Ornis Fennica 82:63–72. 2005

Factors affecting the prevalence of blood parasites of Little Owls *Athene noctua* in southern Portugal

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Received 7 December 2004, accepted 5 April 2005

We studied the relationships between occurrence of blood parasites and host traits in a wild population of Little Owls *Athene noctua* in Mediterranean habitats of southern Portugal. Of 39 owls captured between February and August 1999, 16 (41.0%) were infected with *Leucocytozoon ziemanni*. One individual was infected with *Trypanosoma* sp. and another with a microfilaria. Age was the only variable influencing the prevalence of *L. ziemanni* in a multivariate logistic regression model: adults were more often infected than juveniles (82.4%, n = 17 vs. 9.1%, n = 22). This difference was probably due to the scarcity of appropriate vectors in the area at time of fledging. There was also a trend that infected adults owls had shorter bills than uninfected ones. This may indicate that infection may be associated with individual song characteristics of Little Owls, since a relationship between bill length and song features was found previously in the same area.

1. Introduction

Leucocytozoon species are haematozoan (family Haemosporidia; Desser & Bennett 1993) blood parasites that are commonly found in wild birds, including owls (Korpimäki *et al.* 1993, Muñoz *et al.* 1999) and diurnal raptors (Korpimäki *et al.* 1995, Wiehn *et al.* 1997). They are traditionally considered as only slightly pathogenic to wild birds (Bennett *et al.* 1988, Atkinson & van Riper III 1991), but this can be due to the difficulty in finding the cause of death or sub-lethal effects in wild animals (Grenfell & Gulland 1995). However, they have been reported to cause mortality in conjunction with other debilitating conditions (Peirce & Marquiss 1983, Hunter *et al.* 1997), reduce reproductive rates (Korpimäki *et al.* 1993, Dunbar *et al.* 2003) and affect host fitness

(Atkinson & van Riper III 1991, Stuht *et al.* 1999).

As the maintenance of immune function seems to be energetically and nutritionally costly (e.g. Demas *et al.* 1997, Lochmiller & Deerenberg 2000), prevalence of parasitism may be affected by factors such as energy reallocation during reproduction *per se* (Deerenberg *et al.* 1997, Bentley *et al.* 1998), food supply and resource levels (e.g. Wiehn & Korpimäki 1998, Appleby *et al.* 1999), hunting investment (Wiehn & Korpimäki 1998), and reproductive effort (e.g. Allander & Sundberg 1997, Wiehn *et al.* 1999). Prevalence may also vary with factors like sex (e.g. Zuk & McKean 1996, Fargallo *et al.* 2002) or individual age (e.g. Appleby *et al.* 1999, Garvin & Greiner 2003).

The role of avian parasites as morbidity and mortality factors affecting bird populations may be relevant to conservation issues, particularly when dealing with threatened bird species (e.g. Dobson & McCallum 1997, Newton 1998). A majority of the research on owls is limited to a description of haematozoan species and to the records of prevalence and intensity of infection in birds collected at rehabilitation centres (Mikaelian & Bayol 1991, Krone *et al.* 2001, Murata 2002). The Little Owl *Athene noctua* is a sedentary cavity-nesting owl associated to open and semi-open habitats. Throughout a big part of Europe, Little Owl populations have decreased in the last decades due to habitat changes (e.g. Génot & van Nieuwenhuyse 2002), and it is now listed as a "SPEC 3" species (i.e. a species whose global populations are not concentrated in Europe, but which have an unfavourable conservation status in Europe; Tucker & Heath 1994). To our knowledge, there are practically no studies on the blood parasites of Little Owls (e.g. Peirce 1981). The objective of our study was to examine the occurrence of blood parasites in Little Owls and to investigate the relationships between host traits and the prevalence of haematozoa in a wild population of this species in Mediterranean habitats.

