ten days later (final capture). On each capture, a blood sample was obtained from the brachial vein. One drop of the blood sample was immediately smeared, air-dried and fixed with absolute ethanol. The rest of the blood was conserved in a cold-box to minimise protein degradation until centrifugation in the laboratory (on the same day) to separate sera and cellular fractions and, later, frozen until molecular analyses. After the initial blood sample was obtained, as a part of other previous study on MIs (Martínez-de la Puente *et al.* 2006), birds were assigned to either medicated or control groups. Medicated birds were injected subcutaneously with 0.1 ml of the antimalarial drug primaquine diluted in saline solution (concentration $1 mg \cdot ml^{-1}$; Sigma, St Louis, MO, USA) and controls were injected with the same volume of saline solution. Ten days later, birds were recaptured to obtain the final blood samples that were handled as described above. However, samples differ between the previous (Martínez de la Puente *et al.* 2006) and the present study because we failed to get enough blood for immunoglobulin analyses from some birds in that study but here we include other birds that were not recaptured. Brood size was quantified at each capture.

Blood smears from both captures were stained with Giemsa (1/10 v/v) for 45 minutes and scanned for parasites under the microscope at 100X magnification. The density of infection by *Haemoproteus majoris* gametocytes was calculated as the number of infected cells per 2000 red blood cells (Godfrey *et al.* 1987). Smears from infected individuals were selected to determine the presence or absence of MIs for the same number of erythrocytes. Serum was employed to quantify the level of total immunoglobulins by a direct enzyme linked immunosorbent assay (ELISA), using polyclonal rabbit anti-chicken immunoglobulin conjugated with peroxidase (Sigma, St

Louis, MO,USA). Absorbances were measured using a plate spectrophotometer at λ = 405nm. Details and validation of the method have been described in Martínez *et al.* (2003).

The density of infection at each capture was log-transformed to normalize the distribution. For each sex, the effect of the treatment was analyzed using repeated-measures ANOVA including the initial and final density of *Haemoproteus* in the blood as dependent variables (repeated-measures) and medication treatment as factor. The rest of the statistical analyses were performed by generalized linear models (GLZ) for binomial distributions. For initial capture, analyses were performed including the presence/absence of MIs in smears from infected hosts as dependent variable and the level of total immunoglobulins and the density of the parasite as continuous variables. For the final capture, the presence/absence of MIs was included as dependent variable, the density of the parasite and the level of total immunoglobulins were included as continuous variables and medication treatment as factor.

Results

Females showed a significantly reduced density of infection by *Haemoproteus majoris* when medicated ($F_{1,57}$=5.81, p=0.02). By contrast, the primaquine injection did not reduce significantly the density of infection by *Haemoproteus* in medicated males with respect to controls ($F_{1,53}$=0.32, p=0.57). In addition, the level of total immunoglobulins was significantly higher in females than males at initial capture ($F_{1,127}$=15.01, p<0.001). This was not the case for final samples ($F_{1,107}$=1.80, p=0.18). Brood size is not significantly correlated with immunoglobulins in males or females (p>0.07 in all cases).

Table 1. Samples sizes of *Haemoproteus* hosts of both sexes with and without MIs, tramped at each capture.

	With MIs	Without MIs
Females		
Initial capture	20	46
Final capture	15	39
Males		
Initial capture	16	47
Final capture	13	42

Female hosts

A total of 66 and 54 females infected by *Haemoproteus* were trapped on the initial and final capture respectively and their level of immunoglobulins measured (sample sizes are shown in Table 1). At initial capture, the presence of MIs was significantly and positively related to both the density of infection and the immunoglobulin level (Fig. 1a; density of infection: Wald= 11.21, $p<0.001$; immunoglobulin level: Wald= 4.03, p= 0.045). This was also true for the final capture (Fig. 1b; density of infection: Wald= 12.66, $p<0.001$; immunoglobulin level: Wald= 4.70, p= 0.03). In females, the presence of MIs was not associated to the primaquine treatment at final capture (Wald= 0.68, p= 0.41).

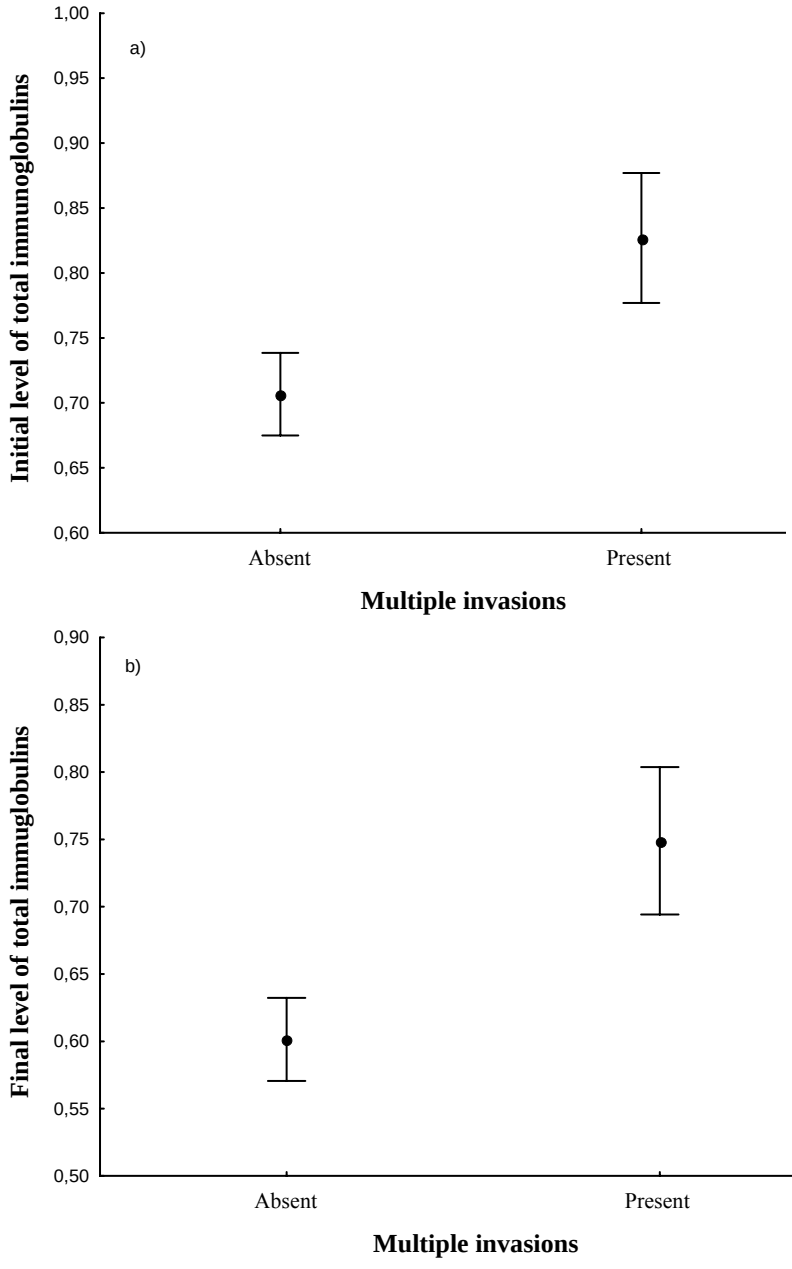

Figure 1. Female initial (a) and final (b) immunoglobulin levels in relation to the presence of MIs. Graphs represent the associations between both variables after statistical control for the effect of the parasite density of infection (initial and final levels respectively) and the primaquine treatment (for final samples). Bars show standard error.

Male hosts

A total of 63 and 55 males infected by *Haemoproteus* were trapped on the initial and final capture respectively and their level of immunoglobulins measured (sample sizes are shown in Table 1). On the initial capture, the presence of MIs was significantly and positively related to the density of infection (Wald= 12.04, p<0.001). No association between the presence of MIs and the immunoglobulin levels was found (Wald= 0.02, p= 0.89). At final capture, the presence of MIs was positively associated with the density of infection in males (Wald= 11.22, p<0.001). No association between the presence of MIs and immunoglobulin levels was found (Wald= 0.18, p= 0.67). The presence of MIs was associated with the primaquine treatment (Fig. 2; Wald= 4.05, p= 0.044), medicated males presenting more MIs than controls.

