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Avian haemosporidian persistence and co-infection in great tits at the individual level

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Abstract

Background: Many studies have tracked the distribution and persistence of avian haemosporidian communities across space and time at the population level, but few studies have investigated these aspects of infection at the individual level over time. Important aspects of parasite infection at the individual level can be missed if only trends at the population level are studied. This study aimed to determine how persistent Haemosporida are in great tit individuals recaptured over several years, whether parasitaemia differed by parasite lineage (mitochondrial cytochrome b haplotype) and how co-infection (i.e. concurrent infection with multiple genera of parasites) affects parasitaemia and body mass.

Methods: Parasite prevalence was determined by polymerase chain reaction (PCR), quantitative PCR were used to assess parasitaemia and sequencing was employed to determine the identity of the lineages using the MalAvi database.

Results: Haemosporidian prevalence was high over sampled years with 98% of 55 recaptured individuals showing infection in at least one year of capture. Eighty-two percent of all positive individuals suffered co-infection, with an overall haemosporidian lineage diversity of seventeen. *Plasmodium* and *Haemoproteus* parasites were found to be highly persistent, with lineages from these genera consistently found in individuals across years and with no differences in individual parasitaemia being recorded at subsequent captures. Conversely, *Leucocytozoon* parasites showed higher turnover with regard to lineage changes or transitions in infection status (infected *vs* non-infected) across years. Parasitaemia was found to be lineage specific and there was no relationship between *Plasmodium* parasitaemia or host body condition and the presence of *Leucocytozoon* parasites.

Conclusions: The findings of this study suggest that different genera of haemosporidian parasites interact differently with their host and other co-infecting parasites, influencing parasite persistence most likely through inter-parasite competition or host-parasite immune interactions. Even-though co-infections do not seem to result in increased virulence (higher parasitaemia or poorer host body condition), further investigation into infection potential of these parasites, both individually and as co-infections, is necessary.

Keywords: *Plasmodium*, *Haemoproteus*, *Leucocytozoon*, Multiple infection, Competition

Background

Haemosporidians are well-known and extensively studied parasites as *Plasmodium* gives rise to malaria in humans and animals, remaining one of the most common diseases in warm climate countries [1]. Investigating interactions between hosts and their parasites as well as the factors governing host susceptibility is key for understanding the epidemiology of the disease and host-parasite coevolution.

The importance of avian haemosporidian parasites (*Plasmodium* sp., *Haemoproteus* sp. and *Leucocytozoon* sp.) as a model system for studying host-parasite evolution and the consequences on ecology and conservation has been increasing over recent decades [2]. A number of studies have shown the costs on life-history traits associated with haemosporidian infection. Avian haemosporidian parasites can affect host body condition [3], reproductive success [4-6] and survival [7-10], with

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extreme cases resulting in the extinction of the avian host [11-13]. Consequently, these parasites can exert strong selective forces on their hosts.

As avian heamosporidians are ubiquitous [8], birds are exposed to a variety of haemosporidian parasites [8,14-21] and the distribution of these blood parasites within and between host populations has the potential to reveal different evolutionary dynamics of host-parasite interactions [22]. Therefore, knowledge of the persistence of parasite communities and their composition across temporal scales is a prerequisite for investigating and determining these host-parasite interactions.

Many studies have investigated haemosporidian community composition across space and time at the population level [22-25] and found that most parasite communities remained stable (but see [23]), even for up to 17 years [22]. However, few studies have investigated haemosporidian communities at the individual level. When studying communities at the population level only, changes happening at the individual level might be overlooked, especially if these changes have fast turn-over. Therefore, while parasite communities at the population level appear stable, at the individual level hosts might be experiencing rapid changes in parasite onslaught. At the individual level Hasselquist *et al* [25] found that the likelihood of retaining the same infection status (infected vs. uninfected) for *Haemoproteus payevskyi* was higher than the probability of experiencing a change in status. Knowles *et al* [26] showed that 26% of *Plasmodium* infection in individual blue tits (*Cyanistes caeruleus*) from the UK could be lost over time, Piersma and van der Velde [27] found that 23% of house martins (*Delichon urbicum*) in the Netherlands showed no haemosporidian infection status changes over time and Latta and Ricklefs [28] found high individual turn-over in haemosporidian infections between years in various host species on the island of Hispaniola.

