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How Does Circadian Rhythm Shape Host-Parasite Associations? A Comparative Study on Infection Patterns in Diurnal and Nocturnal Raptors

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Abstract: Infection patterns of parasites, including their prevalence, diversity and host specificity, can be impacted by many biological and environmental factors, but no study has focused on the circadian rhythms of vertebrate hosts, which may affect susceptibilities and encounter rates between hosts and vectors and further shape host-parasite associations. In this study, we focused on avian haemosporidians, a classical model in studies of host-parasite associations, and investigated the infection patterns in rescued raptors brought to the Beijing Raptor Rescue Center during 2007–2020. We first assessed the association between prevalence and host biotic traits; haemosporidian prevalence was higher in the nocturnal raptors than in the diurnal raptors, and the prevalence of *Haemoproteus* and *Leucocytozoon* in the nocturnal raptors was significantly higher than that in the diurnal raptors. Furthermore, we analysed the phylogenetic relationship and host-parasite network-level differences of haemosporidian parasites in diurnal and nocturnal raptors, and demonstrated that the lineages infecting the diurnal and nocturnal raptors were not clearly separated, but the nocturnal lifestyle led to a more specialized host-parasite network structure. These variations in host-parasite associations may be driven by different susceptibilities of the hosts and the diversity or abundance of vectors during the day and night. Our study provides new insight into host-parasite associations shaped by circadian rhythm and calls for more studies on the underlying mechanisms of parasite infection.

Keywords: raptors; circadian rhythm; haemosporidians; infection patterns

Citation: Gao, K.; Zhou, B.; Yang, L.-X.; Dong, L.; Huang, X.; Deng, W.-H. How Does Circadian Rhythm Shape Host-Parasite Associations? A Comparative Study on Infection Patterns in Diurnal and Nocturnal Raptors. *Diversity* **2021**, *13*, 338. https://doi.org/10.3390/d13080338

Academic Editor: Gary Voelker

Received: 3 June 2021
Accepted: 20 July 2021
Published: 23 July 2021

1. Introduction

Parasites are widely distributed in a variety of organisms, and parasitic infection has a variety of effects on the fitness of the individual host, including death and reduced reproductive success, which further affects the health of the host population [1–4]. For vast parasites, complex host-parasite associations are the result of long-term host-parasite co-evolution [5]. A broader understanding of parasite infection and transmission in different ecological contexts will facilitate more effective management of emerging infectious diseases [6,7].

Avian haemosporida are widely prevalent in wild birds around the world; these parasites are grouped taxonomically into three main genera: *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*. In recent years, they have gradually developed into an important model system in the fields of host-parasite interaction and the evolution of wildlife diseases [8,9]. Previous studies have shown that these blood parasites infect bird species to varying degrees, including specific distributions across different geographic ranges [10–12], lineage-level host specificity in different climatic conditions [13], and variations in prevalence among host families or congeneric species [14,15]. These observed variations can be driven by multiple biological and environmental factors. For example, at the species level, because of differences in host life history, behaviour and environment that underpin

patterns of parasite exposure, parasite prevalence varies greatly among different bird host species [16,17]. At the same time, host species occupying similar niches may be exposed to similar combinations of vector-transmitted pathogens, leading to similar parasite community compositions and prevalence [18–21]. Additionally, host phylogeny also contributes to predicting patterns of haemosporidian infection, and previous studies have shown that closely related hosts share more similar parasite communities and phylogenetic distance is a key predictor of cross-species transmission [22,23].

Despite these variations, some studies have found that host-parasite associations can be disrupted in natural host populations or communities if hosts escape parasitism through the evolution of resistance [24] or through colonization of a new region [25–27]. In addition, *Plasmodium* parasites are transmitted by mosquitoes belonging to the genera Culex, Aedes, Culiseta and Anopheles (Diptera: Culicidae). Biting midges (Diptera: Ceratopogonidae) and hippoboscid flies (Diptera: Hippoboscidae) are the vectors of *Haemoproteus* parasites. *Leucocytozoon* parasites are transmitted by blackflies (Diptera: Simuliidae) [28]. Previous studies have also identified several behavioral differences among these vectors, including circadian rhythms (i.e., an endogenous biological rhythm with a period of approximately 24 h, also known as sleep-wake cycle). For example, most mosquitoes that transmit *Plasmodium* are nocturnal, with peak blood feeding occurring in the midnight [29]; Most blackflies are diurnal, with peak activity levels in the early morning and afternoon [30,31]; Most biting midges are crepuscular or nocturnal, with activity peaks during the evening and the first half of the night, and there are no reports of blackflies attacking birds after dark [32,33].