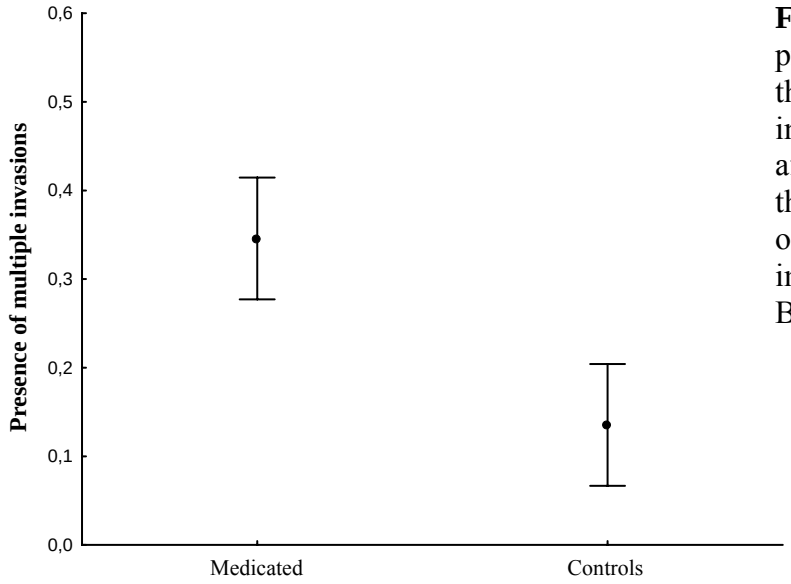

Figure 2. Effect of primaquine injection on the occurrence of multiple invasions in host males after statistical control for the final parasite density of infection and the immunoglobulins level. Bars show standard error.

There was also no significant association between brood size and parasite density or MIs (p> 0.09, data not shown), except for a positive correlation between final density and brood size for males once controlled for treatment effect (brood size: $F_{1,53}$= 4.07, p=0.049; treatment: $F_{1,53}$= 2.24, p=0.14). However, brood size is not significant when introduced in analyses presented in this work and the rest of significant results are maintained, except for the relationship between medication and MIs which change from p=0.044 to p=0.11 when brood size is included.

Discussion

Usually, studies in human and non-human malaria and related diseases have reported the occurrence of MIs is tightly associated to elevated parasitaemias (Wang 1970, Fallis and Desser 1977, Ahmed and Mohammed 1978, Valkiūnas 2005) independently of the host sex (Martínez-de la Puente *et al.* 2006, this study). However, according to the studies of Miller *et al.* (1984) and Ramasamy *et al.* (1999) the host immune system could be also involved in the occurrence of MIs. Our results in female blue tits clearly support this possibility, although this was not the case for males. It is known that sexual differences in behaviour, physiology and, especially, immune defence are important factors determining sexual differences in host parasite load (Zuk and McKean 1996). In addition, the fact that males had lower immunoglobulin levels than females could be the reason, at least in part, for the absence of its effect on promoting MIs in males.

Different possibilities have been proposed to explain the mechanisms whereby antibodies promote MIs. Miller *et al.* (1984) proposed that MIs could be caused by a weak agglutination of merozoites by monoclonal antibodies after the rupture of infected blood cells. Later Ramasamy *et al.* (1999) suggested that cross-linked parasites by bivalent antibodies can recognize erythrocyte ligands and complete invasion together or following dissociation from the antibody in close proximity to the erythrocyte previous to independent invasion of the cell.

In addition, our results imply a differential effect of the primaquine treatment with respect to host sex. Several factors may account for the differential efficacy of the primaquine treatment between sexes that could affect the efficacy of the drug reducing the intensity of infection. Many other reports in medical and veterinarian literature have identified host sex as a key factor affecting drug pharmacokinetics at two different levels, the absorption and the metabolism of drugs (Gordi *et al.* 2002, Pinsonneault and Sadeé 2003; Klein 2004) although, in humans there are no differences in the kinetic parameters of primaquine between sexes (Elmes *et al.* 2006). Also, other factors tightly related to sexual characteristics, such as hormone concentrations and genetic differences, may be involved in the influence of sex on drug pharmacokinetics (Pinsonneault and Sadeé 2003). In addition, males are slightly larger and heavier than females but were treated with the same dose of medication which may account for the differential effect of primaquine between sexes. Although the effect of the primaquine reducing the density of infection in males could result effective for a shorter period in

male versus female hosts, it could be possible that medication benefit males for a longer period if the higher number of MIs contribute to reduce the number of single infected erythrocytes. In this respect, although in females the immunoglobulin level was positively associated with the occurrence of MIs independently of the medication treatment, in males, the drug may be directly involved in the production of MIs or alternatively induce male natural immunity other than immunoglobulin levels to promote MIs. However, the effects of medication on promoting MIs in males should be taken with caution as when reproductive effort measured as brood size is included in analysis, the relationship between MIs and medication is not longer significant. To conclude, our results represent the first evidence for the role of the host immune system in promoting the occurrence of MIs in nature, as was expected according to a host adaptive hypothesis of MIs induction.

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CAPÍTULO 6

Factores que afectan la ocurrencia de invasiones múltiples por *Plasmodium* y otros parásitos emparentados. ¿Una característica omitida de las infecciones?

Las invasiones múltiples (MIs) se producen cuando más de una célula parásita infecta un mismo eritrocito. Estas invasiones múltiples ocurren en diferentes especies de haemosporidios incluyendo *Plasmodium* y otros parásitos emparentados. No obstante, la ocurrencia de estas invasiones múltiples ha sido tradicionalmente considerada en estudios *in vivo* e *in vitro* como un artefacto debido a altas intensidades de infección, influyendo en el hecho de que un considerable número de investigadores piensen que las invasiones múltiples son un producto meramente debido al azar. Por otro lado, otras explicaciones propuestas sobre la ocurrencia de invasiones múltiples incluyen la posibilidad de que estas infecciones sean una estrategia adaptativa del hospedador para reducir el daño parasitario y dificultar el éxito de transmisión del parásito así como una estrategia adaptativa del patógeno con la que favorecer su éxito de transmisión. Aquí realizamos una revisión sobre la literatura relevante presentando evidencias a favor y en contra de estas hipótesis propuestas para explicar la ocurrencia de invasiones múltiples. Aunque la posibilidad de que estas invasiones múltiples sean debidas a altas intensidades de infección ha recibido un gran apoyo, son necesarios más estudios con los que clarificar la posible importancia de las defensas del hospedador y estrategias parasitarias en la ocurrencia de invasiones múltiples en la naturaleza.

Factors affecting multiple invasions of erythrocytes in *Plasmodium* and other malaria-like parasites. A neglected characteristic of infections?

Josué Martínez-de la Puente, Santiago Merino

Multiple invasions (MIs) are produced when the same erythrocyte is infected by more than one parasite cell. These MIs commonly occur in different haemosporidia species including *Plasmodium* and other malaria-like parasites. However, the frequency of MIs has been traditionally considered in studies both *in vivo* and *in vitro* as an artefact produced by high parasite densities, leading most researchers to think that MIs does not have a true biological meaning but they are merely the product of chance. Other proposed explanations for the occurrence of MIs include an adaptive host strategy to reduce parasite damage and hinder parasite transmission success and an adaptive parasite strategy which favours parasite transmission success. Here we review the relevant literature supporting or rejecting these hypotheses proposed to explain the occurrence of MIs. Although the possibility that MIs being due to higher parasite densities has received much support, more studies are clearly necessary to reveal the potential importance of host defences and parasite strategies on the occurrence of MIs in nature.

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Haemospororins, or malaria-like parasites, infect blood-sucking dipterans and several classes of vertebrates. The life cycle of these parasites includes both sexual and asexual phases. Within the vertebrate hosts, after undergoing asexual reproduction, each merozoite invades a red blood cell (RBC), developing into either a gametocyte (gamete precursors) or a meront (schizonts). In some species, such as *Plasmodium,* both meronts and gametocytes can be found infecting RBCs, and in other species, such as *Haemoproteus* and *Leucocytozoon,* meronts do not infect erythrocytes.