While a number of factors can influence the persistence and abundance of a parasite in a host (e.g. host health or immunocompetence [29,30] and environmental condition [31]), the presence of multiple parasites within a host, i.e. co-infection, can critically impact infection dynamics and virulence. Co-infections with different haemosporidian genera can result in within-host competition leading to increased virulence [32]. On this topic, studies of mixed infections in birds have yielded diverse results. Palinauskas *et al* [33] reported increased virulence in experimentally co-infected individuals although this effect was host-species specific. Conversely, Marzal *et al* [34] showed that co-infection results in increased mortality but higher reproductive success in house martins, presumably as a result of increased investment in reproduction. Finally, Davidar and Morton [35] revealed that although single infections of *Haemoproteus prognei* and

filarial nematodes are relatively harmless in purple martins (*Progne subis*), a co-infection of these two parasites almost exclusively result in death of the host.

This study reports data from wild, free-living great tits (*Parus major*), sampled across a three-year period and addresses the following questions: 1) how persistent are the three haemosporidian genera (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*)? 2) Are there lineage-specific (a lineage being defined as a *Cyt b* haplotype) differences in parasitaemia? and 3) what is the frequency of co-infections and do co-infections between *Leucocytozoon* and *Plasmodium* appear to impact *Plasmodium* parasitaemia and host body condition? Taken together, these data will enable a better understanding of the dynamics of parasite infection.

Methods
Great tit captures and handling
A total of 311 nestboxes were installed in temperate broadleaf, mixed forests throughout the Canton of Vaud in western Switzerland. Adult great tits (*Parus major*) were trapped in their nestboxes during three consecutive breeding seasons (2009-2011) by using door traps mounted inside the nestboxes when their nestlings were twelve days old. Body mass was measured to the nearest 0.1 g. A 30 μl blood sample was taken by brachial venapuncture, and collected in lithium-heparin lined Microvettes® (CB 300 LH, Sarstedt, Germany). Only birds captured in more than one season were considered for this study (n = 55). All birds were captured under license from the Swiss Federal Office for the Environment (number F044-0799), and in accordance with the Cantonal Veterinary Authorities of the Canton de Vaud, Switzerland (authorization number 1730).

Molecular analyses
DNA was extracted from blood using the DNeasy tissue extraction kit (QIAGEN) according to the manufacturer's protocol for purification of DNA from blood using the BioSprint 96. After DNA extraction a nested PCR refined by Waldenström *et al* [36] from the original protocol made by Bensch *et al* [14] was performed on all samples. The full method is described in [37] with the following modifications: The PCR cycle profile included an initial denaturation at 94°C for 3 minutes followed by 20 cycles of 94°C for 30 s; 50°C for 30 s; 72°C for 45 s, and with a final extension at 72°C for 10 min. The final PCR amplification was run for 35 cycles with the same thermal profile as described here. For *Leucocytozoon* amplification, initial primers HaemNFI and HaemNR3 and nested primers HaemFL and HaemR2L (amplifying a 480 bp *Cyt b* fragment) were used [38] with the same thermal profile and procedure as described. Reactions were run on a Veriti 96 Well Thermal Cycler (Applied Biosystems). Distilled

RNAse-free water was used as a negative control and one negative control was inserted for every nine samples tested. Genomic DNA from individuals with known malarial infections were used as positive control. Nested PCR products were separated on 2% agarose gels containing ethidium bromide and a sample deemed positive if a fragment of approximately 500 bp was present when the gel was viewed under a UV light.

Nested PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) using the manufacturer's protocol for DNA purification by centrifugation. Purified PCR products were sequenced in both directions using the primers HaemF and HaemR2 for *Plasmodium* and *Haemoproteus* and primers HaemFL and HaemR2L for *Leucocytozoon* with a dye terminator cycle sequencing (BigDye® v3.1) reaction and electrophoresis was carried out on an ABI Prism 3100 sequencer (Perkin Elmer, Norwalk, CT). The sequences were assembled and edited using CodonCode Aligner (CodonCode Corporation). Sequences were then identified by performing a local BLAST search with the MalAvi database [2]. All *Plasmodium* and *Haemoproteus* sequences could be identified (or characterised as multiple infections), while 3.5% of *Leucocytozoon* samples were unidentifiable after sequencing due to poor DNA quality resulting in unreadable chromatographs. Similarly, identification of lineages involved in multiple infections were not possible (38.9% of infected birds suffered multiple *Leucocytozoon* infections, while 3.7% suffered multiple *Plasmodium* / *Haemoproteus* infections).