Similar to vectors, avian hosts also represent different circadian rhythms, and whether hosts with different circadian rhythms are exposed to different vectors and lead to a different host-parasite associations is an important issue for understanding disease transmission, but remains unknown. Birds of prey, also known as raptors, are a group of species that sit at the top of the food chain and play important roles in the ecosystem. Raptors mainly belong to three orders: Strigiformes (Strigidae and Tytonidae), Falconiformes (Falconidae), and Accipitriformes (Pandionidae and Accipitridae). The most extant of species in Strigiformes are nocturnal, and are separated from their sister group, the diurnal Coraciimorph clade in the Paleocene [34]. With high species diversity and different circadian rhythms, raptors and avian haemosporidian parasites are an ideal model to study whether variation in lifestyle can alter host-parasite associations.

In the present study, we first examine the species-level biotic traits (activity pattern, phylogenetic signal, weight and longevity) that influence variation in haemosporidian prevalence between diurnal and nocturnal raptors. Secondly, we analysed phylogenetic relationship of haemosporidian parasites in diurnal and nocturnal raptors. Lastly, we measured host-parasite network-level differences between diurnal and nocturnal raptors with the aim of answering the following questions: (i) Do the prevalence of avian haemosporidians differ among diurnal and nocturnal raptors? (ii) Are the haemosporidian lineages infecting diurnal and nocturnal raptors distinctly different? (iii) Can nocturnal behaviour alter the host-parasite network and result in a more specialized or generalized parasitism structure? Some raptors will be active to some extent at both day and night; therefore, we define activity periods using the time when a species is foraging as this is when they are most likely to be exposed to vectors. Depending on whether the active period was in day or night, raptors were categorized either as nocturnal or diurnal species.

2. Materials and Methods

2.1. Data Collection

During 2007–2020, blood samples (n = 1336) were collected from 35 raptor species brought to the Beijing Raptor Rescue Centre (BRRC) and stored in absolute ethanol at −40 °C until DNA extraction. The BRRC is a conservation project of the International Fund for Animal Welfare (IFAW) in China that provides medical treatment, care and rehabilitation training to injured or weak raptors. The BRRC is located in Beijing, an important node in

the East Asian-Australasian flyway [35]. The majority of raptors received by the BRRC were found in Beijing and adjacent areas. The raptors at the BRRC include mainly injured or weak raptors found by citizens, nestlings that fell out of nests, raptors that entered human settlements accidentally and raptors that were confiscated by enforcement actions. We used the data on birds' body mass (g) and maximum longevity (years) as reported in Healy et al. [36].

2.2. Parasite Identification

Total genomic DNA was extracted using a TIANamp Genomic DNA Kit DP304-03 (Tiangen Biotech Ltd., Beijing, China) following the manufacturer's instructions. Haemosporidian parasites were detected via nested PCR (polymerase chain reaction) amplification of a 479-bp fragment of the mitochondrial cytochrome *b* (cyt *b*) gene [37]. In the first PCR, the HaemNFI and HaemNR3 primers were used to amplify the DNA of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*. The product of the first PCR was then used as a template for the second PCR, using the primers HaemF and HaemR2 to amplify DNA of *Haemoproteus* and *Plasmodium* and HaemFL and HaemR2 L to amplify DNA of *Leucocytozoon*. Each sample was tested three times to check for possible mixed infection or false positivity, and negative controls were used for each PCR run. Positive samples were sequenced using the primers HaemF and HaemFL, and unique haplotypes were assigned to known haemosporidian lineages using the MalAvi database [38]. Parasites with at least one base-pair difference from cyt *b* sequences in MalAvi were defined as novel lineages.

