zoonotic diseases

Article

Ticks and Tick-Borne Zoonotic Pathogens from Wild Birds in Northwestern Coastal Spain

Alberto Espí, Ana del Cerro, Paloma Peón-Torre, José Vicente González-Escudero and Aitor Somoano

MDPI

https://doi.org/10.3390/zoonoticdis3040026

zoonotic diseases

MDPI

Article

Ticks and Tick-Borne Zoonotic Pathogens from Wild Birds in Northwestern Coastal Spain

Alberto Espí [1,2,*], Ana del Cerro [1,2], Paloma Peón-Torre [3], José Vicente González-Escudero [3] and Aitor Somoano [1]

[1] Department of Animal Health, Regional Service for Agrofood Research and Development (SERIDA), 33394 Gijón, Spain; anadc@serida.org (A.d.C.); aitors@serida.org (A.S.)
[2] Translational Microbiology Consolidated Group, Health Research Institute of the Principality of Asturias (ISPA), 33011 Oviedo, Spain
[3] Iberian Ringing Group (GIA)-Asturias-Torquilla, 24006 León, Spain; vicente3@telecable.es (J.V.G.-E.)
* Correspondence: aespi@serida.org; Tel.: +34-653-372-118

Simple Summary: Ticks are parasites that, by feeding on blood, can transmit diseases to people and animals, being a cause for concern for health authorities. Their ability to move is limited, but not that of animals they parasitize, especially migratory birds that travel great distances every year. For this reason, it is important to know the percentage of birds that are parasitized by ticks in different countries that are on their migratory routes, the observed species of birds and ticks and whether they carry disease-causing organisms. That has been the objective of our study, which has taken advantage of the regular work of bird ringers over 2 years in both estuaries and forested areas. The results obtained from the examination of almost 1700 birds show a low percentage (2.5%) of birds parasitized by ticks and only one disease-causing organism in a bird's tick. Despite this, the information is relevant showing that the percentage of birds with ticks is higher in forest areas than in estuaries. This study also allows us to complete the information obtained in previous studies carried out in domestic and wild animals in a region that accounts for most of the Lyme disease hospitalizations in Spain.

Abstract: Migratory and local birds may disperse ticks and their associated pathogens. The aim of this study was to provide information regarding ticks infesting birds in Asturias, a region that accounts for most of the Lyme disease hospitalizations in Spain. From September 2021 to April 2023, trained and experienced bird-banders collected ticks from birds in two estuary and four forest locations. A total of 1698 birds (52 species, 38 genera, and 26 families) were captured. A total of 51 ticks (28 larvae, 20 nymphs, and 3 females) were collected from 43 birds, belonging to three species: *Ixodes ricinus* (31), *Ixodes frontalis* (18), and *Haemaphysalis concinna* (1). The average prevalence of tick infestation was 2.5% and the average tick burden was 1.2 ticks per infested host. The bird species *Turdus merula*, *Parus major*, *Luscinia svecica*, and *Anthus pratensis* were among the most infested. *Anaplasma phagocytophilum* was detected in one *I. ricinus* nymph collected from *Phylloscopus collybita*. We have not detected *Borrelia burgdorferi* s.l., *Rickettsia* spp., *Coxiella burnetii*, or piroplasmids in any of the 51 analyzed ticks. These results suggest low infestation rate in migratory/estuary birds and a higher rate in forest/sedentary ones. Despite this, the detection of pathogens, although with low prevalence, can pose a risk to public health.

Keywords: wild birds; ticks; *Ixodes* spp.; *Anaplasma phagocytophilum*; *Borrelia* spp.; *Rickettsia* spp.; piroplasms; Spain

Citation: Espí, A.; del Cerro, A.; Peón-Torre, P.; González-Escudero, J.V.; Somoano, A. Ticks and Tick-Borne Zoonotic Pathogens from Wild Birds in Northwestern Coastal Spain. *Zoonotic Dis.* **2023**, *3*, 316–333. https://doi.org/10.3390/zoonoticdis3040026

Academic Editor: Stephen K. Wikel

Received: 9 October 2023
Revised: 25 October 2023
Accepted: 31 October 2023
Published: 7 November 2023

1. Introduction

It is known that birds can be infested by ectoparasites such as mites, ticks, fleas, and lice. These ectoparasites, especially ticks, can carry several pathogens, and both ticks and

pathogens can be dispersed by the birds over short, medium, and long distances, posing a constant risk to local host populations in different geographic regions [1–4].

The annual cycle of migratory birds is marked by their seasonal movements between breeding and non-breeding sites. In addition, one of the best documented responses to recent climate change is the altered migratory behavior of birds [5]. The Convention on the Conservation of Migratory Species of Wild Animals (CMS) launched in 2022 the first interactive "Eurasian-African Bird Migration Atlas" [6], a very useful tool not only for conservation but also for the study of the epidemiology of many diseases, as has been demonstrated in the case of avian influenza. In this context, it is essential to know the role of the ticks that infest birds from different locations of the migratory route that goes from Africa to Northern Europe, since the risks vary according to latitudes but there are epidemiological links between the situation in each of those locations.

The Iberian Peninsula, due to its strategic location, is a vital connection point for migratory birds between Europe and Africa. It is the main migratory corridor in Western Europe and hundreds of thousands of birds cross the Strait of Gibraltar twice a year on their migratory journey. The wetlands of the Cantabrian coast, where Asturias is located, play a fundamental role in the provision of essential resources during pre-nuptial migrations (March–April) to northern Europe and post-nuptial migrations (July–September) to southern Spain and Africa. In addition, the temperate climate of this region offers refuge to various species of breeding birds from the north, where winter conditions are adverse. A large group of species that spend the winter in Africa migrate exclusively to the Iberian Peninsula to breed [7,8].

In Spain, several tick-borne microorganisms such as Anaplasma, Borrelia, Rickettsia, and Crimean-Congo hemorrhagic fever virus (CCHFV) were detected in ticks taken from birds, mainly in the inland areas of the country, confirming that birds can disperse vectors and microorganisms [9,10]. However, to our knowledge, no data are available on tick species associated with avian hosts in Asturias, a region located on the northern coast of Spain within the southern limit of distribution of *Ixodes ricinus* and other hygrophilous ticks and which accounts for most of the hospitalizations for Lyme disease in Spain [11].

This study aims to provide information on the avian tick burden on the migratory and sedentary birds that cross the Spanish Cantabrian coast to better understand their role in the tick-transmission cycles of tick-borne zoonotic diseases, especially Lyme borreliosis.

2. Materials and Methods

2.1. Ethical Statement

All birds were trapped during the normal trapping activities of staff members at the GIA Asturias-Torquilla ringing group (G00037), under a general ringing license from the Aranzadi Ringing Office, in accordance with Spanish regulations. Sampling of birds was approved by the Ministry of Rural Environment and Territorial Cohesion of the Principality of Asturias (ref. AUTO/2020/20871, AUTO/2020/20935, AUTO/2020/20937, AUTO/2020/20938 and AUTO/2020/20939 DECO/2021/15449 Y DICO/2022/16272).

2.2. Site Selection

Wild birds were surveyed on three sampling stations belonging to two types of habitats in Northern Spain: postnuptial passage in estuary; wintering in estuary and forest. Six ringing locations conformed these samplings (Figure 1): Villaviciosa estuary—Villaviciosa—43°30′44″ N, 5°23′40″ W and Nalón estuary—Soto del Barco—43°32′11″ N, 6°53′22″ W (postnuptial passage and wintering in estuary); and Fuensanta—Nava—43°30′44″ N, 5°23′40″ W, Les Praeres—Nava—43°20′45″ N, 5°28′57″ W, Somiedo—Sta. María del Puerto 43°1′24.05″ N, 6°13′29.06″ W and Sobrescobio—Ladines—43°12´48.57″ N, 5°25′48.57″ W (Forest).

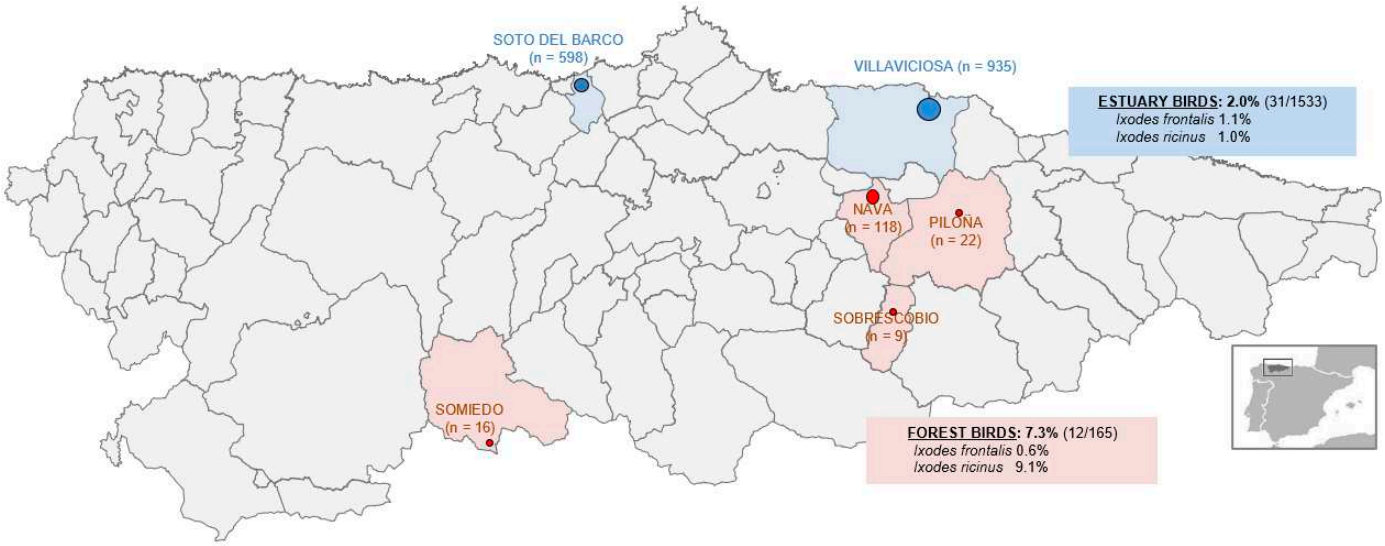

Figure 1. Bird ringing locations in Asturias. Numbers in parentheses represent the number of birds captured from corresponding locations. Blue and red dots represent estuary and forest areas, respectively (the size of the dots in the figure is proportional to the number of birds captured). The text boxes show the prevalence of infestation in the two differentiated areas and for each identified tick species.