2. Material and methods

2.1. Study areas

The study was conducted in two areas located 22 km from each other, in the Baixo Alentejo province, Southern Portugal: S. Marcos da Atabueira (37° 42' N, 7° 56' W) and Cabeça da Serra (37° 37' N, 8° 09' W). S. Marcos da Atabueira comprises 15.7 km^2 of an open pseudo-steppe area, used for pastures or cereal cultivation. Density of Little Owls is *ca.* 2.3 pairs/km^2 (R. Tomé, unpubl. data). Cabeça da Serra area covers 5.6 km^2 and is mostly occupied by open woodland of holm oaks *Quercus rotundifolia*, also used as pasture for cattle or cereal cultivation. The density of Little Owls in this area is very high, reaching *ca.* 7 pairs/km^2 (R. Tomé, unpubl. data). Climate is dry in both areas, with marked inter-annual variation in precipitation (Rivas-Martinez 1981, Reis & Gonçalves 1987). Streams are mostly temporary, drying up during late spring and summer. Nonetheless, a few larger streams and some reservoirs constitute permanent water bodies in the region.

2.2. Field work

Little Owls were captured between February and August 1999 by different methods, including a trap using a live mouse as bait (R. Tomé, unpubl.) and the use of a flashlight and a dip net at night (Bub 1991). Owls were ringed with metal and individual colour rings. Captured birds were classified as juveniles (born in the year of capture) or adults (born in a previous year), based on their plumage features (Cramp 1985). Age of juvenile owls varied, but only 3 individuals were captured at the nest, before fledging (fledging occurs at 30–35 days of age; Génot & van Nieuwenhuyse 2002). Age of fledged juveniles could be assessed by measuring the length of the 3rd left primary (Juillard 1979).

Sex of captured owls was determined through molecular methods (see below). Sex could be determined for all but three individuals. Measurements taken included body weight, wing length (body condition was defined as the residuals of a regression of weight on wing length), length of tail and tarsus, and height and length of bill. The amount of white in the adult plumage was estimated, by ascribing each individual a class ("small", "medium" or "large") depending on the extent of white in the throat, over the bill and in the eyebrows. Likewise, iris colour was also classified for each individual, as "pale", "yellow" or "bright

yellow". Owls were also inspected for ectoparasites (e.g. lice and ticks) and a class of presence/absence of these parasites was ascribed to each individual.

2.3. Blood sampling and analysis

Blood samples were obtained by piercing the brachial vein, drawing blood into a capillary tube and smearing it onto a glass slide. Approximately half the volume of each sample was conserved in "Queen's" lysis buffer at 4°C and later used for DNA extraction following the method described by Sambrook *et al*. (1989). Slides were immediately air-dried. Once in the laboratory, they were fixed in 100% methanol for 10 minutes and stained with Giemsa for 15 minutes. In order to determine prevalence, and identify parasitic species involved, smears were scanned for 30 minutes under oil immersion at 1,000× magnification, constantly changing fields. The number of fields examined was not recorded (Fedynich & Rhodes 1995, Leppert *et al.* 2004).

This methodology was validated for *Leucocytozoon* and *Haemoproteus*, but not for other blood parasites like *Trypanosoma*, *Plasmodium* and microfilaria species (Bennett 1962, Apanius 1991, Holmstad *et al.* 2003). Although more sensitive, PCR based, methods have been recently used in the detection of haemoprotozoans in birds (e.g. Hellgren *et al.* 2004), most published studies still rely on the visualization of parasites in blood smears. Moreover, the scanning protocol we used was considered sufficient to detect low intensity infections (Fedynich *et al.* 1993). Although using a different scanning protocol, Holmstad *et al.* (2003) found a 95% probability of successful detection of *Leucocytozoon* spp. parasitaemia by scanning a single blood smear.

Leucocytozoon spp. infect both erythrocytes and leucocytes (contrary to *Haemoproteus* spp., which infect only erythrocytes). The erythrocytes/leucocytes ratio varies with factors such as physiological and immunological status, and quantification protocols that measure the intensity of infection as the number of infected cells/number of erythrocytes examined have not been yet thoroughly tested for *Leucocytozoon* spp. (Fedynich *et al.* 1995; but see Fedynich & Rhodes 1995). Therefore we only report data concerning prevalence. We followed the terminology suggested by Bush *et al.* (1997), regarding quantitative descriptors of parasite populations.

