Conserv Genet (2010) 11:1479–1487
DOI 10.1007/s10592-009-9978-x

RESEARCH ARTICLE

Factors associated with the geographic distribution of leucocytozoa parasitizing nestling eagle owls (*Bubo bubo*): a local spatial-scale analysis

Joaquín Ortego · Pedro J. Cordero

Received: 16 March 2009 / Accepted: 17 August 2009 / Published online: 4 September 2009

Abstract Knowledge of the factors influencing the patterns and distribution of parasite infections and disease outbreaks is a major question in disease ecology and conservation research. In this study, we use a highly efficient PCR approach to detect blood parasites and investigate the factors influencing geographical variation in the distribution of leucocytozoids parasitizing nestling eagle owls (*Bubo bubo*) over a 3-year study period. We found that both prevalence and intensity of infections by the single lineage of *Leucocytozoon ziemanni* infecting nestling eagle owls in the study area increased with local owl population density and decreased with nest height above sea level. Overall, these results suggest that higher horizontal transmission rates under higher host density conditions together with the presence of adequate habitats for larval development of vectors are relevant factors influencing blood parasite distribution in the study system.

Keywords *Bubo bubo* · Disease ecology · *Leucocytozoon* · mtDNA · Parasite transmission

J. Ortego (✉)
Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), C/José Gutiérrez Abascal 2, 28006 Madrid, Spain
e-mail: joaquin.ortego@mncn.csic.es

P. J. Cordero
Grupo de Investigación de la Biodiversidad Genética y Cultural, Instituto de Investigación en Recursos Cinegéticos, IREC (CSIC, UCLM, JCCM), Ronda de Toledo s/n, 13005 Ciudad Real, Spain

Introduction

Infectious diseases have serious negative consequences on human health and can be an important threat for wildlife populations (Woodworth et al. 2005; Bermejo et al. 2006). For these reasons, a major question in disease ecology is to understand the factors influencing the patterns and distribution of parasite infections and disease outbreaks. Integrative ecological studies on the factors influencing disease risk have important applied implications, especially at a time when human-induced habitat alteration, global climate change, and transportation of vectors and pathogens are favouring the emergence of novel infectious diseases (Woodworth et al. 2005). Several host factors have been found to determine parasite infection, including sex, host population density and host behaviour (Anderson and May 1979; May and Anderson 1979; Møller 1997; Arneberg et al. 1998; Rohner et al. 2000; Wood et al. 2007). However, a number of less studied abiotic parameters have been also identified as major determinants influencing the occurrence of infections and this is likely to be particularly relevant for vector-borne transmitted parasites (Balls et al. 2004; Omumbo et al. 2005; Githeko et al. 2006; Koenraadt et al. 2006; Wood et al. 2007). For instance, in host–parasite-vector systems the presence of adequate conditions for vector development is essential for the spread and persistence of disease. The distribution over time and space of such favourable conditions for vector proliferation is usually much influenced by landscape composition on a local scale (Omumbo et al. 2005; Wood et al. 2007).

Avian haematozoa of the genera *Leucocytozoon* (Haemosporida, Leucocytozoidae) comprise a group of vector-borne transmitted parasites that infect birds (Valkiūnas 2004). In recent years, DNA sequencing has greatly contributed to understanding several aspects of this host–

parasite system, including relevant information on their ecology and evolution (Perkins and Schall 2002; Sehgal et al. 2006; Hellgren et al. 2007, 2008; Krone et al. 2008; Martinsen et al. 2008; Ishak et al. 2008; Ortego and Cordero 2009). Recent phylogenetic information is also of great relevance because several apparent cryptic species may show different transmission times or vectors, and previous studies disregarding this variability may confound the factors influencing parasite transmission (Sehgal et al. 2006; Pérez-Tris et al. 2007; Ishak et al. 2008) or the consequences on host fitness (Marzal et al. 2008; Ortego et al. 2008). However, data on *Leucocytozoon* are still more sparse than for the sister genera *Plasmodium* and *Haemoproteus*, and little is know on the factors influencing their transmission at local (Ashford et al. 1990; Ortego and Espada 2007) or large spatial scales (Hellgren et al. 2007; Pagenkopp et al. 2008).