Villaviciosa estuary showed vegetation of maritime reeds (*Juncus maritimus*), amphibious reedbeds of maritime cirpus (*Scirpus maritimus*), reeds (*Phagmites australis*), and grasslands of marsh ballast (*Elymus pycnanthus*). There are also marshmallow spots (*Althea officinalis*). Two invasive plants have thrived in the area: *Paspalum vaginatum* and *Cotula coronopifolia*. Around these formations that border the Sordo River, there is a wide area in which there are hardly any arboreal elements, except on the banks of some of the drainage channels and in the few existing boundaries.

Nalon estuary was bordered by a meander of the Nalón River, in the internal marsh area. It has plots of agricultural use and areas of reed (*P. australis*) and rush (*J. maritimus*). In the surroundings there are also trees, mainly willows (*Salix* sp.) and alders (*Alnus glutinosa*). The cultivation regime consists of planting maize for feed.

Forests were located at low and medium altitude in mountain areas with predominantly oak and beech arboreal vegetation.

Samplings took place from August 2021 to March 2023. Postnuptial passage sampling comprised fourteen weekly sessions between mid-July and mid-October. The wintering samplings comprised six weekly sessions between November and March. Forest station occurred between mid-March and April.

2.3. Bird Trapping

Bird captures were made with mist nets, also known as Japanese nets (Figure 2). They are used worldwide and are the preferred trapping method, especially to catch live birds. Mist nets are very fine nets that go unnoticed by birds, which, when passing where they are installed, become trapped in the net pockets that are formed with the tensioners, which are horizontal threads that run through them. With this system, the trapped animal does not suffer any harm. Their use is regulated, and they can only be used for scientific purposes [12].

The constant effort station methodology, known by the acronym CES, has been followed: the same number of nets are always available at the same locations and at regular time intervals, which facilitates long-term studies in which biological and demographic parameters can be compared [13].

Figure 2. Members of the GIA-Asturias-Torquilla Ringing Group releasing the small birds caught in the Japanese nets placed in the Villaviciosa estuary (photo on the right: common reed warbler, *Acrocephalus scirpaceus*).

Table S1 compiles all available information for each of the ringed birds including date, type of migration, location, habitat, ring number, species, age, sex, and tick parasitization data. Bird species were classified as ground, low, medium low, medium, medium high, and high feeders. The group of migratory birds captured includes migratory in passage, summer migratory, and wintering migratory birds. The sedentary birds are those that carry out their entire life cycle in these habitats, despite almost all of these species making short movements due to lack or abundance of feeding.

2.4. Ticks Collection

The captured birds were identified following Harrison and Svensson bird field guides [14–16], ringed for individual identification, inspected for attached ticks, and quickly released back in the same area.

Each bird was examined for ticks paying special attention to the areas around the head of eyes, ears, beak, crown, and neck (Figure 3). All attached ticks were removed using fine tweezers and stored in tubes with ethanol (70%) until specific morphological identification in the laboratory. In addition, other ectoparasites occasionally found on birds, such as lice or trombiculid mites, were also collected. Prevalence (percent infested) and mean intensity (mean number per infested host) [17] were calculated for each tick species on each infested bird species (Table 1).

Figure 3. Tick found attached to a common reed warbler (*Acrocephalus scirpaceus*) during post-banding examination.

Table 1. Bird species captured and infested in Asturias (2021–2023). Bird behavior and tick species.

Bird Family	Bird Species	MB	FB	C. Bird No.	Tick No.	BI %	Tick Stage			Tick Species			
							Ad	Nf	Lv	I.r	I.f	I.	H.c
Acrocephalidae	*Acrocephalus paludicola*	m	g	4	0	0.0							
	Acrocephalus schoenobaenus	m	g	233	1	0.4		1					1
	Acrocephalus scirpaceus	m	m	107	3	2.8			3	1	2		
Aegithalidae	*Aegithalos caudatus*	s	m	7	3	42.9		2	1	3			
Alaudidae	*Alauda arvensis*	m (s)	g	1	0	0.0							
Alcedinidae	*Alcedo atthis*	s		1	0	0.0							
Certhiidae	*Certhia brachydactyla*	s	m	25	1	4.0			1	1			
Cettiidae	*Cettia cetti*	s	g	40	1	2.5			1		1		
Cisticolidae	*Cisticola juncidis*	s	m	39	0	0.0							
Corvidae	*Garrulus glandarius*	s	g	2	0	0.0							
Emberizidae	*Emberiza schoeniclus*	m (h)	g	294	1	0.3	1			1			
	Emberiza pusilla	m (h)	m	1	0	0.0							
Fringillidae	*Pyrrhula pyrrhula*	s	m	3	0	0.0							
	Fringilla coelebs	s/m (h)	g	14	0	0.0							
	Chloris Chloris	s/m (h)	g	7	0	0.0							
	Carduelis carduelis	s	g	6	0	0.0							
	Serinus serinus	s	h	1	0	0.0							
Hirundinidae	*Hirundo rustica*	m	h	12	0	0,0							
Laniidae	*Lanius collurio*	m (s)	g	4	0	0,0							
Locustellidae	*Locustella naevia*	m (s)	g	4	0	0.0							
Motacillidae	*Anthus spinoletta*	m (h)	g	29	1	3.4			1		1		
	Motacilla flava	m (s)	g	4	1	25.0		1		1			
	Anthus petrosus	m (h)	g	1	0	0.0							
	Anthus pratensis	m (h)	g	146	9	6.2		2	9	7	4		
	Anthus trivialis	m (s)	m	1	0	0.0							
	Motacilla alba	s/m (h)	g	2	0	0.0							
Muscicapidae	*Luscinia svecica*	m (s)	g	39	2	5.1			2	2			
	Erithacus rubecula	s/m (h)	g	52	2	3.8		1	2	1	2		
	Saxicola rubicola	s	g	46	0	0.0							
	Saxicola rubetra	m	g	20	0	0.0							
Paridae	*Cyanistes caeruleus*	s	h	15	0	0.0							
	Lophophanes cristatus	s	h	5	2	40.0		1	2	3			
	Parus major	s	m	16	2	12.5		2		2			
	Poecile palustris	s	h	23	0	0.0							
	Periparus ater	s	h	15	0	0.0							
Passeridae	*Passer domesticus*	s	g	8	0	0.0							
Phylloscopidae	*Phylloscopus collybita*	m (h)	h	283	8	2.8	1	1	6	2	6		
	Phylloscopus ibericus	m (s)	h	3	0	0.0							
	Phylloscopus trochilus	m	h	31	0	0.0							
Picidae	*Dendrocopos major*	s	h	2	0	0.0							
	Jynx torquilla	m	g	2	0	0.0							
Prunellidae	*Prunella modularis*	s	g	10	0	0.0							
Regulidae	*Regulus ignicapilla*	s	h	31	1	3.2		1		1			
Sittidae	*Sitta europaea*	s	h	3	0	0.0							
Sturnidae	*Sturnus unicolor*	s	g	1	0	0.0							
Sylviidae	*Sylvia communis*	m	m	12	0	0.0							
	Sylvia atricapilla	s/m (h)	m	27	1	3.7		1			1		
	Sylvia melanocephala	s	m	10	0	0.0							

Table 1. *Cont.*

Bird Family	Bird Species	MB	FB	C. Bird No.	Tick No.	BI %	Tick Stage			Tick Species			
							Ad	Nf	Lv	I.r	I.f	I.	H.c
Troglodytidae	*Troglodytes troglodytes*	s	m	16	0	0.0							
Turdidae	*Turdus merula*	s	g	17	4	23.5	1	7		6	1	1	
	Turdus iliacus	m [(h)]	g	2	0	0.0							
	Turdus philomelos	s	g	21	0	0.0							
26 families	52 species	Total		1698	43	2.53	3	20	28	31	18	1	1

MB–migratory behavior (m—migratory, s—sedentary, s/m—sedentary, and migratory, [(h)]—hibernating, [(s)]—summer), FB—feeding behavior (g—ground, m—medium, h—high), not included in the classification the common kingfisher (*Alcedo atthis*) that hunts mainly fish from a perch, C. bird—number of captured birds of particular species, BI—prevalence of tick infestation among particular bird species, tick stages (Ad—adult, Nf—nymph, Lv—larvae), tick species (I.r—*Ixodes ricinus*, I.f—*Ixodes frontalis*, I.—*Ixodes* spp., H.c—*Haemaphysalis concinna*).

