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ECOLOGY OF PARASITISM OF NESTLING EURASIAN EAGLE-OWLS (*BUBO BUBO*) BY *LEUCOCYTOZOON ZIEMANNI*

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The study of blood parasites in birds has increased in the past two decades as a result of their influence on several components of host fitness and evolution. However, the ecology of the interaction between blood parasites and their hosts is still poorly known. For example, we know relatively little about the temporal stability of blood parasite communities (see Fallon et al. 2004 and references therein) and, to our knowledge, data on inter-annual stability of infections in successive offspring cohorts for long-lived territorial bird species are not currently available. In addition, the amount of time between nestling hatching and the appearance of parasite gametocytes in blood samples from chicks is unknown for most bird species and for most blood parasites in the wild (Krone et al. 2001). Furthermore, the chick age at the time of sampling may be relevant to explanations of some recorded absences of blood parasites, such that hypotheses to justify such absences (e.g., elevated host immunocompetence or the absence of vectors for hemoparasite transmission) might be incorrect in some cases (Martínez-Abraín et al. 2004).

Although several aspects of Eurasian Eagle-Owl (*Bubo bubo*) ecology have been studied, most data on blood parasites infecting this species are relatively sparse (Krone et al. 2001, Ortego and Espada 2007). Here, we (1) record blood parasites of nestling eagle-owls in a population from central Spain; (2) estimate the age at which infections become first patent; (3) estimate baseline values of prevalence and intensity of infection in the study population and analyze the relationship between these variables and estimates of fledgling quality and availability of adequate prey; and (4) explore whether the parasitism status is consistent between two successive broods from the same owl pair.

METHODS

Parasite Sampling. The study was conducted from March to early June of 2003 ($N = 10$ nests; 26 fledglings) and 2004 ($N = 27$ nests; 83 fledglings) in an 2400-km^2 area located in Toledo province, central Spain (39°47′N, 4°04′W; see Ortego 2004, Ortego and Díaz 2004 for detailed description). Nests were visited at least two times during the nestling phase. During the first visit we calculated the age of the chicks by their feather development, using previous information from 11 nests for which we ascertained the exact hatching dates (Penteriani et al. 2005). A second visit was conducted when nestlings were 40 d old (near fledging) to obtain blood smears. We collected blood by puncturing the brachial vein of chicks, and drawing blood up into heparinized microcapillary tubes. A drop of blood was immediately smeared on four individually marked microscope slides. Each smear was rapidly air-dried, fixed with absolute ethanol, and later, in the laboratory, stained with Giemsa's solution (1/10) for 45 min. We also preserved blood samples (100 µl) in ca. 1200 µl ethanol 96% at −20°C for molecular sexing. To estimate the age at which infections first become patent, we sampled chicks from different nests with ages ranging 10–55 d old, obtaining a series of blood samples covering all stages of chick development. Fledglings older than 40–50 d ($N = 16$) were located by intensely searching an area within ca. 300 m of the nest and looking for excrement, molted down feathers, and prey remains that usually denoted the fledglings' presence nearby; fledglings were captured by hand. Each nest was sampled 1–3 times when chicks were different ages. Thus, samples came from different nests but this approach allowed us to avoid disturbance associated with an excessive number of visits. The ten nests sampled in 2003 were also studied in 2004.

Diet Sampling. During 2004, we estimated the diet of eagle-owl nestlings by counting the number of carcasses (only entire prey were quantified) of each prey species found in the nests (see Ortego 2004) during repeated visits (2–4) throughout the chick nesting period. The percentage of European rabbits (*Oryctolagus cuniculus*) in the prey in the nest was used as an index of food abundance and quality, as wild rabbits are the most adequate prey species for eagle-owls in Mediterranean ecosystems, and their frequency in the diet is known to affect different aspects of the species reproductive performance (Serrano 2001, Martínez and Zuberogoitia 2001) and is inversely correlated to the frequency of raptors in the diet (Serrano 2000).

Parasite Quantification. We determined the presences or absence of blood parasites at 40% magnification and when we found parasites, their relative concentrations (number of gametocytes/2000 erythrocytes) were estimat-

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ed. The measures were carried out by the same person (FE) and the repeatability of parasite intensity both between replicated smears from a same individual ($r = 0.95$, $F_{1, 9} = 60.05$, $P < 0.001$) and within the same smears ($r = 0.97$, $F_{1, 22} = 88.80$, $P < 0.001$) were very high.