In this study, we use a highly efficient PCR approach to detect blood parasites and investigate the factors influencing geographical variation in the distribution of leucocytozoids parasitizing nestling eagle owls (*Bubo bubo*) over a 3-year study period. Molecular techniques have now revealed that nestling eagle owls are parasitized by a unique lineage (EO1) of *Leucocytozoon ziemanni* (synonym to *L. danilewskyi*; Valkiūnas 2004) in our study population from Central Spain (Ortego and Cordero 2009). The prevalence of this lineage among individual nestlings is 52.7 and 68.9% of the nests had at least one infected nestling (Ortego and Cordero 2009). Leucocytozoids infecting nestling eagle owls are a good model system for exploring the factors influencing transmission of avian haematozoa for several reasons. First, eagle owls frequently have the first encounter with the parasite during the nestling phase and their relatively long nesting period (ca. 50 days) in comparison with other bird species allows reliable sampling of patent infections before fledging, i.e. the parasite has time enough to progress through its life cycle and appear in the bloodstream during the nesting period (Ortego et al. 2007a). Second, sampling nestlings provides precise information on the time and area of parasite transmission in comparison with studies based on adult birds which could have acquired infections before breeding or natal dispersal (Van Riper III et al. 1986; Wood et al. 2007). Finally, nestling eagle owls do not suffer important deleterious short-term effects of infections by *L. ziemanni* (Ortego et al. 2007a; see also Korpimäki et al. 1993), avoiding bias in parasite transmission estimates that may occur if some individuals die after they have encountered the parasite and before they can be sampled (Bensch et al. 2007; Ortego et al. 2007b). For these reasons, the eagle owl is a sensitive sentinel organism for the study of the factors determining haemoparasite transmission at local spatial scales.

Materials and methods

Study population and field procedures

The study area (Toledo province, central Spain, 39°47′N, 4°04′W) covers 2,400 km^2 with meso-Mediterranean climate, mean temperatures ranging from 26°C in July to 5°C in January and 300–400 mm of rainfall concentrated in spring and autumn (see Ortego 2007 for detailed description). From March to early June 2003–2005 we monitored 74 successful nests of eagle owls in the study area. Nests were generally visited at least two times during the nesting phase. In a first visit we calculated the age of the chicks according to their feather development by means of previous information from 11 nests containing chicks with known hatching dates (Marchesi et al. 2002; Penteriani et al. 2005). Laying date was calculated by subtracting 35 days, the average incubation period reported for this species, from hatching date of the oldest sibling from each nest (Snow and Perrins 1998). A second visit was conducted when nestlings were 25–40 days old to obtain blood samples and determine the prevalence and intensity of blood parasite infections. The only blood parasite infecting eagle owls in the study population is *Leucocytozoon ziemanni* (Ortego et al. 2007a; Ortego and Cordero 2009) and all nestlings which eventually become infected by fledging had detectable gametocytes in the blood smears from age 25 days old (Ortego et al. 2007a). Thus, absences of *L. ziemanni* after this age are not likely to be due to unreliable sampling before infections are patent (Ortego et al. 2007a). We collected blood by puncturing the brachial vein of nestlings and transferred it to heparinized microcapillary tubes. Immediately, a drop of blood was smeared on four individually marked microscope slides. Each smear was rapidly air dried, fixed with absolute ethanol and stained in the laboratory with Giemsa′s solution (1/10) for 45 min. We also preserved blood samples (100 µl) in ~1,200 µl ethanol 96% at −20°C for molecular analyses. 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).

Screening for blood parasite infections

We used NucleoSpin Tissue Kits (Macherey–Nagel, Düren, Germany) to extract and purify genomic DNA from the blood samples. Each individual nestling was screened for infections by *Leucocytozoon ziemanni* using a highly efficient nested PCR protocol that amplifies a 524-bp fragment (including primers) of the mitochondrial cytochrome *b* gene of these parasites (Hellgren et al. 2004). This method consists in two rounds: an initial 20