2.5. Tick Identification

Each tick was morphologically identified using a stereomicroscopy (NIKON© SMZ 1270) and the taxonomic keys of Manilla and Estrada-Peña and Mihalca [18,19]. Regarding the genus Ixodes, larvae were identified according to Heylen et al. [20]. Ticks were photographed dorsally and ventrally using the software NIS-elements (NIKON©).

The degree of blood ingested by each tick was estimated as unfed (U), little fed (LF), half fed (HF), or fully fed (FF) following Sandelin et al. [21].

In order to confirm morphologically identification, especially when the ticks had been damaged during removal from the birds, they were subjected to molecular identification using primers targeting the mitochondrial *16S rRNA* gene as previously described [22].

2.6. DNA Extraction and PCR Amplification and Sequencing Analysis

Total DNA was extracted from whole individual tick or pools (2 to 4 ticks per pool according to bird host, tick species, and tick stage) using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for insects. Briefly, ticks were crushed in 180 µL of PBS in a Precellys 24 Tissue Homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) for 2 × 20 s at 5000 rpm using 1.4 mm ceramic (zirconium oxide) beads (Precellys Lysing kit CK14, Bertin Technologies, Montigny-le-Bretonneux, France) for larvae and unfed nymphs and 2.8 mm stainless steel beads (Precellys lysing kit MK28-R) for adults and engorged nymphs. Samples were incubated overnight at 56 °C with proteinase K in a thermomixer (Ohaus, Nanikon, Switzerland) and DNA was obtained in 60 µL of elution buffer. The DNA quality and quantity was measured spectrophotometrically (NanoDrop ND-100, ThermoFisher Scientific, Waltham, MA, USA) and stored at −20 °C until use.

Five µL of tick DNA samples (25–100 ng) was initially screened for the presence of amplifiable DNA by conventional PCR for the mitochondrial *16S rRNA* gene [22]. DNA samples were tested for the presence of *B. burgdorferi* s.l., *Rickettsia* spp., *Anaplasma* spp., *C. burnetiid*, and piroplasmids by previously reported, real-time, and conventional PCR assays [23]. Multiplex real-time PCR was used for simultaneous detection of *Anaplasma* spp. (*16S rRNA* gene) and piroplasmids (*18S rRNA* gene), whereas single real-time PCR were conducted to amplify the *msp2* gene of *Anaplasma phagocytophilum* and IS1111 region of *Coxiella burnetii*. In *A. phagocytophilum*-positive samples, detection of different variants was investigated using a nested PCR targeting the partial *16S rRNA* gene. *Borrelia burgdorferi* s.l. and *Rickettsia* spp. were detected by amplification of partial *flaB* gene and *ompA* gene, respectively, using nested PCR protocols. All PCR primers and probes used in the study and their respective references are provided in Table 2. Real-time PCR amplifications were performed in a StepOne PlusTM system (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) with NZYSpeedy qPCR Probe Master Mix, ROX plus (NZYTech Lda, Lisboa, Portugal) and end-point PCR assays were carried out in a 2720 Thermal cycler

(Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) with Amplitools Master Mix (Biotools, Madrid, Spain). Positive (DNA from the corresponding pathogen tested) and negative controls (nuclease-free water) were included in each assay run. All the PCR products of the expected size were purified using NZYGelpure (NZYTech Lda, Lisboa, Portugal) and sent to Eurofins Genomics (Konstanz, Germany) for Sanger sequencing with the corresponding forward and reverse PCR primers (Table 2). Nucleotide sequences were aligned with Clustal W [24] algorithm using MEGA X package [25] and the obtained sequences were compared with the GenBank® database by using the Basic Local Alignment Search Tool (BLASTN) at the National Center for Biotechnology Information (NCBI). Nucleotide sequences obtained in this study were submitted to GenBank® under accession number OR623250.

Table 2. PCR primers and probes used for amplification and sequencing of tick-borne pathogens in ticks collected from birds.

Target	Target Gene	Oligo Name	Sequence (5′–3′)	References
Ixodidae	*16S rRNA*	16S+3 16S−3	ATACTCTAGGGATAACAGCGT AAATTCATAGGGTCTTCTTGTC	[22]
Anaplasma spp.	*16S rRNA*	Aspp16S-F Aspp16S-R Aspp16S-Pr	GCTATGCCGCGTGAGTGAG AGTTTGCCGGGACTTCTTCTG FAM-CTTAGGGTTGTAAAACTC-MGB	[26]
A. phagocytophilum	*16S rRNA*	ge3a ge10r ge9f ge2	CACATGCAAGTCGAACGGATTATTC TTCCGTTAAGAAGGATCTAATCTCC AACGGATTATTCTTTATAGCTTGCT [a] GGCAGTATTAAAAGCAGCTCCAGG [a]	[27]
A. phagocytophilum	*msp*2	ApMSP2-FN1 ApMSP2-R ApMSP2-Pr	AAGGCAGTGTTGGKTAYGGTATT TTGGTCTTGAAGCGCTCGTA FAM-TGGTGCCAGGGTTGAGCTTGAGATTG-BHQ1	[26] [28]
Coxiella burnetii	IS1111	sIS1pri_f sIS1pri_r Tqpro sIS1	CGGGTTAAGCGTGCTCAGTAT TCCACACGCTTCCATCACCAC FAM-AGC CCA CCT TAA GAC TGG CTA CGG TGG AT-BHQ1	[29]
Piroplasmids	*18S rRNA*	TB-F3 TB-R3 TB-Pr3	TTACTTW[+G]AG[+A][+A]AAYTAGAGTG [b] CTAAGAATTTCA[+C]CTCTGACA [b] JOE-CCAA[+C]Y[+G]TT[+C][+C]TATTAA[+C][+C]ATTA-BHQ1 [b]	[26]
Borrelia burgdorferi s.l	*fla*B	Outer 1 Outer 2 Inner 1 Inner 2	AARGAATTGGCAGTTCAATC GCATTTTCWATTTTAGCAAGTGATG ACATATTCAGAGCAGACAGAGGTTCTA GAAGGTGCTGTAGCAGGTGCTGGCTGT	[30] [31]
Rickettsia spp.	*omp*A	Rp190.70p Rp190.701n Rp190.602n	ATGGCGAATATTTCTCCAAAA GTTCCGTTAATGGCAGCATCT AGTGCAGCATTCGCTCCCCCT	[32] [33] [32]

[a] The primer was used for PCR and/or the sequencing reaction. [b] LNA™ modified oligos (Merck Life Science, Darmstadt, Germany), locked nucleic acids are in brackets with a plus sign.

2.7. Statistical Analyses

Differences in total bird abundance and relative abundances of each bird family and species in each sampling station were assessed by Kruskal–Wallis tests. This test was also used to assess differences in overall parasitization and parasitization per tick species among bird species and sampling stations. Then, Mann–Whitney U-tests with Bonferroni adjustment for multiple tests ($\alpha = 0.05$/number of pairwise comparisons) were used to establish differences between pairs of bird species.

We grouped species of birds by their foraging behavior and also assigned them to a migratory or sedentary group. Tick prevalence and intensity of infestation were compared among these groups.

Statistical analyses were performed with SPSS (IBM SPSS statistics version 20.0).

3. Results

3.1. Bird Species

A total of 1698 birds belonging to 26 families were captured during ringing seasons between September 2021 and April 2023 (Table 1 and Table S1). Of these birds, a total of 90.3% (1533/1698) were captured in estuaries and 9.7% (165/1698) in forested areas, with significant differences in bird abundance between three sampling stations ($H' = 494.31$, $p < 0.01$). Overall, most frequent families were Acrocephalidae ($n = 334$), Phylloscopidae ($n = 317$), Emberizidae ($n = 295$), and Motacillidae ($n = 183$), although family diversity notably varied among sampling stations (Figure 4). We identified 52 different species belonging to 38 genera, the most frequent being the following in general *Emberiza schoeniclus* ($n = 294$), *P. collybita* ($n = 283$), *A. schoenobaenus* ($n = 233$), *A. pratensis* ($n = 146$), and *A. scirpaceus* ($n = 107$). The most frequent bird species were *R. ignicapilla* ($n = 30$), *A. schoenobaenus* ($n = 233$), and *E. schoeniclus* ($n = 291$) in forest, postnuptial passage, and wintering estuary, respectively.

