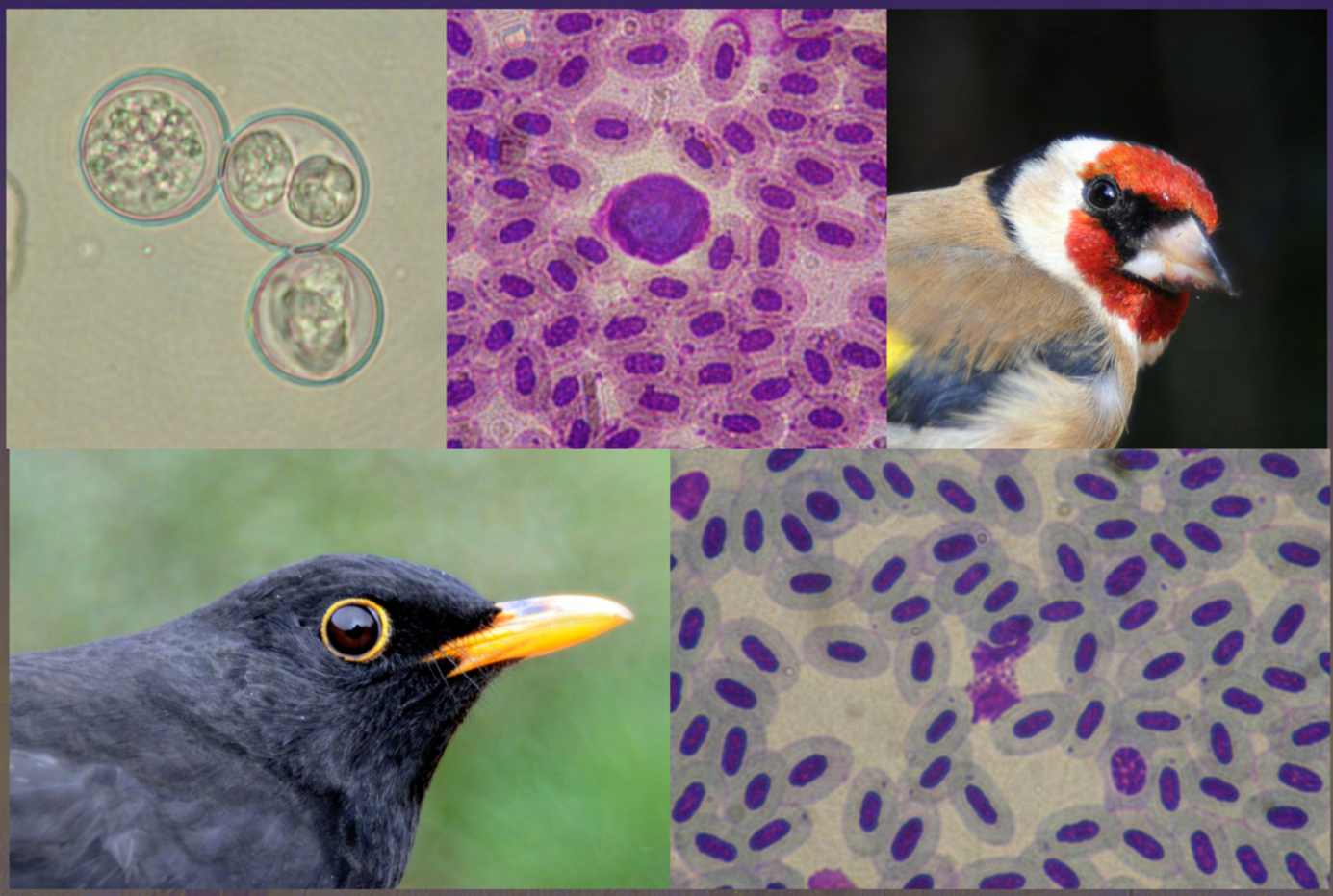
Relaciones eco-fisiológicas hospedador-parásito en aves silvestres

Tesis doctoral



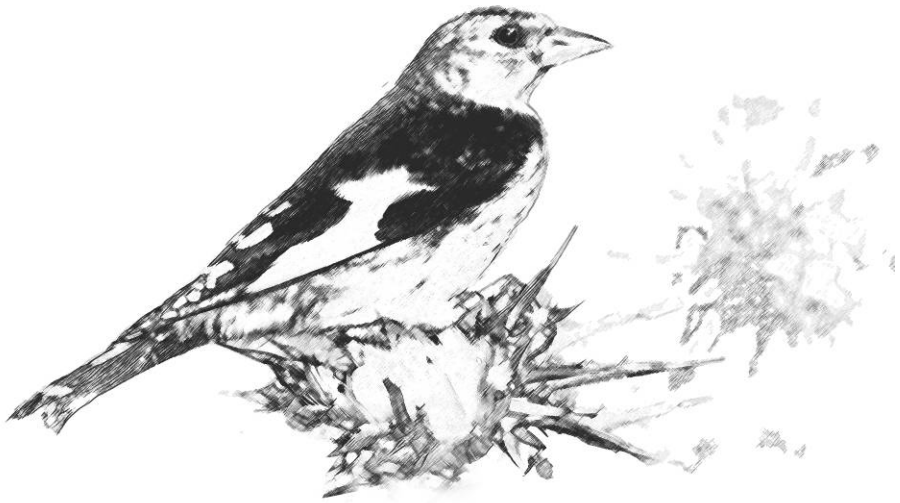
Guillermo López Zamora

Tesis doctoral

Relaciones eco-fisiológicas hospedador-parásito en aves silvestres

Memoria presentada por
Guillermo López Zamora

Para optar al título de doctor por la Universidad Autónoma de Madrid
Programa de doctorado de Ecología



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Per severa, persevera

A mis padres, por su inestimable apoyo para la consecución de esta tesis. Pero sobre todo por su amor incondicional y por su ejemplo en mis seis lustros largos de vida.

Y a Susana, por su paciencia, por su aliento y por postergar con cariño tanto tiempo de nuestra vida común para que pudiera terminar los trabajos de esta tesis.

ÍNDICE

1. INTRODUCCIÓN

Las poblaciones y comportamientos de los animales silvestres están regulados por multitud de complejos factores bióticos y abióticos. Uno de los principales factores bióticos lo conforman los parásitos. A pesar de ser ignorados en muchos estudios del funcionamiento de los ecosistemas, los parasitos representan una importante fracción de la biomasa de los ecosistemas (Kuris et al. 2008), con importantes efectos sobre la regulación de las poblaciones de sus hospedadores (Hudson et al. 1998; Hochachka y Dhondt 2000). Ante la necesidad de sobrevivir y reproducirse en sus ambientes, los animales silvestres han desarrollado una gran variedad de mecanismos de defensa frente a estos (Clayton y Moore, 1997). Las interacciones hospedador-parásito han cobrado un creciente interés en los estudios de ecología evolutiva durante las últimas décadas. En líneas generales, esta disciplina se ha interesado en los posibles costes de la función del sistema inmune para la eficacia biológica y de los conflictos de estos con otras necesidades concurrentes del organismo (Sheldon y Verhulst, 1996; Zuk y col. 1996; Schmid-Hempel y Ebert, 2003). Estos conflictos se han estudiado generalmente como una respuesta plástica individual, bien referida al coste del uso del sistema inmune (Moret y Schmid-Hempel, 2000), o bien a un patrón de co-variación genética traducida en el coste de tener el sistema inmune (Kraaijeveld y Godfray, 1997). En la primera línea, ha surgido un gran volumen de investigación analizando los factores que limitan la inversión de los organismos en el sistema inmune (Norris y Evans, 2000; Segerstrom 2007; McKean y col. 2008). Uno de los aspectos más estudiados de las interacciones parásito-hospedador en las aves es su impacto sobre la selección sexual mediante su influencia en la expresión de ornamentos (Hamilton y Zuk, 1982; Andersson, 1994; Hill 2002). Aunque los parásitos han llegado a ser el foco de un gran interés científico, muchos aspectos de las interacciones entre parásitos y sus hospedadores aún no son bien conocidos. El objetivo de esta tesis ha sido ahondar en el conocimiento de las relaciones ecológicas hospedador-parásito en aves silvestres en el medio natural. Para ello se han estudiado las consecuencias de la parasitación por diversos grupos de parásitos sobre la fisiología y la expresión de ornamentos en aves

silvestres muestreadas en el medio natural. Dentro de este marco, se ha prestado una atención especial al estudio de las relaciones ecológicas entre el virus del Nilo occidental (WNV, por sus siglas en inglés) y las aves silvestres, por ser este un patógeno aviar con potencial zoonósico y por tanto con relevancia en la salud pública. Se trata de un virus endémico en la cuenca mediterránea que ha causado un gran impacto en la dinámica poblacional de muchas aves en América del Norte (LaDeau y col. 2007) y cuyo impacto en las poblaciones locales no se había estudiado.

1.a Parásitos y aves

Un parásito se puede definir como un organismo que vive en o sobre otro del que obtiene parte o todos los nutrientes orgánicos, sufriendo normalmente algún grado de cambio estructural y causando algún grado de daño en el hospedador (Price 1980). Bajo esta definición se engloba un amplio abanico de organismos que comprende desde virus hasta organismos superiores. Aunque la presión evolutiva de los parásitos en los hospedadores es grande, la susceptibilidad del hospedador a la patogenia persiste a lo largo del tiempo. Asumiendo una relación lineal entre intensidad de infestación y eficacia biológica del hospedador, se postuló que la única explicación de este hecho era la ventaja evolutiva de los parásitos sobre los hospedadores derivada de su menor periodo intergeneracional (May y Andersson, 1990), aunque algún estudio reciente sugiere que la eficacia biológica puede ser máxima a niveles medios de parasitación (Stjernman y col. 2008). Para mantener este equilibrio, los hospedadores presentan mecanismos de defensa para luchar contra las rápidas adaptaciones de los parásitos, y así, en una escala de tiempo evolutiva, las interacciones entre hospedadores y parásitos pueden considerarse escaladas armamentísticas sin fin (Morse 1994; Ewald 1994; Poulin 2007).

Las aves en la naturaleza están sometidas a la infección por multitud de parásitos, algunos de los cuales aparecen y desaparecen cíclicamente

(Clayton y Moore, 1997). Generalmente se asume que las infecciones parasitarias son costosas para las aves, pero esos costes son difíciles de cuantificar en la práctica y además no son constantes entre diferentes grupos parasitarios (Booth y col. 1993). Los parásitos pueden tener efectos negativos en la eficacia biológica de las aves como consecuencia de diferentes procesos: (1) afectando su capacidad reproductora (p.ej. Korpimäki y col. 1993; Hudson y Dobson, 1997; Marzal, 2005; Spencer y col. 2005; Tomás y col. 2007; Potti 2008; Bischoff y col. 2009), (2) afectando otras funciones vitales (Brown y col. 1995; Harper 1999; Dawson y Bortoloti, 2000; Navarro y col. 2003; Garvin y col. 2006) y (3) originándole la muerte (Wojzinski y col. 1987; Hunter y col. 1997; Petersen y Roehrig, 2001; Höfle y col. 2004; Chen y col. 2005).

Los principales parásitos aviares pueden clasificarse en los siguientes grupos:

a. Virus
b. Bacterias
c. Protozoos:
    c.1. Hemosporidios
    c.2. Coccidios
d. Helmintos
e. Ácaros
f. insectos

Los distintos grupos de parásitos presentan distintos ciclos vitales y vías de transmisión que pueden determinar la distinta exposición de las aves a grupos diferentes de parásitos en función de su ecología. De este modo, se ha descrito una mayor exposición a ectoparásitos y parásitos sanguíneos en especies coloniales (Tella 2002; Rekasi et al. 1997), mayor exposición a parásitos sanguíneos en especies migratorias (Figuerola y Green 2000) y en las que viven en ambientes de agua dulce (Figuerola 1999) o mayor prevalencia de ácaros de las plumas en especies que viven en grupo durante el invierno (Figuerola 2000).

1.c Los ornamentos en las aves

Entre los ornamentos exhibidos por las aves, aquellos basados en la coloración son los más comunes (Andersson, 1994). Dentro de estos, se distinguen los siguientes grupos principales de ornamentos:

1.c.1 La muda parcial: Numerosas especies de aves ostentan plumajes juveniles diferentes al de los adultos, y la mayor parte de estas realizan una muda postjuvenil parcial, que provoca la ostentación de un plumaje en parte adulto y en parte juvenil durante el primer año de vida (Svensson, 1984; Cramp y Perrins, 1994).

1.c.2. coloración: Los dos principales grupos de pigmentos que colorean el plumaje y los tegumentos de las aves son:

a. Carotenos:

b. Melaninas

1.d Relación entre ornamentos, parásitos y variables fisiológicas

Para explicar la evolución de ornamentos extravagantes masculinos, Darwin (1871) propuso la teoría de la selección sexual. Darwin razonó que los machos con ornamentos grandes se podrían haber favorecido con la selección sexual si las hembras seleccionasen positivamente estos machos como pareja. No obstante, Darwin no propuso una explicación de cómo las hembras se podrían beneficiar con esas preferencias. Posteriormente han surgido numerosas teorías tratando de explicar este hecho (Fisher1930, Zahavi 1975; Grafen 1990). La teoria del handicap de Zahavi propone que los ornamentos masculinos son costosos de desarrollar y/o mantener, y por tanto sólo los machos de mejor calidad deberían ser capaces de afrontar ese coste. Siguiendo este razonamiento, si la calidad de los machos y la extravagancia de los ornamentos se relacionan positivamente, y esta calidad es heredable, las hembras se beneficiarían en su elección consiguiendo una mayor supervivencia de su progenie. Hamilton y Zuk (1982) ampliaron los modelos del *handicap* y sugirieron

que los ornamentos masculinos expresaban la capacidad de resistencia frente a parásitos. Así, los individuos con ornamentos más desarrollados tendrían menor carga parasitaria que los que tuvieran ornamentos más discretos. Esta hipótesis colocó a los parásitos en un punto central de la investigación de la selección sexual.

Hasta la fecha, un gran número de estudios en aves y otros animales han demostrado que los ornamentos masculinos pueden expresar información de la carga parasitaria individual en la línea expuesta por Hamilton y Zuk (Andersson 1994; Hamilton y Poulin, 1997). Aunque su teoría ha recibido mucho apoyo empírico, la idea de Hamilton y Zuk ha sido controvertida. Se ha argumentado que la hipótesis es imposible de falsificar, ya que hay varios factores que pueden explicar la asociación predicha (Read 1990). Por ejemplo, se puede argumentar que los taxones cruciales de parásitos no se incluyeron en el modelo. Igualmente, la asumida transferencia a la descendencia de la resistencia frente a parásitos no se ha estudiado. Aunque las hembras seleccionasen machos libres de parásitos a través de los ornamentos, los beneficios que estas obtuviesen podrían ser igualmente directos, tales como un mayor esfuerzo de alimentación de la progenie en machos con mejor condición física (Hoelzer 1989). De manera alternativa, las hembras también podrían elegir los machos más ornamentados para beneficiarse de la producción de una progenie más atractiva (Fisher 1930; Jones y col. 1998). Algunas pruebas de la hipótesis de Hamilton y Zuk han recibido críticas por la disparidad y elección oportunista de evaluar la inmunocompetencia con parámetros, en ocasiones, poco precisos (Siva-Jothy 1995; Sheldon y Verhulst, 1996; Penn y Potts, 1998).

2. Planteamiento de trabajo

En esta tesis se ha abordado el estudio de las relaciones hospedador-parásito en aves silvestres para profundizar en el conocimiento de los costes fisiológicos de la parasitación y la señalización intra- e inter-específica de estos costes. Dentro de los parásitos estudiados, se ha profundizado en el estudio de la ecología del WNV en las aves silvestres del sur de la Península Ibérica.

2.a Premisas metodológicas

Como el grupo parasitario más prevalente en las aves estudiadas han sido los coccidios, y debido a que estos presentan ciclos circadianos de eliminación de ooquistes, el primer paso de este estudio ha sido el diseño de una metodología que permitiese analizar correctamente tanto la prevalencia como la carga de coccidios en relación a otras variables. Así, el primer capítulo (López y col. 2007) sienta las bases para trabajar con datos repetibles y estimas no sesgadas en los análisis de los capítulos siguientes que incluyen estos parámetros.

2.b Costes fisiológicos de la parasitación y su relación con la ornamentación

El capítulo 2 estudia cómo se relaciona la extensión de la muda parcial postjuvenil (como posible señal no basada en intensidad de pigmentación) con variables hematológicas indicadoras de estatus fisiológico en lavanderas blancas (*Motacilla alba*) silvestres muestreadas en el medio natural. El capítulo 3 explora cómo se relaciona la coloración de la máscara facial del jilguero (*Carduelis cardualeis*), como ornamento del plumaje basado en carotenoides (y por tanto implicado en la selección sexual), con variables hematológicas indicadoras de estatus fisiológico y con prevalencia y carga parasitaria de endoparásitos (hemoparásitos y parásitos intestinales). El capítulo 4 estudia la relación entre la coloración carotenoide del pico del mirlo común (*Turdus merula*), como ornamento dinámico de una estructura tegumentaria, indicadores de estatus fisiológico (variables hematológicas y bioquímicas) y prevalencia y carga

parasitaria de endoparásitos (hemoparásitos y parásitos intestinales). Por último en este bloque, el capítulo 5 explora la relación entre los valores de carotenoides séricos circulantes y la parasitación con endoparásitos (hemoparásitos y parásitos intestinales), con objeto de estudiar cómo la parasitación afecta la disponibilidad de carotenoides para la ornamentación y/o otras necesidades fisiológicas y las relaciones entre distintos grupos de parásitos.

2.b Ecología del virus del Nilo occidental

Debido, en primer lugar a la relevancia para la salud pública que tiene WNV, y en segundo a la baja seroprevalencia encontrada frente al virus en las aves muestreadas, este ha recibido una atención diferenciada del resto de parásitos en esta tesis. En primer lugar, los tamaños muestrales necesarios para realizar estudios de ecología han debido ampliarse notablemente con respecto a los del resto de parásitos considerados en los demás trabajos. El capítulo 6 explora la prevalencia de WNV en relación con la condición migratoria de las aves, con objeto de comprobar si el virus se halla presente en el sur de la Península ibérica de forma endémica o es vehiculado por especies de aves migradoras. El capítulo 7 estudia diversas variables ecológicas y evolutivas (tales como grupo taxonómico, tamaño, colonialidad, gregarismo invernal, época del año, etc) en relación con seroprevalencia frente a WNV en 72 especies de aves muestreadas en el área de estudio. Por último, en el capítulo 8 se estudia si el WNV es una causa de mortalidad o morbilidad en las poblaciones de aves silvestres del área de estudio en un periodo de circulación documentada del mismo.

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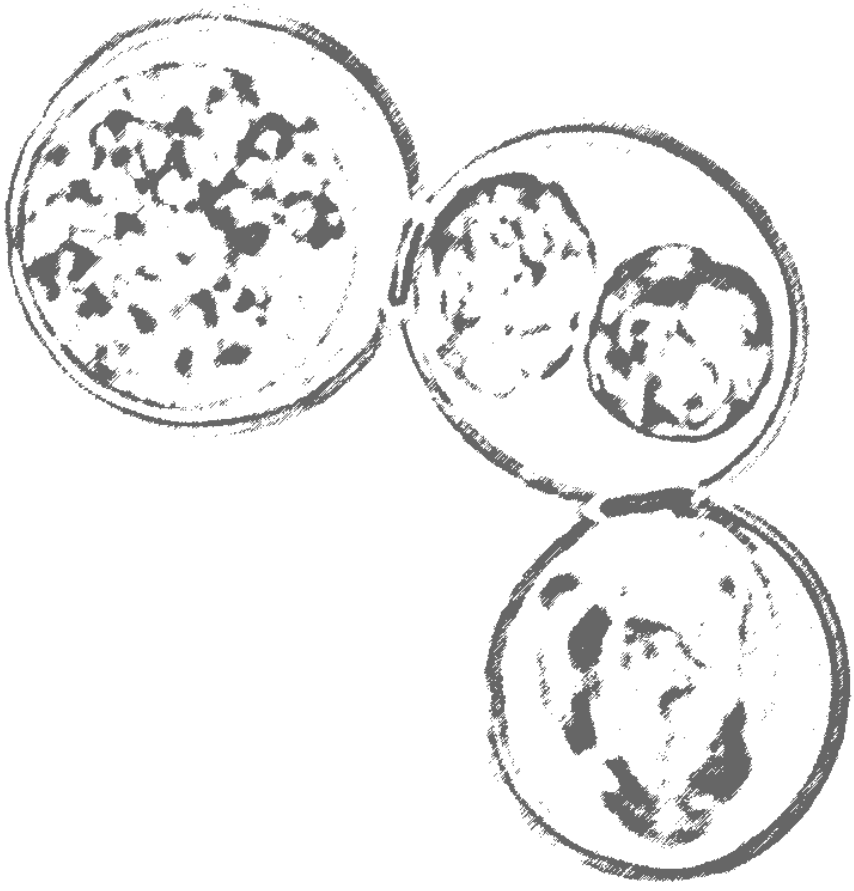
2. PREMISAS METODOLÓGICAS



2.a CAPÍTULO 1: La hora del día, la edad y los hábitos alimenticios influencian la eliminación de ooquistes coccidianos en las aves paseriformes

Resumen:

Los protozoos del orden *Eucoccidia* son uno de los grupos de parásitos intestinales más frecuentes en aves. Las técnicas ordinarias de detección y cuantificación de coccidios han mostrado ser imprecisas para los paseriformes silvestres debido a la existencia de marcados ritmos de eliminación de ooquistes a lo largo del día. Estudios previos han sugerido que estos ritmos deberían tenerse en cuenta al analizar datos de carga y prevalencia coccidiana, pero su patrón y magnitud son aún mal conocidos. En este estudio caracterizamos los ritmos de eliminación de ooquistes en el medio natural analizando 406 muestras de heces de dos especies de paseriformes con diferente dieta: El verdecillo (una especie granívora) y la curruca mosquitera (una especie insectívora). Tanto la prevalencia como la carga de coccidios presentaron un ritmo bimodal, presentando máximos al principio de la tarde. La eliminación de ooquistes permaneció consistentemente alta en la segunda mitad del día, mientras que la prevalencia presentó un pico al principio de la tarde y disminuyó durante el final de la misma. Este patrón se halló en ambas especies. Encontramos una alta repetibilidad en prevalencia y en carga cuando las diferencias entre mañana y tarde se controlaron estadísticamente. Por ello, recomendamos que se tengan en cuenta estas variaciones en la eliminación de ooquistes para definir el periodo de muestreo en análisis de prevalencia o carga coccidiana, y de este modo se limite el muestreo al principio de la tarde o bien se realice control estadístico de este factor.

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Time of day, age and feeding habits influence coccidian oocyst shedding in wild passerines

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Abstract

Protozoan coccidia are one of the most common intestinal parasites in birds. Ordinary coccidian detection and quantification techniques have proved to be inaccurate for wild passerines due to the existence of marked oocyst shedding rhythms throughout the day. Previous studies have suggested that these rhythms should be taken into account when analysing coccidian load and prevalence data, but their pattern and magnitude still remain poorly known. In this study we characterised shedding rhythms in the field by means of 406 samples of faeces taken from two species of passerines with different diets: the European Serin (a granivorous species), and the Garden Warbler (an insectivorous species). Both coccidian prevalence and load were two-phased, with maximums occurring in the afternoon. Oocyst elimination remained consistently high during the second half of the day, whereas prevalence peaked during the afternoon, lowering throughout the evening. This pattern was found in both species. We found a high repeatability of prevalence and intensity when differences between the morning and afternoon were statistically controlled. As a result, we suggest that sampling periods used in the analysis of coccidian prevalence and/or load studies should take into account these differences in times of shedding and be limited to the afternoon, otherwise a statistical control of this factor will be required.

Keywords: Avian; Circadian rhythm; *Isospora*; Parasite; Bird; Coccidiosis; Protozoa

1. Introduction

Parasitism is widespread in nature and its importance in the ecology and evolution of organisms is well known (Thomas et al., 2005). Host-parasite relationships in avian passerine species have become a common focus of research over the last decade. Many studies have centred on ecto- and blood parasites, whereas due to the lack of an accurate quantification method, rather fewer have focused on endoparasites. Coccidian protozoa are intestinal parasites that are found in most vertebrate species and which have been shown to be involved in many ecological avian processes (McGraw and Hill, 2000; Hill, 2002). Most (families *Eimeridiidae* and *Cryptosporidiidae*) are monoxenous, the transmission between individuals taking place via infective oocysts released in faeces. The only non-invasive method of determining the presence and burden of these coccidians is to detect and count oocysts in host faeces (Watve and Sukumar, 1995). This is, however, an inaccurate method for field studies, in which only one sample can usually be taken at a time, because circadian variation in oocyst shedding has been observed in many species. For example, variation is known to occur in some species of the genus *Eimeria* that infect domestic chickens and partridges (Clarke, 1979; Willliams, 1995; Villanúa et al., 2006). Passerines are mainly infected by species belonging to the genus *Isospora* (reviewed by Giacomo et al., 1997; McGraw and Hill, 2000), in which a host-dependent circadian variation in oocyst shedding has also been observed (Boughton, 1933). Although previous studies with passerines have suggested that oocyst discharge is much greater in the afternoon than in the morning, knowledge of this process is still deficient and indeed many of these studies have only

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focused on prevalence (Brawner and Hill, 1999; Hudman et al., 2000; Brown et al., 2001). Other studies have assumed, but not tested, the existence of alternative morning/afternoon states (Schwalbach, 1961; Hudman et al., 2000; Misof, 2005 . Eurasian Blackbirds (*Turdus merula*) and their gastrointestinal parasites: A role for parasites in life-history decisions? ULB, Bonn.), or have been performed on birds kept in captivity for days or weeks at a time (Boughton, 1933; Brawner and Hill, 1999; Dolnik, 1999a). Moreover, the effect that diet and the natural activity rhythms of hosts may have on oocyst shedding rhythms has never been analysed. Since digestive physiology varies with feeding habits (Sturkie, 1986), oocyst shedding may well also be influenced by these variables. Thus, knowledge of patterns occurring in circadian rhythms with different feeding habits will help researchers to collect, analyse, and interpret data correctly. In this paper, we describe coccidian oocyst shedding rhythms in the field over the whole day in two species of passerines with different feeding patterns. Our goal was to achieve an accurate coccidian detection and quantification method in order to establish the best sampling period to use in field studies. We analysed oocyst presence and burden in faeces of both a seed-eater and an insectivorous species of free living passerines.

2. Materials and methods

2.1. Field work

Because of their abundance and diet specificity, European Serins (*Serinus serinus*, Linnaeus 1766) and Garden Warblers (*Sylvia borin*, Boddaert 1783) were chosen as models of seed-eater and insectivorous birds, respectively. Birds were trapped during daylight between March and May in 2004 and 2005 in a tree nursery in the city of Seville (37°23′11″N, 5°57′46″W), with mists nets placed amongst bushes. Birds were individually marked with numbered aluminium rings, sexed and aged (as juveniles or adult birds) according to Svensson (1996). Capture and handle of the birds were carried out with animal ethics approval by the Spanish Environment Ministry. Whereas *S. borin* is strictly migrant in the study area, *S. serinus* is a common breeder. Consequently, juveniles were only present in the samples of *S. serinus*. Birds were kept individually in cloth bags for 20 min to collect faecal samples and were then released. Of the total trapped birds 49.9% of *S. serinus* and 84.2% of *S. borin* produced faeces during the 20-min capture period. Between 0.5 and 1 mg of faeces were placed in individually marked vials containing 5% formol and the collection time was recorded for each sample. Because urine does not contain oocysts and given that its mass would have affected the sample mass, we only analysed the intestinal component of the dropping and rejected the part corresponding to urine. When the two fractions could not be separated, the sample was excluded from the study. A total of 406 samples (252 from *S. serinus* and 154 from *S. borin*) were included in the analysis.