cycles of PCR using the primers HaemNFI and HaemNR3 and a final 35 cycles of PCR using the internally nested primers HaemFL and HaemR3L (Hellgren et al. 2004). Reactions were performed in 25-μL reaction volumes containing 5 ng of template DNA, 1× reaction buffer (67 mM Tris–HCL, pH 8.3, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, EcoStart Reaction Buffer, Ecogen, Barcelona, Spain), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each dye-labelled primer (FAM, HEX or NED) and 0.1 U of *Taq* DNA EcoStart Polymerase (Ecogen, Barcelona, Spain). For the second PCR, 1 μL of the PCR product from the initial PCR was used as template. We routinely used positive (i.e. DNA from individuals with known infections) and negative controls (i.e. samples with ddH_2O instead of genomic DNA as template) to ascertain that the outcome of each PCR run was not affected by contamination (Hellgren et al. 2004). Sample quality was confirmed by amplification of the CHD1 genes in the W and Z chromosomes of eagle owls using the primers 2945F, cfR and 3224R (Ellegren 1996; see above). All negative samples were confirmed by repeated PCR again using positive and negative controls. The PCR programme and thermal profile were as described by Hellgren et al. 2004, with the exception of using a 9 min denaturing at 95°C rather than 3 min at 94°C because we used a hot-start polymerase (EcoStart, Ecogen, Barcelona, Spain). All reactions were carried out on a Mastercycler EpgradientS (Eppendorf, Hamburg, Germany) thermal cycler. Positive or negative infections were scored running 2 μl of the final PCR product on a 2% agarose gel stained with ethidium bromide and determining the presence/absence of a band of the expected size under UV light. In a previous study we found that all infections detected by ocular screening of blood smears gave positive PCR amplifications whereas only 83.17% of positive amplifications are also detected by ocular methods (Ortego and Cordero 2009). Thus, as also established in several previous studies (e.g. Waldenström et al. 2004), the nested PCR protocol is more sensitive than the microscopy-based examinations of blood smears particularly when infections are below the limit of detection of traditional microscopic procedures (but see Valkiūnas et al. 2006). Although serological analyses for detecting antibodies to the parasite are likely to be more sensitive than PCR or microscopy-based examinations, to the best of our knowledge, such techniques have not been developed for *L. ziemanni* (Atkinson et al. 2001a, b; Jarvi et al. 2003). PCR products from 48 positive samples were purified using NucleoSpin Extract II (Macherey–Nagel, Düren, Germany) kits and bidirectionally sequenced on an ABI 310 Genetic Analyser (Applied Biosystems, Foster City, USA). Sequences were edited and aligned using the program BioEdit (Hall 1999).

Determination of intensity of parasitemia

Blood smears were examined to determine the presence or absence of blood parasites at 400× magnification. In positive smears, we estimated infection intensity as number of gametocytes/2,000 erythrocytes (Ortego et al. 2007a). Determination of intensity of infection was carried out by the same person (F. Espada), who had no information about the individual birds except ring number. 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 ($r = 0.97$, $F_{1,\ 22} = 88.80$, $P < 0.001$) was very high (Ortego et al. 2007a).

Habitat features and owl population density

We measured five variables related to landscape composition: (1) nest altitude; (2) index of topographic irregularity; (3) length of watercourses; (4) cover of forested areas; (5) Simpson's index of habitat diversity. These variables were measured within 1,500 m (an area of 7.07 km^2) and 250 m (an area of 0.20 km^2) radius around the studied nests. The 1,500 m spatial scale reflects the main hunting areas for parent eagle owls during the breeding season (i.e. territory size; Ortego and Diaz 2004; Ortego 2007) whereas the 250 m scale is likely to reflect habitat requirements for vector development within their dispersal potential (Thompson 1976; Finn et al. 2006). We determined altitude, length of watercourses, and the index of topographic irregularity from 1:25,000 topographic maps of Spain (I.G.N.). The index of topographic irregularity was calculated as the number of 10 m contour lines crossed by two axes (N–S and W–E) from the plot centre. To calculate the cover of forested areas, we incorporated nest sites into a Geographic Information System (GIS) and measured land use types from digitised 1:100,000 CORINE Land Cover maps using Arc-View software (ArcView 3.2, ESRI, Redlands, USA). The 14 land use types provided by CORINE Land Cover maps were grouped into two categories: (1) cover of forested habitats, which grouped the covers of (a) forests of evergreen sclerophyllous and Lusitanian oaks (*Quercus lusitanica*); (b) transitional woodland-scrubland; (c) coniferous forest; (d) other broad-leaved tree plantations; (e) agro-forestry areas; and (2) cover of non-forested habitats, the sum of the covers of (a) non-irrigated arable land; (b) land occupied mainly by agricultural uses with some areas of natural vegetation; (c) vineyards (*Vitis vinifera*); (d) olive groves (*Olea europaea*); (e) mixture of perennial crops; (f) mixtures of annual and perennial crops; (g) saline grounds; (h) low-density scrub and scrubland; (i) high scrubland formations of medium to high density. Among all these land-uses, we only considered the cover of forested habitats for further

