Co- and mixed-infections of avian haemosporidian parasites in great tits and blue tits of the French Pyrenees

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Thesis Abstract

Avian haemosporidia have played a significant role in advancing our understanding of vectored disease epidemiology and continue to do so. These diverse blood parasites (inclusive of those responsible for avian malaria) are globally distributed, adopt both host-generalist and host-specialist infection strategies, and can be highly pathogenic. Due to the diversity of haemosporidia, many avian populations play host to species rich communities of blood parasites. Interactions between parasites coinfecting a single host can lead to increased pathological costs, between-parasite competition may additionally influence spatial and/or temporal parasite distributions. However, many questions remain regarding the implications of these parasite-parasite interactions and their relevance in nature. In this thesis, I study three genera of avian haemosporidia (*Haemoproteus*, *Leucocytozoon*, and *Plasmodium*) which form a community infecting two geographically overlapping host species, blue tits (*Cyanistes* caeruleus) and great tits (*Parus major*). In chapter one, I apply a novel-PCR detection method, which improves the sensitivity of such techniques, to survey the complete local haemosporidian community infecting these birds. *Leucocytozoon* is the most prevalent genus, infecting >90% of individuals. I identify a significant negative association between *Leucocytozoon* and *Haemoproteus spp.,* which exists despite similar spatial distributions. An interaction is also identified between two clades of *Leucocytozoon*, Clade A and Clade D, as mixed-infections are lower than predicted. In chapter two, I further explore this *Leucocytozoon* clade interaction. Clade A is more prevalent in great tits, while Clade D more prevalent in blue tits, neither impact reproductive measures in their typical host. Clade A has lower prevalence in blue tits, but does carry reproductive costs. Blue tits which have Clade D infections are less likely to be infected by Clade A, which implies a conferred resistance against this great tit-typical parasite. Collectively, these studies reveal complex interactions occurring within a multi-host, multi-parasite community which have important implications for haemosporidia epidemiology and parasite-host co-evolution.

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Thesis Introduction

Parasites are agents of some of the world's deadliest infectious diseases in both human and wildlife populations. Haemosporidian blood parasites cause malaria (*Plasmodium spp.*) and are alone responsible for over 400,000 deaths each year (World Health Organisation 2016). While related parasites in avian populations, have created conservation crises and driven species extinctions (Wikelski et al. 2004, LaPointe et al. 2012, Atkinson et al. 2014). Our ability to alleviate the burden of parasitic disease, in any animal population, necessitates a comprehensive understanding of said parasites. Malaria presents an illustrative case; prior to the 1870's the agents of this disease were unknown and their complex lifecycle, which requires a developmental stage in a dipteran vector, had yet to be elucidated (Cox 2010). Indeed, the very name 'malaria' now lasts as tribute to this historical ignorance, stemming from the Italian 'mala aria' (bad air), a reference to the sulphurous marsh odours formerly held responsible (Reiter 2000). It took the work of Ronald Ross, among others, to conclusively demonstrate mosquitoes' key role in malaria transmission, a discovery which facilitated the life-saving expansion of practical prophylactic measures (Dobson 1999, Cox 2010). Our understanding of parasite lifecycles has advanced considerably since the mid-18th century, with drugs now developed to disrupt specific parasitic developmental stages, and in turn, epidemiological research focuses have broadened (Pullan & Brooker 2008, Gilson et al. 2017).

Recent considerations have expanded to the ecology of parasites within their host environments. One complexity of said environment is the presence of co-infecting parasites (Petney & Andrews 1998). Parasite-parasite interactions, and their influence on epidemiology, have now begun to receive greater attention (Read & Taylor 2001, Pedersen & Fenton 2007, Pullan & Brooker 2008, Johnson et al. 2015). Hosts are rarely infected by a single parasite genotype, instead infections may consist of multiple strains of the same parasite (e.g. multiple clones of *Plasmodium falciparum*), and/or consist of different parasite species (e.g. *P. falciparum* infection alongside helminths) (Kinung'hi et al. 2014). Interactions between co-infecting parasites can arise for various reasons (Poulin et al. 2003, Lello et al. 2004, Knowles et al. 2013). One cause stems from the sharing of limited host resources, here competition may arise and consequently reduce the

growth of the inferior competitor (Mideo 2009). Thus, parasite-parasite interactions can act as drivers to alter both infection costs (e.g. if the superior competitor is less virulent) and parasite distributions, as transmission of the outcompeted parasite is impacted (Read & Taylor 2001). Changes in these factors have clear implications for disease outcomes and control strategies. Furthermore, these interactions are likely to act as selection pressures, influencing the course of co-evolutionary relationships between parasites and their hosts, including selection for disease virulence (Choisy & de Roode 2010, Alizon et al. 2013). Therefore, the importance of understanding these interactions cannot be overstated, it is a vital component of a more complete ability to tackle devastating infectious diseases.

Avian haemosporidia cause considerable conservation challenges and burden life-stock production (Valkiunas 2005). All three of the best-studied genera cause pathology in their hosts; *Haemoproteus* (with chronic costs (Knowles et al. 2010, Martínez-de la Puente 2010, Asghar et al. 2015)), *Leucocytozoon* (particularly pathogenic in fowl (Ruff 1999)), and *Plasmodium* (introduction to Hawaii has devastated the endemic avifauna (van Riper et al. 1986)). Historically, avian malaria has played a key role in our understanding of blood parasites, it was in birds that many of the details of haemosporidian lifecycles were first identified (Valkiunas 2005, Cox 2010). Studies of haemosporidia in avian hosts continue to make generalisable contributions to parasite epidemiology (Pigeault et al. 2015). Within hosts, both mixed infections comprised of clonal species, and co-infections of multiple species (often of different genera) are common (Valkiunas 2005). Widely distributed host generalists such as *Plasmodium relictum*, come into frequent co-occurrence with host specific haemosporidia (Bensch et al. 2009). Combined with diverse localised haemosporidia communities (Hellgren et al. 2009), the potential for interactions between these parasites is highly probable. Experimental infections of mixed *Plasmodium spp.* have revealed increased host-species specific pathology, such as increased parasitemia and mortality (Palinauskas et al. 2011). Studies exploring the costs and ecological consequences of co-infections in natural populations are few and far between, and often contradictory (Evans & Otter 1998, Sanz et al. 2001, Marzal et al. 2008, van Rooyen et al. 2013b). Marzal et al. (2008) reported an association between *Haemoproteus* infection and decreased body conditions in house martins

(*Delichon urbica*), primarily driven by birds harbouring more than one lineage (unique mitochondrial cytochrome *b* type) of *Haemoproteus*. While between-genera interactions have been detected in Alaskan birds, where *Leucocytozoon* and *Haemoproteus* were negatively associated (the authors did not attribute this to environmental variables), raising the possibility of a broad competitive association between genera (Oakgrove et al. 2014).

Over the past three years we have collected samples with aim to explore the haemosporidia community in two geographically overlapping host species, blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*). These two species share many ecological similarities (Morse 1978, Minot 1981). Yet, despite overlapping geographical ranges, both host-species specific and host generalist haemosporidia have been reported between these hosts (Wood et al. 2007, Jenkins & Owens 2011, Glaizot et al. 2012, van Rooyen et al. 2013, Jenkins et al. 2015). For this reason, they serve as a valuable study system in which to explore host-specific parasite-parasite interactions, where to some degree, significant host-specific ecological variables (e.g. variations in nesting sites) can be ruled-out. Previous screening carried out in these populations has indicated that *Leucocytozoon spp.* have high prevalence, providing an opportunity to expand understanding of a much under-studied genus (Hellgren et al. 2004). Representatives of both *Plasmodium* and *Haemoproteus* are also present and determining interactions within these haemosporidian communities will be illustrative of the importance of co-infections for parasite epidemiology.

Included in this thesis are two chapters which share a unified aim: to identify and evaluate between-parasite interactions within these host populations. Chapter one (pg. 9) provides background to the study system by describing the complete haemosporidia community across both host species. It specifically asks whether there are host-specific differences in haemosporidia distributions and, with a focus on parasite genera, whether interactions occur between these parasites. Chapter two (pg. 26) delves further into between-host and parasite-parasite interactions by exploring how lineages of the most prevalent genera, *Leucocytozoon*, can interact to alter host pathologies and population distributions. Here a focus is placed on parasite-conferred resistance, an interesting concept largely unexplored in natural populations. In concluding, our findings will be reviewed in the wider context of avian haemosporidian research

and parasite-host co-evolutionary theory, illustrating the importance and relevance of this research.

Chapter One

Co- and mixed-infections of avian haemosporidian parasites in great tits and blue tits

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Abstract

Parasite-parasite interactions occurring within hosts can have a significant impact on parasite virulence, distribution, and disease outcomes. Avian haemosporidia are a diverse group of blood parasites, which typically co-infect multiple, geographically-overlapping hosts within their ranges. Our understanding of the impact of co-infections on their avian hosts remains, however, incomplete, in large part because it can be technically challenging to detect all the parasite lineages present within a host. Here we develop a novel-PCR detection method of avian haemosporidia (genera *Haemoproteus, Leucocytozoon,* and *Plasmodium*) to determine parasite prevalence in two co-occurring host species, *Cyanistes caeruleus* (N = 529) and *Parus major* (N = 443), over three-years. The prevalence of *Leucocytozoon spp.* was highest, reaching >90% in the two hosts, with the majority of infections involving more than one *Leucocytozoon* lineage. Furthermore, *Leucocytozoon spp.* displayed marked host specificity and we detected evidence of negative associations between *Leucocytozoon* clades, as well as between *Leucocytozoon* and *Haemoproteus spp*. These patterns of association, however, failed to be explained by either within- or between-genera spatial differences in elevational distributions. Our results highlight a number of within-host haemosporidian interactions. Understanding the causes and consequences of these interactions will help inform both the study of disease epidemiology and the co-evolutionary relationships between haemosporidian parasites and their avian hosts.

Introduction

Understanding how parasites interact within their hosts can provide vital insight into disease epidemiology (Alizon et al. 2013, Wuerthner et al. 2017). Typically, the host environment is inhabited by a cast of co-infectors, which give rise to parasite-parasite interactions within the host (Read & Taylor 2001, Poulin 2001, Poulin et al. 2003, Rigaud et al. 2010, Telfer et al. 2010). These interactions can manifest through direct competition and/or indirect antagonistic activity (e.g. activation of host immune mechanisms) (Graham 2008, Mideo 2009), and can therefore have downstream repercussions for the interaction with the host (Forbes et al. 1994, Read & Taylor 2001, Poulin et al. 2003, Lello et al. 2004, Telfer et al. 2010, Knowles et al. 2013). For example, children infected with both malaria and helminths were shown to have increased anaemia compared to those infected with malaria alone (*Plasmodium falciparum*) (Kinung'hi et al. 2014), indicating that parasite virulence can increase when there are co-infections. Interactions between parasites are therefore predicted to have important implications for the ecology of both host and parasite species, which will likely in-turn influence their co-evolutionary trajectories (Rigaud et al. 2010, Alizon et al. 2013).