2.2. Laboratory method

Samples were filtered through a double piece of cotton-lint cheesecloth (which oocysts easily pass through) and then homogenized to obtain a dilution. This was scanned for coccidian oocysts in a McMaster chamber (Williams, 1973). This method of quantification is the most widely used method in passerine coccidia research (Hõrak et al., 2004; Misof, 2004; Hõrak et al., 2006). Since the low concentration of faecal debris made it easy to find oocysts, no oocyst concentration method (such as flotation) was used, in order not to affect oocyst density. The scanning area of the McMaster chamber contains 300 μl of sample, and it is divided by nine parallel lines into 10 rectangular sections. Two scanning areas were examined for each sample. Subsequently, 200 μl of the same dilution was taken from the chamber and dried out in a 54 °C heater; the extract was then weighed to the nearest 0.0001 g in an Ohaus Voyager precision weight (Ohaus, Switzerland). Coccidian load values (expressed as the number of oocysts per milligrams of dry extract of faeces) were obtained by dividing the number of oocysts counted in the chamber by the estimated mass of the scanned sample. Prevalence was calculated as the percentage of individuals releasing oocysts in faeces out of the whole group of sampled birds. Unlike chicken *Eimeria* oocysts found in faeces, most of the oocysts in our sample were already sporulated, allowing identification to genus level. Based on size and number of sporocysts, oocysts were identified as *Isospora*-like. The repeatability of coccidian load values was estimated by blindly counting the load of 10 individuals twice and calculating the intra-class correlation (Lessels and Boag, 1987). Repeatability of coccidian load estimates from the same samples was very high (97%). This gave confidence to the accuracy of oocysts counts.

2.3. Statistical analysis

Because daylight length varied by 2 h and 36 min during the sampling period, hourly data from different days were not comparable. To obtain comparable hourly data, this variation was controlled by comparing the hourly data to total day length. The time of sample collection was transformed in the following way: relative hour = (time of sample collection − time of daybreak)/(time of sunset − time of daybreak). Values for the relative hour ranged from 0 to 1. As coccidian load and prevalence does not necessarily change linearly with time, relative hourly values were standardized by rounding off to the next decimal point. Thus, the standardized hour was analysed as a 10-level factor. Coccidian prevalence and load data did not fit a normal distribution, so traditional parametric statistical methods based on variance analysis could not be performed. Generalized linear models (GLMs) were performed instead. GLMs allow a more versatile analysis of correlation than standard methods, since the error distribution of the dependent variable

and the function linking predictors can be adjusted to the characteristics of the data.

The effects of standardized hour, species and year on coccidian load and prevalence were analysed as independent factors. To test for differences between species in the hourly patterns of oocyst shedding, the interaction between the standardized hour and species was included in the initial models. The coccidian load was analysed with a negative binomial distributed error and a log link, while prevalence was analysed by means of a binomial distributed error and a logit link. Models were fitted using the GENMOD procedure with the III type of sum of squares and backwards stepwise selection procedure using the SAS 9.1 statistical package (Littell, R.C., Milliken, G.A., Stroup, W.W., Wolfinger, R.D., 1996 SAS System for Mixed Models. SAS Institute Inc. Cary, NC, USA). The least significant variable was excluded from the model and the model was refitted to the data until all of the remaining variables contributed significantly to the fit (as judged by partial P-values < 0.05). To test the effect of age on oocyst shedding patterns, a separate analysis using only data for *S. serinus* was carried out, since no yearlings of *S. borin* were captured. In this analysis, the standardized hour, year, age (expressed as yearling/adult) and the age*hour interaction were included as factors.

Because preliminary analysis suggested the existence of a morning/afternoon dichotomy regarding load and prevalence, the repeatability of both batches of data was calculated with and without taking into account the morning/afternoon factor (defined as the first and second half of the day). For these analyses, 48 samples of 23 individuals (nine *S. borin* and 14 *S. serinus*), captured at different hours and on different days, were used. A random factor controlling for individuals was included in the model using the GLIMMIX procedure in SAS 9.1. The repeatability of prevalence data was calculated using the latent variable approach described by Browne et al. (2005). We are not aware of a similar approach for variance decomposition and repeatability calculation for variables with a negative binomial distribution and so the repeatability of the coccidian load data was calculated with log transformed values and a normal distribution error. Given that 14 individuals were captured both in the morning and the afternoon on different days (10 *S. serinus* and four *S. borin*) we used a Wilcoxon non-parametric test to compare the coccidian load during these two periods.

3. Results

3.1. Effects of hour, species and age on coccidian prevalence

Standardized hour was the only variable significantly related to coccidian prevalence in our model (Table 1). No significant differences in prevalence were found either between species or between yearling and adult *S. serinus* ($\chi^2 = 0.21$, d.f. $= 1$, $P = 0.649$). The test of mean differences in the minimum squares showed that prevalence was similar and peaked in periods 6, 7, 8 and 9. This means that coccidian elimination in infected individuals takes place mainly between 1/2 and 9/10 of the daylight period (Fig. 1). The repeatability of coccidian prevalence was low when morning/afternoon was not taken into account (5.26%), but when this factor was controlled for, the repeatability was very high (90.19%).

3.2. Effects of hour, species and age on coccidian load

Both standardized hour and species, as well as the interaction between these two factors, were significantly related to coccidian load (Table 1). No differences in coccidian load were found between years. The test of differences in minimum squares identified two homogeneous periods in the day in both species (standardized hour periods 1, 2, 3, 4 in one group, and 6,7, 8, 9 in the other group; neither of the groups was significantly different from each other in hour period 5). This result shows that oocyst elimination was homogenously low during the first 2/5 day and homogenously high during the second half of the day (Fig. 2). Lower oocyst discharge occurred in *S. borin* (minimum squares mean $\pm$ standard error in log scale: 4.29 ± 0.21; $n = 49$) than in *S. serinus* (4.49 ± 0.15; $n = 109$). The test of differences in minimum squares showed that this difference was limited to period 2 ($\chi^2 = 4.27$, d.f. $= 1$, $P = 0.04$) and to the afternoon periods 6 ($\chi^2 = 75.22$, d.f. $= 1$, $P < 0.0001$), 7 ($\chi^2 = 9.54$, d.f. $= 1$, $P = 0.002$), and 8 ($\chi^2 = 4.21$, d.f. $= 1$, $P = 0.04$). In *S. serinus*, the oocyst load was greater in yearlings (5.38 ± 0.24, $n = 64$) than in adults (4.48 ± 0.21, $n = 41$; $\chi^2 = 7.66$, d.f. $= 1$, $P = 0.005$). Twelve out of 14 individuals trapped both in

Table 1
Models analysing the effects of year, standardized hour and species, as well as their interactions, for both coccidian prevalence and load

Factor	Prevalence				Load			
	Estimate	F	d.f.	P	Estimate	F	d.f.	P
Standardized hour	−2.525 to 1.704	142.36	9	<0.0001	−2.591 to 3.374	78.68	8	<0.0001
Species		0.08	1	0.772	0.658	5.66	1	0.017
Year		0.17	1	0.683		2.51	1	0.113
Standardized hour*Species		9.86	8	0.277	−5.357 to 1.432	43.56	8	<0.0001

Model selection followed a backwards stepwise selection procedure. For variables included in the final model, parameter estimates and statistical significance are given. For variables not included in the final model the statistical significance when added to the final model is given.

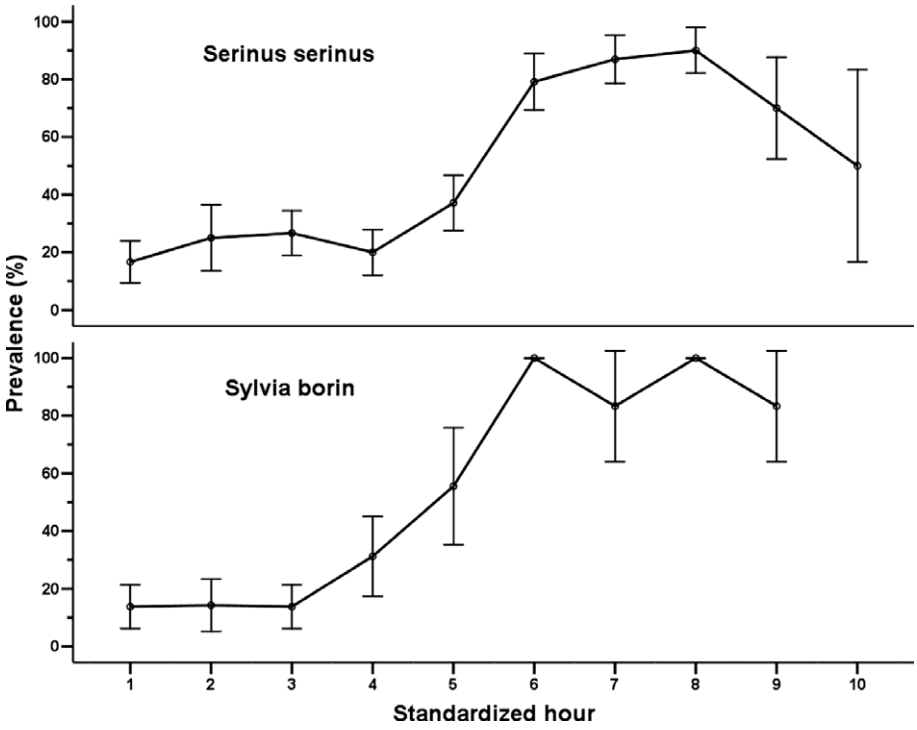

Fig. 1. Estimated coccidian prevalence (expressed as the percentage of individuals releasing oocysts in faeces) throughout the daylight period showed higher values during the second half of the day. Lines represent the S.E.M. for each period.

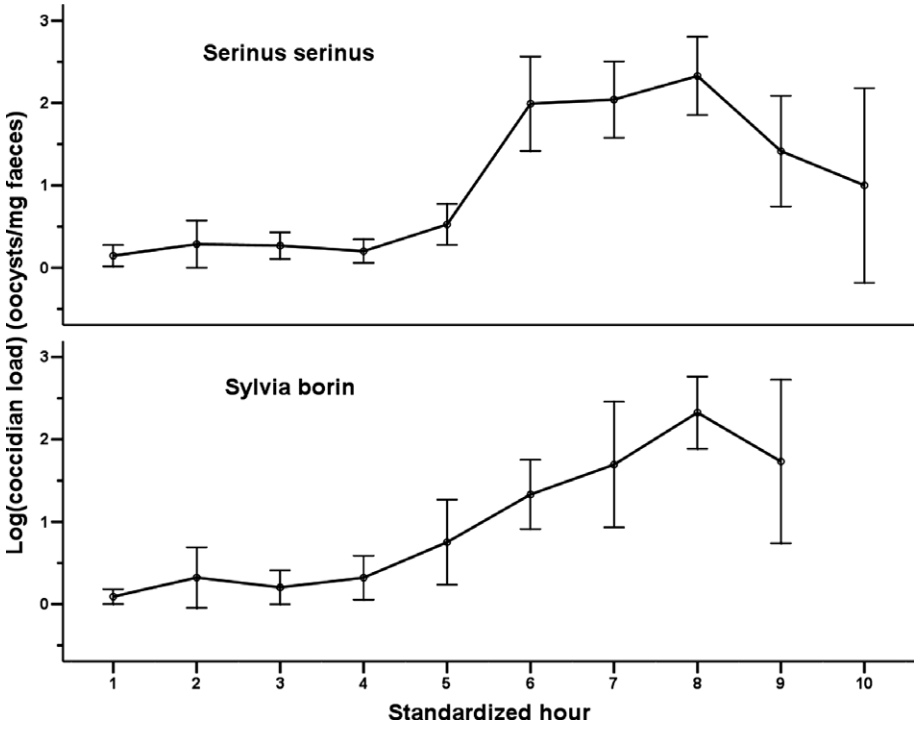

Fig. 2. Estimated coccidian load (expressed as number of oocysts released in each milligram of dry extract of faeces) throughout the daylight period showed a peak during the late afternoon. Mean ± standard error of log transformed (log(value + 1)) load values are represented.

the morning and the afternoon showed higher oocyst discharges during the afternoon; in the other two birds no shedding occurred in either of the periods (Wilcoxon Test, $Z = -3.59$, $P = 0.002$; Fig. 3). Coccidian load estimates were only repeatable after controlling for the morning/afternoon factor (0% without control, 41.92% after

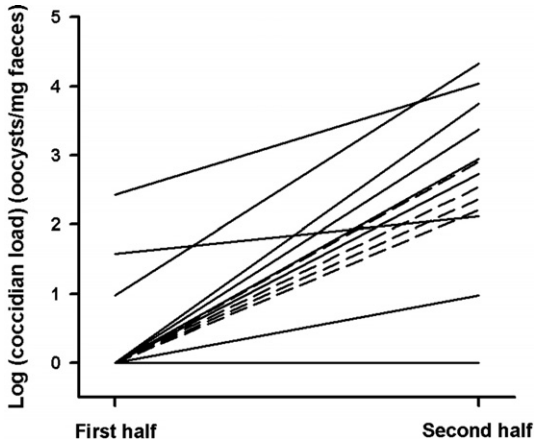

Fig. 3. Differences in coccidian load between morning and afternoon for 14 individuals sampled in both periods. Load values were higher during the afternoon for all individuals except for two, which presented no oocyst shedding in either period (and so their lines overlap). Solid lines represent *Serinus serinus* ($n = 10$) and dashed lines *Sylvia borin* ($n = 4$). Coccidian load data are presented log transformed (log (value + 1)) in order to get a better idea of the differences between individuals.

controlling for the factor). Since recaptures were usually separated by several days (range 0–66 days), it is possible that oocyst loads had changed during this time. For this reason much higher repeatability estimates were found when using data from individuals recaptured within fewer than 6 days (76.35%, controlling for morning/afternoon).

4. Discussion

This research shows that coccidian oocyst shedding in free-living passerines presents clear circadian rhythms, which strongly affect the estimates of both coccidian prevalence and load based on oocyst-counting in chamber. In the wild, these findings are not affected by changes in diet or in the typical activity patterns of birds kept in captivity. We believe this study is the first report of the existence of differences in coccidian load between species with different feeding habits. It also suggests that coccidian load is related to age in wild passerines. The results indicate that prevalence is not related to bird age, a finding that agrees with previous work (Dolnik, 1999a; Hudman et al., 2000; Misof, 2005). Yearling *S. serinus* exhibited greater oocyst discharge in their faeces than adults. This is to be expected given immune system physiology, since the presence of coccidia leads to the acquisition of immunity (Rose and Hesketh, 1982; Lillehoj and Trout, 1996) and so hosts will increase their immune response in cases of consecutive exposure to parasites (Guzman et al., 2003; Ding et al., 2004). This would explain the drop in coccidian load as birds age. Both coccidian load estimates and prevalence estimates revealed circadian bimodal cycles with oocyst discharge peaks in the afternoon. These patterns agree with previous results obtained from captive populations (Dolnik, 1999a; Brawner et al., 2000). The two species studied did not differ with respect to prevalence estimates, but did with respect to load estimates, a fact that indicates that different physiologies possess different oocyst elimination patterns. This difference was not caused by the presence of yearling *S. serinus* in our sample because excluding yearlings from our model did not change the results (results not shown). It is known that coccidian oocyst shedding is controlled by host physiology (Boughton, 1933; Dolnik, 1999a,b), and the physiological differences between feeding habits is probably responsible for this difference. The mechanisms underlying this process, however, are still unclear. A variation in the amount of faeces produced according to time of day could influence these rhythms. Nevertheless, researchers must be aware that highly reliable estimates of both prevalence and load can be obtained if, and only if, time of day is taken into account. Estimates of prevalence and load made without considering time of day were not repeatable, but were highly repeatable when a factor coding for morning/afternoon was included. Another good method for obtaining accurate data seems to be to restrict the sampling period. Coccidian load sampling should be restricted to the second half of the total daylight time, whereas in the case of prevalence, samples should be taken at between 1/2 and 4/5 of the daylight time. This more restrictive period should thus be considered as the best period for obtaining reliable data for both coccidian prevalence and load because even heavily infected individuals could not release oocysts during the morning.

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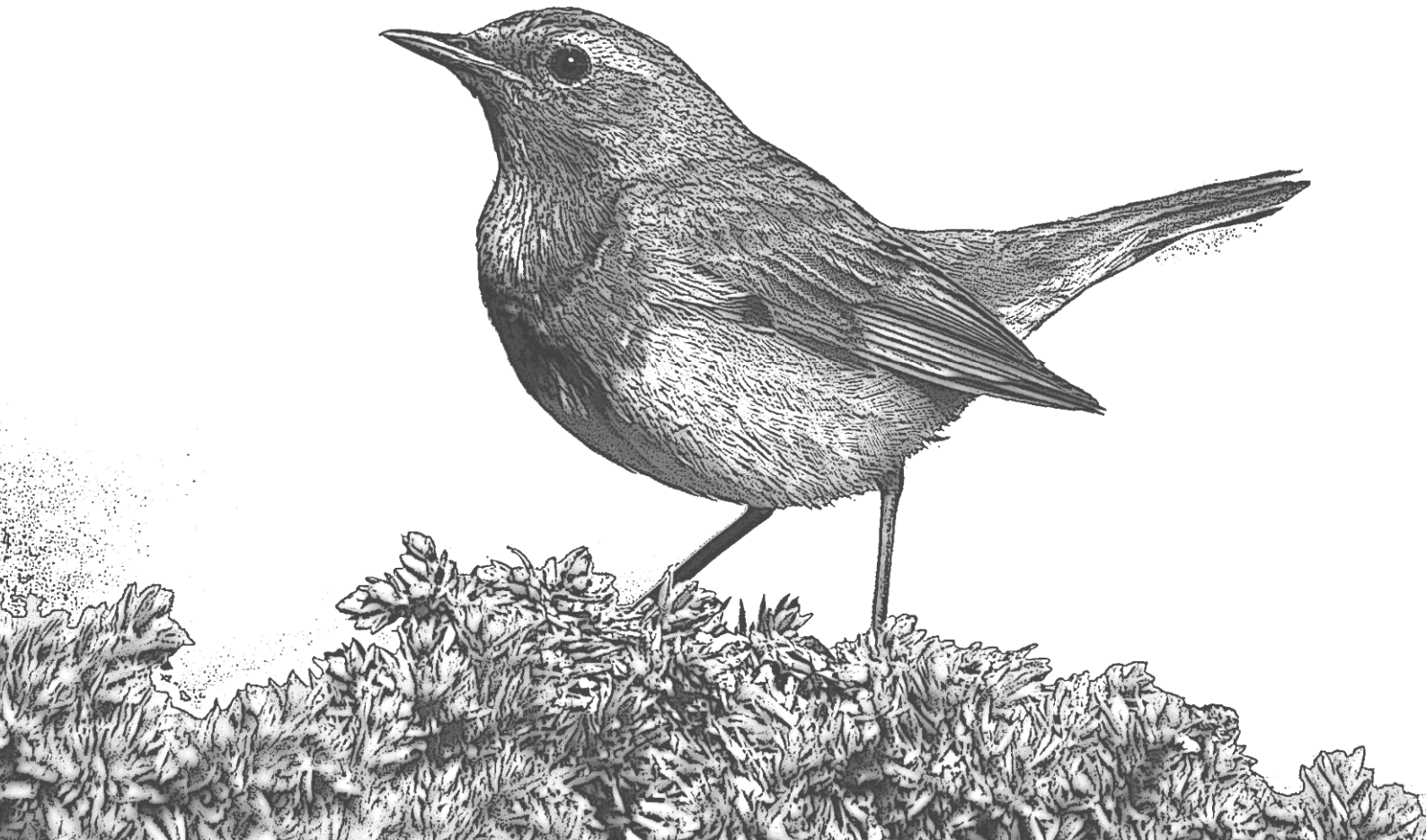
3. COSTES FISIOLÓGICOS DE LA PARASITACIÓN Y SU RELACIÓN CON LA ORNAMENTACIÓN



3.a CAPÍTULO 2: Las lavanderas blancas que muestran muda parcial más extensa están más estresadas.

Resumen:

Los individuos jóvenes de muchas especies de aves paseriformes realizan una muda parcial postjuvenil en la que reemplazan la mayor parte de las plumas coberteras y un número variable de cobertoras y plumas de vuelo. Esta muda genera diferencias de coloración perceptibles en muchas especies, que pueden actuar como señales de estatus. En este estudio analizamos cómo la extensión de la muda parcial se relaciona con diferentes estimadores de condición. Para ello, analizamos 43 individuos juveniles de lavandera blanca (*Motacilla alba*) capturados en un dormidero urbano de la ciudad de Sevilla. La extensión de la muda parcial estuvo positivamente relacionada con la razón heterófilo/linfocito (H/L) circulante (estimador de estrés), pero no lo estuvo con abundancia de leucocitos ni con la masa corporal. Los individuos con plumaje más similar al de los ejemplares adultos pueden estar expuestos a niveles superiores de estrés debido a la agresividad de los adultos territoriales. En consecuencia, el incremento de H/L en nuestro estudio es, probablemente, consecuencia de la extensión de la muda más que una explicación a la variación intraespecífica en la extensión de la muda.

White Wagtails *Motacilla alba* showing extensive post-juvenile moult are more stressed

Guillermo López[1,*], Jordi Figuerola[1], Nico Varo[2] & Ramón Soriguer[1]

López G., Figuerola J., Varo N. & Soriguer R. 2005. White Wagtails *Motacilla alba* showing extensive post-juvenile moult are more stressed. Ardea 93(2): 237–244.

Young individuals of many passerines undergo a partial moult and replace most of their body feathers and a variable number of coverts and minor wing feathers. In many species, this moult generates perceptible coloration differences, which may act as status signals. This study analyses how the extent of partial moult is related to different estimators of condition. A total of 43 young White Wagtails *Motacilla alba* caught in an urban roost in the city of Seville were analysed. The extent of their partial moult was positively correlated to the heterophil-lymphocyte ratio, but not to the abundance of leukocytes or to body mass. Individuals with more adult-like plumage may be exposed to higher stress due to the aggressiveness of territorial adults. Consequently, the increased heterophil-lymphocyte ratio found in the study is probably a consequence of the extent of moult rather than an explanation of intraspecific variation in the extent of moult.

Key words: partial moult, wagtail, stress, heterophil, lymphocyte, H/L

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INTRODUCTION

The functions of avian plumage in animal communication have attracted the attention of evolutionary biologists for many years (Darwin 1871, Butcher & Rohwer 1989, Zahavi & Zahavi 1997). Plumage characteristics may convey information such as (1) the capacity to care for young (reviewed in Andersson 1994), (2) the ability to escape from predators (Papeschi & Dessì-Fulgheri 2003), (3) genetic quality (Fitze *et al.* 2003, McGraw & Ardia 2003) or (4) social status (Badyaev & Ghalambor 1998, Senar *et al.* 2000, Velando *et al.* 2001). Additionally, many plumage characteristics have been shown to act as reliable indicators of health status (McGraw *et al.* 2002, Papeschi & Dessì-Fulgheri 2003), parasite load in the blood (Merilä *et al.* 1999, Figuerola *et al.* 1999), ectoparasite abundance (Doucet & Montgomerie 2003) and the functioning of the immune system (Lindström & Lundström 2000, Saks *et al.* 2003).

Juvenile birds of many species present drab plumages, usually similar to those of females. Although different functions have been proposed for this delayed maturation of plumage, the most widely accepted hypothesis suggests that it may reduce aggression from adults (McDonald 1993, Muehter *et al.* 1997). Many passerines perform a partial post-juvenile moult with great intraspecific

variation in extent (Gosler 1991, Smith 1992, Jenni & Winkler 1994). Although this partial moult has been shown to be relevant in social communication (Senar *et al.* 1998b), little is known about the factors that influence its extent. Patterns of post-juvenile moult have been studied in some passerine species which showed that there is a correlation between the extent of moult among most feather tracks (Jenni & Winkler 1994, Deviche 2000). Furthermore, the extent of moult is related to the intensity of colour (Jenni & Winkler 1994). Intraspecific differences in the extent of partial moult are little studied and are mainly considered to be dependent on energetic or time constraints (Jenni & Winkler 1994). This point of view is based on the assumption that partial moult is 'the best of a bad job', that is, individuals aim to moult as much of their plumage as they can. However, partial moult generates age-related patterns of coloration that seem to have implications for sexual selection and social behaviour (Savalli 1995) and, consequently, the optimal extent of moult may depend on individual condition.

Numerous condition and health indices have been used in ornithology (Hõrak *et al.* 2002). Size-corrected body mass is commonly used as an estimator of condition (Gosler *et al.* 1998); other indices, based mainly on leukocyte variables, are also good indicators of physiological/health status (Ots *et al.* 1998). Leukocytes are important components of the immune system, becoming altered in quantity and composition when an organism is exposed to pathogens or stress (Campbell 1995). Consequently, values for the total number of leukocytes (TLC) have been interpreted as an indication of an individual's current investment in immune defence (Ots *et al.* 1998, Nunn *et al.* 2000). In particular, high TLC values are characteristic of the inflammatory processes that occur in response to microbial infections and injuries (Hõrak *et al.* 2002, Thrall *et al.* 2003). Heterophils and lymphocytes are the most numerous cellular lines in the immune system and they are usually responsible for the change in the total number of leukocytes. Heterophils act as the first defence barrier (Thrall *et al.* 2003), whereas lymphocytes lead the specific defence in the immune system. Given that heterophils and lymphocytes are found so profusely in immune systems, their ratio (H/L) has been widely used as an estimator of stress in birds (Totzke *et al.* 1999, Thrall *et al.* 2003). In avian species, stress generates a decrease in circulating lymphocytes and an increase in circulating heterophils (Davison *et al.* 1983), leading thus to an increase in the H/L ratio. H/L acts as a reliable indicator of stress in passerines (Groombridge *et al.* 2003). Furthermore, in birds H/L is a useful measurement of stress caused by long-term changes in the environment, social rank, or the action of chronic stressors (Gross & Siegel 1983, Davis *et al.* 2000), and is even more useful than a single measurement of plasma corticosterone levels (Vleck *et al.* 2000). It is therefore a good indicator of health for use in behavioural studies in free-ranging birds (Gross & Siegel 1983).

The White Wagtail *Motacilla alba* is a partial migrant passerine which winters in Iberia in large numbers. At the end of summer, first-year birds replace part of their juvenile plumage and show great inter-individual variation in the extent of moult. This species shows both flocking and territorial behaviour during winter (Davies 1976): while some individuals (mainly adult males) defend feeding territories, most juveniles and adult females form larger flocks (Zahavi 1971, Davies 1982). Individuals with young-looking plumage are allowed by owners to feed in their territory (Davies 1981a, b), a fact that suggests that partial post-juvenile moult may play a role in communication in this species. In this study, the relationship between the extent of moult and different estimates of body condition in juvenile White Wagtails is analysed as a means of testing the hypothesis that moult extent is related to individual health status.

MATERIAL AND METHODS

Fieldwork and measurements

A total of 43 young White Wagtails were trapped in an urban roost in the city of Seville (37°23'N,

5°57'W) in November 2003. The wagtails roost in several types of ornamental trees between 5 and 10 m above the ground; the total number of wagtails using the roost has been estimated at over 500 000 birds (Vázquez *et al.* 2001). Birds were caught between 18:00 and 20:00 h in Japanese mist nests placed 8 m above ground level between the trees where the birds spent the night. For each individual, wing (to the nearest 0.5 mm, mean ± SE: 88.35 ± 0.4) and tarsus length (to the nearest 0.1 mm, 23.32 ± 0.16) were measured. Body mass was measured with a digital scale (to the nearest 0.1 g, 21.51 ± 0.23) and age was determined from plumage characteristics (Svensson 1992). Subsequently, the number of moulted greater wing coverts (GC) on the right wing was counted in all the individuals. Given that the extent of post-juvenile moult in White Wagtails is correlated with the number of moulted GC and most of the rest of feather tracks (Jenni & Winkler 1994), this measurement was used as an indicator of the global moult appearance. All measurements were made by the same person (NV).