Plasmodium and *Haemoproteus* parasite quantification was achieved using a real-time quantitative PCR (qPCR) assay described in [37] but using a parasite *Cyt b* TaqMan probe (CY3-CYTb-BHQ2: 5'-CCTTTA GGGTATGATACAGC-3') and a host 18S rRNA probe (FAM-18S-BHQ1: 5'-AACCTCGAGCCGATCGCACG-3'), and calculated in the following manner: Firstly, parasite and host DNA quantity present in a sample was calculated by the equation

$$\alpha = 10^{\frac{1}{m(\triangle Ct)}} \qquad (1)$$

Where α represents the DNA quantity in the sample; m represents the slope of the standard curve; and $\triangle Ct$ is the difference between the mean Ct value of the sample and the intercept of the standard curve. Ct (or threshold cycle) is the fractional cycle number at which the fluorescence is detected to significantly surpass the threshold. This equation then gives a relative quantification value for parasitaemia relative to a standard sample used. Relative parasite density relative to host ($R\alpha$) was then calculated by the equation

$$R\alpha = \frac{\alpha(parasite)}{\alpha(host)} \qquad (2)$$

As a result of the exponential relationship between Ct and $R\alpha$, the $\log_{10}$ of $R\alpha$ was used to linearize this relationship. Parasitaemia is, therefore, unitless. The same reference sample was used for all qPCR standard curves and the efficiency of each qPCR run is taken into account during the calculation, therefore, parasitaemia values for samples collected in different years are directly comparable.

Cloning of multiple infections

Cloning was performed on a randomly chosen subset of ten samples containing multiple double peaks when DNA chromatographs were viewed. This was done to verify that the double peaks observed indicated multiple infections of parasites within a single host or due to problems like amplification errors arising from sequencing. Cloning was performed using the pGEM®-T Easy Vector System (Promega) according to the manufacturer's protocol. Ligation reactions were set up using purified PCR products and cultures of transformed high efficiency competent cells were plated onto LB/ampicillin/IPTG/X-Gal plates. Eight positive colonies were selected at random from every cloned sample. Inserts of positive colonies were sequenced using the SP6 and T7 Promoter Primers (Promega). Due to the cloning procedure being prone to introducing artificial mutations, only lineages that were detected in two or more separate colonies from each sample were considered as lineages involved in co-infection. Cloning confirmed that the double peaks on DNA chromatographs indicated infection with multiple lineages at the same time within a single host.

Statistical analyses

The analyses were performed with the freeware R-Cran Project [39]. A Fisher's exact test was performed to determine differences in the frequency of lineage identity changes and infection status (infected vs. uninfected) changes between *Plasmodium*, *Haemoproteus* and *Leucocytozoon* infections in recaptured individuals. Parasitaemia data were log transformed to achieve normality. A Welch two-sample t-test was used to assess the difference in parasitaemia between the two most prevalent *Plasmodium* lineages, SW2 (*P. polare*) and SGS1 (*P. relictum*), as sample sizes for other recorded lineages were too low to be considered. Paired t-tests were performed to determine whether an individual's parasitaemia changed between years of capture. Linear mixed models (lmer) in the lme4 package [40] were performed on parasitaemia data to determine whether there is a relationship between concurrent infection with *Leucocytozoon* and *Plasmodium* parasitaemia. *Plasmodium* parasitaemia was considered as response variable with bird identity and year of capture as random factors and *Leucocytozoon* infection as fixed effect. To assess the virulence (i.e. affect on host body mass) of co-infection, linear mixed models were

performed on a subset of data containing only occurrences of lineage SGS1 (n = 83) and considering *Leucocytozoon* infections as presence or absence values only. Body mass was considered as response variable with bird identity as random factor and sex and *Leucocytozoon* infection as fixed effects. Likelihood ratio tests (LRT) were used to compare "goodness of fit" between models containing and excluding the fixed effect of interest. Probability values of $p \leq 0.05$ were considered significant.

Results

Great tit recaptures
A total of 328 adult great tits (*Parus major*) were captured over the three breeding seasons (110 great tits in 2009, 158 in 2010 and 60 in 2011). Amongst them, 55 birds were recaptured in one or both subsequent years to their first capture. In 2010 there was a recapture rate of 22.8% (n = 158) (i.e. birds caught in 2010 that were also trapped the previous year) and a recapture rate of 38.3% (n = 60) in 2011 (birds that were previously trapped in 2009 and/or 2010).