Between-parasite interactions can impact both the distribution and/or the virulence of the parasites concerned. For example, parasites exploiting a shared host environment may be facilitative, when one parasite alters this environment to the benefit of other co-infectors (Lello et al. 2004). Conversely, exploitative competition can lead to reductions in parasite prevalence (Knowles et al. 2013). As a result, both negative and positive associations can be found in closely related parasite communities (Lello et al. 2004), as well as between parasites belonging to different domains (Telfer et al. 2010). Where between-parasite competition for resources occurs, theory often predicts an increase in virulence (Alizon et al. 2013). These predictions have received support from studies of clonal malaria infections in mice (infections comprised of different lineages of the same haemosporidian species): Bell et al. (2006) found that the most virulent lineages of *Plasmodium chabaudi* gain a competitive advantage and suppress their less virulent co-infecting lineages. In contrast, cooperation may arise between parasites when their interests overlap (Poulin et al. 2003). In larval helminths infecting crabs, cooperating to alter the host phenotype may be more

beneficial, as the collective alteration of crab neural activity is thought to help these parasitic species reach their terminal hosts (Poulin et al. 2003). Such parasite-parasite interactions are, however, only revealed through the comprehensive survey of the host's parasite community (Telfer et al. 2010, Hellard et al. 2015), and are most likely to occur between parasites known to concurrently infect their hosts.

Avian haemosporidia (encompassing the causal agents of avian malaria) are a species-rich assemblage of blood parasites, which often simultaneously infect individual birds (van Rooyen et al. 2013b, Oakgrove 2014). The three most studied genera of haemosporidia are: *Haemoproteus*, *Leucocytozoon*, and *Plasmodium* (Bensch et al. 2009). Species of all three infect similar host tissues, exploiting and destroying host erythrocytes through successive bouts of asexual reproduction and gametogenesis (Valkiunas 2005). Within the last two decades, advances in molecular detection methods have enabled a boom in the study of haemosporidian ecology (Bensch et al. 2000, Hellgren et al. 2004, Waldenström et al. 2004, Bensch et al. 2009). Their overlapping exploitation of host populations, the relative ease with which they can be detected, and the ability to closely monitor their host populations, position these parasites as ideal candidates to explore parasite-parasite interactions and their influence on the ecology and evolution of both parasites and hosts. Additionally, the cost of the diseases these parasites induce (particularly severe in host populations lacking previous exposure (van Riper et al. 1986)) provides a further imperative to understand how complex infections might alter disease outcomes.

Studies of haemosporidia communities have already provided evidence of between-genera interactions, which can take the form of apparent distributional exclusion in either time (Hatchwell et al. 2000) or space (Forbes et al. 1994, Oakgrove et al. 2014). Additionally, genetic divergence of parasite species infecting the same host species suggests niche adaptations driven by interspecific interactions (Pérez-Tris et al. 2007). Competitive interactions have been found between *Plasmodium* species within a shared vector; for instance, *P. juxtanucleare* reduced the ability of *P. gallinaceum* to complete fertilisation (Paul et al. 2002). When mixed infections of haemosporidia do occur, however, they can result in an increase in virulence and a worsening of the host's prognosis, as found in mixed-infections of avian *Plasmodium spp*. (Palinauskas et al. 2011).

Parasite-parasite interactions within the haemosporidian community may therefore either impact within-host infection dynamics or host population-level parasite distributions.

To study these interactions, we surveyed haemosporidian parasites in populations of great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) breeding on an elevational gradient in the French Pyrenees. These birds have long been used as model species in a range of ecological and behavioural studies, although the ecology of their blood parasites has only recently received greater focus (Wood et al. 2007, Jenkins & Owens 2011, Glaizot et al. 2012, van Rooyen et al. 2013, Jenkins et al. 2015). This wealth of host data and their overlapping habitat use, makes them a valuable system within which to explore the distribution and interactions of their haemosporidian communities. Existing molecular detection methods, which amplify a region of the parasite's mitochondrial genome, are efficient at detecting *Plasmodium* and *Haemoproteus* (Hellgren et al. 2004, Bensch et al. 2009, Pérez-Rodríguez et al. 2013a), however non-target amplification has been reported to occur where *Leucocytozoon* parasites also prevail (Cosgrove et al. 2006, Cosgrove et al. 2008).

In our study system, *Leucocytozoon spp.* prevalence is known to be high, making difficult the detection of *Plasmodium* and *Haemoproteus*. To address this issue, we designed a new amplification method aimed at specifically detecting *Plasmodium* and *Haemoproteus* infections to the exclusion of *Leucocytozoon* infections. Using this novel method, in conjunction with a previous method designed for *Leucocytozoon* amplification (Hellgren et al. 2004), allowed us to survey the avian haemosporidian community with a focus on three areas of interest. First, we described the prevalence of haemosporidian parasites within our two host species to identify host-specific and host-generalist parasites. Second, we identified interactions between the three parasite genera, as well as within-genera interactions between parasite species of the most common genus, *Leucocytozoon*. Third, we tested the extent to which negative association between parasites may be explained by elevation. Because altitude can here be used as a proxy for climatic differences, insights into the distribution of haemosporidian interactions as a function of elevation will improve our predictions of the impacts of climate change on infection (Valkiunas 2005, Altizer et al. 2013).

Methods

Study populations and sampling

We captured adult great tits (443 individuals) and blue tits (531 individuals) in the west of the Pyrénées Ariégeoises National Park in France. Four nest box populations have been established within 14km of one another and cover an elevational range from 430m to 1530m. The landscape is predominantly mixed deciduous woodland, interspersed by small patches of conifers and open fields used for low-intensity pastoral farming. These sites, their individual elevation ranges, contemporary nest box numbers, and positions are as follows: Moulis (430 – 593m; 159 boxes; 42°57'90"N-42°58'36"N, 01°05'31"E-01°05'73"E), Cescau (549 – 1091m; 209 boxes; 42°55'34"N-42°56'46"N, 01°02'47"E-01°03'47"E), Galey (821 – 1193m; 105 boxes; 42°56'64"N-42°57'24"N, 00°54'13"E-00°55'30"E,), Castera (1058 – 1530m; 147 boxes; 42°53'74"N-42°55'07"N, 01°05'40"E-01°03'43"E).

Birds were captured and banded at these sites during the spring (May and June) and autumn through to winter (October - March). During breeding, birds were captured in their nest boxes, whilst mist nets were deployed near seed feeders throughout the rest of the year. Individuals used in this study were captured between May 2015 and May 2017 and therefore this data encompasses three breeding seasons and two overwintering periods. On capture, morphological data was recorded (sex, age, and body mass) and a blood sample was obtained by brachial venapuncture (approximately 35µl). Blood samples were collected into sodium heparinized micro-hematocrit capillaries (Hirschmann Laborgeräte, Germany) and then transferred to centrifuge tubes pre-filled with ~1ml of 96-100% ethanol. Weight was obtained using an electric scale accurate to 0.01g. Sex was determined based on plumage characteristics and the presence or absence of the brood patch. Because they are less sexually dimorphic, the sex of blue tits was not recorded for individuals captured outside of the breeding season. Individuals were categorised into either of two age classes: as yearlings or as post-second year adults based on the pigmentation of the primary coverts. On first capture, all individuals were fitted with a unique 7-digit metal identification

ring and coloured band combination. This allowed for the recording of recaptured individuals.

Molecular analysis

We extracted DNA from our blood samples using the DNeasy Blood & Tissue extraction kit (QIAGEN®) following the manufacturer's protocol pertaining to nucleated blood. We standardised these extractions to working concentration of 25ng/µl. To detect the presence of haemosporidians, each sample was subjected to two nested-polymerase chain reaction (PCR) methods which each target a specific region of the blood parasites' mitochondrial cytochrome *b* gene.

To amplify the DNA of *Leucocytozoon spp.*, we followed the protocol developed by Hellgren *et al.* (2004) which used the external primers HaemNFI (5'-CATATATTAAGAGAAITATGGAG-3') and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3') in the first reaction round, followed by the internal primer pair HaemFL (5'-ATGGTGTTTTAGATACTTACATT-3') and HaemR2L (5'-CATTATCTGGATGAGATAATGGIGC-3') in the second round.

To amplify *Plasmodium* and *Haemoproteus* DNA, we designed new primers based on published sequences of the most common *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* species previously detected in our population and host species (Accession numbers: *P. relictum* (HM031937), *P. circumflexum* (JN164734), *H. majoris* (JN164727), and *Leucocytozoon spp.* (EU627797, FJ168563, & KX832559). These primers were designed to be as specific to *Plasmodium* and *Haemoproteus* sequences as possible, whilst still encompassing the region of the apicomplexan's mitochondrial cytochrome *b* gene now widely used to classify these parasites (Bensch et al. 2009). We adopted this approach as our previous application of *Plasmodium*- and *Haemoproteus*-specific primers (as found in Hellgren et al. (2004)) had led to unintentional amplification of *Leucocytozoon* DNA. This issue has been reported elsewhere (Cosgrove et al. 2006) and is likely most noticeable in our study population due to the very high prevalence rates of *Leucocytozoon* species (>90%). We concur with Cosgrove et al. (2006) that this unintended targeting of *Leucocytozoon* mitochondrial DNA is most probably made possible by the lack of specificity in the first-round forward primer (HaemNFI) and second round reverse primer (HaemR) - which must then act in concert in the second round and enable

amplification. As it is now widely used for comparative and classification purposes, we aimed to target the same region of the mitochondrial cytochrome *b* gene, however this imposed restrictions on our choice of primers. Our solution has been to use two reverse primers considerably more specific to *Plasmodium* and *Haemoproteus* species, whilst the two forward primers are perhaps less so. But this allows that no combination of forward and reverse primer could result in non-target amplifications, and since applying this technique none have been observed.