Sampling protocol

After measurement, a 0.25 ml sample of blood was taken from the jugular vein with 29 G sterile insulin syringes. A drop of blood was used to prepare a smear on a microscopy slide (Bennett 1970), which was air-dried and properly fixed and stained using Diff-Quick solution. The rest of the blood sample was placed into a vial without anticoagulant and then after several hours centrifuged for 10 minutes at 6000 rpm in an Eppendorf Minispin centrifuge to separate serum from cells. Both sera and cells were kept frozen at –20° C until subsequent analysis. All data was collected in the hour after capture and the time each bird was handled (less than five minutes) was almost the same in all cases, and so no important differences in circulating heterophils should have been provoked.

Molecular sexing

The cellular fraction of the blood sample, which was obtained without anti-coagulants, was used to sex the birds. Sex was determined from blood cell DNA, using polymerase chain reaction (PCR) amplification of the CHD genes (Ellegren 1996, Griffiths *et al.* 1998). All the birds were successfully sexed by this method; in 98% of individuals these results agreed with sexing in the field based on plumage characteristics.

Blood smear analysis

TLC was estimated by the method of Lane (1996), that is, by counting the number of leukocytes on twenty 400x light microscope monolayer fields and selecting those with a similar cell density. The total number of white blood cells per microliter was calculated by multiplying this value by 100. This method of estimation is well correlated with estimates obtained by counting in chambers (Wiskott 2002). For each smear, the cellular type (heterophils, lymphocytes, eosinophils, monocytes and basophils) of 100 leukocytes was identified according to Campbell (1995), and the H/L was calculated as the ratio of the numbers of heterophils and lymphocytes (Fig. 1). Blood parasites (intraerythrocytic protozoa and circulating protozoa and nematodes) were searched for at low (100x; 5 min), medium (400x; 10 min) and high (1000x; 10 min) magnification (Deviche *et al.*

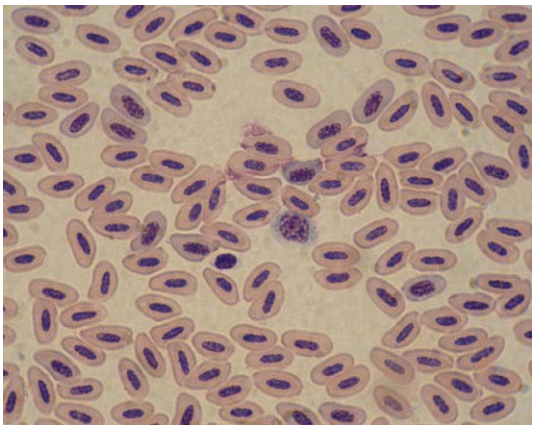

Figure 1. Blood smear of White Wagtail showing abundant erythrocytes and a single lymphocyte (in the middle of this photo by G. López). Stress was estimated from the ratio of numbers of heterophils (not in this picture) and lymphocytes.

2001). In the case of intraerythrocytic protozoa infection, a total of 1500 erythrocytes were counted, getting the genus identification and the intensity of infection. Only one individual (2.3%) was infected by the intraerythrocytic haematozoan *Haemoproteus* sp., with an infection intensity of 0.03% of erythrocytes; the exclusion of this individual from subsequent analyses had no qualitative effect on the results. The repeatability of leucocite variables was estimated by counting the smears of ten individuals twice and subsequently calculating the intra-class correlation (Lessells & Boag 1987). Repeatabilities were very high for both TLC (r = 0.95, $F_{9,10}$ = 35.31, P < 0.0001) and H/L (r = 0.90, $F_{9,10}$ = 18.87, P < 0.0001).

Statistical analyses

In order to discover the effects that the extent of partial moult could have on different condition indices, a MANOVA was used with TLC, H/L and body condition as dependent variables, sex as a factor and number of moulted GCs as a covariate; the interaction between sex and number of moulted GCs was also included. We followed a stepwise backwards selection procedure until all the independent variables increased significantly the fit of the model. We chose tarsus instead of wing length as a measure to standardise body mass for bird size because of its independence from moult processes; the tarsus grows in the nest long before the post-juvenile moult takes place. Nevertheless, results did not change qualitatively when using body condition estimated in relation to wing length. Body condition was estimated as the result of the regression of body mass on tarsus length (r^2 = 0.44). We used the Shapiro-Wilk test to test the fit of the different variables to a normal distribution. Body condition, TLC and log transformed H/L were normally distributed. The distribution of the number of moulted GCs could not be normalised and analyses were done using ranked values (see Conover & Iman 1981). Ranks were assigned to each value as a consecutive number from 1 to 43, from lower to higher values, and whenever identical values occurred we assigned the average of the ranks to each one.

RESULTS

The number of moulted GC did not differ between males and females (mean ± SE: 6.56 ± 0.47; males: 7.67 ± 0.41, n = 15; females: 5.96 ± 0.66, n = 28; t_{41} = 1.29, p = 0.20). An overall relationship between the number of moulted GCs and the indices of condition ($F_{3,35}$ = 5.25, P = 0.004) was found. There were no significant effects of sex ($F_{3,34}$ = 0.6, P = 0.62) or of the interaction between sex and the number of moulted GCs ($F_{3,33}$ = 1.28, P = 0.29). Univariate *a posteriori* tests suggest that H/L (1.63 ± 1.3) was significantly correlated with the number of moulted GCs (r^2 = 0.243, $F_{1,37}$ = 12.01, P = 0.01, Fig. 2), which indicates that individuals with a larger extent of post-juvenile moult had an increased presence of heterophils in relation to lymphocytes. The number of moulted GCs was not significantly related to TLC ($F_{1,37}$ = 3.06, P = 0.09) or to body condition ($F_{1,37}$ = 0.78, P = 0.38).

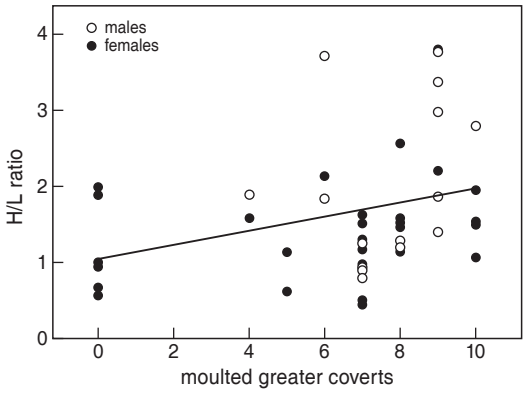

Figure 2. Relationship between H/L and the number of moulted greater coverts in male and female White Wagtails.

DISCUSSION

In this study a correlation was found between the extent of partial moult and H/L. Interestingly, this correlation was positive and contrary to the

hypothesis that the individuals in best condition invest more in moulting. There are at least two possible explanations for this correlation between an indicator of stress and the extent of partial post-juvenile moult. Firstly, individuals in worse condition may invest a relatively greater amount of energy in moulting. Secondly, individuals with more extended moult suffer increased levels of stress during the winter. Contrary to the first explanation, feather moult is directly related to the amount of resources available (Grubb 1991, Carrascal *et al.* 1998). On the other hand, Senar *et al.* (1998a, b) demonstrated that juvenile Siskins *Carduelis spinus* with adult-like plumage received greater aggression from adults than those with delayed moult. As in Siskins, the demonstrated higher permissiveness of territorial adult White Wagtails towards juveniles (Davies 1981b) could explain the increased levels of stress that individuals with more complete moults seem to suffer, since H/L has been shown to correlate better with social stress than with any other estimator (Gross & Siegel 1983). These data suggest that the extent of post-juvenile moult may not only be regulated by energetic or time constraints, but also by social interactions, which could greatly reduce the potential benefits of larger moult extension. Several melanin-derived badges of status are under social control and birds displaying experimentally enlarged badges are exposed to increased levels of aggression and/or mortality (Senar *et al.* 1993, Veiga 1993). Since H/L responds rapidly to food deprivation (Gross & Siegel 1986), both social rank stress and alimentary stress generated by adult intolerance could be responsible for increases in the H/L. Nevertheless, the negative effect of advanced partial moult derived from stress may be the cost paid for obtaining benefits during the breeding season. Accordingly, females prefer to pair with adult individuals rather than with juvenile-looking ones (Samson 1976, Middleton 1979). Consequently, the extent of moult in juveniles could suffer a trade-off between stress derived from social interactions during the winter and pairing success in the next breeding season.

Under this scenario, the correlation between stress and the extent of moult should be negative or not significant at the time of moult. Unfortunately, similar data during the start of moult could not be obtained from our study area because birds come from a variety of different European breeding areas (Belgium, Germany, The Netherlands, Czech Republic and United Kingdom, according to ringing recoveries and subspecies assignment). Nonetheless, a similar study on Siskins showed that the correlation between the extent of moult and body condition was positive just after moult, but negative at the end of the winter (Senar *et al.* 1998b).

The other two estimators, TLC and body mass, have been described as reliable indicators of condition in passerines (Ots *et al.* 1998, Hõrak *et al.* 2002), although no correlation between these factors and the extent of partial moult was found in our study. In the case of TLC, it is known that interstitial liquid variations may strongly alter this value (Campbell 1995, Thrall *et al.* 2003) and that numerous factors may modify the interstitial liquid volume (Sturkie 1986). Another possibility could be the proliferation of certain cellular lines due to injuries, parasites or infections (Campbell 1995). Body mass is also affected by many factors and this could explain the lack of correlation with other variables. For instance, current nutritional status, distances covered to spend the night in the roost, or exposure during the day to different levels of predator abundance (Adriaensen *et al.* 1998, Gosler 1996, Vázquez *et al.* 2001) could be responsible for these changes.

In conclusion, our results suggest that intraspecific variation in the extent of post-juvenile moult is related to health status well after moult completion. Further understanding on how social interactions may affect the costs of moult should clarify the cost-benefit balance faced by juvenile passerines.

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SAMENVATTING

Veel jonge zangvogels ruien voor de winter een deel van de lichaamsveren. De mate waarin dit gebeurt, verschilt sterk van individu tot individu – ook binnen een soort – zonder dat bekend is wat deze variatie veroorzaakt en wat de gevolgen ervan zijn. Er wordt wel verondersteld dat de ruitoestand in de winter te maken heeft met beperkingen in beschikbare energie of tijd tijdens de rui. Individuen die toegang hebben tot meer of beter voedsel, zouden minder last van stress hebben en daardoor in staat zijn meer veren te vervangen dan individuen die in minder gunstige omstandigheden leven. Als dit zo is, dan zou de ruitoestand van jonge vogels een negatief verband moeten laten zien met de mate waarin ze in stress verkeren. Resultaten in de vogelwereld zijn niet eenduidig, want onderzoekers hebben zowel positieve als negatieve trends gevonden.

Om meer duidelijkheid omtrent dit probleem te scheppen vingen de auteurs overwinterende Witte Kwikstaarten *Motacilla alba* op een grote slaapplaats in Sevilla, Zuid-Spanje. Daartoe werden mistnetten op 8 m hoogte tussen de bomen gespannen. Van de gevangen vogels werden biometrische gegevens verzameld en het aantal geruide grote dekveren werd bepaald als maat voor de ruitoestand. Verder werden bloedmonsters genomen om de aantallen te bepalen van witte bloedlichaampjes, waaronder die van heterofielen en lymfocyten. De verhouding van deze twee typen cellen (H/L) wordt wel gebruikt als maat voor de stress waaronder dieren leven (hogere ratio meer stress). In de Witte Kwikstaarten bestond een positief verband tussen de mate van rui en de H/L verhouding, terwijl er geen verband aantoonbaar was tussen de ruitoestand en het totaal aantal witte bloedlichaampjes of het gewicht van de vogel. De verklaring voor de waarnemingen wordt gezocht in het effect van de ruitoestand van de dieren op hun sociale status. Jonge vogels die nog het meest het juveniele kleed hebben, worden het minst door oude vogels lastig gevallen. Jonge vogels die in de winter al het adulte kleed hebben, profiteren daar mogelijk van als ze terugkeren in het broedgebied (onder andere Nederland), maar de prijs daarvoor is een stressvol bestaan in de winter. (CB)

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3.b CAPÍTULO 3: Las máscaras carotenoides de los jilgueros (*Carduelis carduelis*) reflejan información diferente en machos que en hembras

Resumen:

La selección sexual puede jugar un papel importante en la evolución de señales basadas en caroteno. Según la hipótesis de la selección sexual mediada por parásitos, la salud del organismo, la resistencia a parásitos y la expresión de ornamentos estarían interrelacionados. Mientras algunos estudios han analizado la expresión de ornamentos masculinos basados en carotenos en relación a parásitos y capacidad del sistema inmune, pocos se han fijado en los parches de coloración carotenoide expresados en ambos sexos. Nosotros analizamos las relaciones entre la carga de endoparásitos (hemoparásitos y parásitos sitémicos), valores hematológicos y las componentes de color de la máscara roja en jilgueros silvestres, especie que muestra la máscara roja en ambos sexos. Se exploró la calidad inmune y la expresión de color en machos y hembras. La coloración de la máscara facial mostró dimorfismo sexual, presentando los machos máscaras menos naranjas que las hembras. La componente amarilla de la máscara mostró menos intensidad en hembras infectadas con el hemoparásito *Haemoproteus.* El recuento total de leucocitos mostró correlación inversa con la componente amarilla de la máscara en las hembras, sugiriendo que el color de la máscara refleja el estatus inmunitario de las hembras en la temporada de cría. La infección por el coccidio *Isospora* se asoció con una menor reflexión ultravioleta de la máscara facial en las hembras.

Carotenoid-based masks in the European Goldfinch *Carduelis carduelis* reflect different information in males and females

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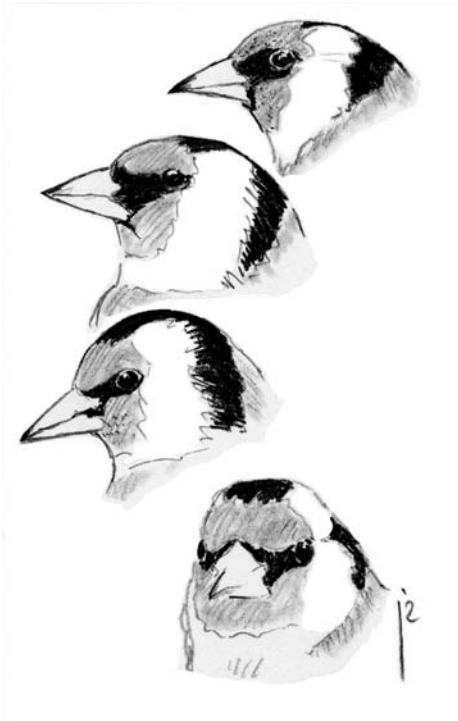

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Sexual selection may play an important role in the evolution of carotenoid-based signals. According to the parasite-mediated sexual selection hypothesis, organism health, parasite resistance and the expression of ornaments are linked. While some studies have analysed the expression of male carotenoid-based ornaments in relation to parasites and immune system capacities, few studies have focused on carotenoid-derived colour patches expressed in both sexes. We analysed the relationships between endoparasite (blood and systemic parasites) loads, haematological values and the components of red mask colour in wild European Goldfinches *Carduelis carduelis*, a species with a carotenoid-based facial mask in both sexes. Both, males and females were assessed for immune quality and face mask expression. Face mask coloration was sexually dichromatic, males have less orange masks than females. The yellow component of the mask showed less intensity in females infected with *Haemoproteus* blood parasites. The total leukocyte count was inversely correlated to the yellow component of the mask in females, suggesting that mask colour reflects the immune status of females during the breeding season. Isospora infection appeared to limit the UV reflection of the red mask of females.

Key words: sexual selection, Goldfinch, bird, carotenoid, ornament, parasites, *Coccidia, Haemoproteus*

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INTRODUCTION

Carotenoid pigments are responsible for many of the brightest colours and most conspicuous signals in birds (Hill 1999, Hill 2002). However, birds are not capable of synthesizing carotenoids from basic biological precursors (Goodwin 1984), and therefore, the expression of carotenoid pigments as colour signals requires the ingestion of carotenoids with food, as well as its absorption, transport, sometimes metabolism and incorporation into the feathers, all processes that are generally considered costly to individuals (Hill 2002, McGraw *et al.* 2005, McGraw *et al.* 2006). Scarcity of carotenoids in the food, or even the energetic cost of enzyme production and manipulation of carotenoids,

remain unclear. Carotenoids are also involved in metabolic pathways related to host immunity and a trade-off between the ornamental and health functions of carotenoids has been proposed (Hõrak *et al.* 2004a, Møller *et al.* 2004). According to this hypothesis, signals based on carotenoid pigments are costly and therefore act as honest indicators of an individual's quality.

The features that each carotenoid-based ornament reflect have been mainly studied in males of very dimorphic species. Carotenoid-based ornaments have been shown to be related to body condition (McGraw *et al.* 2002, Saks *et al.* 2003, Jawor & Breitwisch 2004, Jawor *et al.* 2004), sexual selection (Collias *et al.* 1979, Hill 1991, Drachman 1997) and even parasite loads (Figuerola *et al.* 2003, Hill *et al.* 2004, Hõrak *et al.* 2004). In the case of parasites, studies have reported variously negative (Thompson *et al.* 1997, Brawner *et al.* 2000, Figuerola *et al.* 2003, Hõrak *et al.* 2004b), positive (Burley *et al.* 1991, VanHoort & Dawson 2005) and non-significant (Seutin 1994) relationships between parasites and the expression of a carotenoid-based ornament. The potential causes of these contradictory results could stem from varying methods of study (e.g. correlational vs. experimental, ranges or doses of experimental treatments used), different impacts on parasites on their hosts and environmental-dependent effects of parasites on host condition (Figuerola *et al.* 2003). Work published up to now has generally focused on a single type of parasite, above all blood parasites, and analysis of the relative and combined impact of different types of parasites on ornament expression is still lacking.

Haematozoan parasites are blood-cell parasite protozoa transmitted by blood-sucking arthropods that are quite prevalent in wild passerines (Deviche *et al.* 2001). Coccidian protozoa are widespread intestinal epithelium parasites with a direct biological cycle; transmission results from the ingestion of oocysts liberated in the faeces of an infected individual. Passerines are mainly infected by genus *Isospora* (Hill 2002, Hõrak *et al.* 2006). Both haematozoan and coccidian parasites affect their hosts in a condition-dependent way (Merino *et al.* 2000): they have little impact when resources are abundant (Weatherhead & Bennett 1992, Friend & Franson 2001), but affect negatively the host when resources are scarce (Ots & Hõrak 1998, Ilmonen *et al.* 1999). The mechanisms leading Coccidia to limit the expression of carotenoid-based ornaments may work in at least two different direct ways. First, they may reduce the absorption of carotenoids through the intestine (Tyczkowski *et al.* 1991, Allen 1992) and, second, they may reduce the release of high-density lipoproteins (Allen 1987), which are responsible for the transport of carotenoids in the bloodstream to the tissues. Moreover, a third indirect mechanism related to body condition – to which both carotenoid ornamentation and immunity have shown to be linked – may also be at work (see Smith *et al.* 2007). The mechanisms through which Haematozoa may limit the expression of carotenoid-based ornaments are still unknown.

Most of the relationships described up to now between parasites and the expression of ornaments have been made focusing on conspicuous male ornaments, but little attention have been traditionally paid to such a relation in female ornaments. Roulin (2001), for instance, conducted an experimental study by comparing the degree of female Barn Owl *Tyto alba* ornamentation (eumelanin-based spottiness) with parasite resistance in their offspring raised by foster parents. He found that in females ornamentation positively reflects parasite resistance ability. Some observational studies have also demonstrated that the expression of ornaments is negatively related to the parasite load in female birds (Hõrak *et al.* 2001, Piersma *et al.* 2001).

The aim of this study was to explore the relationship between the colour of the carotenoid-based red mask of European Goldfinches, and a number of indices of condition (haematological parameters and different parasite loads) in both sexes, paying attention to possible inter-sex differences. The study was carried out with free-living birds during the breeding season, just after mate choice had occurred, when individuals were going through the costly task of raising young, because

under these conditions we expected the effect of parasites on their hosts to be maximal. To our knowledge, this is the first study done analysing the relationship between plumage coloration in both sexes and several groups of parasites at a time.

METHODS

The European Goldfinch is a 12-cm long, seed-eating finch that has a unique colour pattern on its head. The front of the face has a conspicuous crimson patch, which is known to be composed of four carotenoid pigments (Stradi *et al.* 1995): a) ε,ε-carotene-3,3'-dione, b) 3-hydroxy-ε,ε-carotene-3'-one, c) 4,4'-dihydroxy-ε,ε-carotene-3,3'-dione (isoastaxanthin), and d) 4-hydroxy-ε,ε-carotene-3,3'-dione.

Although ε,ε-carotene-3,3'-dione and 3-hydroxy-ε,ε-carotene-3'-one are very common yellow pigments in cardueline finches, isoastaxanthin and 4-hydroxy-ε,ε-carotene-3,3'-dione have not yet been found in any other species studied to date. These two pigments provide the red colour in the mask together with the keratin bond arrangement the pigments have in the feathers (Stradi *et al.* 1995). Although both sexes are superficially similar (Cramp & Perrins 1994), small differences in the size of the patch exist, being larger on average in males (Svensson 1996). To our knowledge, differences between the sexes in mask colour have never been investigated.

Fieldwork

In the springs of 2004 and 2005, we trapped 13 adult female and 44 adult male goldfinches in a tree nursery in the Spanish city of Seville (37°23' N, 5°57'W) where these finches are common resident breeders. Birds were captured between sunrise and sunset in 20 twelve-metre long mist-nets. Individuals were marked with numbered aluminium rings. Sex was determined by the presence of a brood patch (only present in females) or a cloacal protuberance (only present in males), and by the colour of the lesser wing-coverts (see Svensson 1996). We also measured body mass (to the nearest 0.1 g) and wing length (maximum chord). Birds were kept individually in clean ringing bags for 20 minutes to collect faecal samples. Faeces were immediately placed in individually marked vials containing 5% formol, and the time of collection was recorded for each sample. To control the mass of faecal samples, we avoided taking the urine-based part of the excretion and only collected the intestinal-based portion. We drew 0.1 ml of blood from the jugular vein using 29 G sterile insulin syringes and prepared smears on a microscopy slide as per Bennett (1970), which were air-dried, fixed and stained using Diff-Quick solution. To confirm field sexing an analysis of the cellular fraction of a drop of blood was performed. Sex was determined from blood cell DNA via a polymerase chain reaction (PCR) amplification of the CHD genes (Ellegren 1996, Griffiths *et al.* 1998). After blood extraction, we took two colour measurements of the frontal area of the red mask in the 57 trapped birds using a MINOLTA CM-2600d spectrometer, which measures the characteristics of reflected light by illuminating the feather surface under standard light conditions. We obtained the reflectance curve of the mask, that is, the light reflection from the UVA (360 nm) to the end of the visible spectrum (740 nm), measured at 10 nm intervals (39 intervals). The UVA reflection is visible to birds and has important implications in sexual selection in some passerine species (Saetre 1994, Siitari *et al.* 2002, Pearn *et al.* 2003). Although 700 nm has been shown as a maximum wavelength for avian visual sensitivity, we included 700–740 nm interval within the analysis because birds indeed present variability in visual spectrum among different species (Bowmaker *et al.* 1997) and, to our knowledge, this spectrum has never been studied in the Eurasian Goldfinch.

Blood smear analysis

For each blood smear, we estimated the total leukocyte count (TLC) by counting the number of leukocytes on twenty 400x light microscope fields of similar density and multiplying this value by

100 (Wiskott 2002). The differential leukocyte count was made by identifying (according to Campbell 1995) the cellular type (heterophils, eosinophils, basophiles, lymphocytes or monocytes) of 100 leukocytes at 1000x magnification. The heterophil-lymphocyte ratio (H/L) was calculated as the percentage of heterophils divided by the percentage of lymphocytes. A total of 15 000 erythrocytes were scanned for blood parasites at low (400x) and high (oil 1000x) magnification (Godfrey *et al.* 1987) and in infected individuals, the blood parasite load was estimated as the percentage of infected erythrocytes. Prevalence was calculated on the basis of the percentage of infected individuals. Only *Haemoproteus* spp. (prevalence: 23.7%) and *Plasmodium* spp. (7.9%) were found in the 38 samples analysed. The repeatability of all variables was estimated by counting twice the smears of ten individuals and calculated as the intra-class correlation (Lessells & Boag 1987). Repeatabilities were high for TLC (95%), H/L (90%), and blood parasite infection (92%).

Coprology

In the laboratory, faecal samples were passed through a double lint filter and mixed to obtain a homogeneous dilution, which was then analysed for coccidian oocysts and other endoparasite eggs using a McMaster chamber. This method only provides an estimation of the real parasite load, although it has been described as the only possible non-invasive method of research on the intestinal parasite of wild animals (Watve & Sukumar 1995). Samples were scored as positive, when coccidian oocysts were observed, and negative, when not. Only protozoan coccidia were found in the sample. Based on size and the number of sporocysts, the oocysts were identified as *Isospora*-like (Baker *et al.* 1972, Grulet *et al.* 1982). Repeatability of coccidian infection estimated from samples of 10 individuals scored twice was very high (97%), giving confidence in the accuracy of oocyst counts.

Colour characteristics

Reflectance curves were analysed by a Principal Component Analysis of reflectances at the 39 different wavelength intervals. Four relevant components (Eigenvalues > 1) were obtained that together summarised 99.97% of variance (Fig. 1). The first component (PC1) summarised reflection in the visible spectrum, mainly between 400 and 530 nanometers (within violets and blues). PC2 was more positive for individuals with more reflection at 650–740 nanometers (reds) and less reflection at lower wave lengths. PC3 was negatively related to reflection in the 550–590 nanometers intervals (yellows), so this component represents the yellow carotenoids reflection. PC4 was mainly influenced by reflection in the non-visible-to-humans portion of the spectra, between 360 and 390 nanometers (UVA radiation). The repeatability of the colour measurements was calculated as the intra-class correlation of the principal components from ten individuals measured twice (Lessells & Boag 1987). The repeatability was very high for all components (PC1: 98%; PC2: 96%; PC3: 97%; PC4: 96%) because of the great accuracy of the spectrometer method (see Figuerola *et al.* 1999).

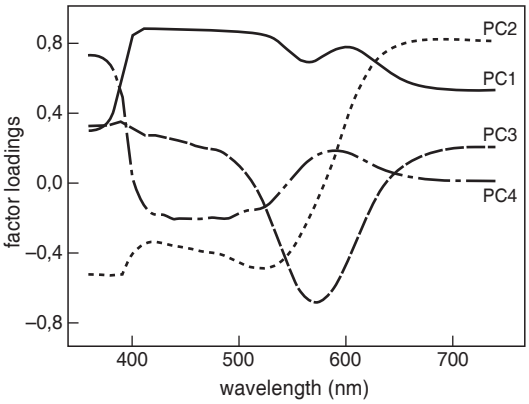

Figure 1. Factor loading of the four principal components calculated from light reflectance at 10 nm intervals between 360 and 740 nm. PC1 represents the blue-violet component, PC2 the red one, PC3 includes the yellows, and PC4 represents the UVA reflection.

Statistical analyses

TLC was log-transformed to fit a normal distribution. H/L did not fit normality by any common

transformation so ranked values were used in the analyses (Conover & Iman 1981). We analysed sexual dimorphism in colouration (PC1, PC2, PC3 and PC4), haematological values (TLC and H/L), and parasite (*Haemoproteus* and *Isospora*) load with ANOVAs including sex as a factor. We analysed the effects of *Haemoproteus* and *Isospora* infections (presence/absence) as factors, and the effects of TLC and ranked H/L on PC1, PC2, PC3, PC4 as dependent variables in two MANOVAs. Due to circadian rhythms affecting coccidian prevalence in passerines (López *et al.* 2007), morning/ afternoon factor was included in the model including *Isospora* infection. All the two-way interactions among covariates and sex and morning/ afternoon factor were included in the models, and stepwise backwards selection procedure was followed until all the independent variables remaining in the model increased significantly the fit of the model.