Chick Condition Parameters and Sex Determination. During the 2004 breeding season, we determined heterophil/lymphocyte ratios, hematocrit values, and residual body mass in 40-d-old fledgling eagle-owls. Heterophil/lymphocyte ratio was assessed by determining the proportion of different types of leucocytes (heterophils, eosinophils, basophiles, lymphocytes and monocytes) on the basis of a count of 100 leucocytes under oil immersion at 1000% magnification. For hematocrit measurement, we placed blood samples in microcapillary tubes which were sealed with plasticine and stored in crushed ice until they were centrifuged at 8000 rpm for 10 min in a microcapillary centrifuge. Hematocrit value was determined with a caliper to the nearest 0.01 mm. We weighed fledglings with a 2.5-kg Pesola scale with a precision of 10 g and took three linear measures of size (tarsus, hallux talon, and bill lengths) using a caliper to the nearest 0.01 mm. To determine a body-size index for fledglings, we carried out a principal component analysis using as input variables the above three size measures. The first principal component explained 81.7% of the variance. A physical condition index was then calculated for each bird using the residuals from a regression of body mass on the scores from the PC1.

The sex of chicks was determined by multiplex polymerase chain reaction amplification of the CHD1 genes in the W and Z chromosomes using the primers 2945F, cfR and 3224R (Ellegren 1996). For this purpose we used DNA extracted from blood.

Data Analyses. To determine whether blood parasite prevalence increased with age, we performed a generalized linear mixed model (GLMM) with binomial error and log-link function using the GLIMIX macro of SAS (SAS Institute 2004). In this analysis we included nestling age and its quadratic term as covariates and nestling sex as a fixed factor. Chick identity nested within nest identity was entered as a random factor. Only nestlings which eventually became infected close to fledging age were included in this analysis ($N = 90$). GLMMs with normal error and an identity link function were used to analyze the effect of blood parasites on chick condition parameters (heterophil/lymphocyte ratio, hematocrit, and residual body mass). The prevalence and intensity of blood parasites were included as a fixed factor and covariate, respectively. Nestlings that showed no infection might have been immune or susceptible but not yet exposed due to absence of adequate vectors. Thus, the association between chick condition parameters and blood parasite intensity was analyzed excluding chicks with negative smears (see Dawson and Bortolotti 2000). Laying date and fledgling sex were also entered as a covariate and fixed factor, respectively, due to their potential influence on chick condition parameters, whereas nest identity was included as a random factor. Finally, we used GLMMs to explore whether the prevalence (binomial error and logit link function) and the intensity of infection (normal error and identity link function) were influenced by the occurrence of wild rabbits in the diet, laying date, and nestling sex. Once again, nest identity was included as a random factor. Models were selected following a backward procedure, by progressively eliminating nonsignificant variables ($P > 0.05$). The significance of the remaining variables was tested again until no additional variable reached significance. Hypotheses were tested using *F*-statistics and all *P*-values refer to two-tailed tests.

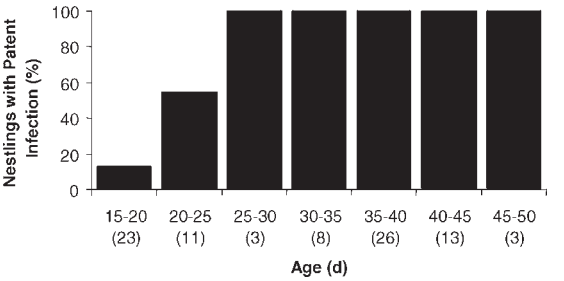

Figure 1. Percentage of nestling Eurasian Eagle-Owls with patent infections in different age intervals, central Spain. Only chicks which eventually became parasitized by the time of fledging were included. The number of chicks studied in each age interval is indicated in parentheses.

RESULTS AND DISCUSSION

The only blood parasite found in our samples was *Leucocytozoon ziemanni*. The prevalence of *L. ziemanni* increased with the quadratic term of age ($F_{1, 90} = 12.85$; $P < 0.001$), but not with the linear ($F_{1, 90} = 0.06$; $P = 0.807$), indicating an initial rapid increase of prevalence that reached an asymptote within the range of studied ages (Fig. 1). However, we found no effect of sex on prevalence in this analysis ($F_{1, 90} = 0.06$; $P = 0.805$). The youngest infected nestling was 18 d old and all the chicks which eventually became infected by fledging had detectable gametocytes in the blood smears from age 25–30 d old (Fig. 1). Thus, in nestling eagle-owls, absences of *L. ziemanni* after this age are not likely to be due to unreliable sampling before the infections are patent. As in other owls (Appleby et al. 1999, Krone et al. 2001), the amount of time between nestling hatching and the appearance of gametocytes in the blood smears is higher than the prepatent periods obtained in experimental research based on controlled inoculations (e.g., see Isobe et al. 1993 for other *Leucocytozoon* species). In altricial birds like eagle-owls, the mother generally broods the chicks until they are able to thermoregulate (approximately 15 d old in eagle-owls; Snow and Perrins 1998), so that vectors may be incapable of reaching them during this period, delaying the event of parasite inoculation (see Davidar and Morton 1993).