Parasite prevalence, persistence and parasitaemia
Fifty-four (98.2%) of all recaptured great tits tested positive for haemosporidian infection in at least one year of capture. A total of three *Plasmodium*, two *Haemoproteus* and 13 *Leucocytozoon* lineages were identified (Tables 1 and 2). Of these, eight *Leucocytozoon* lineages were detected which were previously unencountered in the literature, and these new lineages differed by 2 - 8% from PARUS22, which was the most prevalent and widespread *Leucocytozoon* lineage encountered. *Plasmodium* and *Haemoproteus* parasites were more persistent (i.t.o. infection status and lineage identity changes) than *Leucocytozoon* parasites ($p < 0.0001$, Fisher's exact test) indicating that host-parasite interactions between great tits and *Leucocytozoon* parasites and parasite-parasite interactions between *Leucocytozoon* parasites are more dynamic than *Plasmodium* and *Haemoproteus* host-parasite and parasite-parasite interactions. Ninety-three percent of birds remained infected with the same *Plasmodium* or *Haemoproteus* lineage at subsequent capture, while only 43.8% of birds retained the same *Leucocytozoon* lineage. Parasitaemia of *Plasmodium* infection was dependent on the lineage with which an individual was infected. Individuals infected with lineage SW2 (*Plasmodium polare*) showed higher parasitaemia than individuals infected with SGS1 (*Plasmodium relictum*) (Figure 1). An individual's parasitaemia did not change from one capture to a subsequent capture (paired t-test: 2009-2010: $t_{(24)} = -0.27$, $p = 0.791$; 2010-2011: $t_{(11)} = -0.78$, $p = 0.450$; 2009-2011: $t_{(3)} = -2.25$, $p = 0.110$).

Co-infections
Fourty-four (81.5%) of the infected individuals showed co-infection with either *Plasmodium* or *Haemoproteus* and *Leucocytozoon* at the same time. *Plasmodium* parasitaemia was not affected by co-infection with *Leucocytozoon* (LRT: $\chi^2 = 0.09$, df = 1, $p = 0.759$) and body mass was not significantly affected by concurrent infection with *Plasmodium relictum* lineage SGS1 and *Leucocytozoon* (LRT: $\chi^2 = 0.18$, df = 1, $p = 0.671$).

Discussion
This study examined haemosporidian infection at the individual level in wild free-living great tits across time. Parasite prevalence was high (98%) with only one individual remaining parasite free across both years of its capture. A co-infection rate of 82% was found in recaptured birds. While co-infection can influence parasite dynamics, no relationship was found for the presence of *Leucocytozoon* parasites and *Plasmodium* parasitaemia. The data indicates that *Leucocytozoon* infection is dynamic within a host and between several parasite lineages whilst on the other hand and in agreement with Hasselquist *et al* [25], *Plasmodium* and *Haemoproteus* infection remains constant once a parasite has established itself within a host.

Parasite persistence
The differences observed in the present study with regard to *Plasmodium*, *Haemoproteus* and *Leucocytozoon* lineage turn-over within hosts might be an indication of the differential strategies applied by different parasites when infecting their hosts, and might be explained by two levels of interaction: within-host competition between parasite lineages and host immune defense.

Leucocytozoon parasites in the great tits might not be as effective at competing for resources as *Plasmodium* or *Haemoproteus* parasites, and as a result cannot increase in frequency to such an extent as to gain enough of a competitive advantage over *Plasmodium* parasites to be able to persist within the host's blood in the presence of *Plasmodium*. This would not necessarily imply any transmission disadvantage for *Leucocytozoon*, which might choose to undertake larger-scale sexual reproduction, while *Plasmodium* infection consists of a globally larger number of asexual parasites. This possibility is illustrated by a study on rodent malaria (*Plasmodium chabaudi*) where Taylor *et al* [45] compared infection dynamics of two lineages when inoculated individually into hosts or as co-infections with different initial inoculation frequencies. This was done to test whether competitive interactions between parasites occur in terms of infection of hosts and transmission to vectors. The authors concluded that malaria parasites might have evolved to maximize transmission from mixed-genotype infections, as asexual