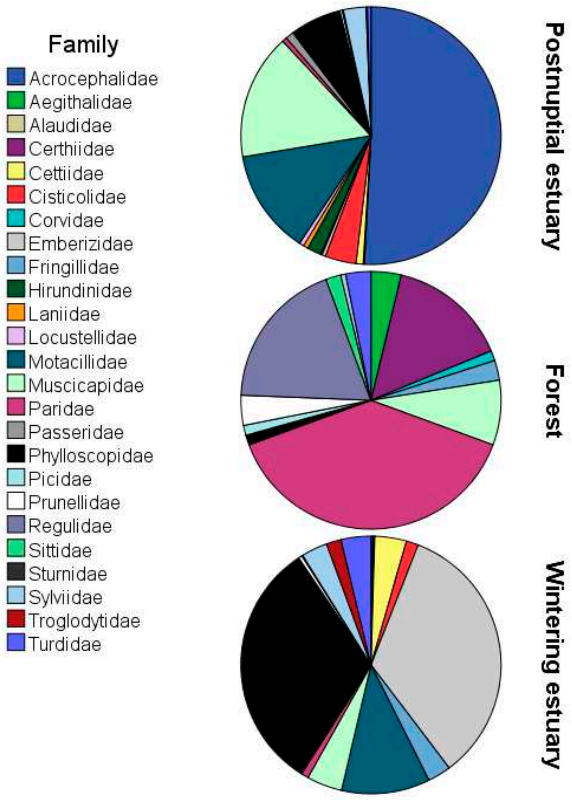

Figure 4. Relative abundances of the families for each sampling.

Out of the 52 different bird species, 27 (1009/1698 birds; 59% of the total captured) were classified as ground feeders, 12 (265/1698; 16%) as medium feeders, 12 (424/1698; 25%), as high feeders, and 1 not included in the classification (*Alcedo atthis*) (Table 1). A total of 22 species (1233/1698; 72.6%) were classified as migratory, 24 (363/1698; 21.4%) as sedentary, and 5 (102/1698; 6.0%) can be migratory or sedentary (Table 1).

3.2. Tick Infestations

A total of 51 bird-attached ticks were removed from 43 individual birds (Table 1 and Figure 5), with tick burden ranging from 1 to 4 ticks per bird.

The prevalence of ticks in birds was 2.5% (43/1698) and the average tick burden was 1.2 ticks per bird (51/43). We found 28 larvae, 20 nymphs, and 3 female adult ticks in the bird species included in analyses.

(**a**) (**b**) (**c**)

Figure 5. Morphology of some tick species identified on birds in Asturias. (**a**) *Ixodes ricinus* adult-female from a *Turdus merula* captured in Villaviciosa estuary in December 2022 (bar: 1000 µm); (**b**) *Ixodes frontalis* female from a *Phylloscopus collybyta*, captured in Navia estuary in November 2022 (bar: 1000 µm); (**c**) *Haemaphysalis concinna* nymph from a *Acrocephalus schoenobaenus* captured in Villaviciosa estuary in July 2022 (bar: 100 µm).

Ticks collected belonged to three species: *I. ricinus* (14 larvae, 15 nymphs, and 2 adult females; from 31 birds), *I. frontalis* (14 larvae, 3 nymphs, and 1 adult female; from 18 birds), and *H. concinna* nymph on a sedge warbler (*A. schoenobaenus*).

The number of ticks for each of the established degree of engorgement was 0 unfed, 9 little fed (3 adults, 3 nymphs, and 3 larvae), 12 half fed (6 nymphs and 6 larvae), and 24 fully fed (5 nymphs and 19 larvae).

Considering all bird species, no differences in overall parasitization were observed between different ringing locations within any of the three sampling stations ($p > 0.05$ in all cases). The same occurred in the case of parasitization by *I. ricinus* (wintering estuary: $H' = 0.00$, $p = 1.00$; Forest: $H' = 6.25$. $p = 0.28$) and by *I. frontalis* (wintering estuary: $H' = 0.00$, $p = 1.00$). However, significant differences in overall ($H' = 19.96$, $p < 0.01$) and in *I. ricinus* parasitization ($H' = 6.54$, $p < 0.05$) (Figure 6) but none in *I. frontalis* ($H' = 2.45$, $p = 0.29$) were observed between different sampling stations. In these cases, forest birds showed higher parasitization than both estuarian stations (Figure 6).

Significant differences in overall parasitization were observed between families from each sampling station (postnuptial estuary: $H' = 33.19$, $p < 0.01$; Forest: $H' = 26.26$. $p < 0.01$) except for the wintering estuary ($H' = 8.64$, $p = 0.73$). Indeed, significant differences in overall parasitization were also observed between bird species captured from each sampling station (postnuptial estuary: $H' = 36.51$, $p < 0.05$; Forest: $H' = 42.29$. $p < 0.01$) except for the wintering estuary ($H' = 21.08$, $p = 0.39$) (Figure 6).

The species *T. merula* showed the highest parasitization (average ± SD) in wintering estuary (1.50 ± 1.91), postnuptial estuary (0.50 ± 0.71), and forest (0.09 ± 0.30) (Figure 7). Nevertheless, the overall parasitization did not vary for each bird species between different sampling stations ($p > 0.05$ in all cases). No differences in *I. ricinus* and *I. frontalis* parasitization were observed between families or species from each sampling station ($p > 0.05$ in all cases).

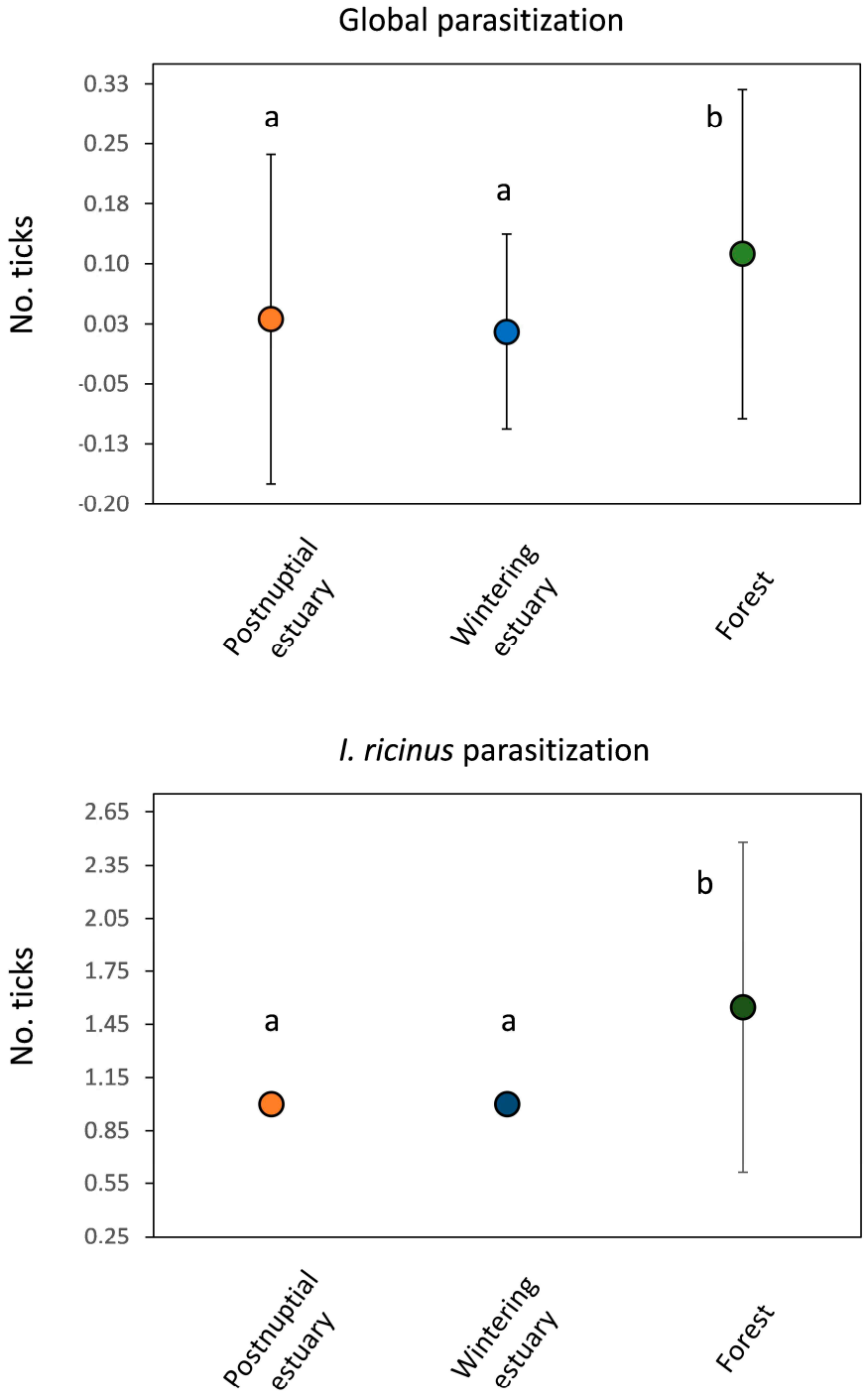

Figure 6. Number of ticks (average ± SD) for each sampling station. Significant differences in number of ticks between sampling stations are indicated by different letters.

The level at which each species feeds did not influence global or specific parasitization in any of the sampling stations ($p > 0.05$ in all cases). No differences in global parasitization were observed between migratory behavior (Forest: $H' = 1.63$, $p = 0.44$; wintering estuary: $H' = 0.87$, $p = 0.83$), except in birds from postnuptial estuary ($H' = 28.23$, $p < 0.01$) (Figure 8). Overwintering and sedentary/migratory birds showed higher global parasitization.