RESULTS

The coloration of the red mask in the European Goldfinch was sexually dichromatic. Sexes differed in reflectance along the whole visible spectrum (PC1, PC2 and PC3), especially within the yellow region (PC3), but not in the UV (PC4) (Table 1).

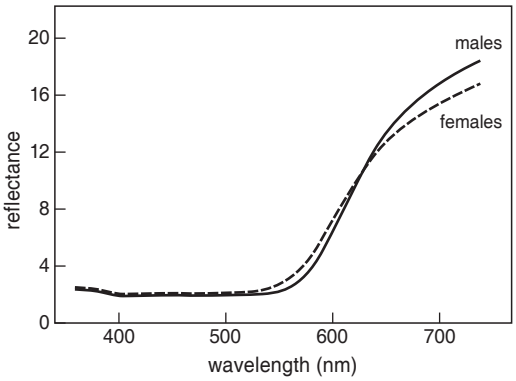

Figure 2. Mean reflection curves of the red mask of the Goldfinch along the UV and the whole visible spectrum by sex.

Reflectance curves showed that males were on average much more red and less yellow than females (Fig. 2). No differences in haematological values or parasite infection were found between sexes (Table 1). None of these variables were related to PC1 or PC2 (Tables 2 and 3). *Haemoproteus* infection, TLC, and their interaction with sex were related to PC3 (Tables 2 and 3, Fig. 3A and B). *Isospora* infection, its interaction with sex, and the interaction between sex and *Haemoproteus* infection were related to PC4 (Tables 2 and 3, Fig. 3C).

Table 1. Mean, SE and sample size for males and females for the colour, parasites and haematological variables analysed. Differences between sexes were tested by one-way ANOVA.

	Males			Females				
	mean	SE	*n*	mean	SE	*n*	*F*	*P*
PC1	–0.174	0.173	33	0.573	0.261	10	4.64	0.05
PC2	0.178	0.172	33	–0.588	0.260	10	4.93	0.03
PC3	0.248	0.132	33	–0.820	0.407	10	10.80	<0.01
PC4	0.016	0.156	33	–0.051	0.426	10	0.03	0.86
Isospora	1.300	0.231	37	1.020	0.282	12	2.37	0.13
Haemoproteus	0.030	0.013	31	0.002	0.002	7	0.92	0.35
TLC	3.771	0.03	31	8.838	0.080	7	0.87	0.36
H/L	0.856	0.040	31	0.704	0.033	7	3.25	0.08

Table 2. Results of stepwise backwards selection procedure MANOVA analysing parasite infection over colour components of the red mask of the Eurasian Goldfinch.

Source	Dependent variable	$F_{1, 32}$	*P*
Sex	PC1	7.21	0.014
	PC2	3.09	0.092
	PC3	0.03	0.872
	PC4	32.91	<0.001
Morning/afternooon	PC1	0.18	0.675
	PC2	1.64	0.214
	PC3	0.45	0.508
	PC4	1.81	0.192
Haemoproteus	PC1	0.47	0.499
	PC2	0.88	0.358
	PC3	9.80	0.005
	PC4	1.72	0.204
Isospora	PC1	1.85	0.187
	PC2	2.64	0.118
	PC3	1.37	0.254
	PC4	20.38	<0.001
Sex x *Haemoproteus*	PC1	0.60	0.446
	PC2	0.26	0.614
	PC3	8.91	0.007
	PC4	4.89	0.037
Sex x *Isospora*	PC1	2.00	0.171
	PC2	1.38	0.253
	PC3	0.26	0.616
	PC4	28.54	<0.001

Table 3. Results of stepwise backwards selection procedure MANOVA analysing haematological values over colour components of the red mask of the Eurasian Goldfinch.

Source	Dependent variable	*Df*	*F*	*P*
Sex	PC1	32	0.81	0.551
	PC2	32	0.58	0.783
	PC3	32	10.38	0.002
	PC4	32	8.24	0.498
Log (TLC+1)	PC1	32	0.49	0.616
	PC2	32	0.01	0.201
	PC3	32	6.36	0.017
	PC4	32	9.69	0.807
Sex x Log (TLC+1)	PC1	32	0.83	0.633
	PC2	32	0.63	0.741
	PC3	32	11.84	0.001
	PC4	32	8.30	0.546
Ranked H/L	PC1	31	0.21	0.651
	PC2	31	1.28	0.266
	PC3	31	2.46	0.127
	PC4	31	0.02	0.885

DISCUSSION

Colour dichromatism has not been reported before in the masks of the European Goldfinch. Our results show that hues differ between sexes: males reflect reds more strongly than females, but reflect yellows and oranges with lower intensity than females. The carotenoids expressed in the mask, are qualitatively the same in both sexes (Stradi 1995). McGraw *et al.* (2002) also found that male American Goldfinches *Carduelis tristis* artificially-fed with *ad libitum* canthaxantin were more colourful than females, due to a higher carotenoid concentration in the feathers. The larger accumulation of red pigments in males than in females seems thus to be the most plausible option for explaining colour differences in European Goldfinches. Testosterone, by means of its capacity to upregulate lipoprotein status, has been proposed as the responsible agent for such differences in the American Goldfinch (McGraw *et al.* 2006), but there are also studies showing opposite outcomes in House Finches *Carpodacus mexicanus* (Stoehr & Hill 2001). Diet differences between sexes, health variations or the effect of other hormones should not be discarded to explain this sexual dichromatism. Even a differential selection of ornaments between sexes could also be an underlying factor regarding the dichromatism. Unfortunately, no information is available on any of these aspects in the Eurasian goldfinch.

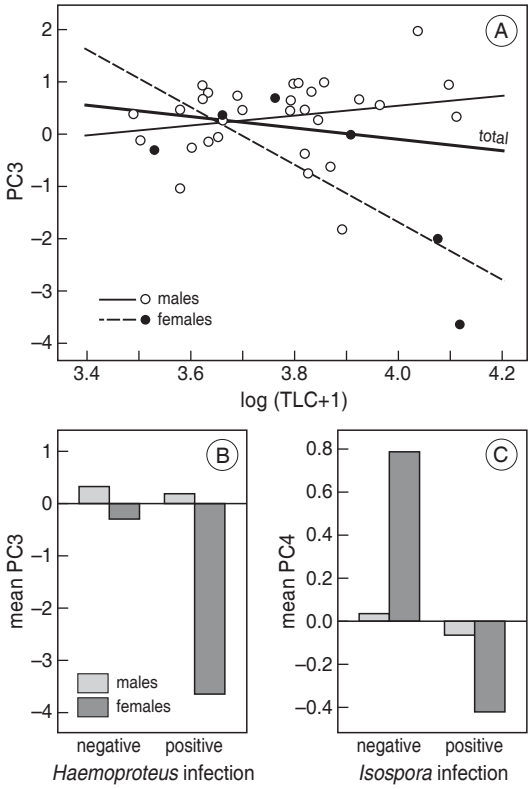

Figure 3. A) Scatter graph of log-transformed TLC over the PC3 values, by sex. B) Mean PC3 in relation to *Haemoproteus* infection state by sex. C) Mean PC4 in relation to *Isospora* infection state by sex.

When interpreting our results relating coloration to health it is important to consider that the study was carried out in the spring. European Goldfinches moult their masks in late summer (Jenni & Winkler 1994), around six months before reproduction takes place. The health status of individuals is expected to change during this time, although the signals involved in sexual selection act in the early spring at the time of mate choice (Cramp *et al.* 1994). The red of the mask is not fully developed at the time of the moult and is only completed during the spring due to the abrasion of melanin derived feather tips (Svensson 1996). We sampled birds at the time of the year when the expression of the ornament is at its fullest, just when the indicator function of a sexual selection signal should be at work.

We found a relationship between presence of different parasites and different colour components of red masks. However, these effects were sex-dependent and only significant for females. Female European Goldfinches infected with *Haemoproteus* blood parasites and those with higher TLC values were more orange, that is with a higher yellow component, than those uninfected or with lower values. A higher intensity in yellow component may be due to 1) a decrease in the intensity of red pigments, or 2) an increase in the intensity of yellow ones. Because red pigments are predominant in the red mask, we think that the first option is more plausible than the second one. In this way, the red of the mask may reflect *Haemoproteus* parasitemia or infection resistance in females. Also, female European Goldfinches with higher TLC values were more orange (with a higher yellow component) than those with lower values. Since high TLC values are linked to chronic or acute infections (Campbell 1995), the red of the mask may reflect immune levels or infection resistance in females. In this way, the most infected females would have less red ornaments, a finding that suggests that red masks act as an honest indicator of general infection in female European Goldfinches. This relationship is not significant in males, probably due to the different reproductive role of each sex, since egg-laying and incubation, a very expensive process, is carried out only by females (Cramp *et al.* 1994). UV reflection was higher in *Isospora* non-infected females than in infected ones in our study. This result seems to indicate that UV reflection acts as an honest indicator of *Isospora* infection in females. Finally, our results suggest that double-infected animals (with *Haemoproteus* and *Isospora*) reflect violets with a higher intensity that those non-infected. This fact could be due to the lack of red and yellow pigments (carotenoids) that those individuals have in their feathers. Although H/L has shown to be related with some aspects of stress and condition in passerines (Ots *et al.* 1998, Groombridge *et al.* 2004), it was not related to any colour variables in our study.

How is it possible that breeding roles had an effect on the relationship between coloration and parasites if plumage was developed several months before breeding? We suggest that under the stress derived from breeding activities females in worst condition or with a less active immune system are less able to control already present infections and/or exclude new infections when exposed to pathogens. A similar process was experimentally demonstrated to work in male Greenfinches *Carduelis chloris* experimentally infected with Sindbis virus (Lindström & Lundstrom 2000).

In conclusion, our study shows that (1) sexual dichromatism exists in the colour of the mask of the European Goldfinch, and (2) the red colour of the mask reflects different signals in both sexes and may be a reliable indicator of parasite infection during the breeding season, at least in females.

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SAMENVATTING

Verondersteld kan worden dat de kleurenpracht van het verenkleed bij vogels afhangt van de gezondheidstoestand van het individu. Of dat zo is werd onderzocht aan de hand van de markante koptekening van Putters *Carduelis carduelis*. Bij vrouwtjes – niet bij mannetjes – werden duidelijke verbanden gevonden tussen de kleur van het rood op de kop en bloedwaarden en de aanwezigheid van parasieten in het lichaam. Individuen die geïnfecteerd waren met de bloedparasiet *Haemoproteus* hadden een minder intensief gekleurde kop (vooral in het gele deel van het spectrum). Daarnaast was de UV-reflectie van de rode koptekening minder wanneer de vogels geïnfecteerd waren door de coccidiose veroorzakende protozoë *Isospora*. Bovendien bleek er een verband te bestaan tussen de kleuring van de kop en de dichtheid aan witte bloedlichaampjes, hetgeen een aanwijzing vormt dat de kopkleur een indicatie is voor de aktiviteit van het immuunsysteem. (BIT)

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3.C CAPÍTULO 4: ¿Se relaciona la coloración del pico de los machos silvestres de mirlo (*Turdus merula*) con parámetros bioquímicos y con el parasitismo?

Resumen:

La regulación de la expresión de los ornamentos basados en caroteno ha llegado a ser un tema central en estudios de señalización de calidad y selección sexual en aves. Los ornamentos con coloración más brillante son preferidos en la selección de pareja, y se supone que señalan (1) resistencia a parásitos, (2) capacidad inmune y/o (3) status de salud. Mientras las plumas se pigmentan durante su crecimiento, las estructuras tegumentarias pueden mostrar coloración dinámica con potencial de reflejar cambios en la condición corporal del individuo. El pico de los machos de mirlo ha llegado a ser un modelo para el estudio de las implicaciones de los integumentos coloreados por carotenoides en aves. La coloración del pico del mirlo ha mostrado relacionarse con la habilidad reproductora y la respuesta inmune, aunque su relación con los parámetros y la fisiología permanece poco clara. En este estudio analizamos las relaciones existentes entre coloración del pico, endoparásitos (hemoparásitos y parásitos intestinales) e indicadores de salud (valores hematológicos y bioquímicos) en 54 machos de mirlo silvestres durante la temporada de cría en el sur de España. La coloración carotenoide del pico se relacionó con la condición corporal, status de salud, estrés y niveles de hidratación y de nutrición. Ni la prevalencia ni la carga de ninguno de los grupos parasitarios estudiados se relacionó con la coloración del pico. Asimismo, los parásitos no mostraron patrones de agregación claros, y sólo Isospora, un cestodo sin identificar y Haemoproteus se relacionaron con alteraciones de las variables bioquímicas. Nuestros resultados sugieren que la infección parasitaria no es la principal fuente de variación en la coloración del pico en el mirlo común, sino más bien otros limitantes fisiológicos.

Estado actual: En revisión en Journal of Ornithology

Is bill colouration in wild male blackbirds (*Turdus merula*) related to biochemistry parameters and parasitism?

Guillermo López, Ramón Soriguer and Jordi Figuerola

Abstract

The regulation of the expression of carotenoid-based ornaments has become a central topic in studies of signalling and sexual selection in birds. Brighter coloured ornaments are preferred during mate choice and are thought to signal (1) resistance to parasites, (2) immune capacity and (3) health status. As integuments have dynamic colouration, they have the potential for reflecting changes in individual condition. The bill of male blackbirds has become a model for the study of the implications of carotenoid-coloured integuments in birds. Blackbird bill colouration has been found to be related to reproductive ability and immune capacity; nevertheless, its relationship with parasites and health remains unclear. The relationships between bill colouration, parasites (blood and intestinal parasites) and health status indicators (standard haematological and plasma biochemistry variables) in free-ranging male blackbirds were analysed during the breeding season. Bill colouration was related to body condition, health status, stress and hydration and nutritional status. The presence or load of the parasite groups studied was not found to be related to bill colouration. Moreover, parasites showed no clear aggregation patterns. Our results suggest that certain physiological constraints rather than parasite infection are the main cause of variability in colouration in male blackbird bills.

Keywords: Carotenoids – Blackbird – Bill – Parasites – Birds – Colouration – Health – Biochemistry

1. Introduction

Parasite-mediated sexual selection theory (PMSS) predicts an inverse relationship between the amount of parasites and the expression of male ornaments (Hamilton and Zuk 1982), thereby explaining the fitness benefits females acquire by selecting more evolved ornaments. Carotenoid-based ornaments in birds take the form of yellowish to reddish colouration in feathers and integuments and are known to play an important role in sexual selection (Andersson 1994; Hill and McGraw 2004). Besides ornamentation, carotenoids also have a number of physiological roles such as the protection and maintenance of the reproductive function, the scavenging of free radicals and the stimulation of the immune system (Lozano 1994; Olson and Owens 1998; Alonso-Álvarez et al. 2008). Given that they are ingested as part of the bird's diet (Goodwin 1984), a trade-off between the

physiological and ornamental use of carotenoids has been proposed (Hill 1999; Alonso-Álvarez et al. 2008). Recently, however, a physiological cost of an excess of carotenoids has been found to exist (Huggins, 2008), which is linked to individual oxidative stress levels (Mougeot et al. 2010; Vinkler and Albrecht 2010). Carotenoids have been shown to be related to circulating levels of a number of biochemical indicators of physiological status (Huggins 2008). Unlike feathers, the expression of carotenoids in integuments is dynamic and may respond to changes in individual condition (Faivre et al. 2003a; Pérez-Rodríguez and Viñuela 2008). Predictions of PMSS have been tested in carotenoid-coloured integuments (Figuerola et al. 2005; Martínez-Padilla et al. 2007; Baeta et al. 2008; Mougeot et al. 2010) and the colouration of ornaments has been found to be related to indicators of health (McGraw and Ardia 2003; Bertrand et al. 2006; Mougeot 2007a; Mougeot et al. 2007b; Mougeot et al. 2009) and reproductive ability (Massaro et al. 2003; Peters et al. 2004; McGraw et al. 2005).

The blackbird (*Turdus merula*), a common medium-sized Palaearctic passerine, has become a model for the study of carotenoids in birds. Males have melanin-pigmented plumage and a carotenoid-pigmented bill that graduates from pale dirty yellow to bright orange (Cramp and Perrins 1994). The bill colouration of male blackbirds has been found to be related to reproductive abilities (Faivre et al. 2001; Préault et al. 2005), survival (Gregorie et al. 2004), physical development (Bright et al. 2004) and immune status (Faivre et al., 2003a; Faivre et al. 2003b). Baeta et al. (2008) showed that individuals with good access to carotenoids in their diets had more colourful bills and a slower replication of the intestinal parasite *Isospora*. However, no relationship has been found between blackbird bill colouration and the presence or intensity of parasites (Hatchwell et al. 2001; Bright et al. 2004). Most studies performed to date have focussed on a single parasite species and only a few have examined different species of the same class of parasites, while none, to the best of our knowledge, has ever investigated the combined patterns of a taxonomically diverse community of parasites. A possible explanation for this is the complexity of parasitic aggregation patterns, which hinders the identification of the potential relationships between colouration and parasites when studying a single group of parasites. Furthermore, none of the previous studies on carotenoid-based pigmentation have taken into account the biochemistry physiological variables that could interact with parasitation in the regulation of the expression of ornamental carotenoids. Plasma biochemical characterisation provides relevant information on the nutrition and condition of the organism or of different organs and, although widely used in clinical diagnosis, is employed only rarely in behavioural ecology (but see for example Alonso-Alvarez et al. 2002; Hegyi et al. 2010).

The goals of this study were to examine (1) how biochemical parameters, haematological values and endoparasites (including both blood and celomic parasites) are related to bill colouration; (2)

how parasite richness and abundance are related to biochemical and haematological values; and to explore (3) possible patterns of parasitic aggregation in wild male blackbirds during the breeding season.

2. Methods

2. 1 Field work

A total of 54 male blackbirds were trapped between March and May in 2004 and 2005 in the city of Seville (37° 23′ 11′′ N, 5° 57′ 46′′W). All birds were individually marked with numbered aluminium rings and their body mass (to the nearest 0.5 g) and wing length (maximum chord to the nearest 0.5 mm) were measured. Birds' ages were determined using Svensson (1984) as first years or adults. All individuals were manipulated in the same way in order to minimize potential differences in the Corticosterone-mediated alteration of the measured biochemical values (Müller et al. 2006). Birds were kept individually in cloth bags for 20 minutes to collect faecal samples, which were collected from 42 individuals. Droppings were immediately placed in individually marked vials containing 5% formol; the collection time was annotated for each sample. Subsequently, 500 µl of blood were taken from the jugular vein using 29 G sterile insulin syringes and birds were then released back into the wild. A drop of blood was smeared on a microscopy slide (Bennett 1970), air dried and stained using Diff-Quick solution. The rest of the blood sample was placed in a vial and a few hours later was centrifuged for 10 minutes at 6000 rpm in an Eppendorf Minispin centrifuge to separate serum. Sera were frozen at –20° C for a maximum of one month until subsequent analysis. As well, the bill colour (curves between 360nm and 740 nm) of all 54 individuals was measured using a MINOLTA 2600 spectrometer.

2.2 Haematology and coprology methods

Samples of droppings were filtered through a double lint cloth and then homogenized to obtain a dilution that was explored for parasite eggs and coccidian oocysts in a McMaster chamber (as per Williams 1973). Subsequently, 200 µl of the same dilution was taken from the chamber and dried in a 54° C heater; the resulting extract was weighed to the nearest 0.0001 g. The number of parasite eggs or oocysts per mg of dry extract of faeces was obtained by dividing the number counted in the chamber by the estimated mass of the scanned sample (see López et al. 2007 for details). The most prevalent parasites were protozoan coccidian *Isospora* (prevalence: 60%) and an unidentified species of cestode (23%), which were included independently in subsequent analyses. The remaining parasites (other cestodes, strigeids and *Ascarididae, Spiruridae* and *Syngamidae* nematodes) were found in prevalences of below 12% and were included together in the analysis as a variable coded as 'other parasites'. The blood slides were searched for blood parasites at high (oil 1000X) magnification. A total of 15,000 erythrocytes were explored in each sample (see Godfrey et al. 1987). *Haemoproteus* spp., *Plasmodium* spp. and *Leukocytozoon* spp. were

detected with prevalences of 50%, 9% and 19%, respectively. Infection intensity could only be estimated in the intraerythrocytic species *Haemoproteus* and *Plasmodium.* The White Blood Cell count (WBC) was estimated by counting the number of leukocytes on twenty similar-density 400X light microscope fields and multiplying this value by 100 (Wiskott 2002) in 41 blood smears. The cellular type (as per Campbell 1995) of 100 leukocytes was estimated at 1000X magnification. H/L, which is considered a reliable assessor of stress in birds (Davis et al. 2008), was calculated as the percentage of heterophils divided by the percentage of lymphocytes.

2.3 Serum biochemistry

Twelve different plasma biochemistry variables were measured from 33 sera collected in 2005. After defrosting the sera, we quantified Aspartate Aminotransferase (AST), Bile Acids (BA), Creatinine Kinase (CK), Uric Acid (UA), Glucose (Glu), Phosphorous (Phos), Calcium (Ca), total proteins (TP), Albumin (Alb), Globulin (Glob), Potassium (K) and Sodium (Na) using a Vetscan (Abaxis, Inc. California) dry and liquid biochemistry-based analyser (see Table 1 for further information).

2.4 Carotenoid chroma

To calculate the bill carotenoid chroma of the bill colouration the difference between reflectance at 700 nm and 450 nm was divided by the reflectance at 700 nm (see Montgomerie 2006, 2008). This parameter only expresses the reflection in the orange section of the spectrum.

2.5 Statistical analysis

WBC, Na and AST were log-transformed - Log (value + 1)- to fit a normal distribution, while H/L, BA and Ca were ranked because there were no common transformations that normalised the data (Conover and Iman 1981). Body condition was estimated as the residuals of the linear regression between body mass and wing length (see Schulte-Hostedde et al. 2005).

A principal component analysis (PCA) was performed with the 12 biochemical variables to identify the principal axis of variation. Five components with eigenvalues >1 were obtained, which explained 76.8% of variance (Table 1). PCB1 was mainly influenced by Glob and TP and so was considered as an indicator of general health status. PCB2 was mainly influenced by AST and CK and was thus considered as a variable related to muscular activity. PCB3 was mainly influenced by Na and K and was considered as an indicator of fluid balance. PCB4 was mainly influenced by Ca and Alb and was considered a variable related to cell hydration level. PCB5 was influenced by UA and Phos and was considered to be a negative indicator of nutritional status.

Similarly, parasitation values were analysed using a PCA to identify the principal axis of variation in our sample. If there was a pattern of parasite aggregation we would expect to find a component that was significantly influenced by several groups of parasites. Three significant components were obtained, which explained 64.5% of the variance (Table 2): PCP1 was negatively

			Principal components				
Value	Method	Evaluation	PCB1	PCB2	PCB3	PCB4	PCB5
Aspartate Aminotransferase (AST)	Modified IFCC reference	Liver structure and muscle status	0.41	0.73	-0.02	-0.31	-0.21
Bile Acids (BA)	Thio-NAD +	Liver function status	-0.44	0.59	-0.02	0.24	0.38
Creatinine Kinase (CK)	Modified IFCC reference	Muscle status	-0.03	0.90	0.05	-0.03	0.02
Uric Acid (UA)	UA-specific Uricase	Renal structure status	0.12	-0.12	-0.41	-0.11	0.71
Glucose (GLU)	Copper-reduction	Sepsis, alimentary status and pancreatic function	0.01	0.13	-0.07	0.54	-0.23
Inorganic Phosphorous (PHOS)	SP + PGM & G-6-PDH	Renal function status and nutritional status	0.26	0.15	0.29	-0.29	0.68
Calcium (Ca)	Arsenazo III	Bone and renal function status	0.26	-0.30	-0.03	0.78	0.05
Total Protein (TP)	Cupric ionization	Gastrointestinal and kidney status	0.91	0.02	-0.05	0.31	0.13
Albumin (ALB)	Bromcresol Green	Liver and kidney status	-0.14	-0.08	0.48	0.76	-0.13
Globulin (GLOB)	Difference TP - ALB	Fluid balance and antigenic stimulation	0.90	0.03	-0.28	-0.10	0.19
Potassium (K)	Pyruvate Kinase	Cell lysis and fluid balance	-0.07	0.25	0.87	-0.04	0.11
Sonium (Na)	ONPG	Fluid balance and antigenic stimulation	-0.18	-0.23	0.74	0.09	-0.15

Table 1. Biochemistry parameters evaluated in the selected blackbirds, their functional relevance according to Hochleithner (1994), and the rotated component matrix (Varimax rotation with Kaiser normalization) of the principal component analysis. Variables which accounted more than 0.6 are marked in grey.

influenced by *Leukocytozoon* prevalence and positively influenced by *Haemoproteus* and the 'other parasite' loads; PCP2 was positively influenced by *Isospora* and the cestode loads; and PCP3 was positively influenced by the *Plasmodium* load.

Relationships between parasite and haematological variables, biochemistry variables and bill chroma were analysed using multivariate general linear models (SPSS 13.0 package). All the two-way interactions between covariates and with factors were included in the models, and stepwise backwards selection procedures were followed until all the independent variables remaining in the model increased significantly ($p < 0.05$) the fit of the model. Given the different sample size of our groups of variables, different MANOVAs were used to avoid losing cases. Due to the occurrence of circadian oocyst shedding in passerine *Isospora*, a morning/afternoon factor was included in all models that included *Isospora* load (see López et al. 2007). First of all, we investigated how biochemistry components, parasite components, body condition and haematological values were related to bill carotenoid colouration. We conducted the following ANOVAs: (1) bill chroma was included as the dependent variable, with biochemistry components and body condition as covariates and age as a factor; (2) bill chroma was included as the dependent variable, with parasite components and body condition as covariates and year, morning/afternoon and age as factors; and (3) bill chroma was included as the dependent variable, with H/L, WBC and body condition as covariates and age as a factor. Secondly, we analysed the relationships between parasite components and biochemical components, body condition and haematological values. The following MANOVAs were performed: (1) parasite components were included as dependent variables, with biochemistry components and body condition as covariates and age and morning/afternoon as a factor; (2) parasite components were included as dependent variables, with H/L, WBC and body condition as covariates and age, year and morning/afternoon as factors.

Component	PCP1	PCP2	PCP3
Log (*Plasmodium* burden + 1)	**0.05**	**-0.09**	**0.89**
Haemoproteus burden	**0.63**	**0.11**	**-0.23**
Leukocytozoon prevalence	**-0.64**	**0.26**	**-0.06**
Log (*Isospora* burden + 1)	**-0.42**	**0.64**	**0.41**
Log (Cestode burden + 1)	**0.16**	**0.84**	**-0.21**
Log (Other parasite burden + 1)	**0.67**	**0.16**	**0.36**

Table 2. Rotated component matrix (Varimax rotation with Kaiser normalization) of the principal component analysis performed on the parasite burdens/prevalence and haematological values of the sampled blackbirds. Variables which accounted more than 0.6 are marked in grey.

3. Results

3. 1 Patterns of parasitic aggregation

Our results do not show any general pattern of aggregation in the different types of parasites in the individuals sampled. This means that the different parasite species analysed in this work were parasitizing different individuals.

Interestingly, haemoprotozoa *Haemoproteus* and the group 'other parasites' were related and positively accounted for PCP1, which might imply 1) the possibility that *Haemoproteus* affects host condition in a way that favours parasitation by other parasite species or (2) a possible association between *Haemoproteus* and one of the parasite species included in the second group. Unfortunately, our sample size did not allow us to investigate this relationship in greater depth. Moreover, *Leukocytozoon* prevalence was negatively related to both groups of parasites, indicating that individuals infected by *Haemoproteus* and 'other parasites' were not infected by *Leukocytozoon*. PCP2 showed an aggregation trend between the intestinal coccidia *Isospora* and the common cestode found in our sample. This aggregation could be due to the fact that infection by one of the species favours infection by the other.