2.3. Phylogenetic Analysis

Sequences of the mitochondrial cytochrome *b* (cyt *b*) gene were aligned using MAFFT 7.471 implemented in PhyloSuite 1.2.2 [39,40]. The best-fit nucleotide substitution model was selected in ModelFinder 2.1.1 using the Akaike information criterion (AICc) [41]. Based on the best-fit substitution model (GTR+I+G), the phylogenetic tree was created using MrBayes 3.2.6 [42]. A Markov chain Monte Carlo (MCMC) model was run for a total of 2×107 generations, with sampling every 1000 generations. Convergence was assessed when the average standard deviation of split frequencies (ASDSF) was less than 0.01 and the effective sample sizes (ESS) were greater than 200 in Tracer 1.7.1 [43]. The first 25% of trees were discarded as "burn in". The remaining trees were used to construct a consensus tree. The phylogenetic tree was visualized using FigTree 1.4.3 [44].

2.4. Statistical Analysis

We built a multivariable generalized linear regression model (glm) to assess the association between prevalence and host biotic traits using the R-package stats. The partial regression coefficient (β) was used as the quantitative relationship parameter. We considered only bird species with 10 or more blood samples, because a minimum sample size of 10 individuals per species gave largely similar prevalence results [15]. The three haemosporidian genera (*Haemoproteus*, *Plasmodium* and *Leucocytozoon*) were analysed separately. If the lineages in mixed infections belonged to two genera, they were included in both genus-specific analyses. To account for phylogenetic signal between host species, we calculated phylogenetic distance according to the consensus tree of host species. We constructed the host phylogenetic tree based on cytochrome *b* sequence data (1124 bp) from NCBI.

To better understand host-parasite network-level differences between diurnal and nocturnal raptors, we measured the network-level specificity index (H2′) for bird-haemosporidian communities and the species-level specificity index (d′) for parasite lineages using the bipartite package [45,46]. Due to structural differences in networks between different bird and haemosporidian communities (i.e., a varying number of host-parasite interactions), we compared observed H2′ and d′ values with those expected for random interactions (but retaining the same connectance as the original network) by generating 1000 null models per network using the bipartite package [46]. We also calculated the standardized effect

sizes (SES) of observed H2′ and d′ values in diurnal and nocturnal raptors following the methods described by Svensson-Coelho et al. [27,47].

3. Results

3.1. Differences in Haemosporidian Prevalence between Diurnal and Nocturnal Raptors

The dataset comprised 1336 bird individuals from 35 species; among them, 897 belonged to diurnal raptors and 439 belonged to nocturnal raptors (Supplementary Materials Table S1). Haemosporidian infections were detected in 329 individuals, with a total prevalence of 24.63%. Among the diurnal raptors, we obtained 51 haemosporidian lineages from 17.95% of individuals (161 total). The majority of these lineages belonged to *Haemoproteus* (25 lineages), while only 16 and 10 lineages belonged to *Leucocytozoon* and *Plasmodium*, respectively. From the nocturnal raptors, we obtained 51 haemosporidian lineages from 38.27% of the individuals (168 total), consisting of 22 *Leucocytozoon*, 19 *Haemoproteus* and 10 *Plasmodium* lineages.

Among the host biotic traits tested in our study, activity pattern is an important explanatory variable; the haemosporidian prevalence in the nocturnal raptors was significantly higher than that in the diurnal raptors (β = −42.44, p = 0.022, 95% CI for β: lower = −77.93, upper = −6.96; Table 1). When the three genera of parasites were analyzed separately, the *Haemoproteus* (β = −24.34, p = 0.038, 95% CI for β: lower = −47.11, upper = −1.57; Table 1) and *Leucocytozoon* (β = −22.62, p = 0.023, 95% CI for β: lower = −41.54, upper = −3.70; Table 1) prevalence in the nocturnal raptors was significantly higher than that in the diurnal raptors. However, there were no significant differences in *Plasmodium* prevalence between the diurnal and nocturnal raptors (β = −0.97, p = 0.867, 95% CI for β: lower = −13.16, upper = 11.22; Table 1).

Table 1. Differences in haemosporidian prevalence between diurnal and nocturnal raptors.