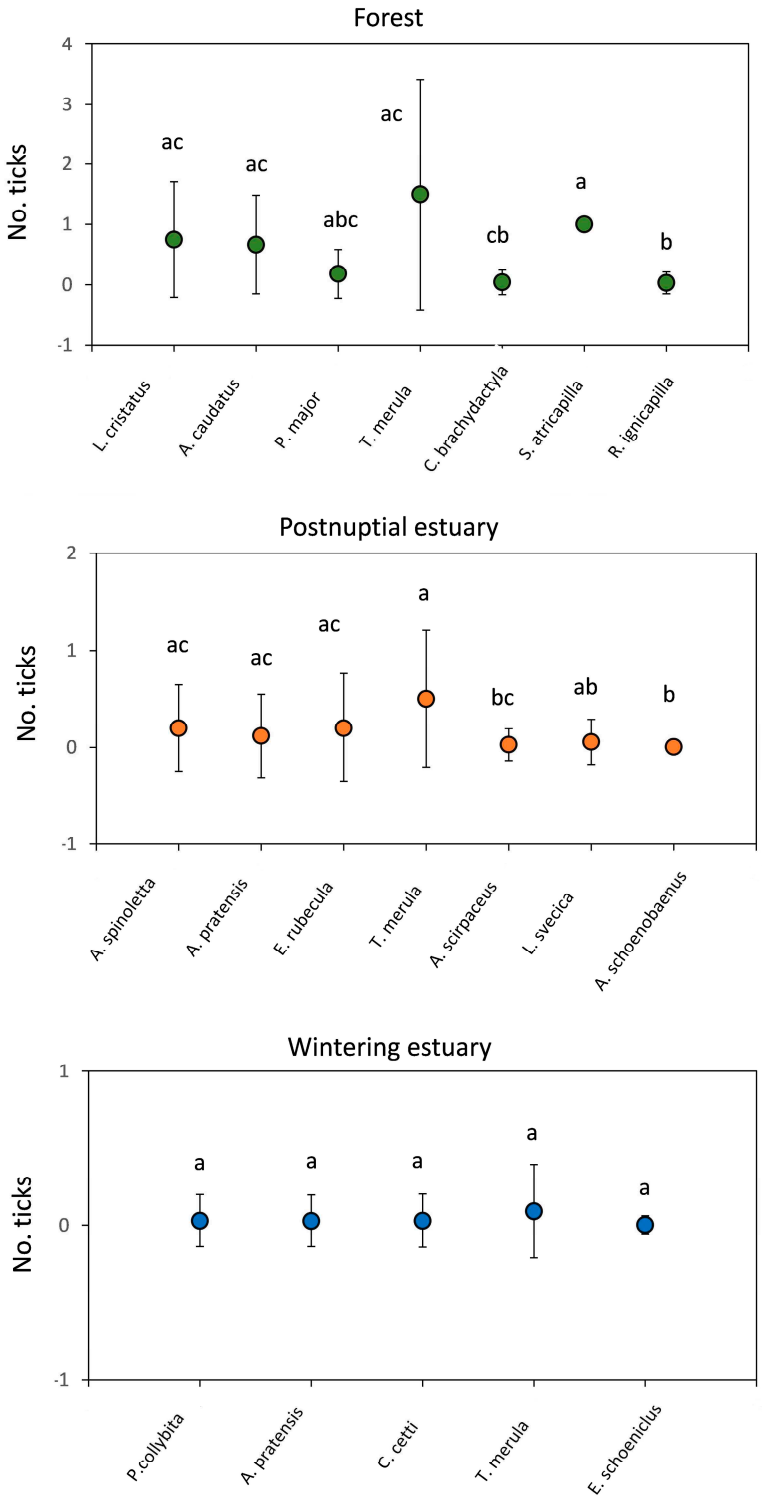

Figure 7. Number of ticks (average ± SD) in each bird species captured in each sampling station. Significant differences in tick number between bird species are indicated by different letters.

Birds, as expected, were mostly parasitized by immature ticks (94.1% of all ticks). Out of the total 51 collected ticks, only 2 *I. ricinus* and 1 *I. frontalis* adults were identified on a common blackbird (*T. merula*), marsh bunting (*E. schoeniclus*), and common chiffchaff (*P. collibyta*).

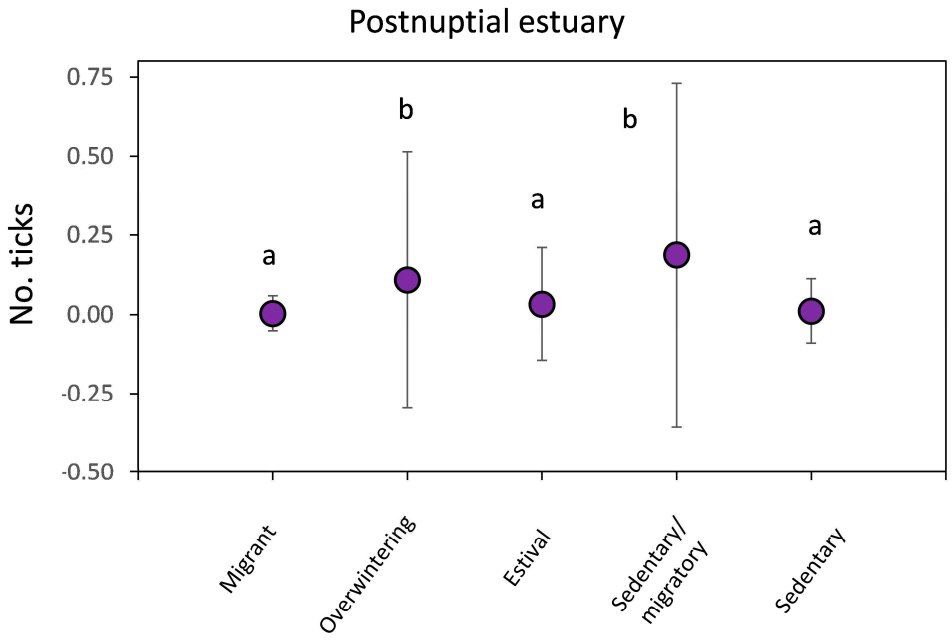

Figure 8. Number of ticks (average ± SD) in birds for each migratory behavior captured in postnuptial estuary. Significant differences in tick number between different migratory behavior are indicated by different letters.

The ticks we collected from the birds were mostly located around the eye (13/18) or the ear hole (3/18) and also on the beak (2/18) (Figure 3), although we could not have this information for all the birds.

3.3. Pathogen Surveillance

A total of 51 ticks (3 adults, 20 nymphs, and 28 larvae) belonging to *I. ricinus* (*n* = 31), *I. frontalis* (*n* = 18), and *H. concinna* (*n* = 1), were analyzed in 39 pools for *B. burgdorferi* s.l., *Rickettsia* spp., *Anaplasma* spp., *C. burnetii,* and piroplasmids detection. *Anaplasma phagocytophilum* was detected in one sample collected from one *Phylloscopus collybita*. Obtained sequence (487-bp fragment of the *16S rRNA* gene) shared > 99% of similarity (100% query cover) with *A. phagocytophilum* sequences associated to questing ticks (MK341075), feeding ticks (MW800887), and roe deer (MN170724) previously reported in North Spain. Alignment of the obtained sequence with the partial *16S rRNA* gene of the human granulocytic anaplasmosis agent (U02521), revealed the presence of *A. phagocytophilum 16S rRNA* variant "I" [34,35] (Figures S1 and S2).

None of the samples tested positive for *B. burgdorferi* s.l., *Rickettsia* spp., *C. burnetii,* or piroplasmids.

4. Discussion

Previous studies carried out in northern Europe have shown that the tick exotic species are mostly those inhabiting the southern and central parts of Europe, rather than those native to Africa or Western Asia, because ticks from those areas seldom stay attached to birds for long enough [36,37]. In Southern Europe, the arrival of infected ticks transported by migratory birds from Africa is a fact that has already been confirmed by previous studies [9]. Cantabrian regions of Spain are located in an intermediate geographical position, without adequate environmental conditions for the settlement and survival of xerophytic ticks of the genus *Hyalomma*. By contrast, this area is the southern limit of distribution of hygrophilous species such as *I. ricinus* [38], a recognized vector of Lyme borreliosis, a highly prevalent human disease in Asturias [11].

Among the 1698 ringed birds, we found significant differences in bird relative abundance between the three sampling stations belonging to two types of habitats: postnuptial

passage in estuary, wintering in estuary and forest. During the post-nuptial passage in the estuary the most abundant family of ringed birds was the Acrocephalidae, while during the wintering, Paridae in the estuary and Phylloscopidae and Emberizidae in the forest were the most frequent families. This highlights that the different proportion of families and species of birds examined due to geographical but also temporal factors would explain an important part of the variability of the results obtained in different studies.