For the *Plasmodium* and *Haemoproteus* targeted nested-PCR, we used Plas1F (5'-GAGAATTATGGAGTGGATGGTG-3') and newly designed Plas1RP (5'-TACTCTCTTGCACCAAAAGC-3') as the external primer pair in the first-round, amplifying a 706bp region (excluding primers) of the parasite's mitochondrial cytochrome *b* gene. HaemFP (5'- ATGGTGTTTTAGATATATGCATG-3') and newly designed HaemRP (5'-ATGTAAAGGAGTAGCATATCTATC-3') act as the internal primers for the second-round, amplifying a 512bp region nested within the first amplicon. Both rounds of this nested-PCR reaction were carried out in volumes of 25µl. In the first round 25ng of total genomic DNA was used and the reagent proportions were as follows: 1X DreamTaq Buffer (Thermo Fisher Scientific), 0.4mM of each deoxynucleoside triphosphate, 0.56µM of Plas1F and 0.04µM of Plas1RP, and 0.625U DreamTaq DNA polymerase (Thermo Fisher Scientific). In the second round 1µl of first round product was used with the reagent proportions: 1X DreamTaq Buffer (Thermo Fisher Scientific), 0.4mM of each deoxynucleoside triphosphate, 0.6µM of both HaemFP and HaemRP, and 0.625U DreamTaq DNA polymerase (Thermo Fisher Scientific). PCRs using the primer pair Plas1F/Plas1RP used the thermal cycle conditions: 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 45 seconds, for 20 cycles. PCRs using the primer pair HaemFP/HaemRP used the cycle conditions: 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds, for 35 cycles. Prior to the cyclic reactions for both PCR profiles, samples were incubated at 94° for 3 minutes, and then incubated at 72°C for 10 minutes at the end of the cyclic reaction.

Reactions were carried out in Applied Biosystems Veriti™ Thermal Cyclers and run with negative controls (sterile Milli-Q water, 1/10 samples). Each reaction run included at minimum one positive control (DNA of verified positive infection

status). Second-round PCR products were separated on 2% agarose gels containing RedSafe™ Nucleic Acid Staining Solution (20,000x) (iNtRON Biotechnology Inc.) and ran at 100V for 60 minutes before visualising under UV. An approximately 500bp (550bp for *Plasmodium*/*Haemoproteus* PCRs) band indicated positive amplification and the presence of haemosporidians. Individuals with negative results were passed through the PCR process again to verify their status.

Nested PCR products which displayed successful amplification were purified in preparation for sequencing. Clean-up reactions used Exonuclease 1 and Antarctic Phosphatase (New England BioLabs®) to degrade unutilised primers and hydrolyse excess dNTPs. Reactions were in volumes of 15µl, containing: 12µl of PCR round-two product, 0.9µl MilliQ water, 0.5µl Antarctic Phosphatase (2.5U), 1.5µl Antarctic Phosphatase Buffer (10X), and 0.1µl Exonuclease 1 (1U). This mixture was incubated at 37°C for 40 minutes, heated to 80°C for 10 minutes, and then cooled to 4°C. Cleaned product was then diluted to nucleic acid concentrations of approximately ≤10ng/µl, 5µl of which was sequenced using the primers HaemFL (for *Leucocytozoon* amplicons) or HaemRP (for *Plasmodium*/*Haemoproteus* amplicons) (Eurofins sequencing service, Eurofins-MWG).

Sequences were assembled, aligned, and analysed using Geneious (Geneious® 9.1.5, Kearse et al. 2012). Parasite lineages were identified by carrying out a BLAST search on the MalAvi database (Bensch et al. 2009). Sequencing results for *Leucocytozoon* lineages could at times not be completely resolved due to slightly shorter sequence reads or more commonly due to multiple infections preventing decisive identification. In most cases, however, it was possible to identify the clade of lineages to which the lineage or constituent lineages belonged. Multiple infections (infection by more than one lineage of haemosporidian) were identified, as they result in double-peaks on the sequence chromatograms. We identified double-peaks using the Heterozygote Plugin (Geneious® 9.1.5), marking each contentious peak with the appropriate IUPAC ambiguity code. We then compared these sequences to a library of every previously detected haemosporidian lineage within our population and through a process of elimination (exclusion of lineages due to the absence of their sequence signals on the target chromatogram), it was possible to determine the

most parsimonious combination of lineages which would produce the peak pattern observed (Drovetski et al. 2014). In many cases, although it was not possible to completely resolve these mixed-infections to just two distinct lineages, we could still identify the presence or absence of entire lineage clades common in our populations.

Phylogenetics and Statistical Analysis

A phylogenetic analysis was carried out to determine the relatedness and describe the diversity of the lineages amplified within our populations. All verified lineages were included and sequences trimmed and standardised to 390bp. *Plasmodium falciparum* (a human malarial parasite (Accession no. AY282930]) was used as the outgroup. To generate this phylogeny a Bayesian reconstruction was performed using the MrBayes plugin (Ronquist & Huelsenbeck 2003) in Geneious and a GTR + I + G substitution model (recommended for our alignment by JModelTest (Darriba et al. 2012)). Two runs were conducted, both of 10 million generations and with sampling set to every 200 generations. Following a 'burn-in' of 25% the remaining trees were used to calculate posterior probabilities. This approach is used elsewhere in studies of avian haemosporidia (Jenkins & Owens 2011, Oakgrove et al. 2014).

All statistical analyses were conducted using RStudio v0.99.902 (RStudio Team 2017). Due to the disparity in the number of recaptured birds compared to individuals captured only once, we excluded additional data for recaptured birds at random, such that individuals only had one record of capture. We tested for interactions between parasite genera using a logistic regression (logit function) with infection status by either *Haemoproteus* (*H. majoris* lineages), *Plasmodium* (*P. relictum* lineages), or *Leucocytozoon*, as response variables. Infection status of the other genera, host age, species, capture elevation, and the interaction term between species and elevation were included as explanatory terms. Great tits and blue tits were analysed together and terms were dropped if it improved model fit. We also compared *Leucocytozoon* mixed infection prevalence's (infection by more than one *Leucocytozoon* lineage). We identified individuals with mixed *Leucocytozoon* infections determined to clade and compared these to predicted prevalence probabilities (calculated from the prevalence rates of the constituent

clades, Table 1), and applied chi-squared tests to expected vs. observed prevalence rates.

Results

Haemosporidian prevalence, diversity and host specificity

In this study, we screened 972 samples from adult great tits (N = 443) and blue tits (N = 529). Haemosporidian infection prevalence was high in both species (98% for great tits and 96% for blue tits: Table 1). *Leucocytozoon* parasites constituted the majority of these infections, >90% of individuals in both species. *Plasmodium* infections mostly involved *P. relictum* and were the second most prevalent, more so in great tits (32%) than in blue tits (16%) *Haemoproteus* prevalence reached 7% in the two host species, with all lineages attributed to the species *H. majoris*. In all, we detected 27 different lineages (23 in great tits and 26 in blue tits), including 20 *Leucocytozoon*, 4 *Plasmodium* and 3 *Haemoproteus* (Figure 1). We identified and verified 3 novel *Leucocytozoon* lineages. *Leucocytozoon* lineages clustered into 2 clades and 2 paraphyletic groups (Figure 1 & Table 1), with within-group pairwise sequence identity ranging from 97.9%-99.5%. We named these groups Clade A – D, and while the utility of these classifications may not extend beyond our populations, they allow us to further our understanding of parasite-parasite interactions; note that closely related lineages can often represent within-species variation and not distinct species (Križanauskienė et al. 2010, Palinauskas et al. 2017).

Interactions between haemosporidian genera and distributions

Haemoproteus-Plasmodium co-infections were rare (great tits = 4, blue tits = 2). A significant negative association was detected between *Haemoproteus* and *Plasmodium* parasites, and infection by either was found to be associated with a decreased probability of infection by the other (Table 2).

Table 1. Great and blue tit prevalence rates of all haemosporidian lineages detected

		Host Species				
		Great tits (443)		Blue tits (529)		Total
Lineage	**Species**	**No.**	**As %**	**No.**	**As %**	**No.**
PARUS1	*H. majoris*	20	4.5%	34	6.4%	54
WW2	*H. majoris*	10	2.3%	3	<1%	13
PHSIB1	*H. majoris*	1	<1%	0	<1%	1
H. majoris	-	31	7%	37	7%	68
Haemoproteus	-	31	7%	37	7%	68
TURDUS1	*P. circumflexum*	3	<1%	3	<1%	6
BT7	*P. circumflexum*	0	<1%	1	<1%	1
GRW11	*P. relictum*	7	1.6%	9	1.7%	16
SGS1	*P. relictum*	82	18.5%	43	8.1%	125
P. relictum	-	138	31.2%	79	14.9%	217
Plasmodium	-	140	31.6%	82	15.5%	224
PARUS20	*Leucocytozoon sp.*	26	5.9%	17	3.2%	43
PARUS21	*Leucocytozoon sp.*	2	<1%	1	<1%	3
PARUS22	*Leucocytozoon sp.*	289	65.2%	101	19.1%	390
*PARU01**	*Leucocytozoon sp.*	1	<1%	1	<1%	2
*PARU02**	*Leucocytozoon sp.*	3	<1%	2	<1%	5
Clade A	*Leucocytozoon sp.*	311	70.2%	121	22.9%	432
PARUS19	*Leucocytozoon sp.*	2	<1%	1	<1%	3
PARUS25	*Leucocytozoon sp.*	5	<1%	3	<1%	8
PARUS74	*Leucocytozoon sp.*	0	<1%	1	<1%	1
Clade B	*Leucocytozoon sp.*	65	14.7%	14	2.6%	79
PARUS4	*Leucocytozoon sp.*	2	<1%	5	<1%	7
PARUS16	*Leucocytozoon sp.*	117	26.4%	1	<1%	118
PARUS17	*Leucocytozoon sp.*	7	1.6%	0	<1%	7
PARUS18	*Leucocytozoon sp.*	9	2.0%	13	2.5%	22
PARUS33	*Leucocytozoon sp.*	9	2.0%	2	<1%	11
PERATE06	*Leucocytozoon sp.*	1	<1%	3	<1%	4
Clade C	*Leucocytozoon sp.*	300	67.7%	157	29.7%	461
PARUS11	*Leucocytozoon sp.*	2	<1%	9	1.7%	11
PARUS12	*Leucocytozoon sp.*	0	<1%	17	3.2%	17
PARUS13	*Leucocytozoon sp.*	1	<1%	79	14.9%	80
PARUS14	*Leucocytozoon sp.*	1	<1%	115	21.7%	116
PARUS15	*Leucocytozoon sp.*	3	<1%	14	2.6%	17
*CYCA01**	*Leucocytozoon sp.*	0	<1%	1	<1%	1
Clade D	*Leucocytozoon sp.*	15	3.4%	392	78.4%	407
Leucocytozoon	-	418	94.4%	500	94.5%	918

Lineage names were identified using MalAvi (Bensch et al. 2009). The three novel lineages identified are indicated with an asterisk and italicized. For classifications in bold we provide pooled prevalence rates (e.g. for *P. relictum*), these are totals including infections which could not be confidently identified to lineage but which could be correctly identified at that classification level.