2.4. Molecular sexing

Sex of owls was determined using a molecular method that amplifies a portion of the CHD1 gene (Ellegren & Sheldon 1997, Griffiths *et al*. 1998), which is located in a region of the avian sex chromosomes that does not undergo recombination (Fridolfsson *et al*. 1998). The primers P2 and P3 (Griffiths & Tiwari 1995) were used to amplify a portion of an exon of the CHD1 gene. The detection of differences between the amplified products of males and females was performed with a single-strand conformation polymorphism approach, following the methods described in Cortés *et al*. (1999) and Palma *et al*. (2001).

2.5. Data analysis

When investigating the relationships between prevalence of blood parasites and body measurements or plumage features, we used data from all captured adult owls. However, in the analysis concerning the relationships between age or sex and prevalence, only data from owls (young and/or adults) captured in different territories were used. By doing so, we avoided some possible pseudoreplication concerning the likelihood of owls occupying the same territories being more or less susceptible to infections. Therefore, we randomly selected one owl from each territory, and excluded from these analyses two adults, from territories where the remaining member of the pair was included, and six juveniles from four territories, where only one sibling from the brood was included. Accordingly, sample sizes varied in the different analysis.

For univariate comparisons, we used two-tailed parametric or non-parametric tests. In some cases, variables were square-root or log-transformed to meet the normality requirements (Sokal & Rohlf 1981, Zar 1996). Contingency analyses were used to compare the distributions of categorical variables (Zar 1996).

We used logistic regression to evaluate simultaneously the effect of different variables and their interactions on parasites prevalence. All variables that differed with $p < 0.25$ in the univariate analysis between infected and uninfected owls were entered in the initial logistic regression model (Hosmer & Lemeshow 1989). Non-significant interactions and main effects were then successively removed from the model, starting from the least significant variable. In this way, only significant effects were included in the final model (Christensen 1990, Valkama *et al.* 1998, Tomé *et al.* 2004). To assess model's performance, we calculated the area under the receiver-operating characteristic curve (AUC) (e.g. Fielding & Bell 1997, Pearce & Ferrier 2000, Manel *et al.* 2001). The AUC varies between 0.5 and 1, and represents the probability of a correct assignment of any pair of presence/absence observations randomly extracted from the data set. This probability tends to 0.5 for random assignments. Since several variables were only measured in adults, logistic regression analyses were performed separately on data from all owls captured and on data from adults.

All analyses were performed using SPSS statistical package (Norusis 1993).

3. Results

3.1. Parasites prevalence

Leucocytozoon ziemanni was the most frequent haematozoan in blood samples of Little Owls, with an overall prevalence of 36.2% (95% confidence interval: 22.5–49.9) (Table 1). When data included only owls from different territories (n = 39), prevalence was 41.0% (95% confidence interval: 25.6–56.4%). The only other haematozoa found in the blood samples were a *Trypanosoma* spp. and an unidentified microfilaria, present in one sample each.

Table 1. Number of captured owls that were infected or uninfected with *Leucocytozoon ziemanni* in both study areas.

	S. Marcos	Cabeça da Serra	Total
Infected	12	5	17
Uninfected	20	10	30
Total	32	15	47

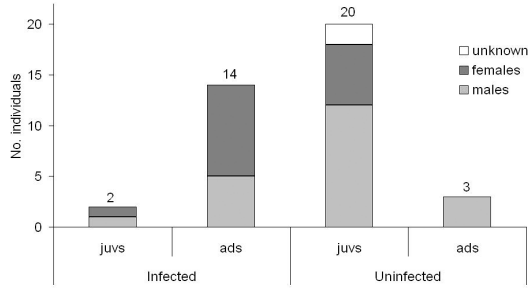

Figure 1. Age and sex classes of Little Owls infected and uninfected with *Leucocytozoon ziemanni* (pooled data from two study areas). Numbers on bars represent sample sizes.