analyses because previous studies suggest that this variable can particularly influence vector proliferation (Little and Earlé 1995; Tella et al. 1999; Ortego and Espada 2007). It should be also noted that the cover of open areas is just the inverse of the cover of forested habitats and, thus, only the latter was included into the analyses. Finally, the Simpson's index of habitat diversity was calculated as: $1-\Sigma p^2$, where p is the proportion of each land use type provided by CORINE Land Cover Maps.

Local population density of eagle owls in the vicinity of each nest was defined as the number of other territories within 1,500 m. We only estimated host density at this large spatial scale because the number of other owl territories within 250 m radius around nest sites lacks sufficient variation as to be adequately analyzed (i.e. practically all nests have no other territory within such spatial scale). We censused eagle owl pairs by means of intensive nest searching, listening to spontaneous vocalizations, visiting the area around potential nest or perch sites to look for moulted feathers, fresh pellets and prey remains, and eliciting territorial calls by means of playbacks of conspecific vocalizations (Marchesi et al. 2002). Eagle Owl lifespan in the field is around 15–20 years and territories are usually highly stable (Snow and Perrins 1998; Penteriani et al. 2004). In this way, host population density can be considered relatively constant and variation in densities through time is likely to be small relative to variation between locations.

Statistical analyses

We analyzed the factors determining blood parasitism in eagle owls using Generalized Linear Mixed Models (GLMMs) implemented with the GLIMIX macro of SAS (SAS Institute 2004). GLMMs allow analyses of data where the response variable is determined by both random and fixed effects. GLMMs are the best tool for analyzing non-normally distributed data such as binary (parasite presence/absence) and count data (parasite counts) that involve random effects (Bolker et al. 2009). These models allow considering the whole variation of the data while avoiding pseudoreplication (Bolker et al. 2009). We performed different GLMMs for each of the two studied spatial scales. Presence or absence of infection in individual birds ($n = 203$) was analyzed using a binomial error structure and logit link, whereas intensity of parasitemia (based on count data) was fitted using a Poisson distribution of errors and a log link function. We analyzed intensity of parasitemia both including ($n = 203$) and excluding individuals with zero values of blood parasites ($n = 89$). All GLMMs were constructed by fitting as covariates laying date, nest altitude, index of topographic irregularity, length of watercourses, Simpson's index of habitat diversity, cover of forested habitats, and number of other eagle owl territories within 1.5 km. Further, nestling sex (fixed factor) and age (covariate) were also included in all analyses. The identities of cohorts and broods (nested within cohort) were included as random effects to control for the potential non-independence of parasitism within cohorts and broods, in the manner of a randomized complete block design to avoid pseudoreplication (Krackow and Tkadlec 2001).

Initially, each GLMM was constructed with all explanatory terms fitted, including first-order interactions and quadratic effects to account for potential nonlinear relationships. Final models were selected following a backward procedure, by progressively eliminating non-significant variables. The significance of the remaining variables was tested again until no additional variable reached significance. The result is the minimal most adequate model for explaining the variability in the response variable, where only the significant explanatory variables are retained. Denominator degrees of freedom (ddf) were computed using Satterthwaite's method. This method provides a numerical approximation of ddf, so the reported ddf are not necessarily whole numbers (SAS Institute 2004). Hypotheses were tested using F-statistics and all P-values refer to two tailed tests.