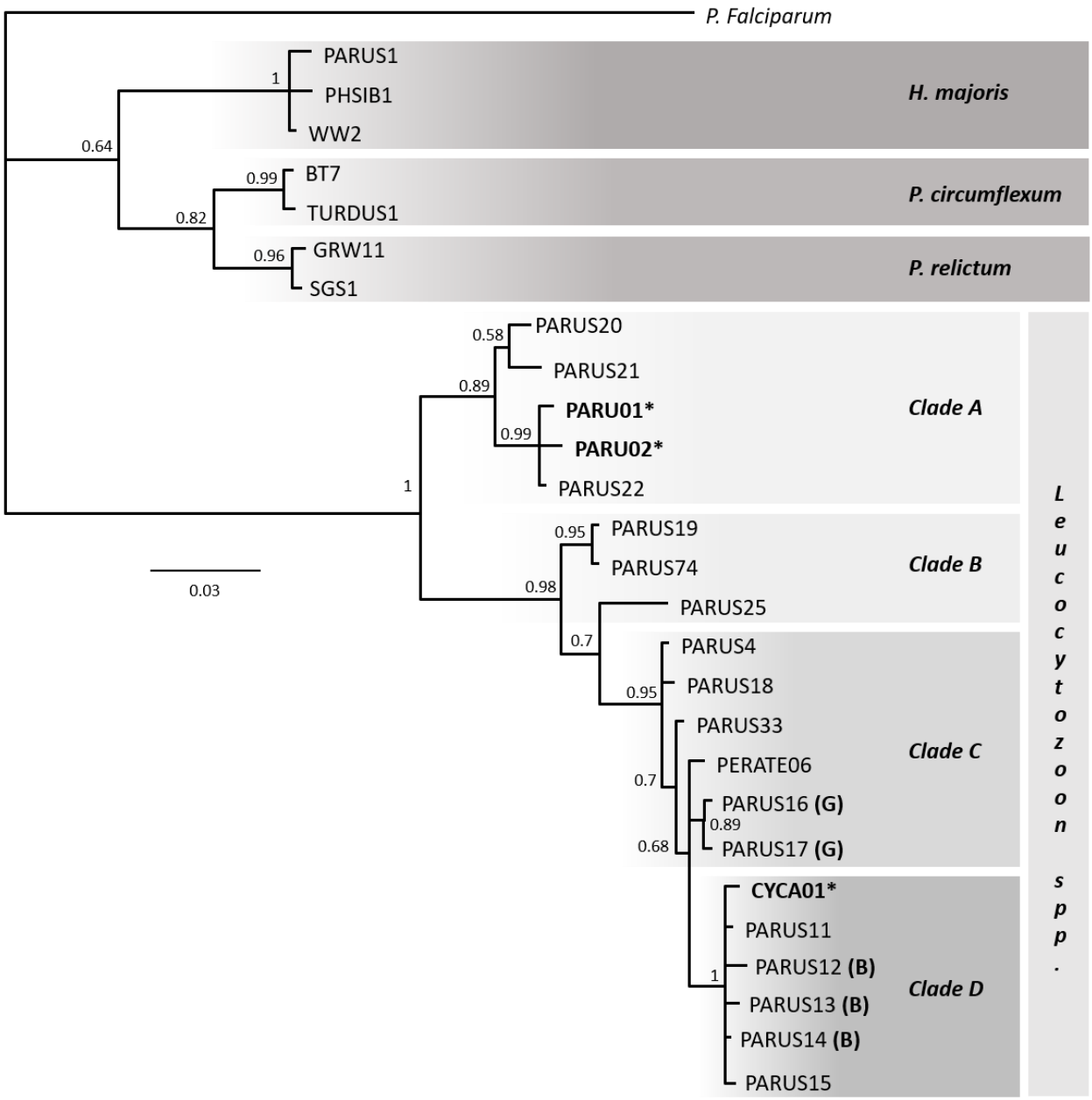

Figure 1. Consensus tree of all haemosporidian lineages created through Bayesian analysis. Species names are provided in italics where known, posterior probabilities shown on branches. Novel lineages are indicated in bold with asterisks. We indicate host preference with either **(B)** for blue tits or **(G)** for great tits when lineages were found predominately in one host species (>95%) (lineages with just one record of detection were excluded from this classification). Colour coding highlights the three genera: *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* (Clades A – D), the four 'clades' of *Leucocytozoon* represent groups of lineages, which received strong posterior probability scores and showed considerable within-group sequence similarity.

Additionally, *Haemoproteus* infection probability significantly decreased in the presence of *Leucocytozoon* infections ($\chi^2 = 24.9, p < 0.001$). Of birds infected by *Leucocytozoon spp.*, 6% were coinfected by a *Haemoproteus* lineage, which contrasts with the 28% infection rate of *Haemoproteus* in *Leucocytozoon*-negative birds. No significant interaction was detected between *Plasmodium* and *Leucocutozoon spp*. ($\chi^2 = 0.55, p = 0.46$). Elevation played a significant role in the prevalence of all three genera, although the direction of this effect differed. Infection probability decreased at higher altitudes for birds infected by *Plasmodium* ($\chi^2 = 16.9, p < 0.001$). Whilst for birds infected by *Haemoproteus* or *Leucocytozoon* lineages, infection probability increased at higher elevations (*H*: $\chi^2 = 5.8, p = 0.016$, *L*: $\chi^2 = 4.74, p = 0.03$, Table 2).

Table 2. Logistic regression results from haemosporidian genus interaction analysis

	Term	Estimate	SE	*OR*	*95% CI*	χ^2	*P*
Haemoproteus	***Leucocytozoon***	***-1.96***	***0.36***	***0.14***	***0.07 : 0.29***	***24.9***	***<0.001****
	Plasmodium	***-1.12***	***0.44***	***0.33***	***0.12 : 0.72***	***8.32***	***0.004****
	Age	***0.89***	***0.26***	***2.43***	***1.46 : 4.1***	***11.49***	***<0.001****
	Elevation	***0.001***	***0.0005***	***1.001***	***1 : 1.002***	***5.8***	***0.016****
	Host (Great tit)	0.13	0.27	-	-	0.21	0.64
	Host (GT) * Elevation	0.00005	0.0009	-	-	0.22	0.89
Leucocytozoon	***Haemoproteus***	***-2.004***	***0.35***	***0.13***	***0.07 : 0.27***	***26.8***	***<0.001****
	Elevation	***0.001***	***0.0007***	***1.001***	***1 : 1.003***	***4.74***	***0.03****
	Host (GT) * Elevation	-0.002	0.001	-	-	1.57	0.46
	Host (Great tit)	-0.05	0.29	-	-	0.02	0.88
	Plasmodium	0.38	0.42	-	-	0.86	0.35
	Age	0.23	0.31	-	-	0.56	0.45
Plasmodium	***Haemoproteus***	***-1.24***	***0.44***	***0.29***	***0.09 : 0.68***	***9***	***0.003****
	Age	***0.57***	***0.17***	***1.77***	***1.26 : 2.48***	***10.9***	***<0.001****
	Host (Great tit)	***1.99***	***0.17***	***7.33***	***2.74 : 19.5***	***45.6***	***<0.001****
	Elevation	***-0.0003***	***0.0006***	***1***	***0.998 : 1.001***	***16.9***	***<0.001****
	Host (GT) * Elevation	**-0.001**	**0.0007**	**0.998**	**0.997 : 1**	**3.63**	**0.057**
	Leucocytozoon	0.31	0.4	-	-	0.55	0.46

Infection status with either *Haemoproteus, Plasmodium*, or *Leucocytozoon* serving as the response term. Host indicates host species, GT is great tit, blue tits served as reference class. Terms retained in the final models are indicated in bold, and those significant at the *<0.05* level are italicized and indicated with an asterisk. Odds ratio (OR) provided for significant terms.

Interactions between Leucocytozoon spp.

Leucocytozoon spp. displayed marked host-specificity (Table 1). Mixed infections comprised of lineages from at least two different *Leucocytozoon* clades were found to match predicted prevalence rates for 5 out of the 6 potential interactions (Table 3a & b). The exception involved Clade A and Clade D, where mixed infections with both were significantly lower than predicted in great tits (χ^2 (2) = 5.9, p = 0.01) and in blue tits (χ^2 (2) = 13.7, p <0.001). Clade A showed a higher prevalence in great tits than in blue tits, whilst Clade D was largely blue tit-specific (Table 1).

3a)	Clade A	Clade B	Clade C	Clade D
Clade A	x	-	-	-
Clade B	10.4% *(10.3%)*	x	-	-
Clade C	49% *(47.5%)*	9.3% *(10%)*	x	-
Clade D	**0.7% *(2.4%)***	0.5% *(0.5%)*	2.3% *(2.3%)*	x

3b)	Clade A	Clade B	Clade C	Clade D
Clade A	x	-	-	-
Clade B	0.4% *(0.6%)*	x	-	-
Clade C	6.8% *(6.8%)*	0.6% *(0.8%)*	x	-
Clade D	**11% *(18%)***	1.9% *(2%)*	22.7% *(23.2%)*	x

Table 3a & 3b. Population prevalence rates of mixed *Leucocytozoon* infections in great tits (3a) and blue tits (3b). Values in parentheses are the predicted prevalence rates for mixed-infection prevalence calculated from the population prevalence of each clade. Significant clade associations are in bold (chi-squared test, p < 0.05).

Discussion

Parasite-parasite interactions within the host environment can alter disease outcomes and are by extension likely to influence host-parasite co-evolution. Here, we describe the haemosporidia community in blue tits and great tits of the French Pyrenes and show a high prevalence and diversity of *Leucocytozoon spp.*, which are as result, involved in between-genera and within-genus

interactions. The design of a novel screening method for detecting *Plasmodium*/*Haemoproteus* allowed us to resolve infections that might otherwise have been overlooked. Previous studies (Cosgrove et al. 2006, Cosgrove et al. 2008) have, indeed, reported difficulties in the detection of *Plasmodium* and *Haemoproteus* in host populations with a high prevalence of the sister genus *Leucocytozoon*. Whilst non-target amplification can be detected through sequencing, it often interferes with read quality and additional steps are required to obtain clear chromatograms (Cosgrove et al. 2006). Improving confidence that infection status has been resolved following electrophoresis provides a more economical approach. We found that the application of our new primer pairs ensured that only sequences originating from *Plasmodium* or *Haemoproteus* parasites were amplified in our population.

Leucocytozoon lineages were the most prevalent in both host species and the diversity of this genus was far greater than that of its sister genera. Our classification of these lineages into four clades was primarily made for ease of analysis, although it is likely that at least one clade reflects a cluster of lineages pertaining to a single species. Clade D encompasses 6 lineages, 5 of which (the sixth was novel to this study) have been detected almost exclusively in blue tits and the closely related African blue tit (*Cyanistes teneriffae*) and range from northern Europe to northwest Africa (Jenkins & Owens 2011, Drovetski et al. 2014, Mata et al. 2015). We often find lineages of this clade together in mixed infections, and as between-lineage sequence divergences are low (<1.1%), Clade D may represent within-species variation attributable to a single highly host-specific *Leucocytozoon* species. While we use here the terms species and clade interchangeably, additional morphological and genetic analysis is required to confidently classify clades into species (Hellgren et al. 2004, Sehgal et al. 2006).