When the chicks were 40 d old, all nestling infections were patent (Fig. 1), and 70% ($N = 10$) and 74% ($N = 27$) of the nests analyzed had at least one infected chick in 2003 and 2004, respectively. Among the individual fledglings from all the nests, we found a prevalence of 58% ($N = 26$) in 2003 and 57% ($N = 83$) in 2004 and the average

Table 1. GLMMs with normal error and identity link function analyzing the effect of prevalence and intensity of infection of *Leucocytozoon ziemanni*, laying date, and sex on different condition parameters of fledgling Eagle Owls. Nest identity was included as a random factor. Only variables included in the models are indicated. Parameter estimates and SE for the levels of fixed factor were calculated considering a reference value of zero for the male level in the variable 'sex' and for presence of parasites in the variable 'prevalence'.

	ESTIMATE	SE	TEST	*P*
Heterophil/lymphocyte ratio				
Intercept	0.346	0.082		
Prevalence	0.162	0.057	$F_{1,83} = 8.23$	0.005
Julian laying date	0.003	0.001	$F_{1,83} = 5.65$	0.020
Nest identity	0	0	—	—
Explained deviance (%)	9.46			
Residual body mass				
Intercept	125.400	39.006		
Julian laying date	−3.380	0.708	$F_{1,83} = 22.79$	<0.001
Sex	95.419	13.457	$F_{1,83} = 50.28$	<0.001
Nest identity	5175.84	1742.08	$Z = 2.97$	0.001
Explained deviance (%)	47.75			
Haematocrit				
Intercept	27.708	1.183		
Julian laying date	0.048	0.022	$F_{1,83} = 4.84$	0.031
Nest identity	3.400	1.695	$Z = 2.01$	0.022
Explained deviance (%)	0.69			

intensities were 25 gametocytes/2000 erythrocytes (SD = 26, range = 5–109, $N = 15$) in 2003 and 34 gametocytes/2000 erythrocytes (SD = 33, range = 2–143, $N = 47$) in 2004. The effect of blood parasite intensity on fledgling condition both including and excluding negative smears gave similar results and only data on the analyses including negative samples are shown in Table 1. As well established for other bird species (Perrins 1970), chick condition, as measured by the heterophil/lymphocyte ratio and the residual body mass, declined with laying date but, contrary to predictions, the opposite pattern was found for hematocrit (Table 1). Females showed higher residual body mass than males (Table 1), a fact probably associated with the reversed sexual size dimorphism in this species. However, no differences between the sexes were found for the heterophil/lymphocyte ratio ($F_{1,83} = 0.13$; $P = 0.721$) or hematocrit ($F_{1,83} = 0.99$; $P = 0.323$). Neither prevalence nor the degree of infection intensity influenced chick condition as measured by the residual body mass (prevalence: $F_{1,83} = 0.12$; $P = 0.733$; intensity: $F_{1,83} = 0.53$; $P = 0.467$) or hematocrit (prevalence: $F_{1,83} = 0.02$; $P = 0.884$; intensity: $F_{1,83} = 0.89$; $P = 0.347$). However, in spite of the fact that the heterophil/lymphocyte ratio is suspected to increase in response to starvation and disease in wild birds (e.g., Moreno et al. 2002), we found a negative association between blood parasite prevalence and this index of physiological stress (Table 1; Fig. 2). This could be reflecting a redistribution of the white blood cells, linked with proliferation of lymphocytes and decreasing levels of heterophil numbers, in response to blood parasite infections (see Figuerola et al. 1999). The contribution of rabbits to the diet of nestling eagle-owls did not influence the prevalence ($F_{1,83} = 0.75$; $P = 0.398$) or the infection intensity (including negative smears: $F_{1,83} = 0.00$; $P = 0.998$; without negative smears: $F_{1,47} = 0.82$; $P = 0.373$). However, we found a weak positive effect of laying date both on prevalence ($F_{1,83} = 3.10$; $P = 0.091$) and the intensity of infection (including negative smears: $F_{1,83} = 5.30$; $P = 0.024$; without