In the vertebrate host, each infected RBC usually contains only one parasite cell but, however, multiply invaded RBCs are also found. Many studies have found MIs in nature with cases reported for all *Plasmodium* species infecting humans (Wang 1970, Poirriez *et al.* 1991, Marzars *et al.* 1997) and even for several *Plasmodium* species infecting non-human hosts (Bungener 1979, Valkiūnas 2005). MIs are also produced by other malaria-like haemospororins including *Haemoproteus* (Ahmed y Mohammed 1978, Lainson y Naiff 1998, Jovani et al. 2004) and *Leucocytozoon* species (Valkiūnas 2005) infecting birds and reptiles. Also, reports of MIs by other blood parasites with intraerythrocytic stages, such as *Hepatozoon* (Mackerras 1959, Bettiol *et al.* 1996, Lainson *et al.* 2003), *Babesia* (Merino 1998, Peirce 2000) and *Karyolysus* (Hussein 2006) exist in parasitological literature.

Traditionally, the frequency of multiple invasions (MIs) has been considered both in studies *in vivo* and *in vitro* as an artefact produced by high parasite densities, with the intensity (density) of infection as the main factor determining their occurrence (Wang 1970, Martínez-de la Puente *et al.* 2006). In this respect, the Poisson distribution has been proposed as an adequate model to predict the abundance of MIs according to the density of gametocytes (Jovani y Sol 2005). Also, differences in the intensity of infection could be the key factor explaining differences in the occurrence of MIs between *Plasmodium* species infecting humans. For example, MIs are more commonly observed in *P. falciparum* infections (Wang 1970) while *P. vivax* MIs have been considered as an uncommon phenomenon (see references in Prasad *et al.* 1990). Therefore the higher densities of infection that usually occur in patients infected by *P. falciparum* as compared to patients infected by *P. vivax* may explain, at least in part, these differences. In fact, when *P. vivax* reaches high parasitaemias (high intensities of infection), MIs are also found (Witzig and Barker 1994, Poirriez *et al.* 1995). Thus, the role of the parasite density (intensity of infection) as the main factor affecting the occurrence of MIs has received considerable support. However other studies suggest

that parasites and host related factors could be also important to determine the occurrence of MIs in nature (Fig 1).

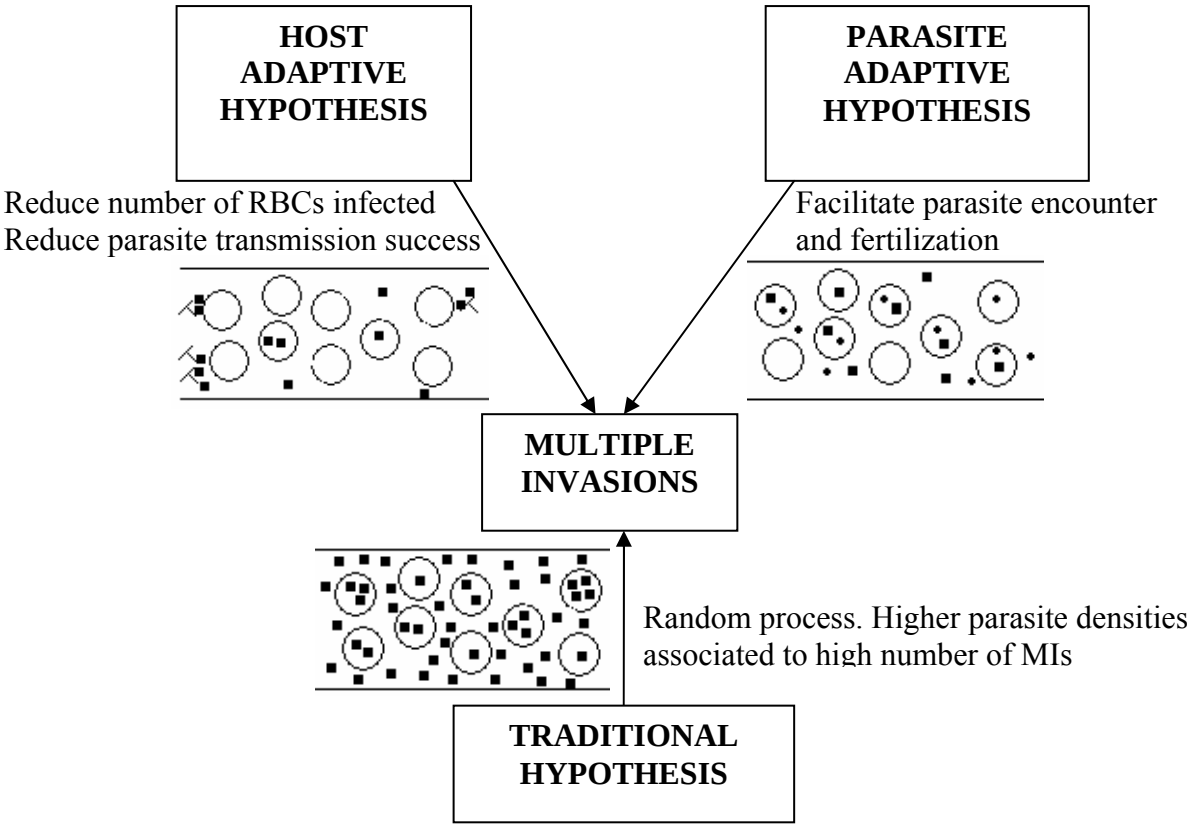

Figure1. Hypotheses proposed to explain the occurrence of MIs. According to the host adaptive hypothesis, host antibodies could cross-link parasites and promote MIs to reduce the number of single invasions and increase the difficulties of parasite to reach maturity at the same time that may reduce the parasite probability of transmission (parasites are represented as small squares and RBCs as empty circles). The parasite adaptive hypothesis implies that parasites promote the occurrence of MIs comprising gametocytes of different sexes (represented as small squares and points) to facilitate parasite encounter and fertilization in the insect vector. Alternatively, according to the traditional hypothesis, the occurrence of MIs could be a random process exclusively dependent of the parasite density of infection, with higher number of MIs as parasitaemia increase.

Recently, Jovani (Jovani 2002) proposed that MIs, even when produced at random, could be a way to increase parasite transmission because infections of male-female gametocytes into the same RBC could ensure an encounter between gametes into the gut of mosquito vector. In this sense, co-invasion of male and female gametocytes could be a parasite adaptive strategy to increase their transmission success (Martínez-de la Puente *et al.* 2006). However, if multiple invasions are an adaptive strategy by the parasite to facilitate gametocyte encounter, MIs should fulfil several predictions

(Martínez-de la Puente *et al.* 2006) according to fertility insurance hypothesis (West *et al.* 2002): i) male and female gametocytes in MIs must reach maturity to be capable to undergo sexual reproduction in the insect vector, ii) MIs must be produced preferentially by gametocytes of different sexes and iii) the frequency of MIs must increase as parasitaemia (intensity of infection) in the vertebrate host decrease, because the higher difficulty of male and female gametocyte to meet in the insect vector increase as the number of gametes being ingested in the vector blood meal decrease. A recent study using an avian malaria-like model did not support these predictions (Martínez-de la Puente *et al.* 2006), perhaps because *Haemoproteus* parasites are able to develop other strategies to assure fertilization in the vector (Merino *et al.* 2004). However, infection by *P. falciparum* parasites from patients who displayed uncomplicated malaria and supported parasitaemias lower than 10% of the total RBC, produced a higher proportion of MIs than did those infections that caused severe or cerebral malaria (Simpson *et al.* 1999, Chotivanich *et al.* 2000) where density of parasites is usually high. This result may support that MIs could be selected for in parasite species or isolates where gametocyte density is always low enough to compromise parasite fertilization success. If those parasites that caused uncomplicated malaria produced mature male-female gametocyte infections, they could maintain transmission and sexual reproduction success at lower parasite densities. In addition, these parasites could increase the lifespan of their host, by reduced cell invasion and destruction, and consequently increase the time available for successful parasite transmission.

On the other hand, different studies have found evidences for the role of immunological defences affecting the occurrence of MIs (Miller *et al.* 1984, Ramasamy *et al.* 1999, Martínez-de la Puente *et al.* 2007). Hosts mediated factors promoting MIs may imply beneficial effects for them due to the reduction of the total number of RBCs infected inside the individual host. In addition, due to the difficulties of parasites to reach maturity in MIs (Inselburg 1983, Martínez-de la Puente *et al.* 2006), the host induction of MIs could render an indirect benefit for hosts by reducing the parasite transmission efficacy and therefore the probability of infection or reinfections in the population of hosts and reservoirs. This possibility could evolve not for a group selection process but for the potential selection of lower virulent parasite strains in hosts when the transmission efficacy of the parasite is reduced (Ewald 1994). That is, if transmission efficacy of the parasite is lowered by host responses, a less virulent, more chronic disease with longer infection (and transmission) time will be favoured.