The high *Leucocytozoon* prevalence rates in our populations mirror those of other studies, which identify these parasites and their blackfly vectors (Simuliidae) as being well adapted to cooler, more mountainous, habitat (Pérez-Rodríguez 2013b, van Rooyen et al. 2013, Oakgrove et al. 2014, Lotta et al. 2016). This adaptation contrasts particularly with *Plasmodium,* which we found to be significantly less prevalent at higher elevations. *P. relictum*, the most abundant species found at our study sites, is a highly successful host-generalist and

distributed throughout the Old World (Palinauskas et al. 2009). Yet its distribution is perhaps constrained by the developmental temperature tolerances within its mosquito vector (LaPointe et al. 2010, Pérez-Rodríguez 2013b). Furthermore, susceptibility to infection by this parasite is reportedly highly host-specific (Palinauskas et al. 2008), in accordance with the ~50% prevalence difference between blue and great tits.

We detected a negative association between *Plasmodium* and *Haemoproteus*. This result is consistent with previous studies (Hatchwell et al. 2000, Oakgrove et al. 2014) though co-infections between these genera may have been underestimated (Loiseau et al. 2010, Bernotienė et al. 2016). We also detected frequent *Leucocytozoon-Plasmodium* co-infections, but a negative association between *Haemoproteus* and *Leucocytozoon* lineages. While positive associations may be expected given the high prevalence of *Leucocytozoon* in our host populations, negative ones are, as a result, noteworthy. Oakgrove et al. (2014) reported a similar inverse relationship between *Haemoproteus* and *Leucocytozoon* genera across 49 Alaskan bird species, with 52% lower *Haemoproteus* infection rates in *Leucocytozoon* positive individuals. In contrast, no such association was found in studies of single host species, with some even detecting a higher prevalence of co-infection than predicted from individual lineage prevalence (Synek et al. 2013, Dunn et al. 2014, Scaglione et al. 2015, Meixell et al. 2016). This suggests that the antagonistic interaction that we detect between *Haemoproteus* and *Leucocytozoon* may be specific to our local haemosporidia (e.g. *H. majoris*). Such negative association between haemosporidia can be driven by a range of factors; for example, there can be seasonal differences in the prevalence of *Leucocytozoon* and *Haemoproteus*, which coincides with the activity of their different insect vectors (biting midges, *Culicoides sp*., for *H. majoris* and blackflies for *Leucocytozoon spp*.) (Atkinson et al. 1988, Hatchwell et al. 2000, Dunn et al. 2014). Although negative associations between *Leucocytozoon* and *Haemoproteus* may also be explained by differences in the spatial distributions of both vectors and parasites, we did not find here an effect of elevation, with the prevalence of both increasing at higher elevations (Bensch et al. 2012). Alternatively, a potentially higher pathogenicity of *Leucocytozoon-Haemoproteus* co-infections could result in increased host mortality (Evans & Otter 1998). Indeed, although the costs of *Leucocytozoon spp.*

infections are often considered to be small in passerines (van Rooyen et al. 2013b, Oakgrove et al. 2014, but see Knowles et al. 2010) and the cost of *H. majoris* to be high (Allander 1997, Merino et al. 2000, Tomás et al. 2007, Knowles et al. 2009, Martínez-de la Puente et al. 2010), the pathogenicity of a co-infection with both remains to be determined.

Within *Leucocytozoon*, we detected a negative association between Clade A and D. Only 15 great tits carried Clade D lineages, but of these just 3 (21%) were also infected by Clade A, which stands in contrast with the 70% overall prevalence rate of Clade A in great tits. Furthermore, Clade D is highly specific to blue tits, whilst Clade A is found in both species - yet at a much higher prevalence in great tits. The negative association between both may be explained by the hypothesis that host-specialist Clade D lineages out-compete generalist Clade A lineages (Mata et al. 2015). Indeed, host generalists are expected to maintain sub-optimal performance as a result of adapting to multiple hosts, whilst host specialists should evolve to optimally exploit their primary host (Rigaud et al. 2010), which may provide a competitive advantage.

By making use of a novel PCR-technique, we confidently surveyed the complete haemosporidia community of two geographically-overlapping hosts and in doing so revealed parasite-parasite interactions occurring within the host environment. A number of unexplored avenues specific to our study system remain. For example, a more comprehensive understanding of local vectors, which is lacking for our study sites beyond generalisations, may help explain some distributional patterns (e.g. host specialisations). Additionally, a better understanding of prevalence seasonality could reveal how temporally robust these parasite-parasite interactions are, providing clues as to whether they are driven more by the parasites themselves or by their insect vectors. More generally, work is required to understand the effects of co- and mixed-infections on parasite pathogenicity. Where these infections carry additional costs, they have the potential to structure host-parasite communities, complicating the epidemiology of individual species, and altering the co-evolutionary interaction between haemosporidia and their avian hosts.

Chapter Two

Enhanced host resistance to a costly parasite is conferred by tolerance towards another

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Abstract

Hosts have evolved a range of defence strategies to mitigate the costs of infection: resistance mechanisms allow hosts to fight infection directly, while those increasing tolerance limit the damage inflicted by parasites. The benefits of evolving tolerance may, in certain cases, extend beyond minimising pathogenesis and even confer indirect fitness benefits when infection with a given parasite precludes infection by another, more costly parasite. Here, we investigate a complex parasite-parasite interaction between two clades of *Leucocytozoon spp.* playing out across two host passerine species, blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*). Our results demonstrate that both host species have likely evolved tolerance to their most common *Leucocytoozon spp*. Furthermore, we show that tolerance in blue tits, to blue tit-specialist parasites (Clade D species), enables parasite-conferred resistance to other costlier parasites (great tit-typical Clade A species). Indeed, only 10% of blue tits that had or were still infected with Clade D were also infected with Clade A, whilst prevalence of Clade A reached 58% in blue tits with no record of Clade D infection. These findings illustrate the implications that different interactions can have within a multi-host, multi-parasite community and highlight the need to take those into account when modelling host-parasite evolutionary trajectories.

Introduction

Parasites abound, exploiting host organisms for resources and inflicting costly damage. In response, hosts have evolved cost-mitigating defence mechanisms. Post-infection defence mechanisms have been defined as employing either of two independent strategies (Simms & Triplett 1994, Roy & Kircher 2000, Råberg et al. 2007). One is resistance, where the parasitic burden is reduced or eliminated through adversarial control of the parasite (e.g. an immune response limits parasite proliferation (Schmid-Hempel 2003)). The other is tolerance, where hosts do not contest the parasite directly, but instead reduce the damage incurred from the infection (e.g. neutralisation of harmful parasite by-products (Pamplona et al. 2007)). Whether hosts evolve resistance or tolerance, and the level to which they do, will depend on the relative costs and benefits of different strategies (Simms & Triplett 1994, Roy & Kircher 2000, Restif & Koella 2004, Råberg et al. 2007, Råberg et al. 2009, Klemme & Karvonen 2017). Since different host strategies are expected to have important and distinct implications for parasite virulence evolution (Roy & Kircher 2000, Gandon & Michalakis 2000, Pitman et al. 2005, Mackinnon et al. 2008, Best et al. 2014), parasites infecting more than one host species may, as a result, experience differing selection pressures across their various host environments.

Whether hosts evolve resistance or tolerance may not only impact the ecology and evolution of the target parasite, but they may also shape parasite-parasite interactions within the host and thereby indirectly, alter host-parasite interactions. Indeed, within a host, concurrent parasite infections are common, with the nature of interactions between parasites ranging from competitive to cooperative (Alizon & Lion 2011). Both theoretical and empirical studies indicate that these interactions may lead to selection for increased parasite virulence (Brown et al. 2002, Bell et al. 2006, Alizon et al. 2013), as evidenced by mixed infections of malarial parasites (*Plasmodium* spp.) that can exacerbate host pathological outcomes (Mayxay et al 2004, Genton et al. 2008, Palinauskas et al. 2011). Less studied is the potential for parasite-parasite interactions that will, instead, benefit the host. Recent theoretical work has explored how parasites may themselves contribute towards host tolerance or resistance to other parasites (Ashby & King 2017), for e.g., when a parasite competitively excludes another more virulent parasite, effectively conferring resistance to its host. Experimental support comes

from an infection of the nematode host, *Caenorhabditis elegans,* with a virulent parasite (*Staphylococcus aureus*) and a less costly gut microbe (*Enterococcus faecalis*), which resulted in *E. faecalis* reducing *S. aureus*-induced host mortality both initially and with increasing effectiveness across subsequent generations (King et al. 2016). In humans, a reduction in parasitemia of the malarial parasite *Plasmodium falciparum* by the parasitic trematode *Schistosoma haematobium* similarly suggests parasite-conferred resistance (Briand et al. 2005, Nacher 2006). Whether parasite-conferred resistance and tolerance is widespread and what their impact is on disease epidemiology within natural host populations, however, remains to be determined.

Here we explore how hosts respond to their most common parasite and examine how infection by these parasites in turn affects interactions with other parasites, in natural populations of blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*). Previous work has revealed that both blue tits and great tits display a high prevalence of *Leucocytozoon* blood parasites, as well as host-specific structuring of the *Leucocytozoon* community (chapter one). First, we test whether hosts are resistant or tolerant to their most common *Leucocytozoon spp.* by measuring the cost of infection on female reproductive output during the breeding season. If hosts have evolved resistance to infection, then infection should be associated with a measurable cost, with infected individuals displaying either the cost of pathogenesis when they are susceptible, or the cost of immunity when they are quantitatively resistant (Gandon & Michalakis 2000, Bonneaud et al. 2017). Conversely, hosts will be tolerant if high prevalence rates are coupled with a lack of detectable cost (Restif & Koella 2004). Second, we measure the cost of *Leucocytozoon* infection in blue tits of the most common great tit- *Leucocytozoon* parasite and examine how the temporal distribution of this parasite may be influenced by parasite-parasite interactions within blue tits. In blue tits, mixed infections of the most common blue tit and the most common great tit *Leucocytozoon spp*. are less common than expected by chance (chapter one). If the cost of mixed infection is greater than that of single infections, this may provide indication as to whether conferred resistance best explains the interaction between these *Leucocytozoon spp.* (Gandon & Michalakis 2000, Ashby & King 2017).