Prevalence	Host Biotic Traits	β	SE	Standardized β	t	*p*	95% CI for β	
							Lower	Upper
Total	(Constant)	173.717	70.669		2.458	0.028	22.146	325.287
	Activity pattern (ref = nocturnal)	−42.444	16.544	−0.873	−2.566	**0.022**	−77.928	−6.960
	Phylogenetic signal	−256.190	172.178	−0.580	−1.488	0.159	−625.475	113.095
	Weight	−0.003	0.003	−0.268	−1.149	0.270	−0.009	0.003
	Longevity	0.066	0.272	0.064	0.243	0.811	−0.517	0.649
Plasmodium	(Constant)	−4.632	24.272		−0.191	0.851	−56.689	47.426
	Activity pattern (ref = nocturnal)	−0.969	5.682	−0.066	−0.171	0.867	−13.156	11.218
	Phylogenetic signal	48.4	59.135	0.361	0.818	0.427	−78.433	175.232
	Weight	−0.001	0.001	−0.191	−0.723	0.481	−0.003	0.001
	Longevity	−0.037	0.093	−0.120	−0.400	0.695	−0.238	0.163
Haemoproteus	(Constant)	131.521	45.35		2.9	0.012	34.254	228.788
	Activity pattern (ref = nocturnal)	−24.340	10.617	−0.808	−2.293	**0.038**	−47.111	−1.569
	Phylogenetic signal	−271.565	110.491	−0.993	−2.458	**0.028**	−508.545	−34.585
	Weight	−0.002	0.002	−0.332	−1.374	0.191	−0.006	0.001
	Longevity	0.191	0.175	0.301	1.097	0.291	−0.183	0.566
Leucocytozoon	(Constant)	66.516	37.685		1.765	0.099	−14.311	147.343
	Activity pattern (ref = nocturnal)	−22.620	8.822	−0.843	−2.564	**0.023**	−41.542	−3.698
	Phylogenetic signal	−59.497	91.816	−0.244	−0.648	0.527	−256.423	137.429
	Weight	0.001	0.001	−0.017	−0.077	0.939	−0.003	0.003
	Longevity	−0.125	0.145	−0.22	−0.859	0.405	−0.436	0.187

Significant p values are shown in bold.

Phylogenetic signal was only positively associated with *Haemoproteus* prevalence (β = −271.57, p = 0.028, 95% CI for β: lower = −508.55, upper = −34.59; Table 1). There are generally no associations between haemosporidian prevalence with the weight and longevity of these raptors, and when the three genera of parasites were analyzed separately, the pattern remains the same (Table 1).

3.2. Phylogenetic Relationship of Haemosporidian Parasites in Diurnal and Nocturnal Raptors

Phylogenetic reconstruction of all the sequenced infections revealed 87 haemosporidian lineages, consisting of 19 *Plasmodium*, 37 *Haemoproteus* and 31 *Leucocytozoon* lineages; 53 lineages were not previously recorded in the MalAvi database, and 15 lineages were shared between the diurnal and nocturnal raptors. Of all 87 parasite lineages, only 15 lineages were shared between diurnal and nocturnal raptors. The three parasite genera clustered in three well-supported clades in the phylogenetic tree, and based on the phylogenetic branches, the lineages infecting the diurnal and nocturnal raptors were not clearly separated (Figure 1).

3.3. Avian Haemosporidian Network Structures in Diurnal and Nocturnal Raptors

The host-parasite network of the diurnal raptors was more generalized (H2′ = 0.624; Figure 2a) than that of the nocturnal raptors (H2′ = 0.739; Figure 2b), and the H2′ values (network-level specificity index) differed significantly from the values expected by chance in both communities (Table 2). There was a trend of more generalized parasite lineages in the nocturnal raptors (mean ± SE: d′ = 0.306 ± 0.257) than in the diurnal raptors (mean ± SE: d′ = 0.529± 0.313), but the difference was not significant. Furthermore, the mean d′ values (species-level specificity index) based on the null model predictions did not differ significantly from the observed values in either community (Table 2).

Table 2. Results of the observed and expected (null model predictions) network-level specialization (H2′), haemosporidian lineage-level specialization (d′) indexes and standardized effect sizes (SES) of observed H2′ and d′ values.

	H2′				**d′**			
	Observed	**Null Mean ± SD**	***p***	**SES (H2′)**	**Observed Mean ± SD**	**Null Mean ± SD**	***p***	**SES (d′)**
Diurnal raptors	0.624	0.171 ± 0.026	**<0.001**	17.3	0.5295 ± 0.313	0.202 ± 0.231	0.202	1.419
Nocturnal raptors	0.739	0.260 ± 0.323	**<0.001**	14.9	0.306 ± 0.257	0.170 ± 0.176	0.385	0.774

Significantly different p values are shown in bold.