Alternatively, the positive association between antibodies and MIs could reflect the fact that parasites in MIs survive poorly and as a result, they stimulate the development of the host immune system more than single-infecting parasites. The first evidence for these possibilities was provided by Miller *et al.* (1984) who studied *Plasmodium knowlesi*, and reported an increase in the occurrence of MIs associated with the presence of the monoclonal antibody (mAb) against to a *P. knowlesi* protein. Subsequent studies, both in laboratory and nature, also support the role of host immune system promoting multiple invasions (Ramasamy *et al.* 1999, Martínez-de la Puente *et al.* 2007) but see (Frazén *et al.* 1989). Two mechanisms for MIs being produced by immune response have been suggested: (i) as the result of the invasion together of parasites cross-linked by the same antibody; or (ii) by several antibody-dissociated merozoites in close proximity to the same RBC (Ramasamy *et al.* 2001). More experimental studies *in vivo* are necessary to reveal the actual effect of antibodies on MIs in nature. In addition, other hosts related factors such as host age (M. A. Peirce pers. comm.) or sex could also play a role in the occurrence of MIs by the association of those variables with both, the parasite intensity of infection and the immune response of hosts. Other factors such as differential susceptibility of RBCs to infection or erythrocyte abnormalities such as thalassemia could also affect the occurrence of MIs.

MIs are a characteristic of infections by haemosporidian parasites in blood cells from vertebrate hosts with potential important consequences for the success of the parasite and/or the host. Although many reports exist for the occurrence of MIs in nature, the majority of them could be considered as case reports and more studies on the role of host immune system or the molecular selectivity affecting the occurrence of MIs should be done in order to clarify their role in these infections. In this respect, it is especially interesting to understand the host-related mechanisms involved in the occurrence of MIs potencially reducing the parasite transmission success and the negative effects of parasites on hosts. To test some of these possibilities, researchers could use samples obtained in other previous studies such as blood smears from human malaria patients. These smears could be employed to study in depth the random or non-random distribution of MIs, because usually blood samples are available before and after antimalaria medication treatments. In those cases, under the parasite adaptive hypothesis, we could expect that the number of MIs should increase as parasite density is reduced by medication.

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CAPÍTULO 7

Las proteínas de estrés aumentan la supervivencia de una población de aves silvestres sometida a presión parasitaria.

Los organismos se encuentran sometidos a diferentes agentes estresantes ambientales en la naturaleza y los mecanismos que mitigan el impacto del estrés han sido por tanto, favorecidos por la selección natural. Uno de estos factores biológicos que producen estrés es el parasitismo, el cual está ampliamente distribuido en la naturaleza y tiene una importancia potencial sobre la supervivencia de los hospedadores. Encontramos un efecto positivo de la manipulación directa de la intensidad de infección por parásitos sanguíneos mediante medicación con el fármaco antimalárico primaquina y del cambio en los niveles sanguíneos de proteínas de estrés (HSP60 y HSP70) sobre la tasa de supervivencia local entre estaciones reproductoras del herrerillo común *Cyanistes caeruleus* L. En el año 2004, la medicación con primaquina redujo la intensidad de infección por *Haemoproteus majoris* en hembras pero el tratamiento no redujo significativamente la intensidad de infección en machos. En consonancia con este resultado, el tratamiento de medicación se relacionó positivamente con la supervivencia local de las hembras hasta la siguiente primavera pero no afectó significativamente la supervivencia local de los machos. Además, cuando incluimos en los análisis el nivel de inmunoglobulinas y el cambio en los niveles de proteínas de estrés, encontramos que el cambio en estos últimos conjuntamente con la interacción entre la medicación y el sexo fueron las principales variables que explicaron la supervivencia local de los adultos. En conclusión, nuestros resultados representan un interesante ejemplo natural sobre el papel evolutivo de las proteínas de estrés (HSPs) en poblaciones silvestres y, hasta donde conocemos, el primer estudio experimental demostrando el efecto negativo de los parásitos sanguíneos y el efecto positivo de las proteínas de estrés en la supervivencia local de una población de aves silvestres.

Blood stress proteins improve survival under parasite pressure in a wild bird population

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Organisms are subjected to different environmental stresses in the wild and mechanisms to buffer the impact of stress have been strongly favoured by natural selection. One of the biotic factors producing stress is parasitism which is almost ubiquitous in nature and potentially important for host survival. By direct manipulation of the intensity of infection by blood parasites and look for survival effects we found a positive effect of the antimalarial drug primaquine and of the change in levels of stress proteins (HSP60, HSP70) from blood on the local survival rate of blue tits *Cyanistes caeruleus* L. between breeding seasons. In 2004, the primaquine treatment reduced the intensity of infection by *Haemoproteus majoris* in females but it was not significantly effective in the reduction of the parasite load in males. In agreement with this fact, the medication treatment was positively associated with female local survival until next spring but the treatment did not affect significantly male local survival. In addition, when the change in the levels of stress proteins and immunoglobulins were included in the analyses, we found that the change in levels of stress proteins together with the interaction between sex and treatment were the main variables affecting adult local survival. In conclusion, our finding represents an interesting field example of the evolutionary role of stress proteins (HSPs) in natural populations and to our knowledge, the first experimental study reporting the detrimental effect of blood parasites and the positive effect of stress protein response on local survival in wild birds.

Functional Ecology, enviado.

Introduction

All living beings are subjected to different kinds of environmental stresses that in different ways and levels compromise survival. Under these conditions natural selection have favoured a sort of mechanisms that allow for reduction and buffering of the effects of these stressors. One of the most thoroughly extended biotic producers of stress for living beings are parasites. These organisms are important in driven the evolution of their hosts and reducing their survival probability. However, the study of parasite-induced mortality is far from easy and should be "estimated through the monitoring of survival in relation to parasite burden or, even better, by experimental manipulation of parasite burden and subsequent monitoring" (Hudson and Dobson 1997). Studies indirectly affecting the parasite load by means of an associated factor such as host reproductive effort have found support for the importance of endoparasites reducing survival in wild birds (Richner *et al.* 1995, Nordling *et al.* 1998, but see Stjernman *et al.* 2004). Also, direct manipulations of the intensity of infection by medication treatments found support for the negative effect of endoparasites on bird survival probability (Hudson and Dobson 1991, Hanssen *et al.* 2003). First, Hudson and Dobson (1991) found that the treatment of red grouse *Lagopus lagopus* L. with an antihelmintic drug increased the probability of survival for treated as compared to control birds. Later, Hanssen and co-workers (2003) also found positive effects of the medication against helminths in female common eiders *Somateria mollissima* L. Although, blood parasites should also reduce survival in birds, to our knowledge, there are not medication studies reporting this association in wild populations. The use of the antimalarial drug primaquine may be an effective method to test for this possibility because several studies have reported the susceptibility of *Haemoproteus* and *Leucocytozoon* blood parasites to sub-lethal doses of this drug, causing a significant reduction of intensity of infection by these parasites in wild birds (Merino *et al.* 2000, Marzal *et al.* 2005, Tomás *et al.* 2005).

In addition, other variables related with infection status also affect adult survival in birds. For example, there is a negative relationship between reproductive effort and survival (Golet *et al.* 1998) that may be mediated by the detrimental effects of reproductive effort on body condition, immunocompetence and the stress response (Sheldon and Verhulst 1996, Nordling *et al.* 1998, Moreno *et al.* 1999, Stjernman *et al.* 2004, Merino *et al.* 2006). Moreover, many studies have identified relationships among immunocompetence, body condition and survival (Saino *et al.* 1997, Christe *et al.* 1998,

Lobato *et al.* 2005, Moreno *et al.* 2005) suggesting that the ability to produce a higher immune response may be indeed associated with higher survival prospects (Møller and Saino 2004) in part due to a higher predation risk of less immunocompetent birds (Møller and Erritzøe 2000) or on those with higher prevalence of infection (Møller and Nielsen 2007). On the other hand, stress, by it association with avian infection status and immune capacity (Tomás *et al*. 2005, Merino *et al.* 2006, Morales *et al.* 2006), may also play a role on avian survival. Previous studies found several evidences for the role of stress as a mechanism affecting general homeostasis and reducing immune-capacity in birds (Merino *et al.* 2006, Morales *et al.* 2006) suggesting that those costs associated to stress could reduce avian survival probabilities. However, it is also true that those molecules involved in stress response play an important role favouring survival and/or longevity in animals by reducing the negative effects induced by stressful situations (Tatar et al. 1997, Sørensen and Loeschcke 2004). One of these molecules, the heat shock proteins (HSPs, or stress proteins), are highly evolutionarily conserved essential molecules maintaining cellular homeostasis under stressful situations including heat (Hercus *et al.* 2003), nutritional stress (Moreno *et al.* 2002) or parasitism (Merino *et al.* 1998, Martínez *et al.* 1999, Merino et al. 2002) (see Sørensen *et al.* 2003 for a review). In addition, in higher vertebrates, stress proteins are considered actively important molecules involved in organismal survival because their role in innate and adaptive immune responses due to their ability to interact with other proteins (Srivastava 2002). These results, together with those from studies on the relation between animal longevity and the stress protein levels (Tatar *et al.* 1997, Hercus *et al.* 2003), suggest that these molecules play a role along with immunocompetence and parasite infection status on the survival probability of wild birds.