Methods

Study population and sampling

Our study populations (great tits and blue tits) inhabit mixed deciduous woodland in the west of the Pyrénées Ariégeoises National Park, France. They are comprised of four nest box sites, which are located within 14km of one another: Moulis (159 nest boxes, 42°57'90"N-42°58'36"N, 01°05'31"E-01°05'73"E), Cescau (209 nest boxes, 42°55'34"N-42°56'46"N, 01°02'47"E-01°03'47"E), Galey (105 nest boxes, 42°56'64"N-42°57'24"N, 00°54'13"E-00°55'30"E,), and Castera (147 nest boxes, 42°53'74"N-42°55'07"N, 01°05'40"E-01°03'43"E). In this region, the woodland is interspersed by manmade clearings and fields. At our sites, there are no large open water bodies, instead fast-flowing streams run through these forests and into the slower flowing Lez river. It is likely these mountain streams provide the breeding sites for the primary vectors of *Lecocytozoon sp.*, blackflies (Simuliidae). However, little is known of the vectors at these sites and species are yet to be determined for the haemosporidians reported here. In these study populations, *Leucocytozoon* parasites display host structuring, *Leucocytozoon* Clade A spp. have higher prevalence in great tits whilst *Leucocytozoon* Clade D spp. are host specific to blue tits (see Chapter One for a phylogeny of this *Leucocytozoon* population). Furthermore, blue tits infected by their common clade, Clade D, are less likely to be infected by the great tit typical Clade A. It is this host-parasite interaction we aim to further determine. In total, 443 adult great tits and 531 adult blue tits were captured and banded between May 2015 and May 2017. This study covers three breeding seasons and two overwintering periods.

During the spring, nest boxes were regularly checked to determine lay date, clutch size, and chick numbers. Nestlings were ringed on day 15 post-hatching and the number of nestlings ringed, minus any mortalities, were recorded as the number of fledglings. Parental adults were captured using nest-traps at day 11 or 15 post-hatching. Non-ringed individuals were ringed (fitted with a unique 7-digit metal identification ring and a combination of coloured bands), and all were measured for morphological data, and sampled for blood. Measures included in this study were: sex, age, and body mass. Sex was determined based on plumage characteristics and the presence or absence of the brood patch.

Individuals were categorised into either of two age classes; as yearlings or as post-second year adults, based on the pigmentation of the primary coverts. An electric scale (± 0.01g) was used to measure mass. Approximately 35µl of blood was collected by brachial venapuncture with sodium heparinized micro-hematocrit capillaries (Hirschmann Laborgeräte, Germany) and stored in ~1ml of 96-100% ethanol. During overwintering periods, birds were caught using mist nets instead of nest-traps. As in spring, morphological data and blood samples were obtained. No sampling took place during the months of April, July, August, and September. If individuals were recaptured, morphological measurements and blood samples were taken if more than 2 weeks had elapsed since last capture.

Molecular analysis of infection

We extracted DNA from our blood samples using the DNeasy Blood & Tissue extraction kit (QIAGEN®) following the manufacturer's protocol pertaining to nucleated blood. We standardised these extractions to working concentrations of 25ng/µl. To detect the presence of *Leucocytozoon sp.*, we used a nested-polymerase chain reaction (PCR) which targets a specific region of the blood parasites' mitochondrial cytochrome *b* gene (Hellgren et al. 2004). All reactions took place in volumes of 25µl using the DNA polymerase, DreamTaq (Thermo Fisher Scientific). For full details of reaction quantities see Chapter One. PCRs were performed in Applied Biosystems Veriti™ Thermal Cyclers. Negative controls (sterile Milli-Q water, one per ten samples) and positive controls (extract from individual of confirmed status) were included in each batch of reactions. Second-round PCR products were separated on 2% agarose gels stained with RedSafe™ (20,000x) (iNtRON Biotechnology Inc.). Samples returning a negative status were verified through a repetition of the PCRs. Those which exhibited successful amplification were purified (ExoAp (Exonuclease 1 and Antarctic Phosphatase (New England BioLabs)) and sequenced using the forward primer HaemFL (Hellgren et al. 2004) (Eurofins sequencing service, Eurofins-MWG). Sequence processing was performed using Geneious (Geneious® 9.1.5, Biomatters). Sequence lineage was identified by carrying out a BLAST search on the MalAvi database (Bensch et al. 2009). Multiple infections (infection by more than one lineage of *Leucocytozoon*) were identified by the presence of double-peaks in chromatograms. Often it was not possible to classify the precise lineages

constituting a mixed-infection, in such cases we instead identified the clade(s) to which the sequences belong based on a phylogeny of previously identified haemosporidia from our populations (Chapter One).

Statistical Analyses

All statistical analyses were conducted with R using RStudio v0.99.902 (RStudio Team 2017). Model reduction was carried out using Akaike information criterion (AIC) to assess model quality, non-significant terms were dropped from a model if this improved model fit. Our first set of analyses aimed to determine whether infection by Clade A or D carried a cost to their primary hosts, and whether Clade A infection carried additional costs in blue tits (where it is less typical). Data was collected on breeding female blue tits (N = 75) and great tits (N = 50), and host species were analysed separately as differences in parasite prevalence's suggest host-specific relationships. We used three breeding measures, clutch size (initial investment), fledgling number (output), and the proportion of initial clutch successfully fledged (return on reproductive investment). We tested whether there was an effect of infection on reproductive investment using two linear mixed-models, with clutch size as the response variable, with female body mass, female age, and lay date as fixed effects and with breeding year as a random effect. When considering blue tits, we included infection with Clade A (0/1) and Clade D (0/1) and their interaction as explanatory terms, while for great tits, we only included infection with Clade A (0/1) as an explanatory term. We tested whether there was an effect of infection on reproductive success using similar linear mixed-models, with fledgling number as the response variable. We used the same infection status explanatory terms as in the clutch size models. Female age, clutch size, and lay date were added as fixed effects and breeding year as a random effect. We tested the effect of infection on the proportion of chicks fledged relative to clutch size, using two generalised linear models, with the proportion of chicks fledged as the response term and with lay date and female age as fixed effects. As before, the same infection status exploratory terms were used for each host species. In this case, we did not use generalised linear mixed models as identifiability issues prevented model convergence when breeding year was included as a random effect; our decision to simply the models was further justified by the very low degree of variance explained by breeding year.

Lastly, we tested whether infection with Clade D (0/1) in blue tits impacted infections with Clade A (0/1) on two temporal scales: (i) historically within individuals, and (ii) seasonally at the population-level. To explore interactions within individuals, we used data for recaptured blue tits (N = 133), and tested whether previous Clade D infection decreased the probability of a current Clade A infection, irrespective of current Clade D infection. We ran a logistic regression model with Clade A infection (0/1) as the response variable, with previous Clade D infection (0/1) and current Clade D infection (0/1) as explanatory terms. Next, to explore the relationship between the prevalence of different clades throughout the year, we made use of capture records for all individuals (blue tits, N = 665; great tits, N = 528) and ran three logistic regression models. Two models considered clade prevalence in blue tits, one with Clade A infection (0/1) as the response term, the other with Clade D infection (0/1). Another model was constructed for prevalence in great tits, with Clade A infection (0/1) as the response term. In all three models month of capture (Jan = 1, Dec = 12) was the explanatory term. We did not remove recaptured individuals from the analysis as we expected their infection status to match any potential patterns of seasonal variation in parasite prevalence. In all models, we only tested for Clade A infections in great tits as prevalence of Clade D was too low (N = 16).

Results

Cost of infection

First, we examined the cost of infection with the most common *Leucocytozoon* clades in each host species. In blue tits, Clade D was the most prevalent clade and infection with Clade D was positively association with clutch size ($\chi^2 = 3.5$, $p = 0.06$, Table 1). Clade D infection was, however, not associated with a difference in the proportion of clutch fledged ($\chi^2 = 0.17$, $p = 0.67$, Table 1) or in fledgling number ($\chi^2 = 0.6$, $p = 0.43$, Table 1). In great tits, Clade A was the most prevalent, and infections with clade A were significantly associated with a greater proportion of the clutch fledged ($\chi^2 = 8.62$, $p = 0.003$, Table 1). Clade A infections were, however, not significantly associated with differences in clutch size ($\chi^2 = 2.1$, $p = 0.15$, Table 1) or fledgling number ($\chi^2 = 2.67$, $p = 0.1$, Table 1). Second, we tested for a cost of infection with Clade A in blue tits and found a negative effect of

infection on the proportion of offspring fledged relative to clutch size, with both mixed-clade infections ($\chi^2 = 12.54, p < 0.001$) and single Clade A infections ($\chi^2 = 4.74, p = 0.03$) associated with a reduced proportion fledged (Table 1).

Table 1. Regression model results for reproductive measures in response to infection

	Term	Estimate	SE	*t Statistic*	χ^2	*P*
Blue tits						
Clutch size	***Lay date***	***-0.06***	***0.02***	***-2.5***	***6.2***	***0.01****
	Clade D	*0.6*	*0.32*	*1.9*	*3.5*	*0.06*
	Clade A	0.12	0.37	0.3	0.1	0.74
	Clade D * Clade A	0.51	0.74	0.7	0.59	0.74
	Age	0.3	0.3	1	0.97	0.33
	Mass	0.27	0.26	1	1.04	0.3
Great tits						
Clutch size	**Mass**	**0.47**	**0.27**	**1.8**	**3**	**0.08**
	Clade A	-0.63	0.45	-1.4	2.1	0.15
	Age	-0.1	0.45	-0.2	0.05	0.82
	Lay date	-0.03	0.02	-1.3	1.58	0.21
Blue tits						
Fledged	***Lay date***	***-0.13***	***0.05***	***-2.9***	***6.68***	***0.009****
	Clade A	-0.87	0.69	-1.3	1.55	0.21
	Clade D	0.49	0.63	0.8	0.6	0.43
	Clade A * Clade D	-2.63	1.42	0.7	5.12	0.16
	Age	0.19	0.62	0.3	0.09	0.77
	Clutch size	-0.19	0.22	-0.9	0.74	0.39
Great tits						
Fledged	***Clutch size***	***0.47***	***0.17***	***2.8***	***7.03***	***0.008****
	Clade A	0.9	0.56	1.6	2.67	0.1
	Age	-0.25	0.56	-0.5	0.13	0.72
	Lay date	0.008	0.03	0.3	0.33	0.56
Blue tits						
Prop fledged	***Clade A * Clade D***	***-1.54***	***0.44***	***-3.5***	***12.54***	***<0.001****
	Lay date	***-0.04***	***0.01***	***-2.8***	***7.79***	***0.004****
	Clade A	***-0.44***	***0.2***	***-2.1***	***4.74***	***0.03****
	Clade D	-0.11	0.19	-0.1	0.17	0.67
	Age	0.22	0.19	1.2	1.39	0.24
Great tits						
Prop fledged	***Clade A***	***0.69***	***0.24***	***2.9***	***8.62***	***0.003****
	Lay date	0.02	0.01	1.5	2.89	0.12
	Age	-0.03	0.24	-0.1	0.01	0.9

Response terms: clutch size and fledgling number (LMM), and proportion of clutch fledged (GLM). Terms in bold retained in final models, significant terms (p <*0.05*) in italics and indicated by *.