Table 1 Haemosporidian lineage observations

Lineage	GenBank accession no.	Morphospecies	*n*
SGS1	AF495571	*Plasmodium relictum* [41]	83
SW2	AF495572	*Plasmodium polare* [42]	9
GRW11	AY831748	*Plasmodium relictum* [43]	1
PARUS1	AF254977	*Haemoproteus majoris* [44]	2
PHSIB1	AF495565	*Haemoproteus majoris* [44]	2
multiple infection	—	*Plasmodium* sp. & *Haemoproteus* sp.	2
PARUS4	AY393795	*Leucocytozoon* sp.	11
PARUS16	—	*Leucocytozoon* sp.	4
PARUS18	—	*Leucocytozoon* sp.	1
PARUS19	—	*Leucocytozoon* sp.	16
PARUS22	—	*Leucocytozoon* sp.	16
PARUS25 *	JX855044	*Leucocytozoon* sp.	2
PARUS26 *	JX855045	*Leucocytozoon* sp.	2
PARUS28 *	JX855047	*Leucocytozoon* sp.	1
PARUS34 *	JX855049	*Leucocytozoon* sp.	1
PARUS35 *	JX855050	*Leucocytozoon* sp.	1
PARUS36 *	JX855051	*Leucocytozoon* sp.	1
PARUS37 *	JX855052	*Leucocytozoon* sp.	1
multiple infection	—	*Leucocytozoon* sp.	25

Number of observations (*n*) of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* lineages across three years in great tit (*Parus major*) individuals. Lineages marked with an asterisk (*) have not previously been encountered in the literature.

abundance did not correlate with transmission success in co-infections. This would therefore imply that different parasites involved in co-infections might differentially invest in either asexual or sexual reproduction, thereby ensuring transmission potential to the parasite occurring in lower numbers in the presence of competition. This may, however, hold true only for mixed infection with lineages belonging to the same genus and which share the same vectors.
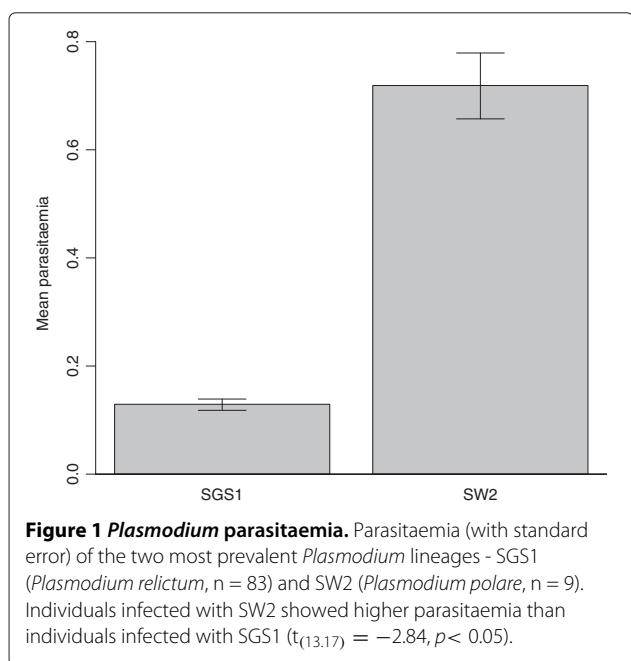
Table 2 *Leucocytozoon* lineages cloned from multiple infections

Lineage	GenBank Accession no.	Number of individuals infected
Lineages also involved in single infections (see Table 1):		
PARUS4	AY393795	2
PARUS16	—	3
PARUS19	—	2
PARUS22	—	5
Lineages involved in multiple infections only:		
PARUS29 *	JX855048	1

Leucocytozoon lineages cloned from a random subset of ten great tits (*Parus major*) showing multiple infections. Only lineages recorded in two or more different colonies per sample were considered. The lineage marked with an asterisk (*) has not previously been encountered in the literature.

Another explanation for the differences in turn-over among lineages could be the host's immune system. Hosts are not passive victims of parasite infections, but actively launch a defensive campaign in the form of the immune system (for a full review on the mechanisms by which haemosporidian parasites evade the host's immune response see [46]). The immune components and defense strategies applied during infection will vary amongst different host species, especially with regard to inflammatory response and adaptive immunity (for a review, see [47]). Little information is available on the evolutionary history of *Leucocytozoon*. One can speculate that in this study system, the bird's immune system might be more competent at fighting off *Leucocytozoon* infection than either *Plasmodium* or *Haemoproteus* infections, indirectly inferring a competitive advantage to the latter and resulting in greater lineage and infection status turn-over.