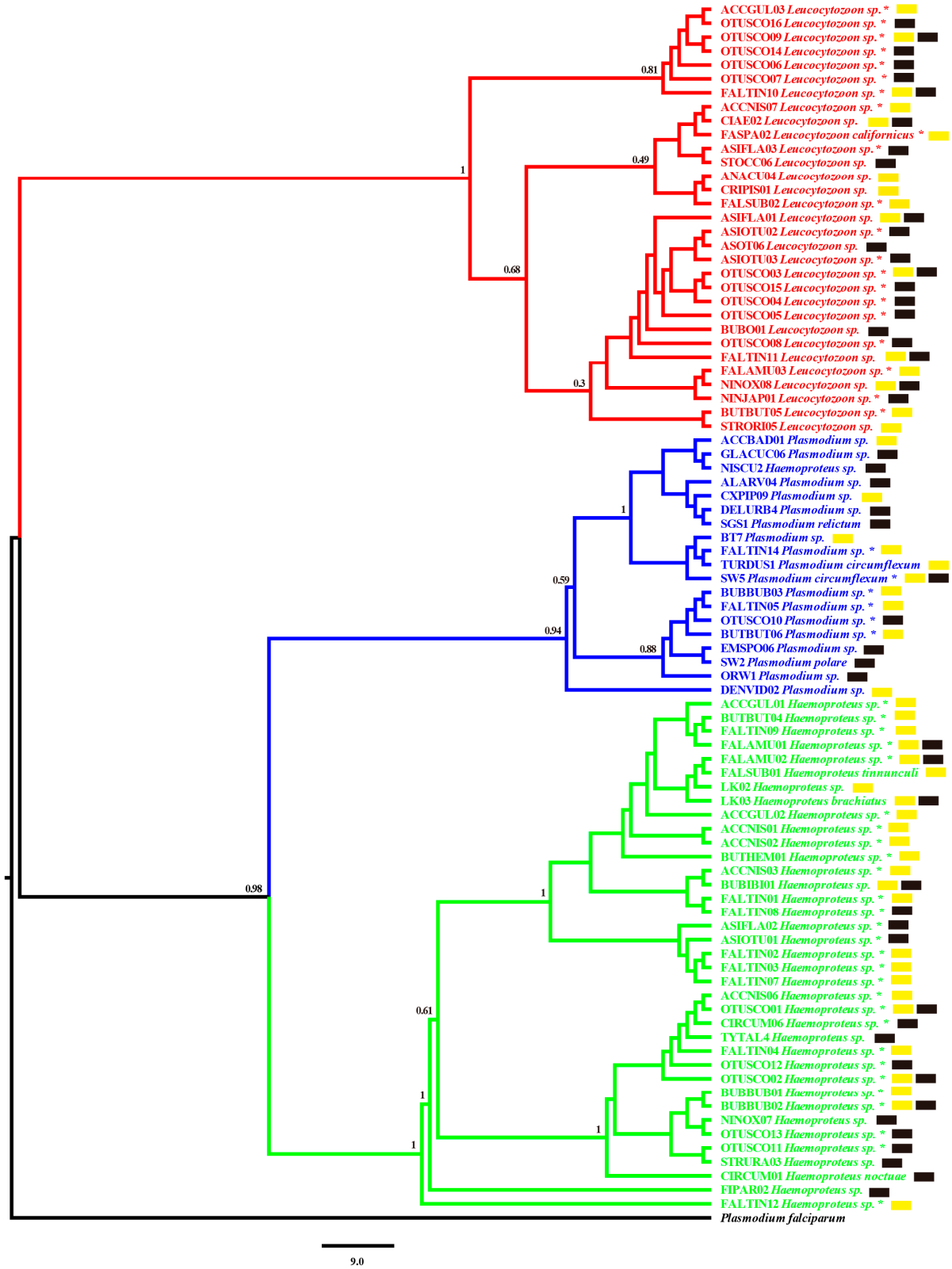
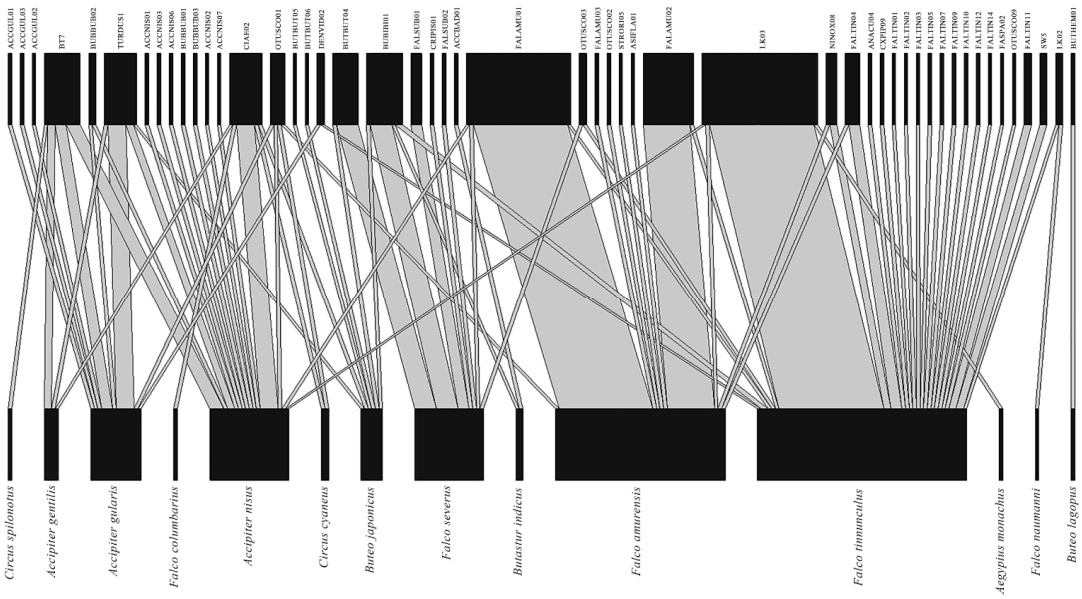