The aim of the present study is to look for the effect of disease and host physiological responses to this pressure on the local survival rate of wild birds using a population of blue tits *Cyanistes caeruleus* and its blood parasites as model. The potential differential effect between host sexes is also studied due to the effect of sex on immune and stress responses, hormone concentrations, parasite load, behaviour and efficacy of antiparasitic treatments (see Martínez-de la Puente *et al.* 2007, Merino *et al.* 2002, Møller et al. 1998, Flajs and Grabnar 2003, Klein 2004).

Material and methods

Our study was conducted on a blue tit (*Cyanites caeruleus* L.) population breeding in nest-boxes, in a Pyrenean oak (*Quercus pyrenaica* Willd.) forest in Valsaín, central Spain (40^o 53' N, 4^o 01' W, 1200 m.a.s.l.).

During the spring of 2004, each adult bird was captured when their nestlings had 3 days of age. Birds were weighed with a Pesola spring balance to the nearest 0.1g and tarsus length measured to the nearest 0.01mm. In addition, a blood sample was obtained from its brachial vein (initial sample, see below). At this capture, birds attending nests of similar clutch size ($\pm$ one egg) and hatching date ($\pm$ one day) were randomly assigned to one of two treatments (medicated or control). Although at the moment of treatment assignment we did not know the infection status of each bird, previous studies in the same population revealed that the prevalence of infection by *Haemoproteus* and *Leucocytozoon* is very high thus allowing a blind assignment of treatment against these blood parasites. This was confirmed based on molecular detection of parasite infection by molecular analyses in the laboratory (see results). Medicated birds were injected subcutaneously with 0.1 mg of the antimalarial primaquine (Sigma, St Louis, MO, USA) diluted in 0.1 ml of saline solution and control birds were injected with the same volume of saline solution. This treatment generated pairs of adult birds attending the same nests with both individuals medicated, pairs with both controls and pairs with one medicated and one control bird. The treatment assigned to the pair did not affect the results (for females: Wald=0.59; p=0.44; for males: Wald=0.10, p=0.75) and was not considered in other analyses. Ten days later birds were recaptured, measured and a blood sample was obtained (final sample). In addition, to explore the effects of the treatment on survival, birds were recaptured until 2007 breeding season in nest-boxes of the same area when their nestlings were 3 or 13 days old.

Blood samples from each bird was obtained by venipuncture. A drop of each blood sample was immediately smeared, air-dried, fixed in ethanol and subsequently stained with Giemsa (Merino *et al.* 2000). An additional drop of blood was collected in a plastic tube and frozen for later molecular detection of parasite infection. The rest of the blood was maintained in a separate plastic tube in a cool box and later centrifuged to separate cell and plasma fractions that were also frozen at -80ºC until protein analyses were conducted (Tomás *et al.* 2004). The plasma fraction was employed to quantify the level of total immunoglobulins following Martínez *et al.* (2003) and cell fractions were employed to quantify the HSP60 and HSP70 levels according to Tomás *et al.* (2004). The change in the level of each protein (immunoglobulins, HSP60 and HSP70) was

calculated as the difference between the final and the initial level. Sample size varies between analyses because in some cases the volume of blood obtained was insufficient to quantify all these physiological parameters. Blood smears were examined to quantify the intensity of infection by *Haemoproteus majoris* as the number of parasites per 2000 erythrocytes scanned at 1000x magnification (Merino *et al.* 2004). The other half of each smear was scanned at 200x magnification in search of *Leucocytozoon majoris* and the intensity of infection by this parasite was calculated as the number of parasites per 100 fields (Merino and Potti 1995). In addition, blood samples from birds that were diagnosed as uninfected by inspection of smears at the initial capture were employed to obtain genomic DNA using the UltraClean DNA BloodSpin kit (MO BIO Laboratories, Inc., Solana Beach, California). In those samples, we carried out molecular analyses to identify potential infections by PCR reactions. This procedure was conducted using 25 μl of reaction volumes containing 20 ng template DNA, 50 mM KCl, 10 mM Tris-HCl, 1.5 MgCl2, 0.2 mM of each dNTP, 1 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): 94º C for 10 min (polymerase activation), 40 cycles at 95º C for 40 sec, 58º C or 60º C during 1 min for *Leucocytozoon* and *Haemoproteus* respectively, 72º C for 1 min, and a final extension at 72º C for 10 min. Primers employed in these reactions were HML (5′-GCT ACT GGT GCT ACA TTT GT-3′) and HMR (5′-CCT AAA GGATTA GAG CTA CC-3′) for *Haemoproteus*. Partial amplification of the cytochrome B gene was accomplished using the newly designed primers LDLd (5′-CAT TCY ACW GGT GCA TCT TT-3′) and LDRd (5′-CTG GAT GWG ATA ATG GWG CA-3′) for *Leucocytozoon*. PCR products were visualized on agarose gels. See Merino *et al.* (2008) for a previous study using this procedure. Blood samples from 1 female and 5 males were insufficient to carry out DNA analyses.

We employed repeated-measured ANOVAs (initial versus final values of each individual) to look for the effect of the medication treatment, host sex and their interaction on the change of the intensity of infection by *Haemoproteus* and *Leucocytozoon*. All variables were log-transformed to normalize when necessary. Survival analyses were carried out by using two different methods. First, for simplicity, we tested for the effect of the medication treatment and sex (see results) on bird local survival by using GLZ models including survival status as a dependent variable and both sex and treatment as factors. The main problem with this approach is that it does

consider neither capture probability nor permanent emigration. Therefore we also estimated survival using capture-recapture models for open populations derived from the Cormack-Jolly-Seber (CJS) model (e.g. Lebreton *et al.* 1992). This method allows us to control for possible bias in survival estimates associated to recapture probability below 1. Models were fitted using program MARK version 5.1 (White and Burnham 1999). The goodness of fit (GOF) test of the data to the CJS model was done using the parametric bootstrap approach in MARK. Model selection was based on a set of *a priori* models designed to consider the possibility of differences in local survival between medicated and the control birds (see below). Thus our starting model included treatment, sex and time (year) effects for local survival, and year effects in recapture probabilities, model $\Phi_{(\text{treatment*year})}\, p_{(\text{year})}$ (Φ = local survival probability; p = recapture probability; * denotes interaction). Sex and primaquine treatment were included as covariates. We used a logit link function in the models, the relationship between survival and a covariate being:

$$\text{Logit}(\Phi) = B_0 + B_1(\text{covariate})$$

$$\Phi = \frac{e^{B_0 + B_1(\text{covariate})}}{1 + e^{B_0 + B_1(\text{covariate})}}$$

where B_0 and B_1 are model parameters to be estimated, with B_1 reflecting the nature of the relationship between the covariate and survival

The set of *a priori* models considered included also models with no year effects on recapture probability ($p_{(.)}$). For local survival, models fitted included interaction between sex, treatment and time ($\Phi_{(\text{treatment *sex* year})}$), and models nested within this model. In addition we also considered a model where local survival differed between the treatment and the control group only in the year of the experiment ($\Phi_{(\text{1 year effect of treatment})}$). Modeling started with recapture rates by holding the parameter structure of survival probabilities as in the global model. When the most likely parameter structure for recapture rates was found, it was used when modeling survival (e.g. Orell and Belda 2002).