The bird species in our study, mostly passerines, showed a remarkable 2.5% tick prevalence, consistent with other studies across Europe, such as the prevalence of 1.7% reported in Greece [39], 3.1% in Germany [40], 4.4% in Poland [41], or 2.0% in Sweden-Denmark [42], but far from the 58.0% reported in a Lyme endemic area in Switzerland [43], 41.1% in Czechia [44], 36.7% in Slovakia and Czechia [45], or 32.4% (Mafra, Lisboa) and 16.7% (Coimbra) reported in two forested areas in western Portugal [46]. A detailed analysis of the epidemiological studies carried out in Europe in the last 30 years (Table S2) revealed great methodological differences between them, both in geographical and climatic characteristics of the studied areas, diversity, and abundance of bird species and other tick host animals, or in the capture period. In this regard, it should be noted that in our study we also found significant differences in overall and in *I. ricinus* parasitization (but no in *I. frontalis*) between different sampling stations. In these cases, forest birds showed higher parasitization than both estuarian stations. We must keep in mind that many of the studied bird species have a clear habitat preference. In fact, of the 52 species identified in our study, 32 were captured exclusively in estuaries and 7 exclusively in forests.

Many studies suggest that the level of parasitism in birds is a consequence of their feeding behavior, which brings them often into contact with subadult ticks but also of the diversity and dominant tick species at each study area [43–51]. However, in our study the level at which each species feeds did not influence global or specific parasitization in any of the sampling stations. The difficulty to classify each bird species into a specific feeding behavior category could be one of the possible causes. The most common is to divide the birds into groups of ground-feeders and non-ground-feeders, with more or less intermediate groups according to the different authors. We consider that a categorization reduced to two groups is not realistic and that, on the contrary, the establishment of an excessive number of groups does not allow us to appreciate trends in terms of tick prevalence and mean tick infestation. Just as an example, indicate that the same species (common chiffchaff—*P. collybita*) is grouped as a "ground-feeder" (two groups) by Klaus et al. [40] in Germany and as a "high foraging level" (four groups) by Norte et al. in [46] in Portugal.

No differences in global parasitization were observed between migratory behavior except in birds from postnuptial estuary. Something similar to what we have mentioned for feeding behavior occurs with the division of bird species into "migratory" or "sedentary", given that in the same species in Asturias (*F. coelebs*, *C. chloris*, *E. rubecula*, *M. alba*, and *S. atricapilla*) we found populations that migrate and others that remain in the region.

Tick burden, considered an important indicator of reservoir competence, had a low range of 0–4 ticks per bird. The mean tick abundance or tick load was 1.2 (51/43) ticks per infested bird. Our results are very close to those obtained in other European countries such as the 1.9 (335/173) ticks per infested bird in Poland [41], 1.8 (1335/748) in Sweden [21], and 0.4 (212/107) in Russia [52], but lower than the 5.1 (2240/562) described in Czechia [44], 3.8 (3195/838) in Germany [40], 2.3 (417/180) and 1.9 (33/17) in two areas at different altitudes in Switzerland [53], or 2.1 (967/465) in Sweden and Denmark [42].

Ixodid tick species in Europe represent five genera: *Ixodes*, *Haemaphysalis*, *Hyalomma*, *Dermacentor*, and *Rhipicephalus*, but the last two usually do not parasitize birds [47]. In our study, the species of ticks identified in the captured birds were almost exclusively belonging to the genus *Ixodes* (98%), with the only exception of one *H. concinna* nymph found in a common warbler (*A. schoenobaenus*). Although *H. concinna* larvae and nymphs occurred more frequently on arboreal birds, its relatively high questing height on the vegetation can explain our finding on a ground-feeding bird species, similar to that described in

other central European countries [48]. The genus *Hyalomma* has not been described for the moment in our region due to its marked xerophytic character, although it has been identified in bordering regions to the south of the Cantabrian Mountains.

In published studies on tick species that parasitize birds [40,41,54], both endophilic (*I. frontalis*, *I. acuminatus*, *I. canisuga*, *I. arboricola*, *I. lividus*, *I. trianguliceps*) and exophilic species (*I. ricinus*, *H. punctata*, *Hyalomma* spp.) are described, although the latter vary much more according to the study areas. We also found endophilic (*I. frontalis* 35.3%, 18/51) and exophilic species (*I. ricinus* 60.8%, 31/51 and *H. concinna* 2.0%, 1/51). Among exophilic species, *I. ricinus* predominance is consistent with their abundance in the vegetation of Asturias where it is by far the most abundant species for all stages as well as the one with a longer period of activity year-round [38]. Among endophilic species, *I. frontalis* also showed notable prevalence values, which is striking if we take into account that almost all birds in our study area need to build a new nest every year due to the winter conditions, whereas the larvae of endophilic tick species are assumed to overwintering in the nest burrows until birds return. Despite the reduced variety of tick species found infesting birds in Asturias, our results agree with those of Heylen et al. [55], who also found the same two species almost exclusively: *I. ricinus* and *I. frontalis* in seven locations in the Netherlands.

Birds, according to many previous studies, were predominantly infested by immature *I. ricinus* stages and rarely by adult females. On the contrary, all *I. frontalis* developmental stages feed on birds; therefore, a greater proportion of adult specimens would be expected for *I. frontalis* compared to *I. ricinus*. Our results do not reflect these differences but rather very similar relative proportions (5.6 and 6.4%, respectively) of adults for both species. Although the number of ticks available does not allow us to deep into this topic, the three adult specimens of ticks came from three different bird families captured in the estuaries at the end of autumn.

I. ricinus is not only the predominant species in forest birds in Asturias, but also shows an infestation prevalence of 9.1% (15/165), well above the 1.0% (16/1533) of this same species in estuary birds. Norte et al. [46] showed that the seasonal pattern of tick infestation on birds was similar to that of questing ticks, at least regarding the immature stages of the species *I. ricinus*. In that sense, *I. ricinus* is the predominant species in the vegetation of Asturias, showing much higher abundances in forest areas than in coastal areas. Regarding *I. frontalis*, this species showed lower prevalence of infestation in both forest areas (0.6%, 1/165) and estuaries (1.1%, 17/1533).

Regarding occurrence of tick-borne pathogens in ticks infesting birds, the only zoonotic bacteria detected was *A. phagocytophilum*. In a previous work carried out in our region [56], we already detected high prevalence of this pathogen (61.0% in roe deer and 80.8% in red deer), suggesting the relevance of deer as reservoir host of *A. phagocytophilum* that could act as source of infection for vector ticks. The genetic variant "I" has been previously identified in Asturias in a questing adult *I. ricinus*. The variant detected is not pathogenic and has already been described in *I. ricinus* ticks collected from roe deer and roe deer tissues as well as in the vegetation in the autonomous community of Galicia [57], which borders Asturias.

The detection of *B. burgdorferi* s.l. among questing ticks and small mammals in Asturias [23,38], as well as the abundance of ticks and of large wild and domestic mammals, indicate a high risk of this infection in the region, where Lyme borreliosis is highly prevalent [11]. Despite this, we have not detected *B. burgdorferi* s.l. in any of the 51 analyzed ticks. The reasons that may explain this are, first of all, the small number of ticks examined despite the high number of birds captured, due to the low prevalence of infestation. In addition, 28 of the 51 analyzed ticks (51.9%) were larvae that are not usually carriers of *B. burgdorferi* s.l., since vertical transmission of this pathogen is very rare, although in some cases they can be infected by co-feeding. Furthermore, only 17 of the remaining nymphs and adults were *I. ricinus*. In a previous study in the same area, we detected *B. burgdorferi* s.l. in 1.4% (12/845) of *I. ricinus* questing nymphs and 9.1% (2/33) of questing adults [38] which, despite being a relevant percentage, makes it improbable to detect this pathogen in a sample of only 15 nymphs and 2 adults of this species. Finally, we must also assess that

most of the birds were captured in coastal estuaries, 1533 birds compared to only 165 in forest areas, and the abundance of ticks is much lower in these coastal areas than in more wooded inland zones. This fact, which we verified in many tick draggings carried out in the vegetation of the region, is also reflected in the lower prevalence of infestation of birds caught in seashore compared to those in forested areas.

5. Conclusions

Ixodes ricinus and *I. frontalis* are the most common tick infesting birds of several species. *Turdus merula*, *P. major*, *L. svecica*, and *A. pratensis* were some of the bird species that presented the highest prevalence of infesting ticks. *Anaplasma phagocytophilum* was detected in one *I. ricinus* nymph collected from *Phylloscopus collybita*. None of the rest of the pathogens tested were detected in any of the analyzed ticks. Updated knowledge of tick bird species and tick-borne pathogens that covers as many areas as possible is essential for understanding their possible impact on public health.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/zoonoticdis3040026/s1, Table S1: List of all the ringed birds and data collected from them prepared by the members of the "Torquilla" ringing group. Table S2: Epidemiological studies of tick infestation in birds in Europe. Figure S1: Phylogenetic relationships were inferred from the partial sequence of *Anaplasma phagocytophilum 16S rRNA* gene by using the Maximum likelihood method (ML) and Kimura 2-parameter model with MEGA X software. Figure S2: Pairwise sequence alignment of the partial *16S rRNA* gene for the human granulocytic anaplasmosis agent (GenBank U025211) and the 487-bp sequence obtained in this study (OR623250). References [21,39–46,49–54,58–60] are cited in the supplementary materials.

Author Contributions: Conceptualization, A.E., A.S. and A.d.C.; methodology, P.P.-T., J.V.G.-E., A.E. and A.d.C.; formal analysis, A.S.; data curation, P.P.-T., J.V.G.-E. and A.E.; writing—original draft preparation, A.E.; writing—review and editing, A.E., A.S., A.d.C., P.P.-T. and J.V.G.-E.; funding acquisition, A.d.C., A.E. and A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by PCTI 2021-2023 (GRUPIN: IDI2021-000102).