Source	Df, error	$F_{1,21}$	p
PCB1	1,21	0.01	0.94
PCB3	1,21	0.02	0.88
PCB4	1,21	0.37	0.55
PCB5	1,21	0.88	0.36
Body condition	1,21	6.37	0.02
PCB1 * PCB4	1,21	5.50	0.03
PCB3 * PCB4	1,21	13.36	0.00
PCB3 * PCB5	1,21	23.86	0.00
PCB1 * Body condition	1,21	9.75	0.01
PCB4 * Body condition	1,21	11.56	0.00
PCB5 * Body condition	1,21	10.64	0.00
PCB2	1,20	0.54	0.54

Table 3. Results of the stepwise backwards selection procedure ANOVA analysing relationships between bill carotenoid chroma and biochemical components.

3.2 Relationships between biochemical components and bill carotenoid chroma

Body condition and the interactions between body condition and (1) PCB1, (2) PCB4 and (3) PCB5, as well as the interactions between (1) PCB1 and PCB4, and (2) PCB3 and PCB4 and PCB3 and PCB5, were significantly related to BCC (Table 3). Interestingly, none of the biochemical components independently showed a significant relationship with bill chroma.

Source	Df, error	$F_{1,21}$	p
Year	1,37	1.59	0.22
Morning / Afternoon	1,37	1.76	0.19
PCP1	1,37	0.07	0.80
Body condition	1,37	0.69	0.41
PCP2	1,34	0.04	0.85
PCP2*Morning/Afternoon	1,26	2,13.	0.16
PCP3	1,24	0.03	0.86

Table 4. Results of the stepwise backwards selection procedure ANOVA analysing relationships between bill carotenoid chroma and parasite components.

3.3 Relationships between parasite components and bill carotenoid chroma

Contrary to expectations, none of the parasite components were related to the bill chroma (Table 4).

3.4 Relationships between haematological values and bill carotenoid chroma

The haematological value H/L was significantly related to bill chroma ($F_{1,39} = 4.44$; $p = 0.04$). Individuals with high values of H/L had paler bills than those with low values of H/L.

Source	Dependent variable	Df (Error)	F	p
Morning/ Afternoon	PCP1	1 (22)	0.65	0.43
	PCP2	1 (22)	0.04	0.85
	PCP3	1 (22)	1.76	0.20
PCB2	PCP1	1 (22)	0.05	0.83
	PCP2	1 (22)	0.02	0.89
	PCP3	1 (22)	3.02	0.10
PCB5	PCP1	1 (22)	0.44	0.51
	PCP2	1 (22)	2.20	0.15
	PCP3	1 (22)	2.23	0.15
PCB2*PCB5	PCP1	1 (22)	1.18	0.29
	PCP2	1 (22)	5.12	0.03
	PCP3	1 (22)	4.87	0.04
PCB1	PCP1	1 (21)	1.80	0.19
	PCP2	1 (21)	5.90	0.02
	PCP3	1 (21)	0.30	0.59
PCB3	PCP1	1 (20)	2.40	0.14
	PCP2	1 (20)	2.97	0.10
	PCP3	1 (20)	1.04	0.32
Body condition	PCP1	1 (18)	2.90	0.11
	PCP2	1 (18)	0.47	0.50
	PCP3	1 (18)	0.03	0.87
PCB4	PCP1	1 (16)	2.36	0.14
	PCP2	1 (16)	0.63	0.44
	PCP3	1 (16)	1.38	0.26

Table 5. Results of the stepwise backwards selection procedure MANOVA analysing relationships between parasite components and biochemical components. Significant relationships are highlighted in grey.

3.5 Relationships between biochemical and parasite components

The interaction between PCB2 and PCB5 was significantly related to PCP2 and PCP3 (Table 5) given that high values of PCB2 and PCB5 were related to low values of PCP2 and PCP3; that is, parasitation by *Isospora*, the quantified cestode and *Haemoproteus* was related to lower muscular activity and poorer nutritional status.

3.6 Relationships between haematological values and parasite components

Haematological parameters did not show any significant relationship with parasite components (Table 6).

Source	Dependent variable	Df (Error)	F	p
Age	PCP1	1 (40)	2.71	0.11
	PCP2	1 (40)	0.17	0.68
	PCP3	1 (40)	0.02	0.88
Morning/ Afternoon	PCP1	1 (39)	2.83	0.10
	PCP2	1 (39)	0.60	0.44
	PCP3	1 (39)	1.11	0.30
H/L ranked	PCP1	1 (30)	0.01	0.93
	PCP2	1 (30)	0.12	0.73
	PCP3	1 (30)	0.00	0.97
Log (WBC + 1)	PCP1	1 (27)	0.39	0.54
	PCP2	1 (27)	0.47	0.50
	PCP3	1 (27)	0.82	0.37
Body condition	PCP1	1 (24)	0.10	0.75
	PCP2	1 (24)	0.24	0.63
	PCP3	1 (24)	0.21	0.10

Table 6. Results of the stepwise backwards selection procedure MANOVA analysing relationships between parasite components and haematological values. Significant relationships are highlighted in grey.

4. Discussion

Our results show for first time a relationship between biochemical indicators of health status and orange bill coloration in male blackbirds. The interaction between the components indicating general health status and the component indicating cell hydration level was negatively related to bill chroma. The orange in male blackbird bills decreased as biochemical indicators of both general health failure and cell dehydration increased. Similarly, the interaction between the component indicating cell hydration level and the component indicating fluid balance was negatively

related to bill chroma. The orange in bills decreased as general dehydration increased. Finally, the interaction between the component indicating cell hydration levels and the component indicating nutritional status was negatively related to bill chroma. In other words, when biochemical indicators of both malnutrition and cell dehydration increased, the orange in male blackbird bills decreased. The interaction between body condition and the components indicating general health status, cell hydration level and nutritional status were also related to bill chroma. This result suggests that the negative relationship between these biochemistry values and the orange in the bills of male blackbirds sampled in our study was body-condition dependent. This finding agrees with the handicap theory (Zahavi and Zahavi 1997), which predicts that only the best-condition individuals can afford the cost of spending more carotenoids on ornamental functions.

The predictions of the PMSS were not supported by our study since no significant relationship was found between bill chroma and any of the components accounting for the parasites evaluated. This result agrees with previous studies that failed to identify any relationship between parasites and bill colouration in male blackbirds (Hatchwell et al. 2001; Bright et al. 2004), possibly because of the different effects that different type of parasites have on host physiology. In this sense, we found that only *Isospora*, the unidentified cestode and *Leukocytozoon* were related to biochemistry values, all being related to low muscular activity and poor nutritional status. This finding suggests that, of the parasite species evaluated, these three species may be the only type of parasites affecting host physiology. Moreover, none of the parasites showed any relationship with the other biochemical values or with haematological values. In line with this finding, the parasites in our sample did not show any clear aggregation patterns other than those between *Isospora* and the unidentified cestode. This is an important result given the traditional focus on a single or small number of parasite species. Several studies have already pointed out that the intensity of infection by different species of parasites is not strongly correlated at intraspecific level (Møller 1991; Weatherhead et al. 1993). Our results show that in many cases the parasitic loads of different groups of parasites are not related and so it is not possible to derive an 'index of parasitism' based on a small number of pathogens. Consequently, conclusions obtained for one group of parasites cannot be extrapolated for the full community of parasites. This limits our capacity to rigorously test parasite-mediated selection hypotheses unless, that is, clear indications of the effects on host fitness exist for a significant fraction of the parasite community. We found a negative relationship between H/L and the orange colour in male blackbird bills. Given that the H/L ratio has been described as an honest indicator of stress (Davis et al. 2008), our results suggest that birds with greater stress levels have paler bills than those with lower stress levels. This finding supports evidence for a relationship

between the orange in bills and health constraints (Faivre et al. 2003a; Baeta et al. 2008). Although high WBC values are thought to be related to the immune function, and despite previous studies that have demonstrated a link between blackbird bill colouration and the immune system (Faivre et al., 2003a; Baeta et al. 2008), our study failed to find a relationship between these parameters. This result may be caused by the different sources of variability in the WBC besides the immune activation.

In conclusion, our results support the idea that carotenoids are part of a trade-off in their use for ornamentation and for satisfying physiological needs, and that the colouration of male blackbird bills signals its immune and nutritional status. As a dynamic ornament, it is likely to be an honest indicator of the actual individual status. Following on from previous studies, our results failed to find a relationship between bill colouration and parasite burden or prevalence, probably due to the complex relationships between parasites, health status and carotenoid-based ornamentation. We conclude thus that biochemistry parameters related to different aspects of health status may help provide a better understanding of the regulation of ornament expression in birds.

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Declaration

All the work performed in the present manuscript comply with the current Spanish laws.

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3.e CAPÍTULO 5: Los niveles plasmáticos de carotenos se relacionan con la infección por parásitos intestinales en paseriformes silvestres

Resumen:

Los carotenos juegan un importante papel en la ornamentación y en la fisiología de las aves, y se ha propuesto la existencia de un dilema en los individuos en la asignación de estos compuestos entre las dos funciones. En este escenario, los parásitos pueden desempeñar un papel preponderante en el mantenimiento de la veracidad del plumaje como sistema de señalización, ya que estos pueden incrementar las demandas fisiológicas de carotenos y/o reducir su absorción en el intestino. Nosotros analizamos la relación entre concentración plasmática de carotenos y riqueza y abundancia de parásitos sanguíneos e intestinales en 22 especies de aves paseriformes muestreadas en las primaveras de 2004 y 2005 en Sevilla. La concentración plasmática de carotenos se relacionó negativamente con la abundancia de parásitos intestinales, pero no lo hizo con los parásitos sanguíneos ni a nivel intra ni interespecífico. Nuestros resultados sugieren un efecto negativo de los parásitos intestinales en los niveles de carotenos circulantes, mientras que confirman que las consecuencias ecológicas del parasitismo difieren diametralmente entre diferentes grupos de parásitos.

Estado actual: Enviado a Ecology

Plasma carotenoid levels in passerines are related to infection by parasites

Jordi Figuerola, Guillermo López and Ramón Soriguer

Abstract

Plumage coloration plays an important role in intra and inter-sexual competition in birds. In addition to ornamentation, carotenoids are important for bird physiology and it has been proposed that a trade-off in their allocation to these two functions occurs. Under this scenario parasites may play a central role in maintaining the honesty of plumage as a signaling system by increasing the demands for carotenoids for infection control and/or by reducing carotenoid absorption in the intestines. We analyzed the relationship between (1) carotenoid concentrations in plasma and (2) blood and intestinal parasite richness and abundance in 22 species of passerines sampled in spring. Loads of different groups of parasites were unrelated so conclusions drawn from examining a particular group of parasites cannot be extrapolated to the whole community of pathogens and parasites inhabiting a host. Both at intra- and interspecific levels plasma carotenoid concentrations were negatively related to the abundance of intestinal parasites, but unrelated to blood parasites. Our results support the existence of a negative relationship between intestinal parasites and carotenoid levels in plasma and suggest that this group of parasites play an important role in the evolution and maintenance of carotenoid-derived sexually selected ornamentations

Keywords: blood parasites – endoparasites – Haematozoa - honest signaling - host-parasite interactions - immune response - plumage coloration - sexual selection

1. Introduction

Hamilton and Zuk (1982) proposed that plumage coloration in birds may act as a reliable indicator of resistance to parasites. Most comparative analyses have supported this hypothesis by reporting a positive relationship between parasite prevalence or richness and interspecific differences in plumage coloration (e.g. Scheuerlein and Ricklefs 2004 and references therein). At intraspecific level positive, negative, and non-significant relationships between parasitism and plumage coloration have been reported (see Møller 1990, Read 1990, Zuk 1992 for a review). However, experiments performed up to now largely support the idea of a negative impact for ectoparasites (Figuerola et al. 2003) and coccidian endoparasites (Brawner et al.

2000, McGraw and Hill 2000, Hõrak et al. 2004) on plumage brightness. Many of the colorations involved in sexual selection are derived from carotenoids (Badyaev and Hill 2000), pigments that cannot be synthesized by birds and thus have to be incorporated from their diets (Olson and Owens 1998). Interestingly, carotenoids are not only used to confer color on feathers and skins, but also are involved in the synthesis of different vitamins and the control of oxidative stress (von Schantz et al. 1999, Blas et al. 2006 but see Constantini and Møller 2008). For this reason, it has been proposed that birds have to face a trade-off between investing carotenoids in showiness or in health-related functions (von Schantz et al. 1999, Peters 2007). Under this scenario, parasites and pathogens may play a central role in the regulation of the honesty of birds' signaling systems. The mechanistic and physiological processes linking parasites, health and ornament expression are now the focus of intense debate and research.

Concentrations of carotenoids differ widely between individuals; individuals with higher concentrations of carotenoids in their blood usually develop brighter plumages (Figuerola et al. 1999) and have a more active immune system (Blas et al. 2006, Aguilera and Amat 2007). Studies of interspecific variation in carotenoids levels are less common. Recently, Tella et al. (2004) analyzed some ecological, morphological and evolutionary factors related to variations in carotenoid concentrations in the blood of 80 wild bird species. Phylogeny, body size, and the presence of carotenoid-dependent colorations were related to interspecific differences in carotenoids. Although Tella et al. (2004) have already suggested that some of the interspecific variation they found in carotenoid concentrations in plasma may merely reflect differences in the incidence of coccidian parasites between species, to our knowledge no study has ever analyzed the relationship between parasitism and interspecific variation in the circulation of carotenoids. At intraspecific level, Mártinez-Padilla et al. (2007) reported an increase in the concentration of carotenoids in the blood after experimentally reducing infection by nematodes in Red Grouse (Lagopus lagopus), while the reduction of coccidian loads has been reported to lead to increased plasma carotenoid levels in Greenfinches (Carduelis chloris) and growing chickens (Zhu et al. 2000, Hõrak et al. 2004).

In this paper, we analyze the relationship of carotenoids and parasitism in 22 species of passerines in terms of two different groups of parasites: haematozoa and intestinal parasites. First of all we tested the relationship between the incidence of different parasite groups and the levels of plasma carotenoids in individual birds. Secondly, we tested the role of interspecific differences in the incidence of parasitism as a means of explaining interspecific differences in the concentration of plasma carotenoids. Overall, we provide evidence that levels of circulating carotenoids are negatively related to the loads of some endoparasites, both on an individual level and specific level of the host species.

2. Methods

We captured 354 individuals of 22 passerine species during the pre-breeding migration period (March-May) in 2004 and 2005 in a tree nursery in a suburb of the Spanish city of Seville (37º 23′ 11′′ N, 5º 57′ 46′′W). Twenty twelve-metre-long mist-nets were operated from sunrise to sunset. Between capture and ringing birds were kept individually in clean cloth bags to allow any droppings produced during this time to be collected. Each bird was marked with a numbered ring and wing length (to the nearest mm) and body mass (to the nearest 0.1 g) were measured. Whenever possible sex was determined on the basis of the plumage characteristics given by Svensson (1996). Subsequently, 0.5 ml of blood was taken from the birds' jugular vein using 29 G sterile insulin syringes. A drop of this blood was used to prepare a smear on a microscopic slide (as per Bennett 1970), which was air dried and then fixed and stained using Diff-Quick solution. The rest of the blood sample was placed in a vial and after several hours centrifuged for 10 minutes at 6000 rpm in an Eppendorf Minispin centrifuge to separate serum from cells. Samples were then stored at –20º C to be used for other studies (see López et al. 2008). After blood extraction, birds were kept individually in the cloth bags for 20 minutes to collect faecal samples and were then released. Between 0.5 and 1 mg of faeces were placed in individually marked vials containing 5% formol and the collection time was annotated for each sample.

Laboratory method

Blood smears were scanned for blood parasites at low (400X) and high (oil 1000X) magnification: a total of 15,000 erythrocytes were explored in each sample (Godfrey et al. 1987). Parasites were identified to genera level and when intraerythrocytic parasitemia occurred, the blood parasite load was estimated as the percentage of infected erythrocytes. Haemoproteus spp. (27.7% prevalence), Plasmodium spp. (16.4%), Leucocytozoon spp. (6.2%), and Trypanosoma spp. (0.8%) were detected in the blood slides.

Droppings were filtered through a double layer of cotton-lint cheesecloth and scanned for endoparasites in a McMaster chamber. A known volume of the sample was dried and the dry weight of the faeces was used to estimate the number of oocysts or eggs per mg of dry faeces (following López et al. 2007). The most frequent parasite species found were Protozoan coccidia of the genera Isospora (45.5% prevalence), although some trematodes and nematodes were also found. Because most species could not be identified, parasites were grouped according to Order: Trematoda: Strigeida (3.1%); Nematoda: Spirurida (13.6%), Capilariida (4.8%), Ascarida (3.4%) and unidentified nematodes (6.2%). All screening of the samples was carried out by one of the authors (GL).

Molecular sexing

The cellular fraction of the blood sample was used to extract DNA for each bird and sex was determined using a polymerase chain reaction (PCR) amplification of the

CHD genes (Ellegren, 1996; Griffiths et al., 1998) with the P2/P8 primers.

Carotenoid quantification

Pigments were extracted from plasma by adding acetone to the plasma samples at a ratio of 1:1 (v/v). The mixture was centrifuged at 13 000 r/min at 16 249g for 10 min to precipitate the flocculant proteins (Negro and Garrido 2000). The supernatant was retained and stored at –20 °C until high-performance liquid chromatography (HPLC) analysis. A Jasco PU-2089 Plus instrument equipped with a quaternary pump (Jasco Analítica Spain, S.L., Madrid) was used for carotenoid analyses, with a reverse-phase C18 column (Phenomenex Synergi 4 μ) and a pre-column of the same material with a particle size of 5 μm. Samples were prefiltered using an OEM nylon filter, 0.45 μm °— 4 mm) and later injected using a Rheodyne 7725i valve equipped with a 20-μL loop (Rheodyne, Rohnent Park, California, USA). The eluent system is that described in Mínguez-Mosquera and Hornero-Méndez (1993), with the only difference being that the flow rate was 1 mL•min–1. Data were acquired between 195 and 650 nm with a multiwavelength detector (MD-2010 Plus, Jasco Analítica Spain, S.L.).

Reference carotenoids were obtained from fresh green plants in J. Garrido's laboratory, as per Mínguez-Mosquera (1997). Known reference dilutions of zeaxanthin, lutein and β-carotene were injected into the HPLC instrument to build a calibration curve at 450 nm. The concentration of individual carotenoids was calculated from HPLC areas recorded at 450 nm. The total carotenoid concentration (g/ml) used in the analyses was obtained by adding together the values for zeaxanthin, lutein, β-carotene and other unidentified carotenoids for each individual.

Statistical analysis

In this paper we aim to explore intraspecific and interspecific patterns of variation in plasma carotenoid concentrations. Thus, the statistical relationships between carotenoids and parasites were tested using two different approaches.

Firstly, patterns of variation between individuals were analyzed by using generalized mixed-effect linear models using the program JMP 5.0. In this analysis, species was included as a random effect and the existence of a relationship between carotenoids and parasites was tested while allowing for species-specific differences in the intercept (but not the slope) between parasites and carotenoid concentrations. In all the analyses sex (male or female), year (2004 or 2005), date of capture (days counted as from March 8) and time of capture (morning or afternoon) were included as fixed factors. The time of capture had an important effect on endoparasite abundance ($F_{1,331} = 223.24$, $P < 0.0001$, see also López et al. 2007) and for this reason an interaction coding for time of capture and parasite abundance was included in the analyses that included intestinal parasites with significant diurnal cycles in egg shedding (coccidians: $F_{1,331} = 338.63$, $P < 0.0001$; Capilariida: $F_{1,331} = 4.74$, $P = 0.03$; $F_{1,331} < 2.05$, $P > 0.15$ for all other parasite

groups). The amount of variance explained by the species factor was estimated from changes in the model deviance.

Secondly, to investigate interspecific patterns of variation we first estimated for each variable and species the least-square means corrected for sex, time of capture, date, and year. Least-square means were then included in a generalized linear model to explore the covariates of plasma carotenoid concentration differences between species. In addition to parasites, the initial model included three other variables that are related to interspecific variation in carotenoid concentrations (Tella et al. 2004): mean body mass, the extent of carotenoid-derived coloration in plumage, and the extent of carotenoid-derived pigmentation in non-feathered parts (following the scoring methodology used by Tella et al. 2004). Phylogeny explains a relevant amount of variance in the concentration of circulating carotenoids (Tella et al. 2004), but most variance occurred at Order level and for this reason we restricted our analyses to passerine species.

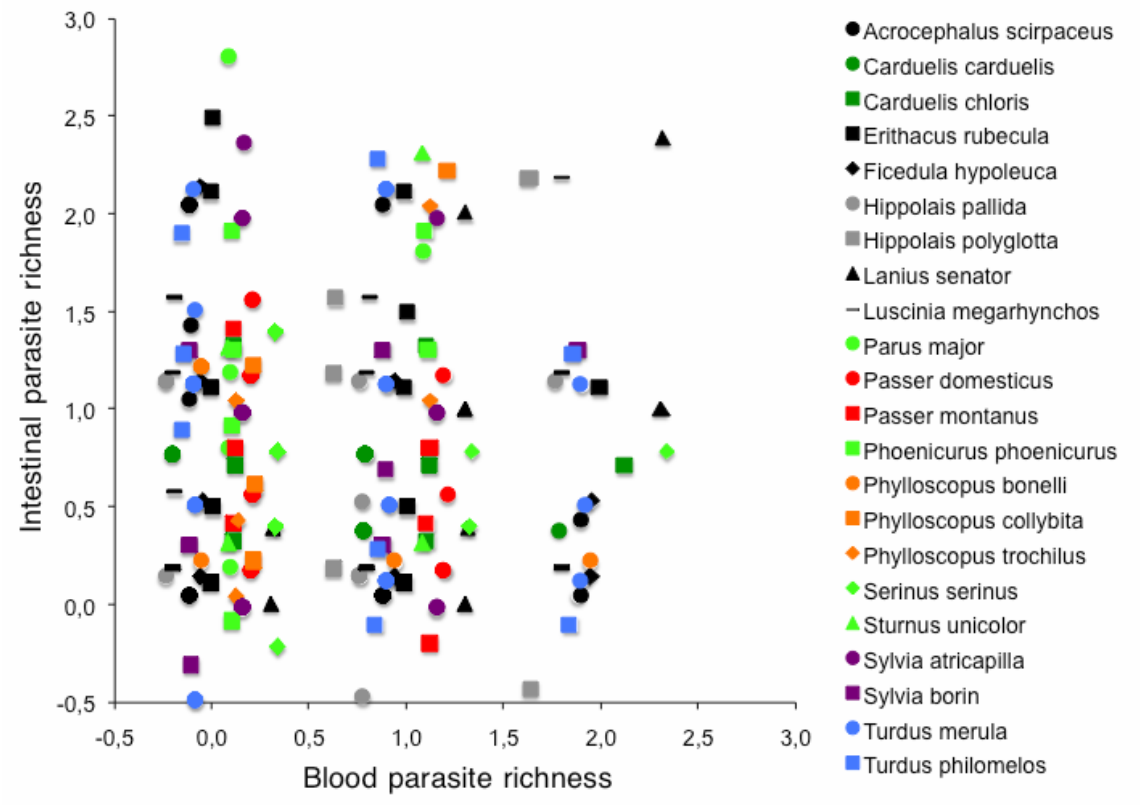

Figure 1. Relationship between intestinal and blood parasite richness for 354 individual birds of 22 different passerine species after controlling for the effects of time of capture (morning or afternoon) on intestinal parasite richness. Species were included in the model as a random factor to allow different intercepts for data from different species but also to provide a common slope for all species.

In all the analyses carotenoid concentrations were considered to be the dependent variable and parasite richness (number of parasite taxons per individual) and parasite abundance as independent variables. Carotenoid concentrations, parasite counts and mean body mass were log-transformed to attain normality. We followed a backwards selection procedure, starting with a model including all the variables (and in the case of intestinal parasites, its interaction with the factor 'time of capture'), removing the least significant variable and fitting the model again until all the variables in the model contributed with $P < 0.10$ to the fit of the model. Only variables with $P < 0.05$ were interpreted as significant.

3. Results

Richness and abundance of intestinal and blood parasites

No relationship was found between the richness of intestinal and blood parasites in individuals ($r2 = 0.01$, $F1,330 = 0.04$, $P = 0.84$, Fig. 1) or in species ($r2 = 0.01$, $F1,20 = 0.25$, $P = 0.63$). Likewise, intestinal and blood parasite abundances were unrelated at either level (inter-individuals: $r2 = 0.00$, $F1,330 = 0.69$, $P = 0.41$; inter-specific: $r2 = 0.09$, $F1,20 = 1.98$, $P = 0.18$).

Factors related to carotenoid circulation

In the analyses at individual level both year (larger concentrations in 2005 than in 2004, $F1,330 = 18.26$, $P < 0.0001$) and date (increases throughout the spring, $F1,330 = 16.11$, $P < 0.0001$) were related to carotenoid concentrations; no differences were found in carotenoid concentrations in relation to either sex ($F1,329 = 0.24$, $P = 0.62$) or time of capture ($F1,329 = 0.01$, $P = 0.92$). Overall, species explained 42.78% of inter-individual variance carotenoid concentrations in plasma.

Analyses of the relationships between plasma carotenoids and parasitism at individual level

Between individuals, plasma concentrations of carotenoids were negatively related to the richness of intestinal parasites (-0.0767 ▯ 0.0298, $F1,327 = 6.64$, $P = 0.01$, Fig. 2a), but were not related to the richness of blood parasites or to the abundance of intestinal or blood parasites ($F1,326 < 2.75$, $P > 0.10$ for all variables). We repeated the analyses, taking into account separately coccidians and Spirurids, and also added data for the other parasite taxons with less than 5% prevalence in a group we named "other intestinal parasites". This second set of analyses confirmed the negative relationship between carotenoids and the presence of "other intestinal parasites" ($F1,329 = 7.51$, $P = 0.007$, Fig. 2b) and a trend for a negative relationship with the presence of Spirurids ($F1,328 = 3.53$, $P = 0.06$). When we repeated the analyses with abundance, both "other intestinal parasites" and Spirurids were negatively related to carotenoid concentrations ($F1,328 = 5.95$, $P = 0.02$, Fig. 2c; $F1,328 = 5.37$, $P = 0.02$, Fig. 2d). Presence and abundance of coccideans were not related to carotenoid concentrations (prevalence: $F1,328 = 1.24$, $P = 0.27$; abundance: $F1,327 = 0.87$, $P = 0.35$).