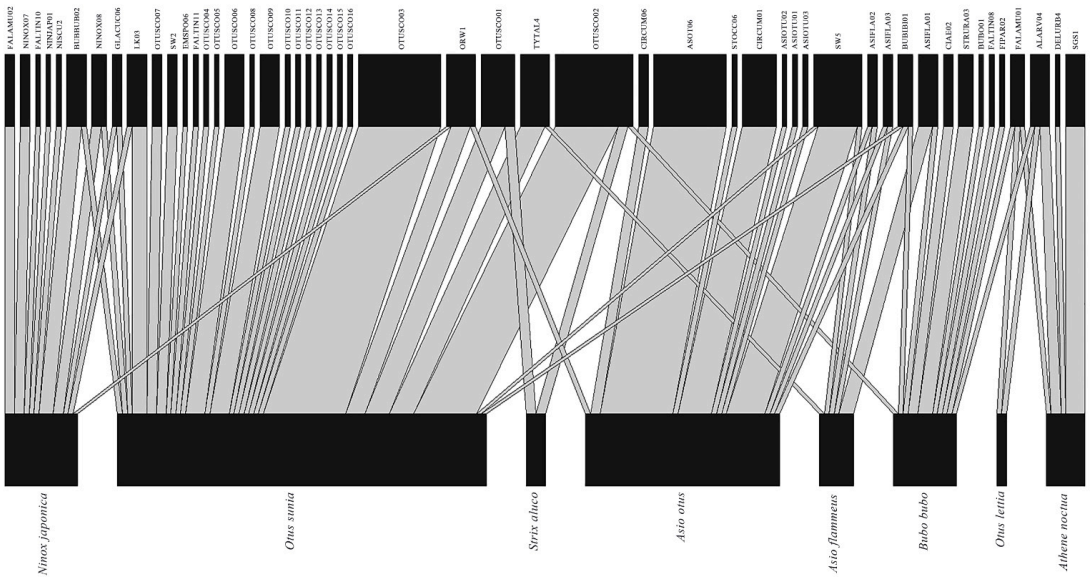