Results

Of the 48 infections sequenced, we have only recovered a single lineage of *L. ziemanni* (lineage EO1; GenBank accession number: EU624137) parasitizing the studied eagle owl population. The probability a nestling was infected by *L. ziemanni* increased with local owl population density and decreased with nest altitude (Table 1; Fig. 1). Similarly, intensity of blood parasitemia (including uninfected individuals in the analysis) also increased with local owl population density and decreased with nest altitude, although the effect of local owl population density was stronger than that of altitude (Table 1; Fig. 2). However, after excluding un-infected individuals from the analysis, only local owl population density remained as significant into the model (Table 1; Fig. 2b). It should be noted that some infections identified using the PCR approach are not detected by ocular examinations of blood smears and, thus, we have no data for intensity of parasitemia in these cases (16.83%). For this reason, we performed an additional analysis, assigning to infections that went undetected in the blood smears a reference value for intensity of parasitemia equal to zero. We re-analyzed the data and once again we found that intensity of parasitemia was positively associated with local owl population density ($F_{1,\ 32.5} = 9.36$; $P = 0.004$). After examining Figs. 1, 2,

Table 1 Results of GLMM analysis for (a) probability of blood parasitism (Binomial error and logit link function) and intensity of blood parasitemia (b) including and (c) excluding un-infected individuals (Poisson error and log link function) in relation to local owl population density, landscape features measured within a 1,500 m radius around nest sites (altitude, irregular topographic index, length of watercourses, habitat diversity, cover of forested habitats), laying date, and nestling sex and age

	Estimate ± SE	Test	*P*
(a) *Presence/absence of L. ziemanni*			
Explanatory terms			
Intercept	3.92 ± 2.10		
Local owl population density	0.83 ± 0.28	$F_{1,\ 71.3} = 8.82$	0.004
Altitude	−0.01 ± 0.01	$F_{1,\ 97.1} = 6.27$	0.014
Rejected terms			
Irregular topographic index		$F_{1,\ 75.4} = 3.29$	0.074
Length of watercourses		$F_{1,\ 63.3} = 0.01$	0.904
Habitat diversity		$F_{1,\ 62.8} = 0.52$	0.472
Cover of forested habitats		$F_{1,\ 52.5} = 1.46$	0.232
Laying date		$F_{1,\ 64.2} = 0.44$	0.509
Nestling sex		$F_{1,\ 139} = 1.99$	0.160
Nestling age		$F_{1,\ 151} = 1.54$	0.217
Covariance parameter estimates			
Nest identity	5.33 ± 1.37	$Z = 3.89$	<0.001
Cohort	0.37 ± 0.80	$Z = 0.46$	0.323
(b) *Intensity of parasitemia (including un-infected individuals)*			
Explanatory terms			
Intercept	5.36 ± 1.41		
Local owl population density	0.52 ± 0.12	$F_{1,\ 54.8} = 17.88$	<0.001
Altitude	−0.01 ± 0.01	$F_{1,\ 115} = 9.42$	0.003
Rejected terms			
Irregular topographic index		$F_{1,\ 61.4} = 1.33$	0.253
Length of watercourses		$F_{1,\ 78.3} = 0.03$	0.855
Habitat diversity		$F_{1,\ 69.2} = 1.33$	0.253
Cover of forested habitats		$F_{1,\ 67.9} = 1.69$	0.197
Laying date		$F_{1,\ 54.2} = 2.31$	0.135
Nestling sex		$F_{1,\ 152} = 1.53$	0.217
Nestling age		$F_{1,\ 153} = 0.42$	0.517
Covariance parameter estimates			
Nest identity	1.13 ± 0.31	$Z = 3.69$	<0.001
Cohort	0	–	–
(c) *Intensity of parasitemia (excluding un-infected individuals)*			
Explanatory terms			
Intercept	2.85 ± 0.22		
Local owl population density	0.20 ± 0.08	$F_{1,\ 37.4} = 6.71$	0.014
Rejected terms			
Altitude		$F_{1,\ 57.6} = 0.81$	0.372
Irregular topographic index		$F_{1,\ 36.7} = 0.80$	0.376
Length of watercourses		$F_{1,\ 43.7} = 0.02$	0.903
Habitat diversity		$F_{1,\ 49.5} = 1.09$	0.301
Cover of forested habitats		$F_{1,\ 53.2} = 0.24$	0.627
Laying date		$F_{1,\ 38.5} = 0.94$	0.339
Nestling sex		$F_{1,\ 79.5} = 0.54$	0.466
Nestling age		$F_{1,\ 59.9} = 0.28$	0.600
Covariance parameter estimates			
Nest identity	0.12 ± 0.09	$Z = 1.33$	0.092
Cohort	0.01 ± 0.05	$Z = 0.24$	0.405