The corrected Akaike Information Criterion (AICc) was used for ranking the fit of models to the data (Burnham and Anderson 1998). This model includes a correction when number of observation is less than 40 times the number of explanatory variables

(and it is the default in MARK). We considered that models with a difference in AICc of less than two units (ΔAIC <2) were similarly supported by the data, whilst ΔAICc >2 was considered an evidence for real difference in the fit of the models to the data (Orell and Belda 2002). Akaike weights (*wi*) determine the relative likelihood of a model within the set of the candidate models considered (Orell and Belda 2002). To cope with model selection uncertainty we used multi model inference based on Akaike weights and model averaging to obtain the estimation of local survival probabilities (e.g. Anderson *et al.* 2000).

Results

A total of 95 female and 92 male birds were included in the experiment during 2004. Forty seven females and 47 males were medicated with primaquine and the rest of the birds were treated as controls. At first capture, we found that the 85% of females and the 88% of males were infected by *Haemoproteus* and 92% of females and 87% of males were infected by *Leucocytozoon*. In fact, with the exception of only one male and one female, all birds captured were infected by at least one of these two parasite species. Repeated-measures ANOVA reveals that the change in the intensity of infection by *Haemoproteus* was significantly related to bird sex ($F_{1,150}$=4.31, p=0.04), with females reducing more their intensity of infection than males. In addition, the interaction between the change in the intensity of infection and the medication treatment approach statistical significance ($F_{1,150}$=3.54, p=0.06). Medicated birds tended to reduce more their infection than controls. The interaction between bird sex and treatment were not significantly related to the change in the intensity of infection ($F_{1,150}$=1.14, p=0.29). These differences in change of the intensity of infection between sexes may be due to a differential efficacy of the treatment between sexes as shown by the effect of primaquine treatment reducing the *Haemoproteus* load in females ($F_{1,77}$=4.25, p=0.04) but not in males ($F_{1,73}$=0.34, p=0.56). No significant associations were found for *Leucocytozoon* (treatment: $F_{1,150}$=1.46, p=0.23, sex: $F_{1,150}$=0.15, p=0.70, treatment-sex interaction: $F_{1,150}$=0.02, p=0.90). In addition, no significant associations were found between intensity of infection by *Leucocytozoon* and medication treatment when each sex was analyzed independently ($F_{1,77}$=0.82, p=0.37 and $F_{1,73}$=0.65, p=0.42 for females and males respectively).

For the 95 females included in the study in 2004, 28 individuals (19 medicated and 9 controls) were recaptured at least once between 2005 and 2007 (Fig. 1). For the

92 males included in the experiment, 26 individuals (11 medicated and 15 controls) survived for at least one year. According to Generalized Linear Model (GLZ) analyses, sex and treatment were not significantly associated to survival but a significant association between local survival and the interaction between both variables exists (treatment: Wald=0.77, p=0.38, sex: Wald=0.001, p=0.97, treatment*sex interaction: Wald=5.56, p=0.02). This result supports a differential effect of the treatment in relation to bird sex. In order to confirm this effect we used capture-recapture methods to estimate unbiased local survival probabilities in relation to bird sex and treatment.

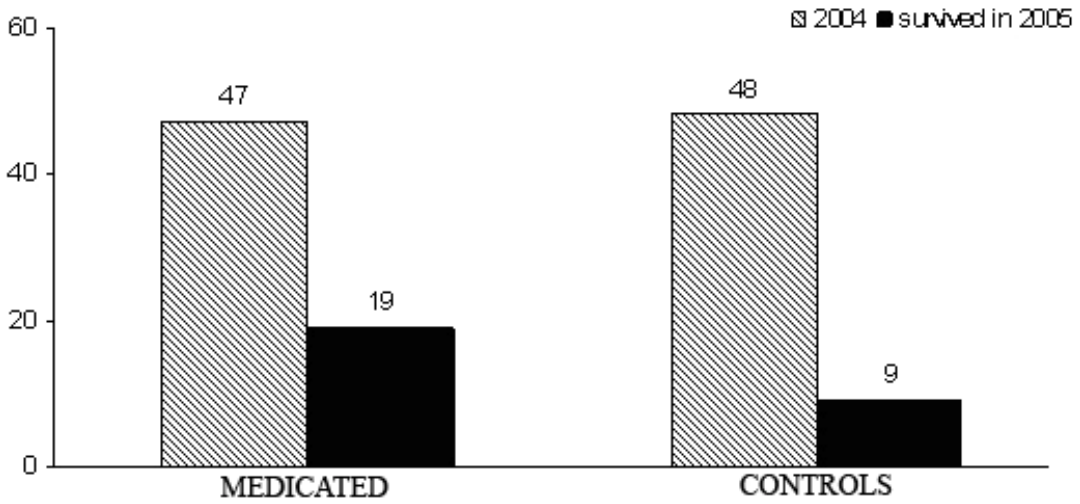

Figure 1. Number of medicated and control females treated in 2004 and survival females in the following year.

The GOF for our starting model (Model 10, Table 1) fitted the data (p = 0.16). A model without time effects on capture probability (Model 9, Table 1) fitted the data 2.3 times better than a model with time effects (Model 10, Table 1) although both models had a similar fit to the data (ΔAICc = 1.69). Thus, we continue modelling considering constant time effects in recapture probability. Recapture probability was high (0.90 ± 0.05).

The best model for survival probability considered that there was an interaction between sex and treatment in the year of the treatment (Model 1, Table 1). The beta coefficients of the log-linear function for survival are shown in Table 2. Only the beta coefficient for the interaction between sex and treatment is different from zero (the 95% confidence interval do not overlapped zero, Table 2). There was a positive interaction effect. This implies that survival probability of females treated with primaquine was higher than survival probability of control females (survival from 2004 to 2005)

however; there was no effect of primaquine treatment on male survival (Table 2). There were other three competing models of the best model including the interaction of sex and treatment on survival probability between 2004 and 2005, though differ in the effect of the treatment in survival in later years (models within 2 AICc units of the best model, (models 2, 3 and 4, Table 1)). We considered model averaging to estimate survival probabilities for males and females (Table 3).

Table 1. Model selection to analyse the effect of treatment with primaquine on blue tit annual survival. For each model, the values for the Akaike's Information Criterion (AICc), the difference in AICc with the best model (ΔAICc), the Akaike weights (*w*) and the number of estimable parameters (np) are shown. Notation follows Lebreton *et al.* (1992): *treatment* = differences in survival between treatments; *year* indicates differences in survival between years; *experiment year*, indicates survival from 2004 to 2005; *year2-3*, indicates survival for years 2005-2006 and 2006-2007 was similar; ( ) = constant effect across years; * denotes interaction between effects; + indicates additive effects. All together 29 models were fitted to the data. Only models with ΔAICc < 4 are shown, together with the models used in modeling recapture probability.

	AICc	ΔAICc	*W*	*np*	*Model explanation*
(1) Φ (*experiment year (sex*treatment), (year2-3 (treatment))* **p** (.)	349.28	0	0.20	6	Survival differs between the first year and the rest of the years. In the first year, survival depends on the interaction of sex and treatment. In the rest of the years survival depends on the treatment.
(2) Φ (*experiment year/sex*treatment), year2 (treatment) year3(treatment)*) **p**(.)	349.44	0.17	0.19	7	Survival differs between years and treatment groups, with an interaction between sex and treatment in the first year.
(3) Φ (*experiment year/sex*treatment), year*)**p**(.)	351.06	1.78	0.08	7	Survival differs between years, with an interaction between sex and treatment in the first year.
(4) Φ (*experiment year/sex*treatment), year2-3*) **p**(.)	351.21	1.93	0.08	6	Interaction between sex and treatment in the survival on the year of the experiment, survival similar for all the individuals in the rest of the years.
(5) Φ (*year*) **p**(.)	351.38	2.10	0.07	4	Time effects on survival.
(6) Φ (.) **p**(.)	352.27	2.99	0.05	2	Constant model.
(7) Φ (*year*treatment*) **p**(.)	352.61	3.33	0.04	3	Survival differs between years and treatment groups.
(8) Φ (*experiment year/sex*treatment), year2 (sex) year3(sex)*)**p**(.)	352.85	3.57	0.03	3	Survival differs between years and sex, with an interaction between sex and treatment in the first year.
(9) Φ (*treatment*sex*year*) **p**(.)	357.58	8.30	0.02	13	Survival depends on the interaction of the year, sex, and experimental group. Constant recapture probability.
(10) Φ (*treatment*sex*year*)**p**(**year**)	359.27	9.99	< 0.01	15	Survival depends on the interaction of the year, sex, and experimental group. Recapture probability is time dependent.