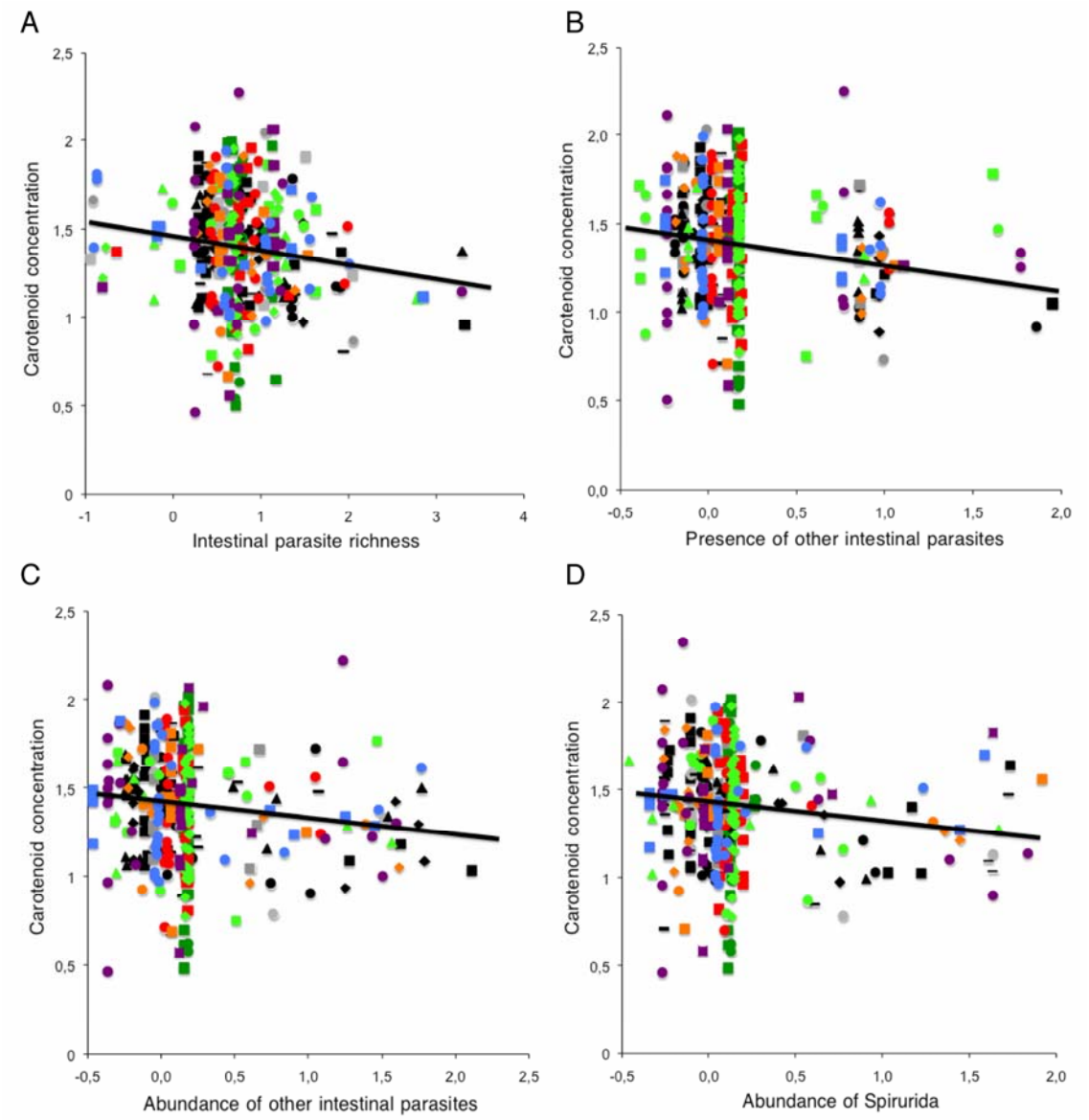

Figure 2. Carotenoid concentrations and parasites. Relationship between carotenoid concentrations (μg/ml) in 354 individuals of 22 species of passerines and (A) intestinal parasite richness, (B) prevalence of other (less frequent) intestinal parasites, (C) abundance of other (less frequent) intestinal parasites and (D) abundance of the intestinal parasites of the Order Spirurida (see legend of Fig. 1 to identify the data from each species).

Interspecific relationships between plasma carotenoids and parasitism

Both the proportion of carotenoids in the plumage (0.0058 ± 0.0019, $F_{1,17} = 9.87$, $P = 0.006$) and in non-feathered parts (0.0806 ± 0.0323, $F_{1,17} = 6.21$, $P = 0.02$) were positively related to carotenoid concentrations. Carotenoid concentrations were unrelated to mean species body mass ($F_{1,18} = 0.02$, $P = 0.88$). In addition, a negative relationship between carotenoid concentrations and the abundance of intestinal parasites was found (-0.4925 ± 0.2350, $F_{1,17} = 4.39$, $P = 0.05$, Fig. 3). This

relationship was not due to the abundance of any particular intestinal parasite group (coccidians: $F_{1,19} = 0.43$, $P = 0.52$; Spirurids: $F_{1,19} = 1.74$, $P = 0.20$; other intestinal parasites: $F_{1,19} = 0.05$, $P = 0.83$). No relationship between intestinal parasite richness ($F_{1,17} = 3.19$, $P = 0.09$), blood parasite richness or abundance was found ($F_{1,18} < 0.08$, $P > 0.78$ for both variables).

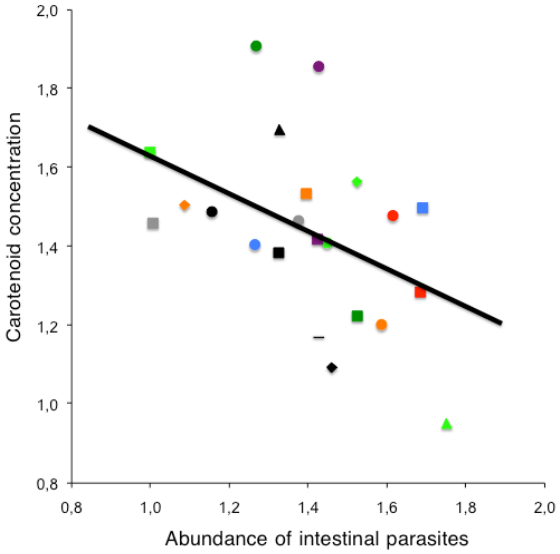

Figure 3. Carotenoid concentrations and parasites. Relationship between the abundance of intestinal parasites and carotenoid concentrations (μg/ml) at interspecific level (see legend of Fig. 1 to identify the data from each species).

4. Discussion

Traditionally, hypotheses concerning the relationships between host ecology and parasites have been tested by focusing on a particular group of parasites (e.g. Haematozoa, intestinal parasites, or ectoparasites). However, the failure to falsify a hypothesis may be due to a lack of success in identifying the group of parasites that most significantly affect host fitness (unless the abundance of the different groups of parasites is highly correlated). Several studies have already pointed out that the intensity of infection by different species of parasites is not strongly correlated at intraspecific level (Møller 1991; Weatherhead et al. 1993, but see Holmstad et al. 2008 for an exception). Our results indicate that species richness and abundance of blood and intestinal parasites are unrelated in analyses at both individual and species level. Consequently, conclusions obtained for one group of parasites cannot be extrapolated for the full community of parasites and so our capacity to rigorously test parasite-mediated selection hypotheses is lessened unless clear indications of the effects on host fitness exists for a significant fraction of the parasite community.

We believe that it is important to highlight that we did not analyze intestinal parasite fauna directly by killing and dissecting the birds. Rather, we used the release of parasite propagules in faeces as a surrogate method for estimating intestinal parasite abundance and richness. Although this is not a direct measurement of parasite load, concentrations of parasite oocysts in faeces do indicate parasite reproductive success (Chapman 1998) in a highly reliable fashion (Lopez et al. 2007). We cannot rule out the possibility that the correlation between blood and intestinal parasites was underestimated. However, the relationships found between intestinal parasites and carotenoid concentrations suggest that we obtained biologically relevant estimates of the composition of intestinal parasite communities.

The second main result of our study is the finding of a negative correlation between intestinal parasite abundance or richness and carotenoid circulation in the blood. These relationships were detected between individuals and between species. There are several non-exclusive factors that can explain these results. Firstly, immune response to parasitism may require the mobilization of carotenoids as scavengers of free radicals released during an immune response, leading to the depletion of carotenoid stores and reduced carotenoid levels in the blood (Alonso-Alvarez et al. 2004, Pérez-Rodriguez et al. 2008 but see Constantini and Møller 2008). Secondly, some intestinal parasites such as coccidians lessen carotenoid absorption in the intestines and thus reduce carotenoid incorporation into the blood (Ruff et al. 1974, Augustine and Ruff 1983, Allen 1987, Tyczkowski et al. 1991). Lastly, food (prey) that is poor in carotenoids may have been more parasitized, thereby exposing individuals and species to a higher amount of parasites. We suspect that this is not the case in our results given that previous analyses by Tella et al. (2004) failed to find any effect of diet on interspecific differences in carotenoid concentrations between species of the same family. However, a lack of information exists on how the consumption of carotenoid-rich foods is related to exposure to parasites at intra- and interspecific levels. The negative relationship between carotenoids and intestinal parasite richness may have at least two potential explanations: 1) the synergistic or accumulative effects of parasites and/or 2) an effect due to only one or just a few parasite taxons and consequently more likely to occur in an individual with a richer parasite fauna. We cannot differentiate between these two possibilities, although when we repeated the analyses separately for the different intestinal groups we found independent negative relationships for Spirurids and for the group 'other nematodes'. None of these groups of parasites is habitually the focus of studies of parasite evolution. In particular, Spirurida, an order of nematodes mainly transmitted by invertebrate vectors, were associated with reduced levels of carotenoids in analyses at individual level. Our results suggest that it would be worthwhile to include this group of parasites in future analyses and experiments on the physiology of carotenoids and on the evolution of carotenoid-derived signals.

In addition to intestinal parasites two other variables were related to inter-individual variation in carotenoid concentrations in the blood: year and date of capture. Carotenoid concentrations were higher in 2005 than in 2004 and increased as spring progressed. As previously commented, carotenoids should be obtained from an animal's diet and in the case of birds both invertebrates and fruit are important sources of carotenoids. Both of these resources undergo significant annual and seasonal oscillations in abundance (Herrera et al. 1998, Jones et al. 2003) that may explain our results. Both the effects of year and season have been already reported in the case of the Great Tit Parus major, the only species in which seasonal and annual variation in plasma carotenoids has been

studied to date (Isaksson et al. 2007). Our results confirm that these factors are applicable to the passerine communities present in our study area. Despite the fact that some studies have reported a higher concentration of carotenoids in males than in females during molting periods (Hill 1995, Figuerola and Gutierrez 1998), our results indicate that this is not the case during the spring, when no molting is occurring. Another potential reason for expecting sexual dimorphism in carotenoid circulation is the deposition of important quantities of carotenoids in eggs that may reduce female carotenoid stores (Saino et al. 2002, Royle et al. 2003). It is important to note, however, that the samples in our study were collected before the start of egg-laying in most of the species studied.

At interspecific level, the extent of carotenoid-derived coloration in feathers and skins was positively related to carotenoid concentrations in the blood, thus confirming the results reported by Tella et al. (2004). However, in contrast to this study, we failed to find any relationship between body mass and carotenoid concentrations, probably because of the smaller numbers and the lesser variation in body mass of the species analyzed in our study.

In conclusion, parasites are related to differences between individuals and species in the concentration of carotenoids in the plasma, suggesting that they may play an important role in the regulation of carotenoid levels. Interestingly, these effects are not generalized for parasites, but are specific to some groups, indicating the need for studies focusing on complete parasite communities and not just on a single group. Additionally, Spirurida were particularly related to reduced levels of carotenoids, suggesting that more attention should be paid to this group of parasites in future studies of host-parasite ecology.

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Declaration

All the work performed in the present manuscript comply with the current Spanish laws.

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4. ECOLOGÍA DEL VIRUS DEL NILO OCCIDENTAL EN AVES SILVESTRES

4.a. CAPÍTULO 6: El tamaño importa: Anticuerpos neutralizantes frente al virus del Nilo occidental en aves residentes y migradoras en España.

Resumen:

La rápida expansión de WNV ha suscitado el interés de comprender la dinámica poblacional y los patrones dispersivos de las enfermedades infecciosas en la fauna silvestre. En este trabajo analizamos diferentes factores ecológicos y evolutivos relacionados con la prevalencia de anticuerpos neutralizantes frente a WNV en 72 especies de aves muestreadas en el sur de España. La prevalencia de anticuerpos fue máxima durante el otoño y el invierno en comparación con los meses de verano. La seroprevalencia estuvo directamente relacionada con la masa corporal y el comportamiento migrador. La mayor prevalencia de anticuerpos observada en migrantes estivales se puede explicar, entre otros factores, por la diversidad de localidades envueltas en sus ciclos vitales o por las áreas geográficas visitadas en las migraciones. La mayor prevalencia observada en especies grandes no se explicaba por la longevidad, ya que la relación permanecía significativa al analizar sólo aves de un año de edad, pero probablemente incluía también una mayor atracción de los vectores hacia las especies mayores. La colonialidad y el gregarismo invernal no estuvieron relacionados con la prevalencia de anticuerpos frente a este patógeno altamente generalista en cuanto a hospedador. Las relaciones evolutivas entre especies no estuvieron relacionadas con las diferencias en la prevalencia de anticuerpos. Nuestros resultados sugieren que las especies más grandes son buenas candidatas para realizar seguimiento de los cambios locales, estacionales y anuales en la seroprevalencia de WNV.

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Size matters: West Nile Virus neutralizing antibodies in resident and migratory birds in Spain

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Abstract

The rapid range expansion of West Nile Virus has raised interest in understanding the population dynamics and dispersal patterns of emerging infectious diseases by wildlife. We analyzed different ecological and evolutionary factors related to West Nile Virus neutralizing antibody prevalence in 72 bird species sampled in southern Spain. Prevalence of antibodies reached its maximum during the autumn and winter in comparison to summer months. Prevalence of antibodies was directly related to body mass and migratory behaviour. The greater prevalence of antibodies observed in summer migrants can be explained, among other factors, by the diversity of localities involved in their life cycles or the geographic areas visited during their migrations. Greater prevalence in larger species was explained by their longevity because the relationship was already significant when analyzing only first year birds, and probably also involved a high attraction to vectors by larger hosts. Coloniality and winter gregarism were unrelated to the prevalence of antibodies against this highly host generalist pathogen. Evolutionary relationships between species were unrelated to differences in the prevalence of antibodies. Our results suggest larger species as good candidates for easy, faster and cheaper monitoring of local, seasonal and annual changes in WN virus serology.

Keywords: Dispersal; Host ecology; Migratory birds; Monitoring program; Reservoir diversity; Virus dispersal; West Nile Virus ecology

1. Introduction

West Nile Virus (WNV) is a member of the *Flavivirus* genus (family Flaviviridae), transmitted by mosquito bites. Humans infected by WNV may develop a variety of signs ranging from mild fever to more severe illnesses such as acute encephalitis,

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poliomyelitis, meningitis, or hepatitis and is fatal in a small percentage (<1%) of cases (Hubálek and Halouzka, 1999). WNV is widely distributed throughout Africa, Asia, Europe, Australia (Kunjin virus). It was first detected in New York in 1999, and in just 6 years has spread throughout all of North America (CDC, 2005). It has been suggested that one of the causes of this rapid expansion is the high mobility of the virus' avian reservoirs (Rappole and Hubálek, 2003) and its wide host range (already detected in more than 285 avian species; CDC, 2005).

In Europe and Africa WNV infection is usually non-fatal for birds (Hubálek and Halouzka, 1999); in the New World, however, the virus has killed many birds (Marra et al., 2004), and reduced populations of more susceptible hosts by up to 45% since WNV arrival (LaDeau et al., 2007). As an example of the different epidemiology in Europe and North America, while experimental infection with WNV of North American birds usually result in high mortalities (i.e. 32.3% of 87 experimentally infected birds of 25 species, Komar et al., 2003), experimental infections done in Europe have reported no apparent mortality due to WNV (9 geese experimentally infected by Malkinson and Banet, 2002). The reasons for this high virulence in North America remain largely unknown; nevertheless, the fact that species from the Nearctic have never been exposed to the virus and the higher pathogenicity observed in the introduced strain (Brault et al., 2004) may explain these differences.

A number of ecological factors can be associated with a higher prevalence or diversity of pathogens in birds: migratory behaviour, coloniality or gregarism, habitat use, mating systems, and immune system capacity (Møller and Erritzoe, 1996; Clayton and Moore, 1997; Figuerola, 1999, 2000; Figuerola and Green, 2000; Tella, 2002). However, to our knowledge, no study has focused on vector-borne generalist pathogens. Despite the thousands of birds that have been tested for WNV or its antibodies in North America, analyses focusing on the relationship between bird ecology and exposure to the virus are still lacking but urgently needed. In this study we take advantage of the differences in the impact of WNV in Europe and in North America to analyze the relationship between bird ecology and phylogeny and prevalence of WNV neutralizing antibodies. The relevance of this study is twofold, on the one hand, the low host specificity of WNV makes this system different from the pathogens used in previous studies (mainly blood parasites and ectoparasites), and may affect the relevance of different ecological factors. On the other hand, given the relevance of WNV for human health and wildlife conservation we also aim to identify the characteristics of the species that can be most useful for monitoring in Europe.

In this paper, we first analyze the relationship between host evolutionary and ecological characteristics and the prevalence of WNV neutralizing antibodies in birds. Second, as we report important differences in the prevalence of antibodies according to host characteristics we used a statistical power analysis to discuss the relevance of our results in relation to WNV monitoring in Europe.

2. Materials and methods

Between January 2003 and February 2005 we captured 1213 individuals belonging to 72 species (49 genera, 22 families, and 8 orders). Birds were captured without damage using mist-nets and walk-in-traps in the Guadalquivir and Odiel Marshes (SW Spain). Blood samples were taken with syringes from the brachial, femoral, or jugular vein, birds were marked with numbered aluminum rings and released after manipulation. The volume of blood extracted depended on the size of the species and never exceeded 1% of body mass (range 0.080–1 ml). Blood was collected in eppendorf tubes, allowed to clot at ambient temperature, and placed into coolers until centrifugation during the same day. All samples were obtained from adult (full grown) individuals to ensure that the antibodies were not the result of the passive transfer of maternal immunity (Gibbs et al., 2005). When possible age was determined (471 first-year individuals and 540 after first-year individuals) according to Prater et al. (1977), Baker (1993) and Svensson (1996).

WNV strain Eg101 and the E6 clone of Vero cells used for virus propagation were obtained from Hervé Zeller (Institut Pasteur de Lyon). The Usutu virus (SAAR 1776 isolate) was obtained through the Centre for Ecology and Hydrology, Oxford, UK, and propagated in Vero cells (American Type Culture Collection, Manassas, VA). Virus titers were

determined by end-point titration following the method used by Reed and Muench (1938). WNV-Neutralizing antibody titers were determined by a micro-virus-neutralization test (micro-VNT) in 96-well plates, adapted from a previously described method (Jiménez-Clavero et al., 2001). A recent study shows that a micro-VNT assay and the standard $PRNT_{90}$ perform comparably in sensitivity at detecting anti-WNV antibodies in birds (Weingartl et al., 2003). Serum samples were inactivated at 56 °C for 30 min prior to the analysis. Dilutions of test sera (25 ml) were incubated with 100 $TCID_{50}$ of WNV strain Eg101 in the same volume (25 ml) for 1 h at 37 °C in Eagle's medium (EMEM) supplemented with L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, followed by the addition of 50 ml of a suspension (2×10^5 cells/ml) of Vero E6 cells in the same medium plus fetal calf serum to a final concentration of 5%. The mixture was further incubated for 6–7 days (37 °C in a 5% CO_2 and saturating humidity atmosphere) until cytopathic effect (cpe) was observed in control wells containing 10 $TCID_{50}$ of virus. The screening of samples was performed at 1:10 and 1:20 dilutions of tested sera (dilutions considered before the addition of virus, that is, in a volume of 25 ml). Only samples yielding positive neutralization (complete absence of cpe) at 1:20 were scored as positives and further titrated by analyzing serial serum dilutions from 1:20 to 1:640. Neutralizing serum titer was considered as the highest value of the reciprocal serum dilution giving a complete absence of cpe.

The specificity of the assay was assessed in two ways. First, by analyzing a panel of sera from an external quality assessment, consisting of serum samples containing antibodies from other four flaviviruses, that proved negative for neutralization titers in our WNV assay, while duplicate testing of all WNV antibody-positive serum samples proved positive (>1:20) for neutralization titers (Niedrig et al., 2007). Second, we also compared the neutralizing antibodies titers of 18 samples tested in parallel for WNV and Usutu virus (a closely related JEV group avian virus). In none of the cases showed higher antibody titers more specific to Usutu than to WNV (see Figuerola et al., 2007a, for more details). We cannot discard that the serology to WNV observed in some of the samples, particularly those from birds flying from Central Europe (e.g. *Turdus philomelos, Sylvia atricapilla*) could be attributed to cross-reacting antibodies to other flaviviruses (particularly TBEV) that can be prevalent in Central Europe. However, this seems unlikely since the technique has shown no cross reactivity to TBEV-positive sera.

In a first model, we investigated the effects of taxonomic relationships on West Nile Virus prevalence by using Generalized Linear Mixed Models (GLMM). GLMM allows a more versatile analysis of correlation than standard regression methods, because the error distribution of the dependent variable and the function linking predictors to it can be adjusted to the characteristics of the data (Littell et al., 1996). Our response variable was the antibody status (1 present, 0 absent), and we used a binomial distributed error and a logistic link function, to ensure linearity, and statistics adjusted to model dispersion. Binomial errors are adequate to analyze binary response variables. Goodness-of-fit of the model was assessed by checking the overdispersion parameter and the Generalized Chi-Square statistic (Littell et al., 1996). Period (a three levels factor, summer: birds captured in June–August, autumn: September–November and winter: December–March) and age (first-year or adult bird, not including unknown age birds in the analyses) were included as fixed factors in the analyses. Species was included as a repeated subject effect (i.e. observations of a same species are correlated) and the interaction between species and period was included as a random factor. The statistical significance of each nested taxonomic level (Genera, Family and Order) was tested using Z-statistics for random effects using the macro GLIMMIX for SAS 8.2 (Littell et al., 1996). As age had no significant effect ($F_{1,27} = 1.88$, $P = 0.18$, $N = 957$), we report the results of analyses excluding this variable to include the full dataset and range of species.

In a second model, we analyzed the relevance of different ecological factors. Species body mass (log transformed mean values to fit a normal distribution as judged by checking the normal quantile plot), migratory behaviour (resident or migratory species), breeding sociality (solitary or colonial breeders), and winter sociality (solitary or gregarious species) were included as fixed factors in the analyses. Values for

Table 1
Model analyzing the relationship between host ecology, period of capture and presence of West Nile Virus (WNV) neutralizing antibodies in the blood of 1213 individuals birds captured in south-west Spain

	Estimate ± S.E.	F	d.f.	P
Body mass	0.746 ± 0.288	6.72	1,69	0.01
Migratory behaviour	2.032 ± 1.084	0.71	1,69	0.40
Period		3.55	2,29	0.04
Summer	0			
Autumn	2.194 ± 1.286			
Winter	2.287 ± 1.133			
Coloniality		0.67	1,68	0.42
Winter gregarism		2.82	1,68	0.10
Body mass × Migratory behaviour		0.71	1,68	0.40
Body mass × Period		1.21	2,1135	0.30
Body mass × Coloniality		0.65	1,68	0.42
Body mass × Winter gregarism		3.23	1,68	0.08
Migratory behaviour × Period		3.71	2,29	0.04
Migratory species in autumn	−1.021 ± 1.366			
Migratory species in winter	−3.653 ± 1.431			
Others	0			
Migratory behaviour × Coloniality		1.63	2,67	0.20
Migratory behaviour × Winter gregarism		1.41	2,67	0.25
Period × Coloniality		1.28	3,26	0.30
Period × Winter gregarism		0.99	3,26	0.41
Coloniality × Winter gregarism		1.55	2,67	0.22

Final model was obtained after backwards variable selection. Only variables with $P < 0.05$ are interpreted as statistically significant and parameter estimates are given. For variables not included in the model no parameter estimate is presented and the F and P values correspond to the values when added to the final model.

these variables were taken from literature (Cramp, 1982–1994) and were validated by four independent ornithologists according to the ecology of the species in Spain. For each individual we also included the period of collection to control for seasonal differences in the prevalence of antibodies. To test the relationship between ecological factors and antibody prevalence we followed a stepwise-backward selection procedure starting from an initial model including all the two-way interactions between factors.

We estimated the sample size necessary to detect increases of 10, 20, 30 and 40% in WNV seroprevalence with the program G-Power (Buchner et al., 1997). Effect sizes were calculated for prevalences between 1 and 55%, and sample size necessary to obtain a power of 0.80 when using a Chi-Square test was estimated. A power of 0.80 indicates that a significant result ($P < 0.05$) will be obtained in 80% of the analyses of datasets with statistical differences of that magnitude, and is the threshold value usually used in ecology (Bausell and Li, 2002).

3. Results

Of the 1213 individuals tested, 126 (10.4% of individuals from 24 out of 72 species) had WNV neutralizing antibodies, with titers ranging from 1:20 to over 1:640 (see Electronic Appendix A). Important interspecific differences in the presence of WNV neutralizing antibodies were found, with prevalences ranging from 0 to 42.9%. However, taxonomic levels were unrelated to these differences in prevalence (Genera, $Z = 0.80$, $P = 0.21$; Family, $Z = 1.11$, $P = 0.14$; Order, $Z = 0.58$, $P = 0.28$).

Multivariate analyses indicate that antibody prevalence was unrelated to host sociality (Table 1). Prevalence of antibodies changed seasonally (Table 1) with significantly higher prevalences in autumn (mean ± S.E.: 10.29% ± 12.71) than in summer (a test of Least-Square means difference, 2.09 ± 7.72, $t_{29} = 2.46$, $P = 0.02$), and intermediate prevalences in winter (3.27% ± 9.12, contrast with autumn, $t_{29} = 1.85$, $P = 0.07$; contrast with summer, $t_{29} = 0.61$, $P = 0.55$).

Although migratory behaviour was not directly related to antibody prevalence (migrants, 5.23% ± 7.41; residents, 3.32% ± 8.13), a significant interaction with season was found (Table 1). Prevalence did not change with season in resident species ($F_{2,29} = 2.11$, $P = 0.14$) but only in migratory species ($F_{2,29} = 6.15$, $P = 0.006$). When comparing migrant and resident species within each period, in summer migrants (i.e. species wintering in Africa) tended to have higher prevalences of antibodies that residents (5.56% ± 8.58 vs. 0.77% ± 8.85; $t_{29} = 1.88$, $P = 0.07$). Winter migrants (i.e. coming from central and northern Europe) tended to have lower prevalences than resident species (1.48% ± 10.49 vs. 7.06 ± 12.16; $t_{29} = 1.70$, $P = 0.09$). Large species (as estimated from their body size) had higher prevalences of antibodies (Table 1, Fig. 1).

As age may affect the relationship between antibody prevalence and body mass, the analyses were repeated using only less than 1 year old birds (417 individuals), confirming that the relationship between prevalence and body mass was significant also when considering only birds of the same age ($F_{1,43} = 5.61$, $P = 0.02$).

Power analyses indicate that the sample size necessary to detect significant changes in seroprevalence depends dramatically on initial seroprevalence (Fig. 2). For example, 4857 individuals are necessary to detect a 40% increase in seroprevalence when initial seroprevalence is 1% (i.e. in our study *Passer domesticus* had a prevalence of 0%), but only 74 individuals are necessary when focusing in species with 40% prevalence (i.e. *Fulica atra*, with 42.6% prevalence or *Larus ridibundus*, with 42.9%).

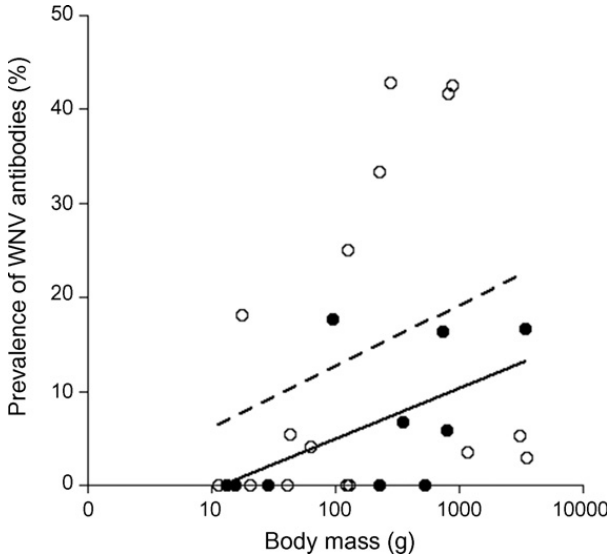

Fig. 1. Prevalence of West Nile Virus (WNV) neutralizing antibodies in relation to body size (grams) in resident (418 individuals) and migratory (795 individuals) birds sampled in south-west Spain. For illustration purposes a regression line has been plotted for migratory and resident species. Open symbols and dotted line correspond to migratory species and filled symbols and continuous line to resident species. Only species with at least ten individuals sampled have been included in the plot.