Proportion of age and sex classes in captured owls did not differ between study areas (respectively: n = 39, $\chi^2 = 0.00$, df = 1, $p = 1.00$; n = 37, $\chi^2 = 0.24$, df = 1, $p = 0.63$). Similarly, parasite prevalence did not differ between areas (Fisher's Exact Test, $p = 1.00$; Table 1), and therefore owls captured in both sites were pooled in subsequent analyses.

The majority of adult owls (82.4%) were infected with *L. ziemanni*, while almost all juveniles (90.9%) were uninfected (Fig. 1). This difference was highly significant (Table 2). The youngest infected bird was an already fledged juvenile, which was only 27 days old.

Table 2. Variables that differed in the univariate comparisons (p-values < 0.25) between owls with and without *L. ziemanni* and were included in the initial logistic regression models.

Variable	n	Test statistic	P
Age	39	$\chi^2 = 18.35$; df = 1	< 0.001
Sex	38	$\chi^2 = 4.26$; df = 1	0.039
Sex[1]	17	Fisher's Exact Test	0.082
White over bill[1]	18	Fisher's Exact Test	0.080
Tail length[1]	18	Mann-Whitney U = 13.00	0.256
Bill length[1,2]	19	Mann-Whitney U = 8.50	0.031

1. Only adults included.
2. Bill height was significantly correlated with bill length ($r = 0.725$, $p = 0.001$) and was not analysed separately.

Among adults, prevalence did not vary significantly between seasons (February–April; May–June; July–August; Fisher's Exact Test, $p = 0.27$) or time of capture (morning; afternoon; evening; Fisher's Exact Test, $p = 1.00$).

Females were more often infected with *L. ziemanni* than males (62.5% vs. 28.6%; Fig. 1, Table 2). Males predominated amongst juveniles (65%) and females amongst adults (53%), although this difference was not significant ($c^2 = 0.59$, df = 1, $p = 0.44$). When only adults were considered, prevalence did not differ between sexes (Table 2), although females were again more infected than males (100% vs. 62.5%; Fig. 1).

Ectoparasites were present in 15 (38.5%) of the owls, but prevalence of *L. ziemanni* did not differ between owls with or without ectoparasites ($c^2 = 0.05$, df = 1, $p = 0.82$).

3.2. Prevalence and individual features

Adult owls infected with *L. ziemanni* had significantly shorter bills than uninfected individuals (Table 2 and Fig. 2). Bill size did not vary between sexes. None of the remaining characteristics of eye colour (Fisher's Exact Test, $p = 0.27$), body condition ($t = 0.52$, $p = –0.66$), white marks on plumage or body measurements differed significantly between infected and uninfected owls (Table 2).

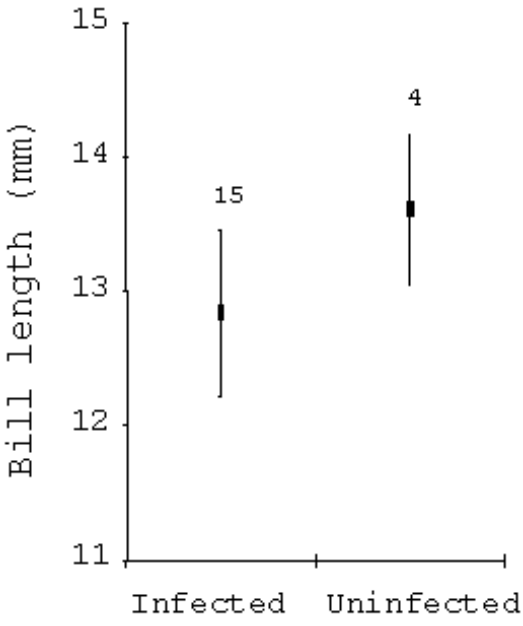

Figure 2. Mean bill length (± SD) of Little Owls infected and uninfected with *Leucocytozoon ziemanni*. Numbers on error bars represent sample sizes.

3.3. Multivariate analysis

Age and sex were the two variables entering the initial model of the logistic regression including data from both juvenile and adult owls. In the final logistic regression model, age was the only significant variable explaining prevalence of *L. ziemanni* (Wald $\chi^2 = 15.47$, df = 1, $p < 0.001$). This model had an overall percentage of correct classifications of 87.2%, varying between 87.0% (parasite absences) and 87.5% (parasite presences). The accuracy of the model was confirmed by an AUC of 0.872.