we noticed that the association between parasitism and nest altitude may be particularly influenced by some nests (20 chicks from 7 broods) located 800 m above sea level in some scattered and isolated mountains within our relatively flat study area. To address this, we re-analyzed the data excluding these nests from the database and we found that both the probability ($F_{1,\ 63.6} = 5.81$; $P = 0.019$) and intensity of parasitism ($F_{1,\ 62.3} = 6.25$; $P = 0.015$) were still negatively associated with nest altitude. Other variables, quadratic terms or interactions between independent variables were not associated with parasite prevalence or intensity at any of the two studied spatial scales ($P > 0.05$ in all cases; Table 1). It should be noted that final models are equal for both the 1,500 and 250 m scales because the variables retained (i.e. altitude and local owl population density) do not vary with spatial scale (see above). Final models were identical when they were constructed using a forward procedure, indicating that the results obtained by the backward procedure are not over-parameterized.

Discussion

Here, we show that the geographical distribution of the single lineage of *Leucocytozoon ziemanni* infecting nestling eagle

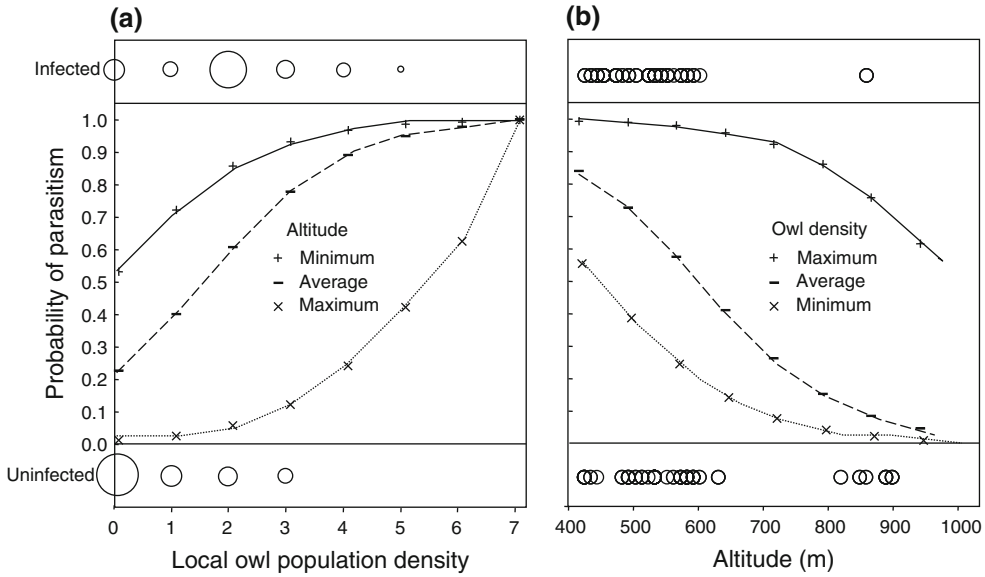

Fig. 1 Probability of parasitism by *Leucocytozoon ziemanni* as a function of local owl population density (number of eagle owl territories/7.07 km^2) (panel **a**) and nest altitude (panel **b**) as predicted by the GLMM. Model predictions have been generated for a range of altitudes and owl densities, respectively. Values for local owl population density and altitude cover the variability observed in the study population. *Circles* indicate actual values for local owl population density and nest altitude in parasitized and non-parasitized nestlings. For number of eagle owl territories, circle size is proportional to sample size and the *smallest circle* represents a nestling

owls is associated with landscape features and local host population density at the studied local spatial scales. The probability of parasitism was positively associated with local owl population density, confirming the results obtained in a preliminary single-year study (Ortego and Espada 2007). This association suggests that higher host densities favour more efficient horizontal transmission rates in the study system. Analyses of intensity of blood parasitemia have now revealed that host population density is also an important factor influencing intensity of infections by *L. ziemanni*. As found in other systems, including human malaria, higher host densities can increase horizontal transmission rates and favour infections by different genetic strains of parasites which can ultimately result in increased virulence and higher parasitemias (Paul et al. 1998). Although we only detected a single lineage of *L. siemanni* parasitizing the studied eagle owl population, a degree of polymorphism within this lineage would be unresolved at the level of the mitochondrial gene fragment analyzed (Schall and Vardo 2007). For this reason, more detailed genetic analyses (e.g. using highly polymorphic nuclear markers such as microsatellites; Schall and Vardo 2007) could help to resolve the possible link between clone diversity, intensity of parasitemias, transmission rates and host population densities (Vardo and Schall 2007; Paul et al. 1998).