It must also be noted that the possibility exists that hosts might be able to clear parasites of all genera equally efficiently, but repeat infection probability of *Plasmodium* parasites (which occur in greater abundances than other parasites within the study population) is higher than for *Leucocytozoon* parasites which show a greater lineage diversity, but at lower prevalences. This could result in an artefact illusion that *Plasmodium* infection is more

Figure 1 *Plasmodium* parasitaemia. Parasitaemia (with standard error) of the two most prevalent *Plasmodium* lineages - SGS1 (*Plasmodium relictum*, n = 83) and SW2 (*Plasmodium polare*, n = 9). Individuals infected with SW2 showed higher parasitaemia than individuals infected with SGS1 ($t_{(13.17)} = -2.84, p < 0.05$).

persistent. This possibility could be tested by serial sampling throughout the season.

Lineage specific differences

The general life-cycle and process of Haemosporida infection are well-known and accepted as a whole [8], but very little is known about the exact infection dynamics within hosts. Parasitaemia experienced by great tits in this study was lineage dependent, with SW2 infection resulting in higher parasitaemia than SGS1. This is interesting, as Valkiūnas [8] reports that infection with *P. polare*, which is the morphospecies to which SW2 belongs, always results low parasitaemia. This observed discrepancy with the literature could point to sampling time coinciding with peak parasitaemia in SW2, but at this time no other explanation can be given. Such lineage-specific differences between parasites in parasitaemia as observed in this study is in concurrence with Zehtindjiev *et al* [48] who found that prepatent period, peak parasitaemia and parasitaemia during chronic infection differed by parasite lineage in great reed warblers (*Acrocephalus arundinaceus*). Comparably, Palinauskas *et al* [49] found that pre-patent period and peak parasitaemia also differed among firstly, different host species and secondly, individuals within the same species experimentally infected with the same parasite lineage. It therefore appears that infection dynamics are idiosyncratic and can depend on many factors and their interactions. One such confounding factor influencing infection dynamics is that hosts face the onslaught of many parasites at the same time, and rarely have to cope with single infections in nature, i.e. within-host competition as a result of mixed infections.

Co-infection

The data from this study show that co-infections of malaria parasites are common, and our estimate in the great tit of 82% is consistent with the high frequencies of infection with multiple parasites in natural populations of other European bird species reported by Valkiūnas *et al* [50]. Despite this high prevalence of co-infections, there does not appear to be a negative effect of co-infection on *Plasmodium* parasitaemia. This could be evidence for a more synergistic/benign type of interaction as summarized in [33], where a parasite induces prolonged infection or better establishment of another parasite. In addition, the possibility that the latter could influence *Leucocytozoon* parasitaemia, cannot be excluded, as this was beyond the scope of this investigation. Another important consideration is that, while this study only considers a co-occurrence of parasites, effects might vary with the number of parasites involved in co-infections.

The large number of *Leucocytozoon* multiple infections observed, as opposed to multiple *Plasmodium* and *Haemoproteus* infections, might indicate *Leucocytozoon* parasites to be opportunistic parasites which can only establish themselves once a host's immune system is weakened by prior infections thereby facilitating their establishment. This is in accordance with Cornet and Sorci [51] who investigated the virulence consequences of parasite-induced immunosuppression on the risk of contracting opportunistic diseases in *Gammarus pulex*. Their study suggests that parasite exploitation of the host depends on the risk of contracting opportunistic diseases. Parasites should therefore limit their suppression of the host's immune response if opportunistic infections will result in host death, but should not have to change their exploitation if the risk associated therewith is low. Accordingly, opportunistic *Leucocytozoon* lineages might not impose significant virulence costs to their hosts so as to allow for infection opportunity. As *Leucocytozoon* parasitaemia was not tested in this study, it is impossible to know if multiple *Leucocytozoon* infections result in higher parasitaemia than single infections and is a definite avenue for future investigation. If this is a case of facilitated, opportunistic infection, it would be evidence that not all lineages have equal infection potential.

Conclusion

Most studies only consider *Plasmodium* and *Haemoproteus* parasites when investigating avian haemosporidian infections and as a result *Leucocytozoon* is underrepresented in the literature. This study has focussed not only on *Plasmodium*, *Haemoproteus* and *Leucocytozoon* infection across time at the individual scale, but also considered multiple infections of these parasites and what that might imply for Haemosporida coevolution with their hosts. This study has demonstrated the complexities

of interactions (host-parasite and parasite-parasite) and dynamics of parasite communities and their hosts in natural study systems which could affect the ecology of either or both organisms involved.

Authors' contributions
PC and OG participated in the design of the study, assisted with data collection and manuscript drafting. FL contributed to data collection and analyses. JR collected data, performed molecular analyses, statistical analyses and drafted the manuscript. All authors read and approved the final manuscript.

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