Figure 1. Phylogenetic relationship of 87 mitochondrial cytochrome *b* (cyt *b*) parasite lineages of raptors from the genera *Leucocytozoon*, *Haemoproteus* and *Plasmodium*. Posterior probability values are shown above nodes. The scale bar represents 0.03 substitutions per nucleotide position. The *Leucocytozoon*, *Haemoproteus* and *Plasmodium* lineages are shown in red, blue and green, respectively. The names of parasite species are given if they were identified at the lineage level. The asterisks indicate newly described lineages. The coloured boxes represent the hosts in which certain lineages were detected, i.e., detected in diurnal (**yellow**) or nocturnal (**black**) raptors.



(**a**) Diurnal raptors: H2′ = 0.624



(**b**) Nocturnal raptors: H2′ = 0.739

Figure 2. Host-parasite networks of comparable avian and haemosporidian communities in the diurnal (**a**) and nocturnal raptors (**b**). The network-level specificity index (H2′) is shown above each network. The width of each link corresponds to the number of interactions between a particular host and haemosporidian lineage.

4. Discussion

In this study, we investigated the prevalence, lineage diversity and network-level specificity of diurnal and nocturnal raptors. We demonstrated that nocturnal raptors harboured higher parasite prevalence than diurnal raptor species, especially of *Haemoproteus* and *Leucocytozoon*. Phylogenetic signal was only positively associated with *Haemoproteus* prevalence, and there were generally no association between haemosporidian prevalence with weight and longevity of these raptors. Furthermore, we demonstrated that the lineages infecting the diurnal and nocturnal raptors were not clearly separated, but that a nocturnal lifestyle led to a more specialized host-parasite network structure.

A larger number of studies have investigated the biological and environmental factors that may drive variation in haemosporidian parasite prevalence [15,48,49], but the circadian rhythms of vertebrate hosts, which may affect susceptibility and encounter rate between

hosts and vectors and further shape host-parasite associations, was poorly investigated. Our study provided a new approach for comparing parasite prevalence between diurnal and nocturnal raptors distributing in the same urban habitat, which may help with understanding the transmission of malaria and related infectious diseases. We demonstrated that activity pattern was an important explanatory variable in haemosporidian parasite prevalence, and we found a higher haemosporidian prevalence in owls (the only avian lineage of nocturnal raptors) than in diurnal raptors for the first time (Table 1). One possible explanation for this finding could be the higher susceptibility of nocturnal raptors due to the strong human disturbance of urban habitats, which is thought to negatively influence many nocturnal animals [50,51]. Another potential explanation could be the higher abundance of active vectors during the night and nocturnal exposure owing to a broader range of parasites [31]. Future studies investigating vector abundances and diversity can pinpoint the underlying mechanisms.

We found that the prevalence of *Haemoproteus* and *Leucocytozoon* was significantly higher in the nocturnal raptors than those in the diurnal raptors. However, there were no significant differences in *Plasmodium* prevalence between the diurnal and nocturnal raptors. We hypothesized that the higher prevalence of *Haemoproteus* parasites in nocturnal raptors may be due to cooler temperatures at night, which may support the survival and development of both *Haemoproteus* parasites and their associated vectors [52]. This also agrees with previous studies that found higher *Haemoproteus* prevalence at higher elevations with low temperatures [53,54]. In addition, the higher prevalence of *Leucocytozoon* parasites in nocturnal raptors supported previous research showing that owls are the main targets of blackflies [55]. Another potential explanation for this trend in *Leucocytozoon* prevalence could be the uneven sampling of host species. For example, the lower sampling of host individuals of some nocturnal raptors may lead to an overestimated prevalence. However, sampling bias may have little effect on the result, as several other *Leucocytozoon* studies have observed a high haemosporidian parasite prevalence in owls [56–59]. Thus, the high prevalence of *Leucocytozoon* in owls is commonly reported, and the underlying mechanism for this phenomenon deserves further research.

We found phylogenetic signal only positively associated with *Haemoproteus* prevalence. This finding supports previous genetic study which showed that *Haemoproteus* have high phylogenetic host specificity and closely related host species tend to share similar *Haemoproteus* lineages compared to other parasites [22,60]. With host weight and longevity, we found no significant association between these variables and haemosporidian infection. A plausible explanation for the non-significant relationship here could be that raptors species are well protected from insect bites regardless of body mass and longevity, probably due to thicker tarsus skin or solitarily roosting behavior. Furthermore, we demonstrated that the lineages of parasites infecting diurnal and nocturnal raptors did not have clearly distinguished phylogenetic relationships. Given that owls were separated from their sister group (the diurnal Coraciimorphae clade) and became nocturnal in the Paleocene [34], the observed similar phylogenetic relationship between diurnal and nocturnal raptors could be a result of parasite species accumulation over time.