The association between infection by *L. ziemanni* and nest height above sea level suggests that higher altitude may be limiting vector proliferation (Van Riper III et al. 1986). A possibility to explain why altitude plays an important role in determining infection by *L. ziemanni* may be the effect of altitude on temperature. Low temperatures at high altitudes can reduce the development, survivorship and lifetime fecundity of vectors and this can ultimately decrease the chance of parasite transmission (Zahar 1951; Su and Mulla 2001; Balls et al. 2004; Afrane et al. 2006). On the other hand, blood parasites of the genera *Leucocytozoon* are transmitted by blood-sucking blackflies that require running water for larval development (Malmqvist et al. 1999; Ojanen et al. 2002) and these microhabitats are much limited in owl territories located at a higher altitude (Ortego 2007). This seems to be the case of nests placed on the top of some scattered and isolated mountains in our study area: only nestlings from one out of seven nests located in these sites were infected by *L. ziemanni* and they showed very low intensities of parasitemia (Figs. 1, 2). Surprisingly, parasitism was not associated with the length of watercourses around nest sites, a variable directly associated with fresh water availability. However, it should be noted that altitude and length of watercourses were highly inter-correlated (Pearson correlation; $r = -0.621$, $P < 0.001$). We re-analyzed the data and we found that after excluding nest altitude from the models, length of watercourses becomes positively associated with both probability ($F_{1,\ 74.8} = 7.73$; $P = 0.0007$) and intensity ($F_{1,\ 111} = 7.44$; $P = 0.007$) of infections by *L. ziemanni*. Thus, the negative association between parasitism and altitude seems to be mediated in part by reduced freshwater availability around nest sites located at higher altitude.