Between clade interactions

In total, 111 blue tits were recaptured at least once. Concurrent infection by Clade D and Clade A in blue tits revealed a negative association ($\chi^2 = 12.6, p = 0.001$; see also chapter one). 66% of recaptured blue tits had been previously found positive for Clade D. We found that a prior infection with Clade D had a significant negative effect on the probability of current infection with Clade A ($\chi^2 = 7.9, p = 0.005$), even when current Clade D infection status was accounted for (Table 2). In blue tits with no prior or current Clade D infection, Clade A prevalence was 58.3%, whereas in non-infected blue tits with a prior Clade D infection, Clade A prevalence decreased to 47.6%. In hosts with both a record for prior and current Clade D infection (e.g. a persistently detectable Clade D infection), Clade A prevalence was 10.4%.

Table 2. GLM results for associations between Clade D & Clade A infections in blue tits

	Term	Estimate	SE	*OR*	*95% CI*	χ^2	*P*
Blue tits							
Clade A	***Previous Clade D***	***-1.2***	***0.43***	***0.3***	***0.13 : 0.7***	***7.9***	***0.005****
	Current Clade D	***-1.58***	***0.45***	***0.2***	***0.08 : 0.49***	***12.6***	***0.001****

Logistic regression, Clade A infection probability as response. Terms in bold retained in final models, significant terms (p <*0.05*) in italics and indicated by *. Odds ratio (OR) provided for significant terms.

Monthly prevalence rates for Clade A in blue and great tits, and for Clade D in blue tits, varied throughout the year (Figure 1a & b). Significant linear trends in prevalence rate across the year were detected for Clade A and Clade D in blue tits (Figure 1a). Clade A was found to significantly decrease in prevalence as the year progressed ($OR = 0.88, \chi^2 = 19.3, p < 0.001$), whilst Clade D significantly increased in prevalence across the same period ($OR = 1.07, \chi^2 = 7.9, p = 0.005$) (Table 3). In great tits, no significant effect of month was found on prevalence of Clade A (Table 3, Figure 1b).

Table 3. GLM results exploring infection status and capture month in blue & great tit

	Term	Estimate	SE	*OR*	*95% CI*	χ^2	*P*
Blue tits							
Clade A	***Month***	***-0.12***	***0.03***	***0.88***	***0.83 : 0.93***	***19.3***	***<0.001****
Clade D	***Month***	***0.07***	***0.03***	***1.07***	***1.02 : 1.13***	***7.9***	***0.005****
Great tits							
Clade A	Month	0.03	0.02	-	-	-1.2	0.26

Logistic regression, current Clade A or D infection probability as response to month of the year in blue tits and great tits. Terms in bold retained in final models, significant terms (p <*0.05*) in italics and indicated by *. Odds ratio (OR) provided for significant terms.

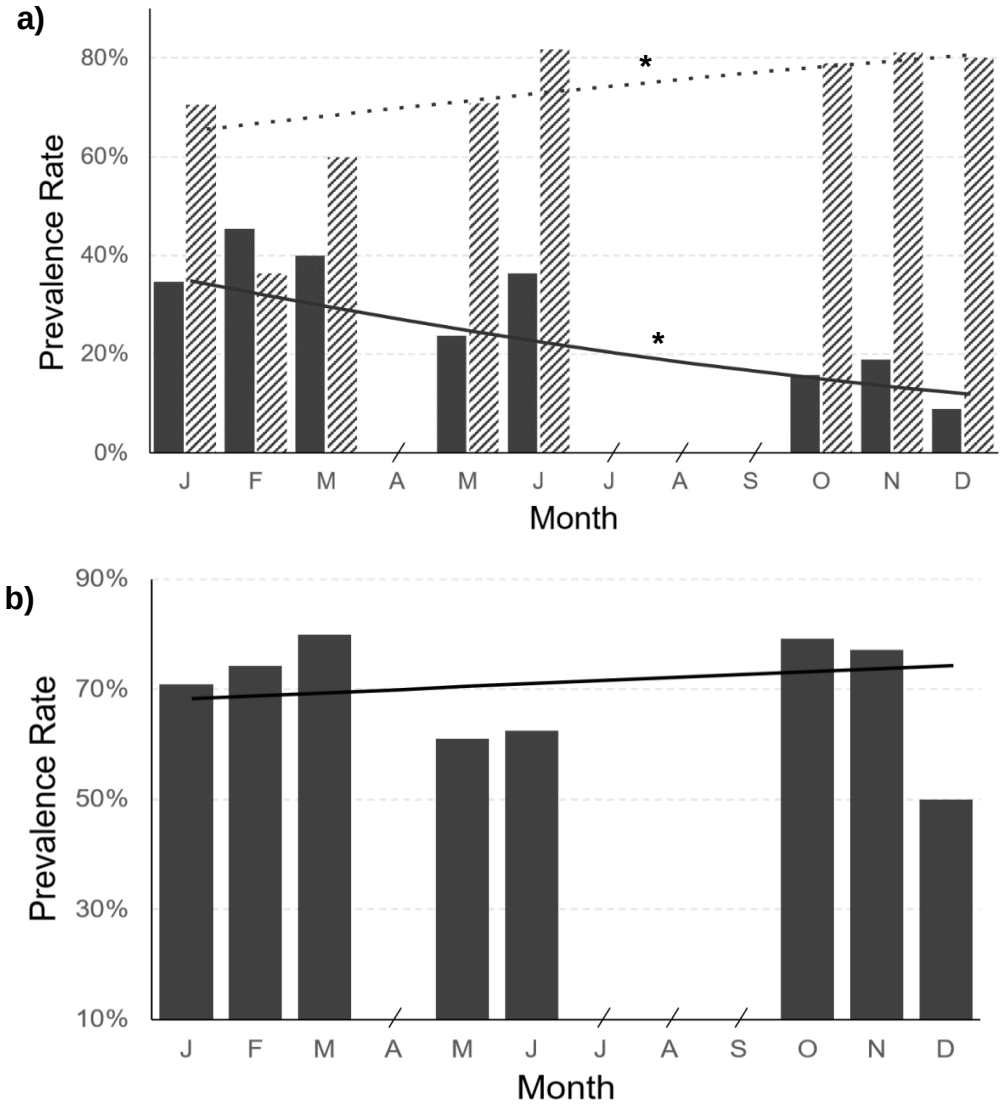

Figure 1. Monthly prevalence rates for Clade A and Clade D in blue tits (a) & great tits (b): Bars represent pooled monthly prevalence rates for *Clade A* (solid bars) and *Clade D* (dashed bars). Lines follow predicted prevalence rates from logistic regression models (Table 2), for *Clade A* (solid line) and *Clade D* (dashed line). Significant (p <0.05) regression lines indicated by asterisks.

Discussion

In this study, we have found evidence of host tolerance to their largely host-specific common *Leucocytozoon spp.*, and yet the *Leucocytozoon spp.* typical to great tits (Clade A) are associated with reproductive costs in blue tits. Furthermore, we have identified a potential example of parasite conferred quantitative resistance in blue tits (Ashby & King 2017), as Clade D lineages (specific to blue tits) are associated with a lower prevalence of Clade A spp. This interaction has epidemiological consequences for blue tits, both in terms of seasonal parasite prevalence patterns and increased infection costs.

Although Clade A and Clade D prevalence rates were high in their typical host species (great and blue tits respectively), neither was associated with significant reproductive costs in their primary host. Tolerance to infection is predicted to be favoured when prevalence is high but infection has a low virulence (Restif & Koella 2004). Female blue tits infected with Clade D displayed a marginally greater clutch size than non-infected females, and no difference in fledging numbers or in the proportion of the clutch fledged. While we cannot rule out here a pattern of terminal investment in response to infection (Clutton-Brock 1984, Bonneaud et al. 2004), the high prevalence of Clade D in blue tits provides further support to the hypothesis that blue tits are instead tolerant to Clade D. Similarly, female great tits infected with Clade A parasites fledged a greater proportion of their clutch than non-infected females, suggesting that they could tolerate infection. Due to the low virulence recorded here and elsewhere for *Leucocytozoon* parasites in passerines, and due to high prevalence rates, it seems probable that tolerance mechanisms play a role in these hosts (Restif & Koella 2004, Råberg et al. 2009, van Rooyen et al. 2013, Oakgrove 2014).

While Clade A infection did not appear to carry a cost in great tit, it was associated with a reduced return on reproductive investment (proportion of chicks fledged) in blue tits. Although Clade A-infected blue tits did not display a reduced number of fledging relative to non-infected individuals, such sustained misallocation of resources could ultimately reduce life-time reproductive success. As Clade A appears tolerated by great tits, it's of note that some theoretical models suggest that tolerance in one host may ultimately lead to the selection for higher parasite growth rates, which could increase costs to susceptible hosts lacking in tolerance (Miller et al. 2006, Best et al. 2014). Therefore, parasite-host interactions between

great tits and Clade A could be influencing the costs of Clade A infection in a less typical host, the blue tit.

Blue tits perhaps show natural qualitative resistance to Clade A (Clade A is less prevalent in blue tits generally), however this resistance appears somehow enhanced by Clade D infection. Individual blue tits with historic Clade D infections were less likely to have a Clade A infection. Indeed, the more persistent a Clade D infection was (detected across multiple captures) the less likely an individual was to be infected by Clade A. Although they were not common, mixed infections of Clade A and D were costly to blue tits (lower proportion of clutch fledged), more so than infection by either clade alone. A cost to the interaction between these two clades could manifest for a couple of reasons. If Clade D directly excludes Clade A due to resource competition the presence of both may exacerbate the use of host resources, limiting host investment in reproduction. Alternatively, if Clade D typically confers qualitative resistance to Clade A in blue tits, perhaps only the most virulent Clade A genotypes are capable of persistence in the presence of Clade D. We have not yet elucidated the mechanistic cause of the negative association between these parasites, leaving uncertainty as to whether true conferred resistance is detected. A key determinant is whether improved blue tit resistance to Clade A results from the non-inheritable contribution of Clade D, or instead reflects host-priming by Clade D and as such reflects an inheritable blue tit trait (Ashby & King 2017). Immune priming (e.g. improved host immune response due to prior infection) can be non-specific and, if it's the mechanism at play here, would have distinct epidemiological implications (Cheng et al. 2014, Tate 2017).