Both the diurnal and nocturnal raptors had a much higher value of $H2'$ than that observed by previous studies on the host-parasite networks of passerine birds (Figure 2) [27,47], and the networks of the diurnal and nocturnal raptors presented a more specialized structure than expected by chance (Table 2). These results suggest that as the top predators, raptors occupy unique ecological niches and are less frequently infected by passerine parasites, leading to more specialized associations between raptors and their haemosporidian lineages [59]. For example, according to our dataset, most of the identified lineages are restricted to raptors (67 out of 87), while only 20 lineages can infect raptors and passerines [38]. Furthermore, we found that the host-parasite network of the nocturnal raptors was more specialized than that of the diurnal raptors. One possible explanation for this trend could be the lower diversity of active vector species at night [30,31], leading to lower infection rates of nocturnal raptors. For example, the most common and generalist parasite

lineage in diurnal raptors (*Leucocytozoon* lineage CIAE02) seems to be a specialist of nocturnal raptors (Figure 2). Another possible explanation for this trend could be the presence of more migratory species among the diurnal raptors than among the nocturnal raptors in our dataset, as migratory species can serve as important carriers of avian haemosporidians and thus lead to a more generalized network [61,62].

In addition to the host biotic traits that we have investigated above, there are many other factors that may be associated with variation in infection patterns of parasites. For example, compared to the juvenile birds, adults might harbour higher prevalence because they have been exposed to parasites for longer times and accumulated more chronic infections [63]. In addition, birds of different sexes may have different prevalence due to sex-associated hormones can directly influence the susceptibility [64]. In addition, the host habitat is also an important factor that may impact parasite diversity and prevalence, because the vectors of avian haemosporidian parasites may have particular habitat preferences [65]. Lastly, it has been observed that prevalence increases in reproductive or migration seasons when hosts are assumed to have relatively poor body condition [66,67]. However, because our data comes from a raptor rescue center, we could not examine some of the ecological factors (habitat, reproductive behaviour, nest type, etc) that influence variation in avian haemosporidian prevalence. Future studies should consider these cofactors simultaneously to reach a comprehensive understanding.

5. Conclusions

Overall, we demonstrated that nocturnal raptors experience higher levels than diurnal raptors of parasitism from haemosporidian blood parasites. As the only birds with a nocturnal lifestyle, the host-parasite network of the nocturnal raptors was more specialized than that of the diurnal raptors. These variations may be driven by different host susceptibilities, abundances of vectors and frequencies of parasite associations with others. However, without investigating vector abundances and diversity, we are unable to pinpoint the underlying mechanisms driving these changes. Future research should focus on analyses of vector communities along with bird and haemosporidian communities to improve our understanding of this complex host-vector-parasite system.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d13080338/s1, Table S1: Summary of tested samples and parasite infection in this study.

Author Contributions: Formal analysis, K.G.; funding acquisition, L.D., X.H. and W.-H.D.; investigation, K.G., B.Z. and L.-X.Y.; writing-original draft, K.G.; writing-review & editing, L.D., X.H. and W.-H.D. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the National Science Foundation of China (No. 31772444 to L. Dong, No. 32000292 to X. Huang, and No. 210100191 to W.H. Deng).

Institutional Review Board Statement: All animal experiments were carried out in accordance with the guidelines and approval of the Animal Research and Ethics Committee of College of Life Science, Beijing Normal University (permissions, CLS-EAW-2021-021).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analysed during this study are included in this published article and its supplementary information files. All obtained cytochrome *b* sequences in this study have been deposited to the MalAvi database.

Acknowledgments: We gratefully acknowledge the support of the Beijing Raptor Rescue Centre (BRRC). We thank C. Dai, S. Zhang, L. Zhou, Z.S. Li and Y.G. Shang for their assistance in collecting samples. We thank H.M. Wen for DNA data analysis.

Conflicts of Interest: The authors declare no conflict of interest.

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