Institutional Review Board Statement: Ethical review and approval were waived for this study due to the fact that birds were captured during the normal trapping activities of members of the "Torquilla" ringing group, belonging to GIA (Iberian Ringing Group). See also Section 2.1. Ethical statemen for more details.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data supporting reported results can be found in the Supplementary Materials cited above.

Acknowledgments: We would like to thank all the volunteers bird-ringers for their collaboration, pivotal in this study.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Brinkerhoff, R.J.; Folsom-O'Keefe, C.M.; Tsao, K.; Diuk-Wasser, M.A. Do birds affect Lyme Disease risk? Range expansion of the vector-borne pathogen *Borrelia burgdorferi*. *Front. Ecol. Environ.* **2011**, *9*, 103–110. [CrossRef]
2. Hornok, S.; Karcza, Z.; Csörgo, T. Birds as disseminators of ixodid ticks and tick-borne pathogens: Note on the relevance to migratory routes. *Ornis Hung.* **2012**, *20*, 86–89. [CrossRef]
3. Vuong, H.B.; Canham, C.D.; Fonseca, D.M.; Brisson, D.; Morin, P.J.; Smouse, P.E.; Ostfeld, R.S. Occurrence and transmission efficiencies of *Borrelia burgdorferi* OspC types in avian and mammalian wildlife. *Infect. Genet. Evol.* **2014**, *27*, 594–600. [CrossRef] [PubMed]
4. Sparagano, O.; George, D.; Giangaspero, A.; Spitalska, E. Arthropods and associated arthropod-borne diseases transmitted by migrating birds. The case of ticks and tick-borne pathogens. *Vet. Parasitol.* **2015**, *213*, 61–66. [CrossRef]

5. Jones, T.; Cresswell, W. The phenology mismatch hypothesis: Are declines of migrant birds linked to uneven global climate change? *J. Anim. Ecol.* **2010**, *79*, 98–108. [CrossRef] [PubMed]
6. Convention on Migratory Species (CMS). Eurasian-African Bird Migration Atlas. 2023. Available online: https://migrationatlas.org/ (accessed on 3 October 2023).
7. Gordo, O.; Sanz, J.J.; Lobo, J.M. La migración de las aves sobre la península Ibérica. *Quercus* **2009**, *280*, 14–22. Available online: https://dialnet.unirioja.es/servlet/articulo?codigo=2981989 (accessed on 11 September 2023).
8. Moncada-Lorén, M. España, uno de los principales corredores migratorios del mundo por su ubicación geográfica. *National Geographic España*. 11 May 2018. Available online: https://www.nationalgeographic.es/animales/2018/05/espana-uno-de-los-principales-corredores-migratorios-del-mundo-por-su-ubicacion-geografica (accessed on 11 September 2023).
9. Palomar, A.M.; Portillo, A.; Santibáñez, P.; Mazuelas, D.; Arizaga, J.; Crespo, A.; Gutiérrez, Ó.; Cuadrado, J.F.; Oteo, J.A. Crimean-Congo hemorrhagic fever virus in ticks from migratory birds, Morocco. *Emerg. Infect. Dis.* **2013**, *19*, 260–263. [CrossRef]
10. Palomar, A.M.; Santibáñez, P.; Mazuelas, D.; Roncero, L.; Santibáñez, S.; Portillo, A.; Oteo, J.A. Role of birds in dispersal of etiologic agents of tick-borne zoonoses, Spain. *Emerg. Infect. Dis.* **2012**, *18*, 1188–1191. [CrossRef]
11. Alguacil, M.A. Carga hospitalaria de enfermedad de Lyme en España (2005–2019). In *Boletín Epidemiológico Semanal (ISCIII)*; Instituto de Salud Carlos III: Madrid, Spain, 2022; Volume 30. Available online: https://revista.isciii.es/index.php/bes/article/view/1214 (accessed on 21 August 2023).
12. Sutherland, W.J.; Newton, I.; Green, R. Techniques in Ecology & Conservation. In *Bird Ecology and Conservation: A Handbook of Techniques*; Oxford University Press: Oxford, UK, 2004. [CrossRef]
13. Robinson, R.A.; Julliard, R.; Saracco, J.F. Constant effort: Studying avian population processes using standardised ringing. *Ringing Migr.* **2009**, *24*, 199–204. [CrossRef]
14. Harrison, C. *Guía de Campo de los Nidos, Huevos y Polluelos de las Aves de España y de Europa*; Omega: Barcelona, Spain, 1991.
15. Svensson, L. *Identification Guide to European Passerines*, 4th ed.; British Trust for Ornithology: Stockholm, Sweden, 1992.
16. Svensson, L.; Mullarney, K.; Zetterstrom, D. *Guía de Aves de España, Europa y Región Mediterránea*; Omega: Barcelona, Spain, 2010.
17. Durden, L.A.; Oliver, J.H.; Kinsey, A.A. Ticks (Acari: Ixodidae) and spirochetes (Spirochaetaceae: Spirochaetales) recovered from birds on a Georgia Barrier Island. *J. Med. Entomol.* **2001**, *38*, 231–236. [CrossRef]
18. Manilla, G. Ixodida. In *Fauna d'Italia: Acari*; Edizioni Calderini: Bologna, Italy, 1998.
19. Estrada-Peña, A.; Mihalca, A.D.; Petney, T.N. (Eds.) *Ticks of Europe and North Africa. A Guide to Species Identification*; Springer International Publishing: New York, NY, USA, 2017.
20. Heylen, D.; de Coninck, E.; Jansen, F.; Madder, M. Differential diagnosis of three common *Ixodes* spp. ticks infesting songbirds of Western Europe: *Ixodes arboricola*, *I. frontalis* and *I. ricinus*. *Ticks Tick-Borne Dis.* **2014**, *5*, 693–700. [CrossRef]
21. Sandelin, L.L.; Tolf, C.; Larsson, S.; Wilhelmsson, P.; Salaneck, E.; Jaenson, T.G.T.; Lindgren, P.E.; Olsen, B.; Waldenström, J. *Candidatus* Neoehrlichia mikurensis in ticks from migrating birds in Sweden. *PLoS ONE* **2015**, *10*, e0133250. [CrossRef] [PubMed]
22. Black, W.C.; Piesman, J. Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 10034–10038. [CrossRef]
23. Del Cerro, A.; Oleaga, A.; Somoano, A.; Barandika, J.F.; García-Pérez, A.L.; Espí, A. Molecular identification of tick-borne pathogens (*Rickettsia* spp., *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato, *Coxiella burnetii* and piroplasms) in questing and feeding hard ticks from North-Western Spain. *Ticks Tick-Borne Dis.* **2022**, *13*, 101961. [CrossRef] [PubMed]
24. Thompson, J.D.; Higgins, D.G.; Gibson, T.J. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **1994**, *22*, 4673–4680. [CrossRef]
25. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]
26. Hurtado, A.; Barandika, J.F.; Oporto, B.; Minguijon, E.; Povedano, I.; García-Perez, A.L. Risks of suffering tick-borne diseases in sheep translocated to a tick infested area: A laboratory approach for the investigation of an outbreak. *Ticks Tick-Borne Dis.* **2015**, *6*, 31–37. [CrossRef] [PubMed]
27. Massung, R.F.; Slater, K.; Owens, J.H.; Nicholson, W.L.; Mather, T.N.; Solberg, V.B.; Olson, J.G. Nested PCR assay for detection of granulocytic ehrlichiae. *J. Clin. Microbiol.* **1998**, *36*, 1090–1095. [CrossRef]
28. Courtney, J.W.; Kostelnik, L.M.; Zeidner, N.S.; Massung, R.F. Multiplex real-time PCR for detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*. *J. Clin. Microbiol.* **2004**, *42*, 3164–3168. [CrossRef]
29. Schets, F.M.; de Heer, L.; de Roda Husman, A.M. *Coxiella burnetii* in sewage water at sewage water treatment plants in a Q fever epidemic area. *Int. J. Hyg. Environ. Health* **2013**, *216*, 698–702. [CrossRef]
30. Clark, K.; Hendricks, A.; Burge, D. Molecular identification and analysis of *Borrelia burgdorferi* sensu lato in lizards in the southeastern United States. *Appl. Environ. Microbiol.* **2005**, *71*, 2616–2625. [CrossRef] [PubMed]
31. Johnson, B.J.B.; Happ, C.M.; Mayer, L.W.; Piesman, J. Detection of *Borrelia burgdorferi* in ticks by species-specific amplification of the flagellin gene. *Am. J. Trop. Med. Hyg.* **1992**, *47*, 730–741. [CrossRef] [PubMed]
32. Regnery, R.L.; Spruill, C.L.; Plikaytis, B.D. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. *J. Bacteriol.* **1991**, *173*, 1576–1589. [CrossRef] [PubMed]