There are a range of epidemiological consequences resultant from this between-clade interaction. For example, we found that this interaction between the two clades altered prevalence patterns in blue tits. In terms of seasonal prevalence, Clade D was sustained at higher rates during the late autumn, coinciding with the lowest prevalence rates of Clade A. Perhaps indicative, Clade A prevalence in great tits revealed no such pattern. As Clade A is common to great tits (Drovetski et al. 2014 even identified it as great tit specific), it seems reasonable to assume that prevalence patterns in great tits would largely drive prevalence patterns in neighbouring hosts (i.e. decreased prevalence in great tits would decrease infection risk to the wider host community). This pattern of host species-specific

Clade A seasonality therefore reveals that the drivers of prevalence patterns in blue tits are specific to this species, and not typical of Clade A epidemiology in the wider host community. Implications also extend to host-parasite co-evolution, antagonistic interactions such as these (e.g. host resistance and Clade D 'competition' against Clade A), are often predicted to select for increased virulence of the parasites involved (Gandon et al. 2001, Pitman et al. 2005, Mackinnon et al. 2008, Choisy & de Roode 2010, Alizon et al. 2013). Clade A parasites may therefore face host-dependent selection pressures, whilst Clade D would face context-dependent selection within its single host.

In conclusion, we have identified host-specific *Leucocytozoon spp.* capable of conferring to their host improved resistance against a costlier clade of a sister-parasite species. This interaction may have important implications for parasite-host coevolution within these overlapping host species (Ashby & King 2017). For example, selective pressures acting on Clade A in blue tits may influence Clade A infections in great tits. Also, where tolerance appears probable in a host (e.g. Clade D in blue tits), it remains to be determined how an antagonistic interaction between the tolerated parasite and a co-infector will influence the stability of tolerance as a defence mechanism. These findings highlight the limitations of current theory, and more elaborate models will be required to capture the true complexities of host-parasite interactions (Boots et al. 2009, Ashby & King 2017). Many mechanistic aspects of these interactions remain unexplored. Important future considerations include; the role of vectors in facilitating between-host species parasite transfer, whether true tolerance is observed (e.g. costs measured across a continuum of parasite load (Råberg et al. 2007, Råberg et al. 2009)), and how the interaction between Clade A and D is mechanically facilitated in blue tits (Mideo et al. 2009).

Thesis Discussion

Through application of a novel PCR technique we have revealed the diversity of the haemosporidia community in two geographically overlapping passerine species. *Leucocytozoon* parasites are reported to be both the most prevalent and lineage rich, and between-genus interactions are revealed. We have recorded the presence of both host-generalist and -specialist haemosporidia, and in addition to this, within-host interactions between *Leucocytozoon spp.* in blue tits.

Both blue and great tits are widely distributed throughout Europe, covering a range of elevations and habitats (Cramp & Perrins 1993). It is likely that vector abundance, and therefore parasite distributions, will vary considerably across these ranges, raising questions regarding the wider relevance of the interactions reported here. However, studies which have surveyed blue and great tit haemosporidia across their ranges have not found evidence of phylogeographical structuring (Jenkins & Owens 2011, Drovetski et al. 2014,). Suggesting localised host-parasite adaptation in these species is less common, and that the interactions we detect may be extrapolated to other overlapping blue and great tit populations. For example, we find considerable lineage similarities to a recent study of haemosporidia in great tits breeding in the Canton of Vaud (Switzerland) (van Rooyen et al. 2013). In total, van Rooyen et al. (2013) identify 18 unique *Leucocytozoon* lineages, in this study we have found 20, of which 8 are common to both studies. Considering that 6 lineages identified in our study are of the mostly blue tit specific Clade D, this shows a considerable degree of lineage overlap between two distant study populations (approximately 590km apart). However, these authors also report much higher *Plasmodium* prevalence, and lower *Leucoccytozoon* infection rates overall, illustrating how local habitat features (e.g. large water bodies) influence vector distributions and therefore haemosporidian community interactions (van Rooyen et al. 2013). To our knowledge, no study has specifically recorded *Leucoccytozoon* in blue tits across years. Some have, however, sampled haemosporidia in this species as part of the broader avian community (Jenkins & Owens 2011, Drovetski et al. 2014, Mata et al. 2015). Jenkins and Owens (2011) report that representatives of Clade D are found at multiple sites throughout the blue tit range (in England, Scotland, Germany, and Spain). However, in contrast, Drovetski et al. (2014) surveyed

haemosporidia in both north western Portugal and eastern Europe (Caucasia) and do not report Clade D (although Mata et al. 2015 did detect Clade D spp. in a similar region of Portugal). At those same sites Clade A is found in great tits, and even in blue tits (Drovetski et al. 2014). Revealing that, whilst haemosporidian lineages in these two hosts are widely represented throughout their ranges, some localisation could exist, limiting the co-occurrence of *Leucocytozoon* Clade A and Clade D spp.

In both chapters of this thesis we detected interesting interactions between haemosporidia, all were negative associations. At the genus-level we found that *Haemoproteus* lineages were negatively associated with both *Plasmodium* and *Leucocytozoon spp.*, on this point it's worth highlighting that the lineages of *Haemoproteus* and *Plasmodium* detected in this study are almost exclusive to two species; *H. majoris*, and *P. relictum*. Therefore, to some degree these interactions are species-specific. There is also an important methodological point to consider; shortcomings have been reported in the ability of PCR-detection techniques to accurately identify mixed *Haemoproteus-Plasmodium* (*H.-P*.) infections (Perez-Tris & Bensch 2005, Bernotienė et al. 2016). Bernotienė et al. (2016) carried out a comparison of five PCR detection techniques by applying them to blood samples of known infection status, observing that *H.-P.* mixed infections were sometimes entirely missed, with typically only one of the constituent genera correctly identified. In these methodologies (as in ours) *H.-P.* sequences are amplified together, this is due to sequence similarity shared by the two genera (Bensch et al. 2000). However, it is thought that the primers used can sometimes show greater affinity towards sequences of one genus (e.g. closer complementarity) and preferentially amplify their lineages, to the exclusion of the other genus (Perez-Tris & Bensch 2005). Oakgrove et al. (2014), found a negative association between *Haemoproteus* and *Plasmodium* in their study of Alaskan passerines, and identify this methodological issue as a potential cause. If mixed *H.-P.* infections are under-reported then this would clearly lead to the negative associations observed. In this study, we have designed a new nested-PCR technique for the amplification of *H.-P.* sequences. It wasn't, however, designed to resolve this issue – although the primers were constructed to target highly conserved regions common to both contemporary *Haemoproteus* and *Plasmodium* lineages sequences. As such, we too interpret the *H.-P.* interaction

we detected with caution, but instead draw attention to the less contentious interaction between *Leucocytozoon* and *Haemoproteus* (sequences of which are detected in two separate PCR reactions).

Leucocytozoon and *Haemoproteus*, are vectored by representatives of different Dipteran families. Both the blackflies which vector *Leucocytozoon spp.*, and the biting midges which vector *Haemoproteus spp.*, differ in their habitat specialisations (e.g. blackfly reproduction occurs in unpolluted, running streams, whilst biting midges oviposition in moist soil) (Pérez-Rodríguez et al. 2013b). Differences in vector habitat use are likely to influence infection probabilities if hosts exhibit specific preferences in habitat occupation, this provides one potential explanation for the negative associations between these genera (Scheuerlein & Ricklefs 2004). However, where the distributions of these two haemosporidian genera have been linked to abiotic environmental variables (e.g. precipitation, temperature, latitude, and elevation) they are often found to display similarities (van Rooyen et al. 2013, Pérez-Rodríguez et al. 2013b, Oakgrove et al. 2014). For example, we found that both genera increased in prevalence at higher elevations. Spatial co-occurrence could increase competition between these two genera, particularly if co-infection probability increases and similar host resources are parasitized. So, whilst local habitat distributions may differ between *Leucocytozoon* and *Haemoproteus spp*., across larger geographical scales similarities exists. Further work is therefore required to resolve why these haemosporidia are sometimes found to be inversely distributed within their host species.

How far generalisations can be applied to haemosporidian genera remains, in many ways, untested. Take for example, temporal and spatial vector distributions, for both measures species-specificity exists within all vector families (Tschuor et al. 2009, Russell & Hunter 2010, Černý et al. 2011). These vectors also show host specificity, with blood meals revealing feeding preferences for particular avian hosts (Hellgren et al. 2008). Additionally, as demonstrated here and elsewhere, host susceptibility, pathology, and periodicity, can be both host-species specific and parasite-species/lineage specific (Palinauskas et al. 2008, 2009, Knowles et al. 2011, Dimitrov et al. 2015). This is not to say generalisations can't apply, for example *Plasmodium spp.* are more typically host generalists, also exhibiting less specific associations with their mosquito vectors (Pérez-

Rodríguez et al. 2013b). But caution should be taken when, for example, assuming infection costs can be studied at the genus level (Tomás et al. 2007).

In this study, we identified a rather specific negative association between *Leucocytozoon* parasites in blue tits. Although we focused on this interaction in blue tits, Clade D was also detected at very low prevalence in great tits, and as individuals with these Clade D infections were less likely to be Clade A positive, it's possible that whatever interactions occurs between these parasites in blue tits is also applicable to this species. In chapter two we advanced the hypothesis that this between-parasite interaction reflects some form of competition between Clade A and D (e.g. Clade D conferred resistance to Clade A in blue tits, perhaps through competitive exclusion). However, an alternate hypothesis is worth consideration. As we detected an increased cost to mixed Clade A – D infections in blue tits, it could be that these infections are highly virulent and that those infected face mortality or incapacitation, reducing their probability of capture. This would have important implications for blue and great tit ecology, as the mortality/incapacitation rate required to explain the prevalence patterns observed would be quite considerable. High mortality rates or loss of reproductive opportunities would have impacts on population dynamics, and so both explanations raise important questions regarding interspecific competition between these host species. Great and blue tits are known to compete for nest sites, and overlap in prey choice (Morse 1978, Minot 1981). A costly interaction between their specific haemosporidian parasites could certainly play a role in mediating this competition. Host-specific parasite interactions have previously been advanced as a contributing factor in blue tit – great tit interspecific competition (Richner et al. 1993).

To conclude, this thesis aimed to identify how far interactions between co-infecting haemosporidia may play an influential role in parasite epidemiology. We have identified important haemosporidian associations which deserve further exploration, for example – a generalisable negative association between *Leucocytozoon* and *Haemoproteus* parasites in passerines could significantly impact disease outcomes in shifting parasite communities (e.g. range expansions due to climate changes). Questions also remain concerning the nature of between-clade *Leucocytozoon* interactions, such as how selection pressures which appear host dependent may alter parasite virulence. These questions paint

the interactions identified here in the broader context of parasite host co-evolution, and demonstrate the potential use of this system to explore empirical answers to theoretical predictions.

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