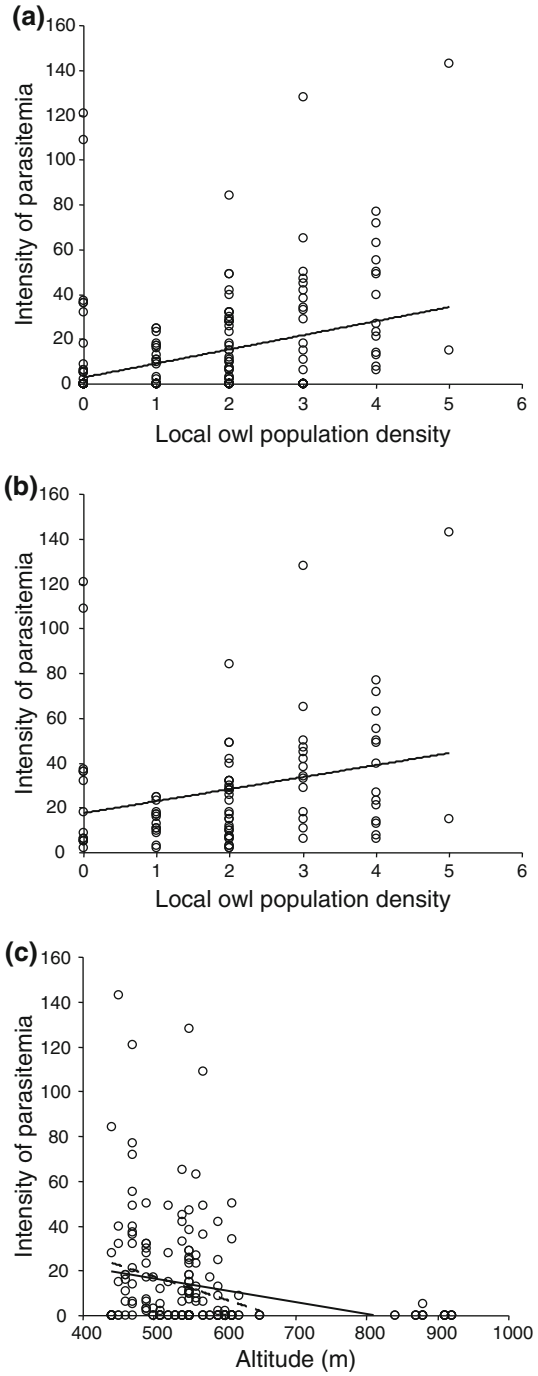

Fig. 2 Relationship between intensity of blood parasitemia by *Leucocytozoon ziemanni* (number of gametocytes/2,000 erythrocytes) and local owl population density (number of eagle owl territories/7.07 km^2) both including **a** and excluding **b** un-infected individuals and **c** nest altitude including un-infected individuals. *Dashed regression line* in panel (**c**) shows the association excluding nests 800 m above sea level

These results are in accordance with previous studies suggesting that the presence of freshwater availability for vector development is crucial in determining blood parasite transmission and distribution (Wood et al. 2007). This pattern may be particularly patent in relatively dry regions such as our study area where freshwater availability is likely to be a much more limiting factor controlling blackfly proliferation than in northern latitudes (e.g. Malmqvist et al. 1999; Ojanen et al. 2002).

Final remarks

This study leaves us with some questions that call for further work. First, it should be acknowledged that a potential overlooked factor influencing *L. ziemanni* distribution among nestling eagle owls is the infection status of the parents and the relative importance of vertical transmission of infections to the offspring (Ashford et al. 1990). However, although the possible relevance of vertical transmission of parasites raises an interesting question, it is difficult to address in the study system as regular capture and manipulation of adult eagle owls is unfeasible. Data on host specificity of the studied lineage is also an interesting issue to be addressed in the future. *L. ziemanni*, the only leucocytozoid species recognized in owls, probably constitutes a complex of cryptic species shared by a variable number of host species (Krone et al. 2008; Ishak et al. 2008; Ortego and Cordero 2009). Phylogenetic analyses and data on sequence divergence derived from recent studies suggest that the lineage infecting eagle owls could be shared with other coexisting owl species and horizontal transmissions may be relevant (Krone et al. 2008; Ishak et al. 2008; Ortego and Cordero 2009). Thus, not only local conspecific population density but also densities and community relationships between different owl species could be particularly relevant to explain the geographic distribution of the studied parasite lineage (Bensch et al. 2000; Ricklefs et al. 2005). In any case, it is worth mentioning that we have only found a single lineage parasitizing eagle owls in comparison with the numerous lineages recovered from other owl species, which may indicate high host specificity (Ishak et al. 2008). Finally, future studies should also focus on determining several unknown aspects of vector ecology such as dispersal, host specificity, and detailed data on their habitat requirements and distribution (e.g. Zahar 1951; Van Riper III et al. 1986; Hellgren et al. 2008). In any case, irrespective of the possible effects of parental parasitism status and interspecific transmission rates on distribution of *L. ziemanni* among nestling eagle owls, our results suggest that conspecific density and landscape features related to vector proliferation are important factors influencing the transmission rates of this lineage in the study system.

Acknowledgments We wish to thank F. Espada for determining blood parasite prevalence and intensities of infections in the blood smears. Four anonymous referees and the associate editor of

Conservation Genetics provided valuable comments that improved the manuscript. This work received financial support from the projects: CGL2005-05611-C02-02/BOS (Ministerio de Educación y Ciencia), PAI05-053 (Junta de Comunidades de Castilla-La Mancha), and PCI08-0130-3954 (Junta de Comunidades de Castilla-La Mancha). We manipulated, bled, and banded nestling eagle owls under license from the Spanish institutional authorities (Environmental Agency of Junta de Comunidades de Castilla-La Mancha and the Ringing Office of the Ministry of Environment) and we followed general ethical guidelines for animal welfare and nature conservation. During this work J.O. was supported by a post-doctoral JAE-Doc (CSIC) contract. We performed all the laboratory work at the Laboratory of Genetics of the IREC and fragment genotyping was performed by the Centro de Investigaciones Biológicas (CSIC) of Madrid.

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