Table 2. A) Beta coefficients for the model with the best fit to the data. The logit function used to estimate survival was Logit (Φ) $=B_0+B_1$ (year)+ B_2 (sex)+ B_3 (treatment)+ B_4 (sex*treatment). B) Beta coefficients for the model that best fit the data considering also changes in condition, immunoglobulin levels and HSP60 and HSP70 stress proteins. The logit function used to estimate survival was Logit (Φ) $=B_0+B_1$ (year)+ B_2 (sex)+ B_3 (treatment)+ B_4 (sex*treatment) B_5 (HSP70). The values for year are coded as 1 for survival in the experimental year and 0 for survival in the other two years (2005-2006, 2006-2007); sex was coded as 1 for females and 0 for males; birds treated with primaquine were coded as 1, and value 0 was for control birds.

A)			**95% Confidence Interval**	
Parameter	**Beta**	**SE**	**Lower**	**Upper**
B_0	-0.02	0.30	-0.60	0.56
B_1	-0.55	0.35	-1.24	0.13
B_2	-0.83	0.47	-1.75	0.09
B_3	-0.58	0.34	-1.24	0.08
B_4	1.71	0.60	0.53	2.89
B)			**95% Confidence Interval**	
Parameter	**Beta**	**SE**	**Lower**	**Upper**
B_0	0.10	0.33	-0.56	0.76
B_1	-0.67	0.40	-1.45	0.11
B_2	-0.92	0.55	-1.99	0.15
B_3	-0.61	0.38	-1.35	0.14
B_4	0.02	0.01	0.003	0.03
B_5	1.71	0.61	0.95	3.35

Table 3. Survival probabilities ± standard error (SE) and 95% Confidence Interval for control and medicated blue tits. Estimation has been done using model averaging.

			95% Confidence Interval	
Parameter	**Survival probability**	**SE**	**Lower**	**Upper**
Control female	0.22	0.08	0.12	0.42
Medicated female	0.42	0.09	0.24	0.60
Control male	0.39	0.07	0.23	0.53
Medicated male	0.27	0.06	0.18	0.46

Models exploring the possible effects of the change of immunoglobulin levels, HSP60 or HSP70 on survival included the capture-histories of 146 individuals because it was not possible to get these variables for all the individuals captured (see methods). All together we fitted 49 models. First we considered the best model considering sex and treatment effects. The bootstrap GOF test for the starting model ($\Phi_{\text{(treatment *sex* year)}}$ $p_{\text{(year)}}$; AICc = 300.72) fit the data (p = 0.72). The model without time effects on capture probability fitted the data best than the previous model although both models had a

similar fit to the data (ΔAIC = 1.78). We continue modelling considering constant capture probability. The best model among the set off models considered included an interaction between sex and treatment and additive effects of stress protein HSP70 in the survival of the year of the experiment (Model 1, Table 4, Fig. 2) and treatment effects in later years. Only the beta coefficients for the interaction term between sex and treatment and for the effect of the change in HSP70 levels were different from zero (Table 2). In both cases the term was positive indicating that the treatment increase survival of females and a positive relationship between the change in HSP70 and survival. There was another model within two AICc units of the best model. This model only differed with the previous one in the fact that it did not include any effect of survival in later years. This was not surprising because for the best model the effect of the treatment was not different from zero (value of the beta coefficient, see Table 2). Other models are also shown (Table 4).

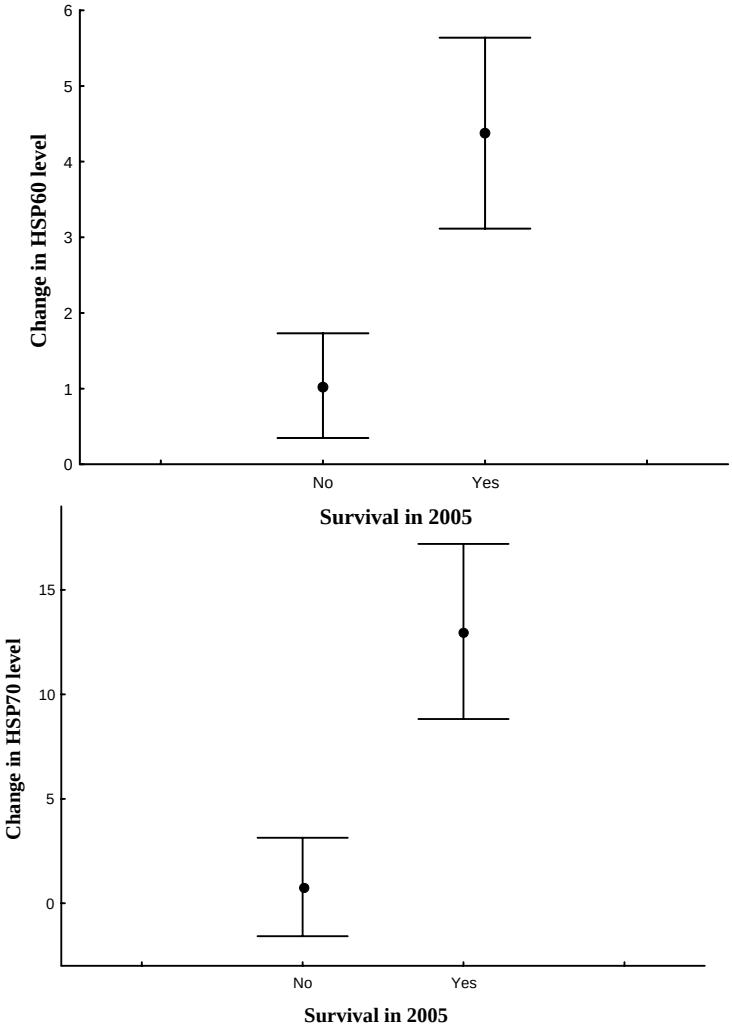

Figure 2. Mean ± SE values of the change in the HSP70 level (a) and HSP60 level (b) calculated as the difference between the final and the initial level (expressed in arbitrary units as area per mean density of the band) during the 2004 breeding season in relation to survival until 2005 breeding season.

Table 4. Model selection to analyse the effect of parasites, immunoglobulins and stress proteins on blue tit annual survival. For each model, the values for the Akaike's Information Criterion (AICc), the difference in AICc with the best model (ΔAICc), the Akaike weights (*w*) and the number of estimable parameters (np) are shown. Notation follows Lebreton *et* al. (1992) (ref 34): *treatment* = differences in survival between treatments; *year* indicates differences in survival between years; *experiment year*, indicates survival from 2004 to 2005; *year2-3,* indicates survival for years 2005-2006 and 2006-2007 was similar; *HSP70* = change in stress protein HSP70 levels; *HSP60* = change in stress protein HSP70 levels (.) = constant effect acro ss years; * denotes interaction between effects; + indicates additive effects. All together 29 models were fitted to the data. Only models with ΔAICc = 4 are shown, together with the models considering survival as a function of the different covarites considered.