33. Roux, V.; Fournier, P.E.; Raoult, D. Differentiation of spotted fever group rickettsiae by sequencing and analysis of restriction fragment length polymorphism of PCR-amplified DNA of the gene encoding the protein rOmpA. *J. Clin. Microbiol.* **1996**, *34*, 2058–2065. [CrossRef]
34. Silaghi, C.; Hamel, D.; Thiel, C.; Pfister, K.; Passos, L.M.; Rehbein, S. Genetic variants of *Anaplasma phagocytophilum* in wild caprine and cervid ungulates from the Alps in Tyrol, Austria. *Vector-Borne Zoonotic Dis.* **2011**, *11*, 355–362. [CrossRef] [PubMed]
35. Overzier, E.; Pfister, K.; Herb, I.; Mahling, M.; Bock, G.; Silaghi, C. Detection of tick-borne pathogens in roe deer (*Capreolus capreolus*), in questing ticks (*Ixodes ricinus*), and in ticks infesting roe deer in southern Germany. *Ticks Tick-Borne Dis.* **2013**, *4*, 320–328. [CrossRef]
36. Capligina, V.; Salmane, I.; Keišs, O.; Vilks, K.; Japina, K.; Baumanis, V.; Ranka, R. Prevalence of tick- borne pathogens in ticks collected from migratory birds in Latvia. *Ticks Tick-Borne Dis.* **2014**, *5*, 75–81. [CrossRef]
37. Ciebiera, O.; Jerzak, L.; Nowak-Chmura, M.; Bocheński, M. Ticks (Acari: Ixodida) on birds (Aves) migrating through the Polish Baltic coast. *Exp. Appl. Acarol.* **2019**, *77*, 241–251. [CrossRef]
38. Espí, A.; del Cerro, A.; Somoano, A.; García, V.; Prieto, J.M.; Barandika, J.F.; García-Pérez, A.L. *Borrelia burgdorferi* sensu lato prevalence and diversity in ticks and small mammals in a Lyme borreliosis endemic Nature Reserve in North-Western Spain. Incidence in surrounding human populations. *Enferm. Infecc. Microbiol. Clin.* **2017**, *35*, 563–568. [CrossRef]
39. Diakou, A.; Norte, A.C.; Lopes de Carvalho, I.; Núncio, S.; Nováková, M.; Kautman, M.; Alivizatos, H.; Kazantzidis, S.; Sychra, O.; Literák, I. Ticks and tick-borne pathogens in wild birds in Greece. *Parasitol. Res.* **2016**, *115*, 2011–2016. [CrossRef]
40. Klaus, C.; Gethmann, J.; Hoffmann, B.; Ziegler, U.; Heller, M.; Beer, M. Tick infestation in birds and prevalence of pathogens in ticks collected from different places in Germany. *Parasitol. Res.* **2016**, *115*, 2729–2740. [CrossRef]
41. Zając, Z.; Kulisz, J.; Kunc-Kozioł, R.; Woźniak, A.; Filipiuk, M.; Rudolf, R.; Bartosik, K.; Cabezas-Cruz, A. Tick Infestation in Migratory Birds of the Vistula River Valley, Poland. *Int. J. Environ. Res. Public Health* **2022**, *19*, 13781. [CrossRef]
42. Olsen, B.; Jaenson, T.G.T.; Bergstrom, S. Prevalence of *Borrelia burgdorferi* sensu lato-infected ticks on migrating birds. *Appl. Environ. Microbiol.* **1995**, *61*, 3082–3087. [CrossRef] [PubMed]
43. Humair, P.F.; Turrian, N.; Aeschlimann, A.; Gern, L. *Ixodes ricinus* immatures on birds in a focus of Lyme borreliosis. *Folia Parasitol.* **1993**, *40*, 237–242.
44. Dubska, L.; Literak, I.; Kocianova, E.; Taragelova, V.; Sychra, O. Differential role of passerine birds in distribution of *Borrelia* Spirochetes, based on data from ticks collected from birds during the postbreeding migration period in central Europe. *Appl. Environ. Microbiol.* **2009**, *75*, 596–602. [CrossRef] [PubMed]
45. Taragelová, V.; Koči, J.; Hanincová, K.; Kurtenbach, K.; Derdáková, M.; Ogden, N.H.; Literák, I.; Kocianová, E.; Labuda, M. Blackbirds and song thrushes constitute a key reservoir of *Borrelia garinii*, the causative agent of Borreliosis in central Europe. *Appl. Environ. Microbiol.* **2008**, *74*, 1289–1293. [CrossRef]
46. Norte, A.C.; de Carvalho, I.L.; Ramos, J.A.; Gonçalves, M.; Gern, L.; Núncio, M.S. Diversity and seasonal patterns of ticks parasitizing wild birds in western Portugal. *Exp. Appl. Acarol.* **2012**, *58*, 327–339. [CrossRef]
47. Hasle, G. Transport of ixodid ticks and tick-borne pathogens by migratory birds. *Front. Cell. Infect. Microbiol.* **2013**, *3*, 48. [CrossRef]
48. Hornok, S.; Flaisz, B.; Takács, N.; Kontschán, J.; Csörgo, T.; Csipak, A.; Jaksa, B.R.; Kováts, D. Bird ticks in Hungary reflect western, southern, eastern flyway connections and two genetic lineages of *Ixodes frontalis* and *Haemaphysalis concinna*. *Parasites Vectors* **2016**, *9*, 101. [CrossRef]
49. Papadopoulos, B.; Humair, P.F.; Aeschlimann, A.; Vaucher, C.; Buttiker, W. Ticks on birds in Switzerland. *Acarologia* **2001**, *42*, 3–19.
50. Movila, A.; Gatewood, A.; Toderas, I.; Duca, M.; Papero, M.; Uspenskaia, I.; Conovalov, J.; Fish, D. Prevalence of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* and *I. lividus* ticks collected from wild birds in the Republic of Moldova. *Int. J. Med. Microbiol.* **2008**, *298*, 149–153. [CrossRef]
51. James, M.C.; Furness, R.W.; Bowman, A.S.; Forbes, K.J.; Gilbert, L. The importance of passerine birds as tick hosts and in the transmission of *Borrelia burgdorferi*, the agent of Lyme disease: A case study from Scotland. *IBIS* **2011**, *153*, 293–302. [CrossRef]
52. Movila, A.; Alekseev, A.N.; Dubinina, H.V.; Toderas, I. Detection of tick-borne pathogens in ticks from migratory birds in the Baltic region of Russia. *Med. Vet. Entomol.* **2013**, *27*, 113–117. [CrossRef]
53. Poupon, M.A.; Lommano, E.; Humair, P.F.; Douet, V.; Rais, O.; Schaad, M.; Jenni, L.; Gern, L. Prevalence of *Borrelia burgdorferi* sensu lato in ticks collected from migratory birds in Switzerland. *Appl. Environ. Microbiol.* **2006**, *72*, 976–979. [CrossRef]
54. Norte, A.C.; da Silva, L.P.; Tenreiro, P.J.Q.; Felgueiras, M.S.; Araújo, P.M.; Lopes, P.B.; Matos, C.; Rosa, A.; Ferreira, P.J.S.G.; Encarnação, P.; et al. Patterns of tick infestation and their *Borrelia burgdorferi* s.l. infection in wild birds in Portugal. *Ticks Tick-Borne Dis.* **2015**, *6*, 743–750. [CrossRef]
55. Heylen, D.; Fonville, M.; Docters Van Leeuwen, A.; Stroo, A.; Duisterwinkel, M.; Van Wieren, S.; Diuk-Wasser, M.; De Bruin, A.; Sprong, H. Pathogen communities of songbird-derived ticks in Europe's low countries. *Parasites Vectors* **2017**, *10*, 497. [CrossRef] [PubMed]
56. García-Pérez, A.L.; Oporto, B.; Espí, A.; del Cerro, A.; Barral, M.; Povedano, I.; Barandika, J.F.; Hurtado, A. Anaplasmataceae in wild ungulates and carnivores in northern Spain. *Ticks Tick-Borne Dis.* **2016**, *7*, 264–269. [CrossRef]
57. Remesar, S.; Díaz, P.; Prieto, A.; García-Dios, D.; Fernández, G.; López, C.M.; Panadero, R.; Díez-Baños, P.; Morrondo, P. Prevalence and molecular characterization of *Anaplasma phagocytophilum* in roe deer (*Capreolus capreolus*) from Spain. *Ticks Tick-Borne Dis.* **2020**, *11*, 101351. [CrossRef]

58. Sormunen, J.J.; Klemola, T.; Vesterinen, E.J. Ticks (Acari: Ixodidae) parasitizing migrating and local breeding birds in Finland. *Exp. Appl. Acarol.* **2022**, *86*, 145–156. [CrossRef]
59. Toma, L.; Mancuso, E.; d'Alessio, S.G.; Menegon, M.; Spina, F.; Pascucci, I.; Monaco, F.; Goffredo, M.; Di Luca, M. Tick species from Africa by migratory birds: A 3-year study in Italy. *Exp. Appl. Acarol.* **2021**, *83*, 147–164. [CrossRef]
60. Wallménius, K.; Barboutis, C.; Fransson, T.; Jaenson, T.G.; Lindgren, P.E.; Nyström, F.; Olsen, B.; Salaneck, E.; Nilsson, K. Spotted fever *Rickettsia* species in *Hyalomma* and *Ixodes* ticks infesting migratory birds in the European Mediterranean area. *Parasit. Vectors* **2014**, 7. [CrossRef] [PubMed]