When only adult owls were considered, sample size decreased considerably and logistic regression could be used merely as an exploratory analysis. In this case, none of the selected variables (Table 2) resulted in a significant final model (variable closest to significance: bill length, Wald $\chi^2 = 2.69$, df = 1, $p = 0.101$).

4. Discussion

To our knowledge, this is the first study dealing with haematozoan infection in a free-living population of Little Owls. *Leucocytozoon ziemanni*, the only haematozoan species frequent in this population, has been reported in previous studies on owls, infecting e.g. Tengmalm's Owls *Aegolius funereus* (Korpimäki *et al.* 1993), Tawny Owls *Strix aluco* (Appleby *et al.* 1999, Krone *et al.* 2001) and Long-eared Owls *Asio otus* (Krone *et al.* 2001). It has also been reported in Little Owls admitted at rehabilitation centres (e.g. Muñoz *et al.* 1999). *Trypanosoma* spp. were also detected before in Little Owls, but, to our knowledge, there are no previous records of microfilaria in this species (Muñoz *et al.* 1999).

Although more than 80% of the adult Little Owls were infected in our study areas, the prevalence was inferior to those recorded in other owl species in e.g. Finland (92–100% in Tengmalm's

Owls; Korpimäki *et al.* 1993, Ilmonen *et al.* 1999) and U.K. (100% in Tawny Owls; Appleby *et al.* 1999). Prevalence of blood parasites in European owls varies considerably, depending on species, geographical region and season (Krone *et al.* 2001). Although species-specific physiological and immunological characteristics may account for differences in prevalence levels (e.g. Forero *et al.* 1997, Deviche *et al.* 2001), geographic variation in prevalence is probably related to differences in parasite-specific vector abundance (e.g. Bennett *et al.* 1995, Merilä *et al.* 1995, Sol *et al.* 2000).

The main documented vector of *Leucocytozoon* species are blood sucking black flies (Diptera; *Simuliidae*) (e.g. Atkinson & van Riper III 1991, Greiner 1991). These insects use freshwater streams to reproduce (e.g. Super & van Ripper III 1995) and their immature stages are restricted to running water (Urquhart *et al.* 1987). Therefore, black fly populations are conditioned by environmental factors such as climate, precipitation and topography (e.g. Thul *el al.* 1980, Dunbar *et al.* 2003). The predominantly dry climate in our study areas, with variable inter-annual precipitation, reduces the availability of suitable habitat for vector populations compared to what occurs in Central or Northern Europe. As a consequence, ornithophilic black flies seem to be scarce in our study sites during spring and summer (Bloise 1999, Grácio *in press*, R. Tomé, unpubl), although they are known to occur in the region at least in May, June and October (Grácio 1984). This probably accounts for the lower prevalence we found in Little Owls, compared to that in other owl species at higher latitudes in Europe. Likewise, Bennett *et al.* (1982) also recorded lower blood parasite prevalence in passerines of Western Europe than in those of Scandinavia and Russia, possibly due to smaller vector populations. Nevertheless, the fact that a big proportion of adult Little Owls was infected in our study sites, suggests that the few streams present are sufficient to produce black flies.

The prevalence of *L. ziemanni* in Little Owls was age-related. Parasites were present in only a very small proportion of juveniles, while a large majority of adult owls was infected. This pattern has been found in other studies of haematozoa in wild birds (e.g. Korpimäki *et al.* 1993, Appleby *et al.* 1999, Garvin & Greiner 2003), and is usually associated to a lack of suitable insect vectors during juvenile fledging (Bennett *et al.* 1975, O'Dell & Robbins 1994) and/or to a longer exposure to vectors throughout life (Bennett & Fallis 1960, Greiner 1975). A scarcity of vectors probably caused the low prevalence we found amongst juveniles. The pre-patent period of *Leucocytozoon* species (*ca.* 1 week; Weatherhead & Bennett 1991, Rintamäki *et al.* 1999) would have allowed the detection of more infected individuals, since practically all juveniles captured have fledged more than one week before.