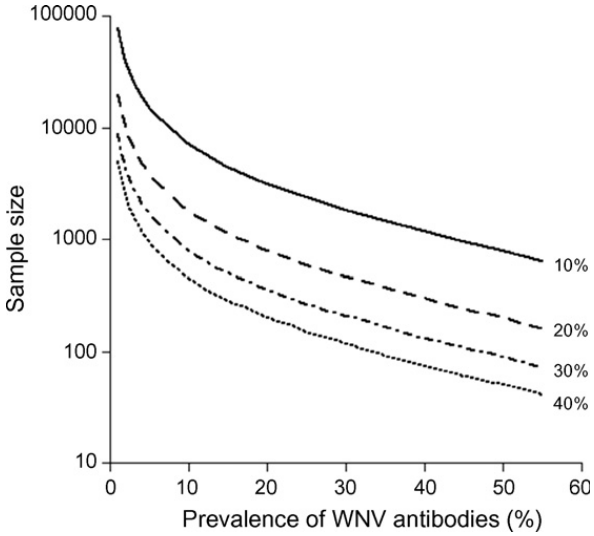

Fig. 2. Sample size necessary to detect with a Chi-square test and a power of 0.80 increases by 10, 20, 30 and 40% in the prevalence of West Nile Virus (WNV) antibodies.

4. Discussion

In Spain, clinical signs of WNV disease in birds has only been reported recently (Höfle et al., 2008). WNV neutralizing antibodies had been reported in horses (Jiménez-Clavero et al., 2007), chicks of different colonial breeding waterbirds (Figuerola et al., 2007a), and the rapid seroconversion of common coots during a capture–recapture study has also confirmed the local circulation of WNV in the study area (Figuerola et al., 2007b). Infections with clinical symptoms in humans were reported in 2004 in Badajoz (Spain) and Algarve (Portugal) (Esteves et al., 2005; Kaptoul et al., 2007), overlapping with the collection of samples for this study. Previous records give a seroprevalence of up to 30% in humans in some towns in the Ebro Delta (Lozano and Filipe, 1998) and of 16.5% in northwest Spain (González and Filipe, 1977), presumably with maximum epidemic activity during the 1970s.

However, these results were obtained by haemagglutination-inhibition, a technique burdened by its cross-reactivity with a range of flaviviruses. Recent studies with highly specific neutralization assays show that the prevalence of WNV antibodies in humans living around wetlands in Spain is currently very low (Bofill et al., 2006).

The presence of serum antibodies neutralizing WNV in adult birds indicates previous contact (infection) with WNV or a closely antigenically related flavivirus, and survival to the initial infection. Consequently, for a wild bird population with low pathogenicity WNV infection (such as those usually found in the Old World, Zeller and Schuffenecker, 2004), the higher the prevalence of WNV neutralizing antibodies, the higher the exposure to the virus. This scenario is not applicable when WNV infection results in high mortality, as observed in North America.

The results suggest that evolutionary relationships are of little importance in explaining variations in exposure to WNV. This contrasts with the initial studies that identified Corvidae (McLean et al., 2001), Mimidae, and Cardinalidae (Ringia et al., 2004) as bird families that are particularly exposed to WNV infection. In our study both Rallidae (6.7–42.6%) and Laridae (25.0–42.9%) presented very high antibody prevalence, although our results suggest that these high prevalences were related to the ecology of the species sampled (migratory species of medium and large size), rather than to the birds' taxonomy.

In North America, the American Crow appears to be particularly susceptible to mortality by WNV (Komar et al., 2003). This has led some researchers to suggest that Corvidae in general might be very at a risk for exposure to the virus. Interestingly, none of the 35 individuals of *Corvus monedula* (the only Corvidae included in our study) had WNV antibodies, even when captured together with individuals of other species with high prevalences. It is important to note that this low (zero) prevalence of antibodies in *Corvus monedula* is not likely to result from the rapid death of infected individuals, given that all attempts we have done to the moment to detect the virus in several hundreds dead water-birds had failed (data not shown). We suggest that the high incidence of West Nile in American Crow can result not only from the transmission by mosquitoes but also from the consumption of corpses of birds dying during the viraemic phase of the infection. In this case the utility of Corvids for monitoring WNV circulation in the wild could be reduced in Europe.

No effect of winter or breeding sociality on antibody prevalence was found. Although a high prevalence of blood parasites had been reported among social living species (Tella, 2002), the low host-specifity of WNV may make the density of birds the relevant parameter affecting risk of exposition, regardless whether or not it consists of conspecifics. Interestingly, migratory species showed higher antibody prevalence than resident species, but only when comparing summer migrants with residents. Although local circulation of the virus is taking place (since resident species also have antibodies), this higher prevalence observed in migratory birds suggest that these birds spend part of their lives in areas in Africa where the circulation of the virus may be higher than in the surveyed area in Spain. For example, a recent serosurvey in horses detected extremely high prevalences of antibodies (up to 97%) in some sub-Saharan countries (Cabre et al., 2006), areas visited by many European long distance migratory species. Our analyses support the view that species of larger body mass may have increased opportunity for exposure to WNV. Given that we analyzed antibody prevalence in free-living and apparently healthy individuals, this conclusion is not merely a bias caused by the difficulties in finding carcasses of smaller species (Marra et al., 2004), a problem associated with studies based only on dead birds. The direct relationship between prevalence of WNV antibodies and body mass can be explained by several non-exclusive factors. Larger species live longer (Calder, 1984), however we have demonstrated that the relationship between seroprevalence and body mass is also significant when analyzing only first-year birds. We suggest that the larger prevalence of antibodies in larger species is the result of their larger surface area and higher CO_2 production (Nagy, 1987), and can host and attract a higher number of ectoparasites (Soliman et al., 2001), mosquitoes, and other biting arthropods that transmit the virus.

In conclusion, we suggest that migratory birds of large body mass may provide a means for monitoring WNV prevalence on a large geographical scale (e.g. migratory flyways). Additionally, resident species of large body mass may provide a better description of

local WNV prevalence. Further, the past allegations regarding a greater probability of infection by WNV by particular taxonomic groups should be more carefully explored given our findings and the fact that many of those studies were based on the examination of only a few species, and on dead birds, making difficult to separate the effects of exposure, susceptibility and carcass detection probability. From a conservation standpoint, it perhaps would be more beneficial to focus our attention on the effects of WNV on species of greater body mass and on migratory species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2008.04.023.

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4.b CAPÍTULO 7: La prevalencia de anticuerpos neutralizantes frente al virus del Nilo occidental en España se relaciona con el comportamiento de las aves migradoras.

Resumen:

El virus del Nilo occidental (WNV) es un flavivirus aviar capaz de infectar caballos y humanos que se transmite por insectos chupadores. En Europa y África han surgido esporádicamente infecciones y brotes epidémicos que han causado enfermedad y muerte en humanos, y que han sugerido dos hipótesis no excluyentes acerca de la circulación de WNV en Europa: (1) la existencia de un ciclo selvático endémico que ocasionalmente provoca infección en humanos o equinos y (2) la introducción esporádica del virus por aves migradoras desde áreas de África (u otros emplazamientos) donde WNV es endémico que cause focos epidémicos locales que eventualmente produzcan infección en humanos o equinos. Para investigar estas dos posibilidades, usamos test de seroneutralización de micro virus para examinar la prevalencia de anticuerpos neutralizantes frente a WNV en 574 ejemplares de 25 especies de aves capturados en 2004 en la ciudad de Sevilla. Las especies migradoras trans-saharianas presentaron prevalencia y títulos superiores a los presentados por especies residentes o migradoras de corta distancia. Este resultado sugiere que los migradores trans-saharianos pasan parte de su ciclo vital en áreas con mayor circulación de WNV (o de flavivirus muy próximos filogenéticamente) antes de su llegada a España. Por otro lado, las seroprevalencias halladas en aves residentes sugieren un bajo nivel de circulación del virus en el área de estudio. Aparte de la cuestión de la circulación local, parece por tanto que el riesgo de introducción de cepas africanas de WNV por aves migratorias en España merece un estudio de campo y experimental más profundo.

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Prevalence of West Nile Virus Neutralizing Antibodies in Spain Is Related to the Behavior of Migratory Birds

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ABSTRACT

West Nile virus (WNV) is a bird flavivirus capable of infecting horses and humans that is transmitted by blood-sucking vectors. In Europe and Africa, sporadic infections and outbreaks causing human illness and deaths have occurred and have led to 2 mutually nonexclusive hypotheses regarding the circulation of WNV in Europe: (1) the occurrence of endemic sylvatic cycles that occasionally result in human or equine infection, or (2) sporadic seeding of WNV by migratory birds from areas where the virus is endemic in Africa or elsewhere that cause local epizootic foci and eventually lead to infection in humans. To investigate these 2 possibilities, we used a micro virus-neutralization test to examine the prevalence of WNV neutralizing antibodies in 574 individuals belonging to 25 species of birds captured in spring 2004 in Seville (southern Spain). Trans-Saharan migrant species had both higher prevalences and antibody titers than resident and short-distance migrants. This result suggests that trans-Saharan migrants spend part of their life cycles in areas with greater circulation of WNV, or a closely related flavivirus, before their arrival in Spain. On the other hand, seroprevalences assessed in resident birds suggest a low level of WNV circulation in the studied locality. Aside from the question of local circulation, it thus seems that the risk for introduction of strains of WNV from Africa by migratory birds merits further field and experimental studies in Spain. Key Words: WNV—Virus dispersal—Bird migration—Long-distance dispersal.

INTRODUCTION

West Nile virus (WNV) is an arbovirus (arthropod-borne virus) belonging to the Japanese encephalitis group (family Flaviviridae, genus *Flavivirus*). Virus transmission between hosts can occur both by means of a vector (usually mosquitoes or ticks, Chevalier et al. 2004, Lawrie et al. 2004) or, less commonly, by direct transmission (Banet-Noach et al. 2003). Although WNV causes illness and outbreaks of disease in humans and equines (Autorino et al. 2002, Durand et al. 2002, Del Giudice et al. 2004, Sanchez-Seco and Navarro 2005), the natural hosts of the virus are wild birds, which act as amplifying reservoirs (Zeller and Schuffenecker 2004). WNV normally causes an asymptomatic infection in Eurasian birds (Petersen and Roerigh 2001), and its presence in Eurasia has been known since 1958 (Bárdos et al. 1959). This situation differs greatly from the situation in the Americas, where the introduction of WNV in a single event in 1999 (Asnis et al. 2000) generated a fast and explosive spread over the whole continent which caused numerous outbreaks and mortality in humans, horses (Asnis et al. 2001, Blitvich et al. 2003), and wild native birds (Kramer and Bernard 2001). Despite WNV's origin in the Old World, it has been studied more intensively in North America since its introduction in 1999 than in Europe, as judged by the number of papers included in the ISI Web

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of Knowledge (129 vs. 693 up to November 2007). The intensity of circulation and the rapid spread of WNV in America, as well as the sensibility of American birds to the disease, are thought to have converted migrant birds into important vectors for the transport of the virus throughout the continent. Peterson et al. (2003), for instance, found that migratory birds could explain the spread of WNV throughout North America. However, Rappole et al. (2003, 2006) did not find any basis to support this idea, as the dispersal patterns of WNV in North America are better explained by short-to-medium-distance dispersive movements of resident birds. The known ecology and epidemiology of WNV in Europe have provided 2 potential hypotheses to explain the disease's outbreak dynamics (Hubálek 2000). First, a sylvatic circulation cycle could exist locally, which would maintain the virus in enzootic foci throughout the year and, under proper conditions, cause outbreaks in humans due to enhanced virus circulation. Second, migrant birds may act as virus carriers, repeatedly seeding the infection from areas where the virus is endemic in habitats suitable for the infection to progress in Europe in differing areas and years (Hubálek 2000, Malkinson et al. 2001, 2002, Malkinson and Banet 2002). To assess whether long-distance migrant birds are more exposed to WNV than resident birds, which would support the second of the 2 hypotheses, we carried out a seroprevalence study in small passerines. We hypothesized that long-distance migrant birds have a higher risk for exposure to WNV and thus may show higher seroprevalence than short-distance migrant or resident birds.

MATERIALS AND METHODS

We trapped 574 birds belonging to 25 different species (mainly passerines; Table 1) during prenuptial migration (March, April, and May)

Table 1. Species, Number of Sampled Individuals, and Titers for Individual Birds with WNV Neutralizing Antibodies Sampled Between March and May 2004 in Seville, Spain

		Titers						
Species	*Sampled* (n)	*20*	*40*	*80*	*160*	*320*	*640*	*Migratory status*[a]
Acrocephalus scirpaceus	5							T
Alectoris rufa	1							R
Carduelis carduelis	10							R
Carduelis chloris	26							R
Erithacus rubecula	1							M
Ficedula hypoleuca	19							T
Galerida cristata	2							R
Lanius senator	17				2			T
Luscinia megarhynchos	3							T
Merops apiaster	1							T
Muscicapa striata	5							T
Oriolus oriolus	1							T
Passer domesticus	79							R
Passer montanus	1							R
Phoenicurus ochruros	1							M
Phoenicurus phoenicurus	2		1					T
Serinus serinus	54							R
Streptopelia decaocto	23							R
Sturnus unicolor	1							R
Sylvia atricapilla	59							M
Sylvia borin	183	7	2	2	1	2	1	T
Sylvia communis	3							T
Sylvia hortensis	2							T
Turdus merula	74	3	1					R
Upupa epops	2							M

[a]Each species was scored as resident (R), short-distance migrant (M), or trans-Saharan migrant (T).

in 2004 in a forestry nursery near Seville city (37° 23′ N, 5° 57′ W). We chose this place because of their proximity to human inhabited areas, where the potential for mosquito cofeeding in humans and birds is likely to be high. Additionally, passerines present high levels of virosis when infected with WNV and may be good candidates for virus amplification and dispersal (Komar et al. 2003, Owen et al. 2006). Birds were captured in 20 12-meter-long mist nets operating from sunrise to sunset. Individuals were marked with numbered aluminum rings and their body mass (to the nearest 0.1 g) was recorded. For each individual we drew a blood sample from the jugular vein using 29 G sterile insulin syringes (always less than 1% of the body mass). The blood was placed in a vial, kept for several hours at ambient temperature (15°C–25°C) to allow clotting, and then centrifuged for 10 minutes at 6000 rpm in an Eppendorf Minispin centrifuge to separate the serum from the blood clots. The sera were frozen at −20°C until subsequent analysis. The presence of WNV neutralizing antibodies was determined in each serum by a micro virus-neutralization test (micro-VNT) in 96-well plates as previously described (Niedrig et al. 2006, Figuerola et al. 2007a). Serum samples were inactivated at 56°C for 30 minutes prior to analysis. Dilutions of test sera (25 μL) were incubated for 1 hour at 37°C with 100 tissue-culture infectious doses $(TCID)_{50}$ of WNV strain Eg101 in the same volume (25 μL) in Eagle's medium (EMEM) supplemented with L-glutamine, nonessential amino acids, sodium pyruvate, penicillin (100 U/mL), and streptomycin (100 μg/mL). Then, 50 μL of a suspension (5×10^5 cells/mL) of Vero E6 cells was added to the same medium, along with fetal calf serum, to reach a final concentration of 5%. The mixture was further incubated for 6 days at 37°C until a cytopathic effect (cpe) was observed in control wells containing 10 $TCID_{50}$ of virus. The screening of samples was performed at 1/10 and 1/20 dilutions of the tested sera (dilutions considered before the addition of virus, i.e., in a volume of 25 μL). Samples yielding positive neutralization (absence of cpe) at one or both of the dilutions tested were confirmed and further titrated by analyzing serial serum dilutions from 1/10 to 1/640. Controls for cytotoxicity in the absence of virus were included for every sample at 1/10 dilution. Cytotoxic samples were excluded from the analyses. The neutralizing serum titer was considered to be the highest value of the reciprocal serum dilution giving a complete absence of cpe. Birds were scored positive when neutralization at 1/20 or higher dilutions occurred. The specificity of the VNT employed in our study has been analyzed previously using a panel of sera with specificity for different flaviviruses (WNV, yellow fever virus, dengue virus, and tick-borne encephalitis virus), and titers of 10 or higher were only detected in sera from WNV-infected individuals (Figuerola et al. 2007a). Additionally, previous studies have shown a higher specificity to WNV in neutralization tests run in parallel against Usutu virus (another bird *Flavivirus*), confirming that the flavivirus causing the immune reaction is more closely related to WNV than to other flaviviruses (Figuerola et al. 2007b). Each species was scored as resident, short-distance migrant (wintering in southern Europe or in Africa north of the Sahara), or trans-Saharan migrant (long-distance migrants wintering south of the Sahara) as per Cramp and Perrins (1994). WNV neutralizing antibody prevalence was analyzed with the GENMOD procedure (SAS Institute 2000a) in the SAS 9.1 statistical package. Deviances from the model were scaled with the square root of the ratio deviance/degrees of freedom to correct for overdispersion. Log-transformed values of antibody titers from individuals with antibodies were compared using a *t*-test for unequal variance groups with JMP 5.0 (SAS Institute 2000b).

RESULTS

For species with more than 20 individuals sampled, the prevalences ranged from 0% (*Carduelis chloris*, 95% CI: 0%–13.2%; *Passer domesticus*, 0%–4.6%; *Serinus serinus*, 0%–6.6%; *Streptopelia decaocto*, 0%–14.8%; *Sylvia atricapilla*, 0%–6.1%) to 8.1% (*Sylvia borin*, 4.7%–13.2%). Intermediate prevalences were found in a resident species (5.4%, 1.5%–13.3%, *Turdus merula*), suggesting that local circulation of WNV exists.

Antibody prevalence was related to migra-

tion distance ($\chi^2 = 19.28$, df = 2, $p < 0.0001$). None of 63 sampled short-distance migrants presented WNV neutralizing antibodies. Antibodies to WNV were more prevalent in trans-Saharan (18 of 240 individuals, 4.5%–11.6%) than in resident birds (4 of 271, 0.4%–3.7%, a posteriori least square means comparison, $\chi^2 = 10.23$, df = 1, $p = 0.001$; Fig. 1A). Most of the sera from trans-Saharan birds correspond to *S. borin*, however, this is not likely to introduce a bias in the analyses because the GLM method used controls for differences in sample size and because a trend for higher prevalence in trans-Saharan species remains even when the analyses are repeated without *S. borin* ($\chi^2 = 5.56$, df = 2, $p = 0.06$). Antibody titers were also higher in trans-Saharan migrants than in resident birds ($t_{17.54} = 2.90$, $p = 0.01$; Fig. 1B).

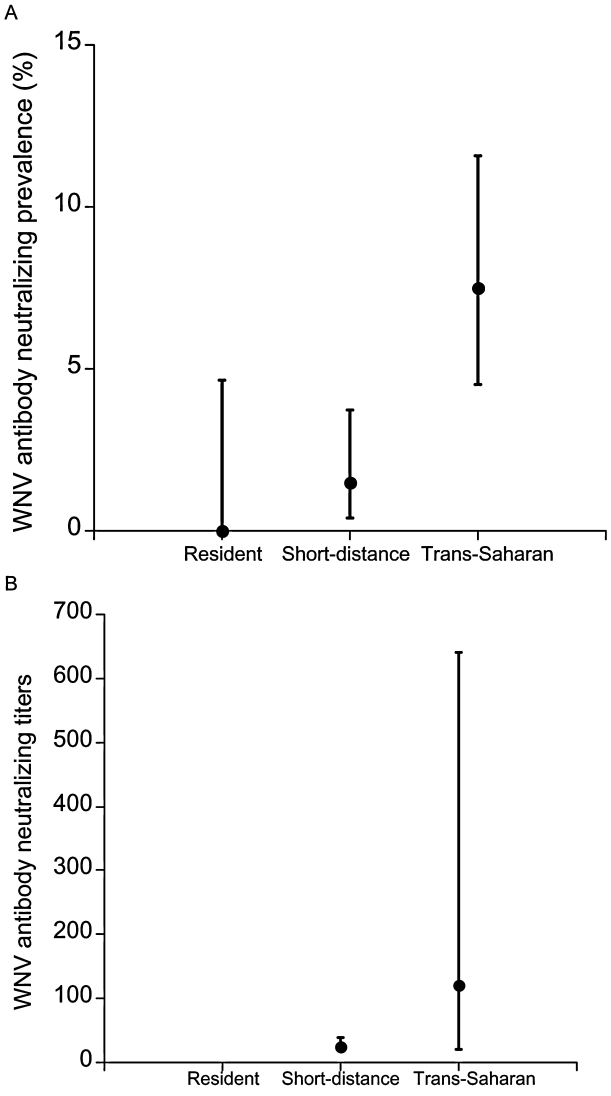

FIG. 1. Mean prevalence ± 95% confidence interval (**A**) and mean and range of titers (**B**) of antibodies against WNV in resident, short-distance migrant, and trans-Saharan migrant bird species trapped during spring migration in Spain.

DISCUSSION

Our results confirm that long-distance migrants are exposed during their migratory journeys and/or their winter stay in Africa to higher levels of WNV circulation, or a closely antigenically related flavivirus, than the levels found in their breeding grounds in Europe. In particular, species wintering south of the Sahara (Figs. 1 and 2) presented higher seroprevalences than species wintering in northern Africa and Spain. In addition, higher WNV antibody titers were found among trans-Saharan migrants, probably reflecting a recent (or repeated) exposure to the virus in individuals with higher titers. Although some studies done in captivity suggest that WNV antibodies remain detectable for more than a year (Gibbs et al. 2005), detailed analyses of animal serology both in the laboratory (Komar et al. 2003) and in the field (Figuerola et al. 2007b, Cabre et al. 2006) suggest a rapid reduction in antibody titers after exposure to the virus. In addition, both trans-Saharan and short-distance migrants captured in this study were on their journeys toward their breeding grounds (some in Spain, but also in northern Europe). There is no a priori reasons to expect differences in the geographical distribution of both groups during the breeding season, and both groups of species breed in sympatry and winter in allopatry. For this reason, we favor the hypothesis of a higher exposure to the virus in Africa rather than during the previous breeding season in Europe.

Among resident species, the case of *Passer domesticus* merits further discussion. Given the anthropogenic behavior of this species, it has often been proposed as a key species in the understanding of transmission to humans and as a focal species for WNV monitoring (Komar et al. 2001, Jourdain et al. 2007). However, none of the individuals sampled in our study presented antibodies, unlike the case of another

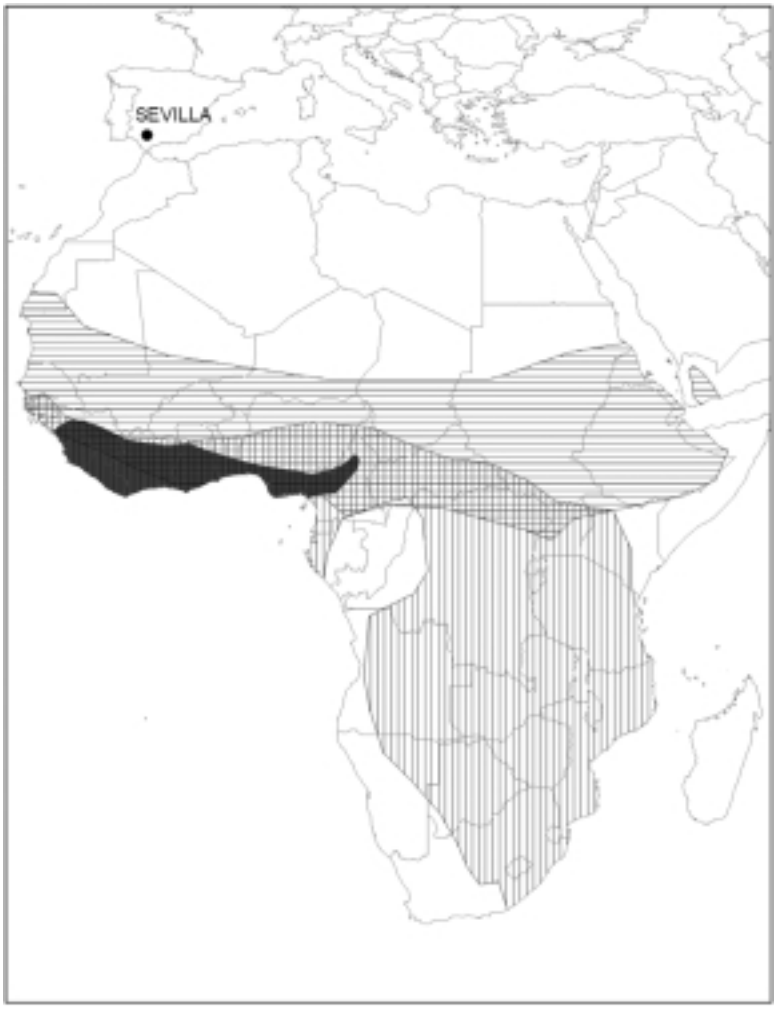

FIG. 2. Wintering areas of trans-Saharan migrant species captured with WNV neutralizing antibodies between March and May 2004 in Seville, Spain: *Ficedula hypoleuca* (gray area), *Sylvia borin* (vertical lines), and *Lanius senator* (horizontal lines), based on Cramp and Perrins (1994).

resident species of larger size, *Turdus merula*. Interestingly, recent detailed analyses of the dynamics of WNV in relation to bird ecology identified the conspecific species *Turdus migratorius* as a likely key species in explaining the transmission of WNV to humans in North America (Kilpatrick et al. 2006). Probably, we still lack the basic knowledge of the nature of the WNV-vector-host interactions needed to understand the regulatory factors of these interactions; thus, interspecific comparative studies are still necessary to understand the ecological factors regulating WNV-vector-host interactions.

Malkinson and Banet (2002) compared WNV strains isolated from birds in eastern Europe that had arrived from Africa with others isolated in Africa and suggested that migratory routes can explain the occurrence of West Nile foci in Europe. The winter distributions of the 3 species of trans-Saharan migrant passerines that show seropositivity for WNV neutralizing antibodies overlap in a region of Africa that includes parts of Liberia, Ivory Coast, Ghana, Togo, Benin, Nigeria, and Cameroon. The highest seroprevalence (8.2%) was found for *Sylvia borin*, a species with a geographically wide winter distribution in Africa extending from 13°N to 35°S. A recent serosurvey of horses in sub-Saharan Africa reporting a high seroprevalence of WNV neutralizing antibodies (up to 97% in some countries, Cabre et al. 2006) further supports these conclusions. While it is true that some birds may be infected while on migration, the low seroprevalence of antibodies in short-distance and resident birds suggests that exposure to WNV occurs mainly south of the Sahara.

Do our results imply that there is a high risk for infection by WNV caused by the arrival of

migratory birds? While this is a possibility, we consider that it is highly unlikely. First, we have previously shown that local circulation of WNV occurs in Spain without necessarily leading to illness among humans (Figuerola et al. 2007b), thus supporting the hypothesis that WNV—or a closely related cross-reacting flavivirus—remains essentially undetected and sylvatic in Europe. Second, for successful long-distance dispersal of WNV by migratory birds, a successive series of highly unlikely events must occur over a short period of time: (1) a WNV-infected mosquito must infect an immunologically naïve bird; (2) the bird then must survive the infection, accumulate fat reserves, and cross the Sahara in a continuous or intermittent flight of 40 hours over 2000 km of inhospitable terrain (Schmaljohann et al. 2007); (3) the bird must then be bitten after arrival by a susceptible vector while still viremic (viremia lasts less than 7 days in birds; see Komar et al. 2003); and, finally, (4) the infected vector must then feed and infect other susceptible birds in the new locality. Most of the parameters needed to calculate the likelihood of this process are still unknown. Recently, Owen et al. (2006) have demonstrated that individuals of 2 passerine species show migratory restlessness while still viremic, although no information is available yet on the impact of WNV on the capacity of birds to accumulate energetic reserves and perform serious exercise while viremic, or on the effect of the stress produced by migration on viremia. The parameterization of the different factors that affect transmission rates of WNV at local and long-distance scales is essential if we are to understand the real role of migratory and resident bird species in the dispersal dynamics of the virus.

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4.c CAPÍTULO 8: Incidencia del virus del Nilo occidental en aves que ingresan en centros de recuperación en el sur de España.