	AICc	ΔAICc	*w*	*np*	*Model explanation*
(1) Φ (*experiment year (sex*treatment+HSP70), (year2-3 (treatment))* ***p*** (.)	288.52	0	0.17	7	**Survival differs between the first year and the rest of the years. In the first year, survival depends on the interaction of sex and treatment and of the change of levels of HSP70 stress protein. In the rest of the years survival depends on the treatment**
(2) Φ (*experiment year (sex*treatment+HSP70), (year2-3)*) ***p*** (.)	290.44	1.92	0.07	7	**Survival differs between years and treatment groups, with an interaction between sex and treatment and additive effects of HSP70 in the first year.**
(3) Φ (*experiment year (sex*treatment+HSP70*treatment), (year2-3 (treatment)*) ***p*** (.)	290.51	2.00	0.06	8	**Survival differs between years, with an interaction between sex and treatment and between HSP70 and treatment.**
(4) Φ (*experiment year (sex*treatment+HSP60), (year2-3 (treatment)*) ***p*** (.)	290.62	2.11	0.06	7	**In the first year, survival depends on the interaction of sex and treatment and of the change of levels of HSP60 stress protein. In the rest of the years there is an effect of the treatment on survival.**
(5) Φ (*experiment year (sex*treatment+HSP70 +HSP60), (year2-3 (treatment)*) ***p*** (.)	290.66	2.14	0.06	8	**In the first year, survival depends on the interaction of sex and treatment and of the change of levels of HSP60 and HSP70 stress protein. In the rest of the years there is an effect of the treatment on survival.**

(6) Φ (*experiment year (sex*treatment+HSP70*sex), (year2-3 (treatment))* ***p*** (.)	290.68	2.16	0.058	8	**Survival differs between years, with an interaction between sex and treatment and between HSP70 and sex affecting survival between 2004-2005.**
(7) Φ (*experiment year (HSP70), (year2-3*) ***p*** (.)	291.03	2.51	0.05	4	**Survival in the experimental year depends on the change in HSP70 levels.**
(8) Φ (*experiment year (treatment +HSP70), (year2-3)*) ***p*** (.)	291.46	2.94	0.04	5	**Survival between 2004-2005 depends on the change in HSP70 levels and treatment.**
(9) Φ (*HSP70*) ***p*** (.)	291.87	3.36	0.03	3	**Survival depends on the change in HSP70 levels.**
(10) Φ (*experiment year (sex*treatment+HSP60*sex), (year2-3 (treatment))* ***p*** (.)	292.29	3.77	0.02	8	**Survival differs between years, with an interaction between sex and treatment and between HSP60 and sex affecting survival between 2004-2005 and medication in later years**
(11) Φ (*experiment year (treatment * HSP60), (year2-3)*) ***p*** (.)	292.57	4.06	0.02	6	**Survival between 2004-2005 depends on the interaction between the change in HSP60 levels and treatment.**
(12) Φ (*experiment year (sex*treatment), (year2-3 (treatment))* ***p*** (.)	292.59	4.08	0.02	6	**Survival differs between the first year and the rest of the years. In the first year, survival depends on the interaction of sex and treatment. In the rest of the years survival depends on the treatment.**
(13) Φ (*HSP60*) ***p*** (.)	292.71	4.20	0.02	3	**Survival depends on the change in HSP60 levels.**
(14) Φ (*immunoglobulin*) ***p*** (.)	294.22	5.71	0.01	3	**Survival depends on the change in immunoglobulin levels.**
(15) Φ (*condition*) ***p*** (.)	294.55	6.04	0.00	3	**Survival depends on the change in condition.**
(16) Φ (*sex*) ***p*** (.)	296.91	8.39	0.00	3	**Differences in survival between sexes**
(17) Φ (*treatment*) ***p*** (.)	296.97	8.46	0.00	3	**Survival depends differs between treatments.**

Discussion

Our results indicate a differential effect of medication in the reduction of the intensity of infection by *Haemoproteus majoris* between sexes. It is known that host sex is an important factor determining the intensity of infection by parasites (Møller *et al.* 1998), males usually supporting higher levels of infection (Klein 2004). In fact, differences in behaviour, physiology and immune defence are important factors determining sexual differences in parasite load (Zuk and McKean 1996). In addition, although reports on the pharmacokinetics of treatments against parasites in wild animals of different sexes are scarce, a considerable medical and veterinarian literature also reports effect of sex on kinetics of drugs (Klein 2004, Pinsonneault and Sadée 2004) including studies with antimalarial drugs (Gordi *et al.* 2002). In general, the efficacy of antiparasitic treatments is strongly influenced by the sex of the patient (Flajs and Grabnar 2003, Klein 2004) principally due to sexual differences in the absorption and metabolism of drugs (Klein 2004, Gordi *et al.* 2002). Different factors may be involved in the influence of sex on drug pharmacokinetics, including those factors tightly related to sex such as hormone concentrations and genetic characteristics (Pinsonneault and Sadée 2004). At least in part, all of these factors may be implicated in the different efficacy of the primaquine treatment between bird sexes reported here. In addition, pharmacokinetics may account for the observed differences in primaquine efficacy if the drug is present for a shorter period in males, thereby allowing parasitaemias to recover before the second blood sample.

Host-parasite interactions may play a key role affecting host population dynamics (Tompkins and Begon 1999). Although host-parasite interactions are mediated by temporal variation in the virulence of parasites, in susceptibility of hosts or in environmental conditions, parasites impact on the fitness components of their hosts, including fecundity and survival, is demonstrated by experimental manipulations of parasite loads (Hudson and Dobson 1991, Tompkins and Begon 1999, Merino *et al.* 2000, Hanssen *et al.* 2003, Marzal *et al.* 2005). Previous medication studies on birds have found clear negative effects of endoparasites on bird survival (Hudson and Dobson 1991, Hanssen *et al.* 2003) however these studies employed helminth parasites as a model of study. For that reason, to our knowledge, here we report for the first time an adverse effect of blood parasites in wild bird survival by a direct manipulation of the intensity of infection by a medication treatment, the better way to test for the parasite infection effects (Hudson and Dobson 1997). Reductions of parasite loads may imply

beneficial effects for hosts in terms of reduction of the adverse effects of parasitism, the quantity of resources drained by the parasite and in the quantity of resources being devoted by hosts to immune defence (de Lope *et al.* 1998, Martínez *et al.* 2004, Tomás *et al.* 2005). Both, infection status and immunological response of hosts may induce an increase in metabolic rate (Martínez *et al.* 2004) that, as consequence, could reduce their survival, directly or indirectly, by increasing their susceptibility to be infected by other pathogens or captured by predators (Hudson *et al.* 1992, Møller and Nielsen 2007). All of these possibilities could be mediating the effect of the anti-parasite treatment on local survival of female blue tits, the only sex in which the primaquine medication was effective in reducing the intensity of infection by *Haemoproteus*.

In addition, we found a clear association between bird local survival rate to the year following the experiment and host stress response together with the interaction between sex and treatment. Our results indicate that birds with the capacity to increase their stress protein levels during the reproductive period could increase their probability of local survival. Stress may represent an important factor affecting survival in blue tits and the synthesis of these proteins to cope with stress means an essential mechanism in maintaining cellular homeostasis by correcting misconfigurations in protein structures (Sørensen *et al.* 2003). However, both stress and the synthesis of stress proteins are traded against different aspects of bird health and condition (Merino *et al.* 1998, Moreno *et al.* 2002, Tomás *et al.* 2005, Morales *et al.* 2006), suggesting that the balance between benefits and costs of stress protein synthesis may play an important role in the ecology and evolution of birds (Sørensen *et al.* 2003). In this respect, Morales *et al.* (2006) found that females of the passerine bird *Ficedula hypoleuca* with higher levels of both HSP60 and HSP70 mounted a lower cell mediated immunological response and those females with higher HSP60 levels also mounted lower humoral responses against a tetanus vaccine. In addition, a measure of nestling growth was negatively associated to higher levels of HSP60 in birds (Moreno *et al.* 2002, Merino *et al.* 2006). However, although the synthesis of stress proteins may be costly, it may also be beneficial if it boosts other responses which finally render a better fitness under stress conditions. In this respect, several laboratory studies support the positive association between increases in the synthesis of stress proteins and survival or longevity by exposure to both lethal and non-lethal stresses (Tatar *et al.* 1997, Hercus *et al.* 2003, Sørensen and Loeschcke 2004). The increase in the expression level of HSP70 under stressful situations was associated with an increase in longevity in transgenic *Drosophila*

melanogaster with higher number of copies of the HSP70 gene (Tatar *et al.* 1997). Hercus *et al.* (2003) also found that *D. melanogaster* females repeatedly exposed to a heat stress increase in lifespan and those flies had higher levels of HSP70 expression. The importance of stress proteins favouring animal survival is also supported by their key role as informers of the MHC (Major Histocompatibility Complex) molecules with respect to intracellular pathogens, thus playing an important part on T-cell response (Srivastava 2002). Based on this role of stress proteins maintaining functional cellular machinery by their effect in a diversity of vital functions including folding and unfolding of proteins, degradation of proteins, assembly of multisubunit complexes, thermotolerance and buffering the expression of mutations, we can conclude that the increase in the level of stress proteins in surviving birds may benefit them when affected by different environment stressors and therefore increase their probability of local survival between years with evident important fitness consequences for this short-lived passerines.

In conclusion, our results show experimentally, to our knowledge for first time, that blood parasite infection has detrimental consequences in terms of local survival prospects in a wild bird population and represent the first field evidence of an association of stress proteins with survival in wild vertebrates.

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