We found a trend for higher prevalence in females than amongst males. Although males usually show higher susceptibility to parasite infections (Zuk 1990, Zuk & McKean 1996) and weaker immune response (Grossman 1985, Olsen & Kovacs 1996) than females, prevalence of haematozoa may be, at least under certain conditions, higher in females. While our work was not designed to investigate this question, other studies showed that this difference could result from immunodepressive effects of reproductive effort in females (Korpimäki *et al.* 1995, Wiehn *et al.* 1999, Wilson *et al.* 2001).

Bill length was the only individual characteristic varying between parasitized (shorter billed) and unparasitized (longer billed) adult Little Owls. A relationship between bill length and characteristics of male territorial song has been found earlier in this species in the same area. Males with longer bills produced longer song units (Cardoso *et al.* 1998) and more aggressive responses to playbacks (Chumbinho 2002). Territorial song is generally accepted to represent an honest signal of male individual quality amongst birds (e.g. Andersson 1994, Ryan 1997). Hence, it is possible that Little Owls showing more aggressive territorial vocal behaviours are also better quality and less parasitized individuals. How a physical trait of continuous growth, like the bill, may be related to prevalence of blood parasites is still to be determined.

Prevalence of blood parasites in Little Owls was strongly related to age, with adults more parasitized than juveniles. This difference should arise from a scarcity of appropriate vectors at time of fledging. The relationship of prevalence with other factors, such as sex and bill length, was mainly indicative and should be interpreted cau-

tiously, due to relatively small sample sizes. Nevertheless, our results provide suggestions, albeit indirectly, that infection may be related to the characteristics of territorial song in Little Owls. An influence of parasite load on territorial song features has been found, for example, in the Tawny Owl (Redpath *et al.* 2000). Further, more focused, investigation are needed to explore the possible consequences of parasite infection in such traits that reveal individual quality.

Acknowledgements. We thank all those who assisted in the field work, especially Albert Ros, Ana C. Cardoso, Ana C. Chumbinho, Carlos David Santos, Carolina Bloise, Inês Moreira and José Pedro Tavares; Miguel Pereira for his help in the examination of smears; Alexandra Sá Pinto for her important assistance in the laboratory work; Câmara Municipal de Castro Verde and Liga para a Protecção da Natureza for logistic support; A.J.S. Grácio for providing important information on the local ecology of black flies; Pedro Segurado for useful advice on logistic regression; Paulo Catry, Rui Rebelo and two anonymous referees for valuable comments on an earlier version of this manuscript. R.T. was funded by Fundação para a Ciência e a Tecnologia (Programa PRAXIS XXI, BD/5036/95).

Veriloisten esiintyminen minervanpöllöissä

Tutkimme minervanpöllön (*Athene noctua*) veriloistartuntoja Etelä-Portugalissa vuonna 1999. Helmi- ja elokuun välisenä aikana pyydystetystä 39:stä pöllöstä 16:lla (41 %) oli *Leucocytozoon ziemanni* -tartunta. Yhdellä yksilöllä oli veressään *Trypanosoma*-siimaeliöitä ja toisella mikrofilarioita. Ikä oli ainoa muuttuja, joka vaikutti *L. ziemanni*n yleisyyteen logistisessa monimuuttujaregressiomallissa: aikuisilla linnuilla oli tartunta useammin kuin nuorilla (82,4 %, n = 17 vs. 9,1 %, n = 22). Tämä ero johtuu luultavasti siitä, etteivät lentopoikaset ole vielä joutuneet alttiiksi *L. ziemanni*a levittäville vektoreille. Infektoituneilla aikuisilla pöllöillä oli lyhyempi nokka kuin infektoitumattomilla aikuisilla. Tämä saattaa viitata siihen, että minervanpöllöjen veriloistartunnat liittyvät yksilöllisiin laulun ominaisuuksiin, sillä nokan pituuden on osoitettu vaikuttavan pöllöjen lauluun samalla alueella.

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