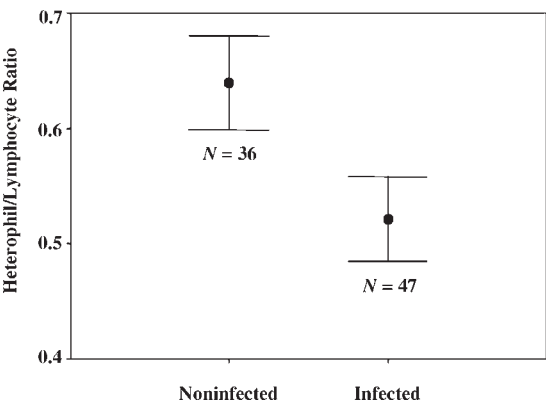

Figure 2. Mean (±SE) heterophil/lymphocyte ratio in infected and noninfected fledgling Eurasian Eagle-Owls, central Spain, 2004.

negative smears: $F_{1,47} = 4.73$; $P = 0.035$), indicating that vector populations increase or that chick immune responsiveness decreases as the breeding season advances. Finally, we found no effect of sex on parasite prevalence ($F_{1,83} = 0.05$; $P = 0.816$) or intensity of infection (including negative smears: $F_{1,83} = 0.01$; $P = 0.961$; without negative smears: $F_{1,47} = 0.15$; $P = 0.701$). Although the effect of the prevalence of *L. ziemanni* on the H/L ratio indicates a certain immunological response to this parasite, nestling eagle-owls probably do not suffer important deleterious short-term effects of infections, a fact probably associated with the very high food availability in the study area (Ortego 2004) and the scarce pathogenesis of *L. ziemanni* in such favorable trophic conditions (reviewed in Remple 2004).

The ten nests sampled in 2003 were also studied in 2004. Two nests were unparasitized and seven showed at least one parasitized chick in both years. Only one nest changed its parasite status, having parasitized chicks in 2003 but not in 2004. Our pairs were not banded, but in eagle-owls both survival (15–20 yr) and mate fidelity are likely to be high (Snow and Perrins 1998), and we suspect that most pairs did not change between 2003 and 2004. As well-documented in previous studies, the prevalence of blood parasites is probably determined by the presence of suitable ornithophilic simuliid vectors (Family: Simulidae) for transmission of *L. ziemanni* (Sol et al. 2000). In the study area, vector populations could be relatively stable among years within the same owl territory, leading to scarce inter-annual variation in prevalence. Other factors, such as inter-pair differences in chick immunocompetence due to genetic or environmental factors, could also account for the general consistence in parasitism status between years. However, lineage turnover among the parasites might be higher than inter-annual changes in prevalence, and this remains a subject for future research (e.g., Fallon et al. 2004). Overall, our results suggest that *L. ziemanni* has few or no short-term detrimental consequences on fledgling eagle-owls, although negative effects on post-fledgling survival cannot be ruled out and may reduce lifetime parental fitness, particularly for those pairs settled in territories where blood parasitemias are persistent over years.

ECOLOGÍA DEL PARASITISMO DE LOS PICHONES DE *BUBO BUBO* POR PARTE DE *LEUCOCYTOZOON ZIEMANNI*

RESUMEN.—En el presente estudio analizamos diferentes aspectos de la ecología del parasitismo de *Leucocytozoon ziemanni* sobre pichones de *Bubo bubo*, determinando la edad a la que las infecciones se hacen patentes por primera vez, la relación entre la prevalencia e intensidad de infección y entre los diferentes parámetros de condición física y dieta, y el grado de estabilidad de las infecciones en dos cohortes sucesivas de pichones de la misma pareja de búhos. La prevalencia de *L. ziemanni* mostró un rápido incremento desde los 18 días de edad, que se estabilizó a partir de los 25 a 30 días. La prevalencia y la intensidad de infección no se correlacionaron ni con el hematocrito ni con la masa corporal residual. Sin embargo, el cociente de heterófilos/linfocitos fue menor en los pichones parasitados, un hecho que probablemente refleja una redistribución de los niveles relativos de células blancas en respuesta a *L. ziemanni*. Tanto la prevalencia como la intensidad de infección aumentaron con la fecha de puesta, mientras que el porcentaje de conejo (*Oryctolagus cuniculus*) en la dieta y el sexo de los pichones no presentaron ningún efecto. La prevalencia no varió en 9 de los 10 territorios muestreados durante dos años consecutivos, un hecho probablemente asociado con la presencia/ausencia de vectores transmisores de *L. ziemanni* en ciertos territorios o con el diferente grado de inmunocompetencia de la progenie de ciertas parejas debido a factores genéticos o ambientales.

[Traducción del equipo editorial]

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