Resumen:

El virus del Nilo occidental (WNV) es un flavivrus neurotropo transmitido por mosquitos que afecta a aves y secundariamente a otros vertebrados en Eurasia, África y América. WNV ha causado frecuentes episodios de mortalidad masiva de aves silvestres en su expansión por el continente americano, llegando a ser un factor regulador en la dinámica poblacional de muchas especies de aves silvestres. Por otro lado, a pesar de su bien documentada circulación por la cuenca del Mediterráneo, raramente se han descrito mortalidades relacionadas con WNV en aves silvestres en este área, y sólo se han descrito brotes esporádicos en caballos. Las causas que subyacen detrás de esta diferencia de patrón epidemiológico no han sido nunca adecuadamente descritas. Inicialmente se sugirió que las cepas de WNV circulando en el Mediterráneo y en América podrían tener diferente patogenicidad, mientras que una hipótesis alternativa propone que brotes y mortalidad originada por WNV podría haber pasado inadvertida en Europa. Para investigar estas hipótesis, muestreamos tejidos de 119 cadáveres de aves silvestres y suero de otras 227 (para buscar anticuerpos) que llegaron a centros de recuperación entre 2004 y 2006 en Andalucía. No hallamos flavivirus en ninguna de las muestras de tejido analizadas. La seroprevalencia de WNV fue 2,2%, similar a la encontrada en 800 aves aparentemente sanas de las mismas especies muestreadas en el medio natural. Nuestros resultados sugieren que la circulación de WNV durante el periodo de estudio no tuvo repercusiones en términos de enfermedad ni mortalidad en las aves.

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Incidence of West Nile Virus in Birds Arriving in Wildlife Rehabilitation Centers in Southern Spain

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Abstract

West Nile virus (WNV) is a neurotropic mosquito-transmitted flavivirus that in Eurasia, Africa, and the Americas primarily affects birds and secondarily other vertebrates. WNV has caused frequent massive episodes of wild bird mortality during its expansion throughout the Americas, and has become a regulating factor in the population dynamics of many wild bird species. On the other hand, WNV-related mortalities in wild birds have rarely been reported in the Mediterranean Basin despite its well-documented circulation, and only sporadic outbreaks in horses have been documented. The causes underlying this contrasting epidemiological pattern have never been properly described. An initial suggestion is that Mediterranean and American strains possess different pathogenicities, whereas an alternative view proposes that WNV-related disease and mortalities may have been overlooked in Europe. To test these hypotheses, between 2004 and 2006 in southern Spain we sampled tissue from 119 wild bird carcasses to detect WNV and other flaviviruses, as well as blood from 227 wild birds arriving in wildlife rehabilitation centers to test for WNV seroprevalence. No flavivirus was found in the tissue samples. The prevalence of WNV-neutralizing antibodies was 2.2%, similar to that of 800 healthy birds of the same species that were captured in the field. Our results suggest that WNV circulation during the study period did not result in any detectable effects in terms of bird morbidity or mortality.

Key Words: Birds—Infectious disease—Mediterranean—Outbreak—Spain—West Nile Virus.

Introduction

West Nile virus (WNV) is a zoonotic mosquito-borne flavivirus (family *Flaviviridae*) that belongs to the Japanese encephalitis serocomplex. Birds are recognized as one of major vertebrate hosts of WNV, although WNV also has the potential to infect other vertebrates, including mammals (Hayes 1989). WNV is a neurotropic virus that provokes acute neurological disease in birds, which can cause a variety of symptoms such as ataxia, disorientation, tremors, and convulsions (Steele et al. 2000, Erdélyi et al. 2007, Beasley and Barrett 2009). Humans and horses are considered accidental dead-end hosts, and infection in these species is associated with a febrile illness that can progress to a lethal encephalitis with symptoms such as cognitive dysfunction and flaccid paralysis (Sejvar et al. 2003, Castillo-Olivares and Wood 2004, Hayes and Gubler 2006).

WNV is mainly maintained in nature in an enzootic cycle between mosquitoes and birds (Komar 2003, Hayes et al. 2005), in which seasonality is linked to vector ecology (Zeller and Schuffenecker 2004). Despite not being host-specific (Beasley and Barrett 2009), WNV incidence and prevalence differ among avian species and populations, and are primarily determined by intrinsic ecological factors (Kilpatrick et al. 2007a, Savage et al. 2007, Figuerola et al. 2008). Mosquito species of the genus *Culex* are the most competent WNV vectors participating in the maintenance of the cycle (Hamer et al. 2008, Reisen et al. 2008), although a large number of other mosquito species also have the potential for transmitting the virus (Turell et al. 2005). Besides vector-mediated transmission, direct bird-to-bird transmission has also been documented (Banet-Noach et al. 2003).

Since WNV was first identified from a human case of febrile illness in Uganda in 1937, it has been found to occur in Africa

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and Eurasia, and to produce some sporadic outbreaks in horses and humans (Murgue et al. 2001a, Gubler 2007). Further, since 1999, when it was first detected in the United States (Gubler 2007, Artsob et al. 2009), WNV has rapidly expanded throughout the Americas and caused recurrent outbreaks in birds (Mostashari et al. 2003, Kilpatrick et al. 2007b), horses (Trock et al. 2001), and humans (Lindsey et al. 2008, Artsob et al. 2009), and is currently considered to be an important emerging pathogen. Due to its pathogenicity, WNV has a negative impact on the population dynamics of many resident American bird populations (LaDeau et al. 2007). By contrast, the sanitary impact of WNV in Europe and Africa seems to be low, and most of the sporadic outbreaks in these continents have been detected in horses (Murgue et al. 2001b, Autorino et al. 2002, Schuffenecker et al. 2005, Cabre et al. 2006). In the Mediterranean Basin, no massive WNV outbreaks have ever been detected in wild birds (Zeller and Schuffenecker 2004). Two major nonexclusive hypotheses are used to explain these differing epidemiological patterns in WNV: (1) the pathogenicity or resistance of birds to the virus strains currently circulating throughout the Americas may be different in the Mediterranean Basin; (2) avian mortalities caused by WNV may have been overlooked given the traditionally low impact of WNV on human health in Europe. In fact, WNV-related avian mortality was not considered an indicator of viral activity until the 1998 epidemics in Israel and subsequently in North America (Malkinson et al. 2002, LaDeau et al. 2007).

In Spain, some authors have provided evidence of local circulation of WNV in recent years (Bofill et al. 2006, Bernabéu-Wittel et al. 2007, Figuerola et al. 2007a, 2007b, Kaptoul et al. 2007), and the virus has been detected in captive large eagles in inland Iberia (Höfle et al. 2008, Jiménez-Clavero et al. 2008). Despite this documented circulation in the area, no WNV outbreak involving large numbers of dead or ill wild birds has ever been detected in the Iberian Peninsula. To study the impact of WNV on wild avian populations in southern Spain, we sampled a large number of wild birds in rehabilitation centers in Andalusia during a period of known WNV circulation in the region. We analyzed birds of different taxons inhabiting both wetland and dry inland areas (Fig. 1). To be sure that WNV was circulating in the study area and, if so, whether this circulation was provoking an increase in morbidity or mortality in local birds, we conducted (1) molecular analyses to detect WNV and other flaviviruses in the tissue of dead birds, and (2) serology tests to detect WNV antibodies in living individuals. If circulating WNV was a cause of morbidity or mortality in birds, we would expect to find polymerase chain reaction (PCR)–positive birds among the arrivals in the rehabilitation centers. Alternatively, if WNV was not a cause of increased morbidity or mortality, we would only expect to detect serology-positive but not PCR-positive individuals.

Materials and Methods

Between November 2004 and February 2006, we sampled 346 wild birds of 33 different species (see Table 1), both alive ($n = 227$) and dead ($n = 119$), in the official network of rehabilitation centers belonging to the Department of the Environment of the Andalusian Regional Government (Andalusia, southern Spain). These rehabilitation centers treat wild birds found injured or sick throughout Andalusia and release them back into the wild. Thus, we expected to find a higher WNV prevalence in these individuals than in healthy wild birds. We divided the sampled species into six groups: raptors ($n = 72$ alive, 0 dead), owls ($n = 67$ alive, 12 dead), ducks ($n = 34$ alive, 32 dead), herons and storks ($n = 28$ alive, 26 dead), waders and gulls ($n = 23$ alive, 37 dead), and other species ($n = 3$ alive, 12 dead). All groups included both migratory and resident species.

A sample of about 1 mL of blood was taken from the brachial or jugular vein of all living sampled individuals using 2 mL syringes with 25G needles. Subsequently, blood was deposited in a vial without anticoagulant and 3 h later was centrifuged in an Eppendorf® Minispin® centrifuge to separate the serum from the blood cells. The sera thus obtained were employed to run a micro-virus-neutralization test in 96-well plates. WNV strain Eg101 and the E6 clone of Vero cells used for virus propagation were obtained from Hervé Zeller (*Institut Pasteur de Lyon*). This technique, already described in Figuerola et al. (2007a), provides high specificity for WNV, and also can provide a light cross-reactivity with closely related flaviviruses. Blood cells were stored at -80°C until subsequent analyses. Additionally, we determined the antibody prevalence in healthy wild birds captured in different localities of the Doñana National Park. Data for some of these individuals have already been reported in Figuerola et al. (2008). To ensure that the data from both sets of birds were comparable, we only considered information from birds of species analyzed in both the rehabilitation centers and captured between November 2004 and February 2006 ($n = 800$ individuals belonging to seven species). The prevalence between wild (presumably) healthy and captured ill individuals was compared with the GENMOD procedure of SAS 9.2 (SAS Institute Inc. 2008) in a generalized linear model, with the number of individuals with antibodies as the response variable, the number of individuals analyzed as the binomial denominator of the response variables, and species as a repeated subject. A binomial error distribution with a logit link was used for the analysis.

A total of 349 tissue samples from different organs collected from dead birds were analyzed for RT-nested-PCR to detect possible infection. To search for WNV, brain ($n = 98$), cardiac muscle ($n = 31$), and feather pulp ($n = 15$) were the main tissues sampled. However, other tissues were also sampled when possible to detect (1) possible WNV infections (Steele et al. 2000) and (2) potential infection by other flaviviruses located in the different target organs. Thus, samples were also collected from liver ($n = 27$), spleen ($n = 22$), lung ($n = 27$), kidney ($n = 27$), ovary ($n = 9$), oviduct ($n = 6$), testicle ($n = 12$), thyroid ($n = 19$), parathyroid ($n = 14$), pancreas ($n = 21$), blood cells ($n = 19$), and the Fabricius bursa ($n = 2$). Tissue samples were placed in liquid nitrogen at -196°C immediately after collection and kept until analysis. The nucleic acid was extracted using Rneasy Mini Kit (Qiagen, Izasa, Spain) following the manufacturer's instructions. The extracted RNA was analyzed using a generic RT-nested-PCR, which detects both generic flaviviruses and specific WNV sequences (Sánzhez-Seco et al. 2005). Both positive and negative controls of the PCR functioned correctly.

Results

The overall WNV seroprevalence of our sample was 2.2%, with 5 positive individuals out of the total of 227 analyzed

Table 1. Serology Results of the West Nile Virus Micro-Virus-Neutralization Test Performed Between November 2004 and February 2006 on 227 Live Birds Sampled in the Andalusian Wildlife Rehabilitation Centers, and on 800 Healthy Individuals Sampled in the Doñana Area

		Rehabilitation centers		*Healthy birds in the field*	
Species	*R/M*	*Positive at 1:10/1:20* (n)	*Prevalence at 1:20 (%)*	*Positive at 1:10/1:20* (n)	*Prevalence at 1:20 (%)*
Falco tinnunculus	R	2/2 (22)	9		
Gyps fulvus	R	0/0 (16)	0		
Falco naumanni	M	1/0 (15)	0		
Buteo buteo	R	0/0 (4)			
Milvus migrans	M	0/0 (4)			
Circaetus gallicus	M	0/0 (3)			
Milvus milvus	M	0/0 (2)			
Hieraaetus pennatus	M	1/0 (2)			
Circus aeruginosus	R	0/0 (1)			
Pernis apivorus	M	0/0 (1)			
Accipiter gentilis	R	1/0 (1)			
Aegypius monachus	R	0/1 (1)			
Total raptors		5/3 (72)	4		
Athene noctua	R	0/0 (20)	0		
Bubo bubo	R	2/0 (18)	0		
Tyto alba	R	0/0 (16)	0		
Strix aluco	R	1/0 (12)	0		
Asio otus	M	0/0 (1)			
Total owls		3/0 (67)	0		
Anas platyrhynchos	R	0/0 (25)	0	6/4 (195)	2
Aythya nyroca	R	0/0 (7)	0	0/0 (1)	
Marmoronetta angustirostris	R	0/0 (2)		1/0 (2)	
Total ducks		0/0 (34)	0	7/4 (198)	2
Ciconia ciconia	M	2/2 (21)	10	5/3 (21)	14
Bubulcus ibis	R	0/0 (3)		1/1 (12)	8
Ardea cinerea	M	0/0 (1)		0/0 (1)	
Ardeola ralloides	M	0/0 (1)			
Platalea leucorodia	R	0/0 (1)			
Plegadis falcinelus	R	0/0 (1)			
Total herons and storks		2/2 (28)	7	1/1 (13)	8
Fulica atra	R	2/0 (13)	0	264/114 (569)	20
Larus ridibundus	M	0/0 (7)	0		
Burhinus oedicnemus	R	0/0 (1)			
Himantopus himantopus	R	0/0 (1)			
Larus genei	M	0/0 (1)			
Total waders and gulls		2/0 (23)	0	264/114 (569)	20
Corvus corax	R	0/0 (2)	0		
Coturnix coturnix	M	0/0 (1)	0		
Total others		0/0 (3)	0		
Total		12/5 (227)	2	272/119 (780)	15

Intraspecific prevalences are indicated when sample size was larger than five individuals. The migratory behavior of every species is indicated (R, resident; M, migratory).

birds (two white storks *Ciconia ciconia*, two common kestrels *Falco tinnunculus*, and one black vulture *Aegypius monachus*). Four positives had titers between 1:20 and 1:80, and a single individual (a kestrel) had a titer of 1:320. Seroprevalence in this sample was not significantly different from that obtained from healthy birds captured in the field ($\chi^2 = 2.93$, $p = 0.09$; rehabilitation center mean ± standard error of the mean: 2.78% ± 13.81%; field: 15.23% ± 13.04%). The apparent large differences in the mean were due to one species, the common coot *Fulica atra*, which was very prevalent among the field samples (20%) but not in the rehabilitation centers (0%), probably due to the different geographical origin of the samples. If we exclude this species from the analyses, the differences in antibody prevalence in the rehabilitation centers (3.39% ± 14.66%) and the field (3.45% ± 8.50%) is nil ($\chi^2 = 0.00$, $p = 0.97$). A further 12 of these positive birds had titers of 1:10, probably due to either a previous contact with the virus, a cross reaction with another WNV closely related flavivirus, an incipient infection with WNV, or even an interindividual difference in the immune response. The proportion of individuals displaying titers of 1:10 or higher was 19% for white storks, 18% for common kestrels, and 15% for common coots. Griffon vultures, little owls, and mallards showed no prevalence despite the large number of samples analyzed. Herons and storks (14%) and raptors (11%) were the groups that had the highest

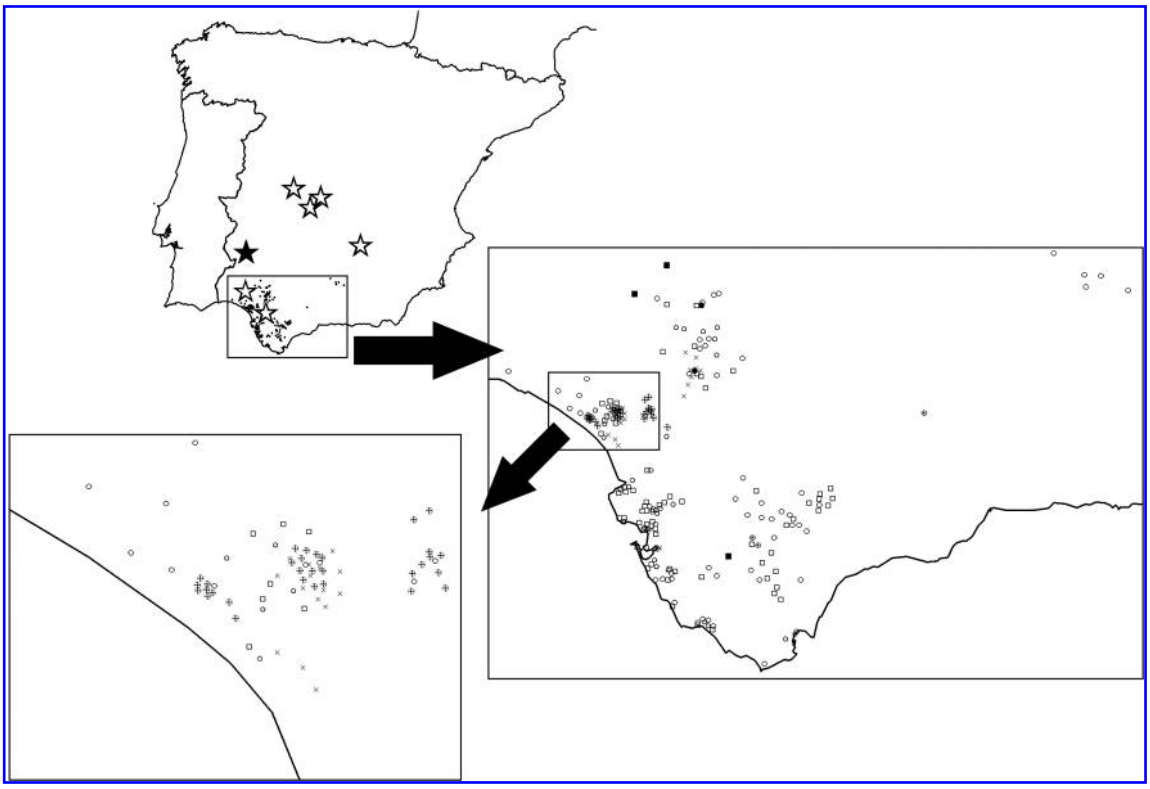

FIG. 1. Geographical origin of the wild live birds sampled in the Andalusian rehabilitation centers in this study. Raptors are represented as squares, owls as circles, herons and storks as pentagons, ducks as crossed circles, waders and gulls as crosses, and other species as dotted circles. Inside the study area, the Doñana wetland area is depicted separately. Bold symbols represent West Nile virus–seropositive cases. Stars represent locations where West Nile virus circulation has been reported in Spain: solid stars represent reports in humans (Kaptoul et al. 2007) and bold stars in birds and horses (Figuerola et al. 2007b, Jiménez-Clavero et al. 2007, Höfle et al. 2008, Jiménez-Clavero et al. 2008).

seroprevalence, whereas there were no positive cases at all in ducks.

Neither WNV nor any other flavivirus genomes were found in any of the tissue samples analyzed. In other work carried out on mosquitoes in the same region using the same methodology as this study, flavivirus sequences have been found (Aranda et al. 2009, Sánchez-Seco et al. 2009).

Discussion

WNV circulation in the Mediterranean Basin has only rarely been associated with wild bird mortality (Dauphin et al. 2004, Zeller and Schuffenecker 2004, Jourdain et al. 2007), an exception being the outbreak occurred in Israel that resulted in detectable mortalities of up to 40% in some stork flocks (Malkinson et al. 2002). Despite the fact that other studies have reported a lack of detectable mortality in the field, this study is the first to specifically analyze the presence of WNV and antibodies against WNV in ill and injured birds arriving in rehabilitation centers. As expected, we did find WNV antibodies in the birds arriving rehabilitation centers, but their prevalence was no higher than that found in healthy birds captured in the field. This result confirms that the virus was circulating in the avian population during the study period. However, we found neither WNV nor any other related flavivirus in the tissues of a large sample of wild birds with health problems in southern Spain. The lack of WNV in tissue suggests that WNV was not a cause of increased morbidity or mortality in birds entering wildlife rehabilitation centers, despite the circulation of WNV in the area. Although isolated cases with related mortality were described during the same period (Höfle et al. 2008, Jiménez-Clavero et al. 2008), our results suggest that the WNV strains currently circulating in the area are not likely to have any effect on avian population dynamics, unlike the situation in the Americas (LaDeau et al. 2007). Recently, experimental infections have confirmed the lower pathogenicity of these WNV isolates on mice under laboratory conditions (Sotelo et al. in press), and similar results have also been obtained using a new avian model for wild birds (Sotelo et al. unpublished data). The 2.2% seroprevalence obtained in our study is slightly lower than those previously found in Spain (3.8%–10.4%; Figuerola et al. 2007a, Figuerola et al. 2008, López et al. 2008) and in other European countries, such as France (4.8%; Jourdain et al. 2008) and the Czech Republic (5.9%; Hubálek et al. 2008). In contrast to the findings in Europe, seroprevalences found in the Americas have usually found to be higher: that is, 25.6% in Argentina (Díaz et al. 2008) or 1.8% to 95% in the United States (Medica et al. 2007, Wilcox et al. 2007, Dusek et al. 2009, Nemeth et al. 2009).

The interspecific differences in seroprevalence observed in this study, above all those found in species occupying the same habitat (such as mallards and coots in the field, 2% vs. 20% antibody prevalence), could be due to intraspecific dif-

ferences in (1) susceptibility to WNV, (2) WNV-vector biting preferences, and (3) differences in avian ecology that result in a different exposure to the vectors (Kilpatrick et al. 2007a, 2007b). Identifying the causes of these interspecific differences in our study is difficult due to the large geographical heterogeneity in the origin of the samples, which means that the individual birds sampled would have been exposed to varying abundances of mosquitoes and different risks of contact with the virus. However, the possibility that the lower prevalence of antibodies in mallards as opposed to coots was due to an increased mortality in WNV-infected mallards could be discarded given that (1) we failed to detect WNV in the brain of 32 dead mallards taken in this study and (2) the results of Marra et al. (2004) described that none of 12 experimentally inoculated mallards developed signs of clinical illness and that the infection cleared up in less than 7 days. In terms of the migratory behavior of the individuals with antibodies, out of the five seropositive cases, only white storks are partially migratory, whereas kestrels and black vultures are both resident. Moreover, four out of five positives were found during winter (December–February), including both white storks (which suggest that they were resident). These results demonstrate that the circulation of WNV took place in the study area and allow discarding that the prevalence found in this study is only due to infections in Africa of migrating birds. The spatial distribution of the seropositive birds also suggests that WNV has been circulating not only in Doñana, as has been previously reported in southern Spain (Figuerola et al. 2007b), but also in other localities in the Seville and Cádiz areas.

Conclusion

On the basis of birds arriving in the rehabilitation centers, our results suggest that WNV circulation in southwest Andalusia has not led to any increased morbidity or mortality among wild birds.

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Disclosure statement

No competing financial interests exist.

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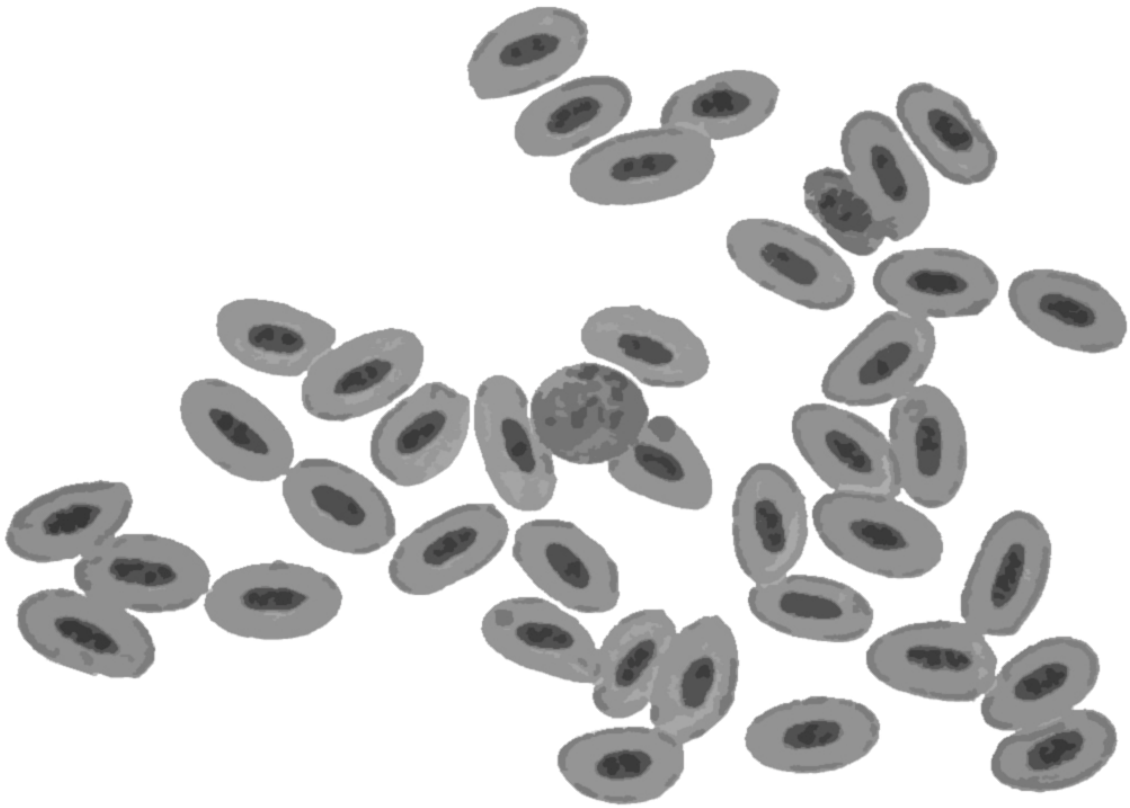
5.CONCLUSIONES



1. Los ciclos circadianos de eliminación de ooquistes que presentan los coccidios del género Isospora en aves paseriformes Dificultan la cuantificación de las cargas parasitarias. Sin embargo, la máxima eliminación se produce durante la tarde, y para tener datos comparables de prevalencia o carga de coccidios basta restringir el muestreo a ese periodo o introducir en los modelos estadísticos un factor mañana/tarde.
2. La extensión de la muda parcial postjuvenil en la lavandera blanca conlleva un coste en términos de estrés, estimado a partir de la composición leucocitaria, probablemente asociado a las presiones sociales derivadas de un mayor o menor grado de similitud con el plumaje adulto.
3. La máscara facial carotenoide del jilguero presenta dimorfismo sexual, siendo más roja en los machos y más anaranjada en las hembras.
4. Durante el periodo reproductor, el color de la máscara facial carotenoide del jilguero se relaciona con la capacidad inmune y con la carga de *Haemoproteus* e *Isospora* en las hembras, no presentando relación con las variables estudiadas en los machos.
5. La coloración carotenoide del pico de los machos de mirlo común se relacionó con la condición corporal, status de salud, estrés y niveles de hidratación y de nutrición, pero no con la presencia ni la carga de hemoparásitos ni parásitos intestinales.
6. Tanto a nivel intra- como interespecífico las consecuencias ecologicas del parasitismo dependieron del grupo de parásitos análizados. Los resultados generados con un grupo de parásitos no pueden ser extrapolados a la comunidad de parasitos y patogenos que explotan un huesped.
7. Los niveles plasmáticos de carotenos están inversamente relacionado con la riqueza de parásitos intestinales en aves paseriformes a niveles intra- e interespecíficos.

8. El tamaño de las aves y el comportamiento migrador están directamente relacionados con la seroprevalencia del virus West Nile en el sur de España. Las especies de mayor tamaño presentaron mayores prevalencias independientemente de la edad de los individuos y se proponen como mejores candidatos para establecer programas de vigilancia frente al virus West Nile.
9. Las especies de aves migradoras trans-saharianas poseen una seroprevalencia de WNV significativamente mayor que las residentes o las migradoras de corta distancia, lo que sugiere que pasan el invierno en zonas con mayor circulacion del virus West Nile que en Andalucía..
10. La comparación de las viremias y seroprevalencias en aves silvestres capturadas en el campo con las ingresadas en los centros de recuperación sugiere que la circulación del virus West Nile en Andalucía no tiene asociado incrementos en término de morbilidad o mortalidad.

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