An epidemiological study of haemosporidian infections in blue tits (Cyanistes caeruleus) and great tits (Parus major) along an elevation gradient

Impact of avian haemosporidian disease in a changing world

Volume 1

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Glossary of Terms

**Acquired immune response:** A targeted vertebrate immune response developed through build up of memory to specific antigens to which the organism is exposed. White blood cells, lymphocytes (T cells and B cells) as well as dendritic cells and antibodies comprise the acquired immune system.

**Acute stage:** Stage of parasitic infection typified by very high levels of parasitaemia and rapid destruction of red blood cells resulting from sudden influx of parasitic cells into the circulating blood stream.

**Avian host:** An infected or susceptible bird in which haemosporidian parasites are able to complete the sexual stages of their life cycle.

**Chronic stage:** Stage of parasitic infection during which haemosporidian parasites persist within the host for extended periods, but at lower infection intensities. In some cases, parasites may even be absent from circulating peripheral blood during this stage.

**Disease system:** A complex network of biologically relevant populations and/or communities that are involved in the transmission and life cycle of a specific parasite.

**Ectotherm:** An organism that does not produce sufficient body heat internally and so relies on external sources of heat for survival.

**Energetic cost:** Requires the expenditure of metabolic products.

**Epidemiology:** Study of the factors determining and influencing the prevalence and distribution of a disease across habitats and species and related health impacts as well as the causes and potential prevention and control measures.

**ExoSAP protocol:** Exonuclease I - Shrimp Alkaline Phosphatase clean up of PCR products in preparation for sequencing of DNA fragments. The clean up uses ExoSAP for the enzymatic removal of excess nucleotides and primers left over from PCR reactions that can disrupt DNA sequencing.

**Haemosporidian parasite:** A highly prevalent and diverse group of minute intracellular protozoan parasites within the phylum Apicomplexa that typically undergo alternating cycles of sexual replication within vertebrate hosts, and asexual replication within diptheran vectors.
Immunoglobulin: A class of proteins that are present in the serum and cells of the immune system, which function as antibodies acting to recognise and counteract invading antigens.

Mitochondrial lineage: Variation observed in the DNA sequence of an amplified mitochondrial gene that may represent a unique species, but which hasn’t been formerly described or functionally distinguished.

Parasitaemia: A measure of the number of haemosporidian parasites present in the host reticuloendothelial system and the peripheral blood circulation, which indicates infection intensity.

Passerines: Birds within the large order Passeriformes that includes over half of all avian species. Species within this order include all songbirds and share the common characteristic of having feet that are adapted to perching.

PCR: Polymerase Chain Reaction. A molecular technique that amplifies targeted DNA fragments to produce hundreds, even thousands of identical copies. A widely applied technique across modern biological science and research.

Phenotypic: The physical observable characteristics of an individual resulting from interactions between its genotype and the environment.

Prevalence: How often a parasite is found to occur within a given host population or community, or the proportion of individuals found to carry infection of a certain type.

Simpson’s Index of Diversity: A measure of species diversity that accounts for both species richness and the diversity or prevalence of each species. The index calculates the probability that two randomly selected individuals within a community will be different species.

Species range: The sum of habitats and locations where a particular species is found to occur.

Susceptibility: The capacity or likelihood of transmission and subsequent infection of a particular parasite or disease to a host individual.

Trade-off: When investment in one activity or process results in reduced investment or functioning in another as a result.
Vector: An organism, typically a biting insect that transmits parasites between susceptible hosts.

Preface Instruction

This thesis is presented in two chapters. Chapter 1 introduces a review of the current literature on avian haemosporidian parasites, and the factors that influence prevalence and distribution patterns within avian host communities. Current limitations to research on avian haemosporidia carried out to date are discussed, and future priorities to improve understanding and knowledge of the disease system recommended. The use of avian populations and their infecting haemosporidian parasites as a model system to predict climate change outcomes on vector-mediated diseases is described. This leads on to the second chapter, which describes a correlational study using molecular and reproductive data recorded from nest box populations of blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*). The study assesses fitness costs to hosts associated with three genera of avian haemosporidian parasites (*Leucocytozoon, Haemoproteus and Plasmodium*) along a climatic gradient. This is used to assess likely implications of broader climate change shifts to this complex vector-mediated disease system.
Chapter 1
Impact of avian haemosporidian disease in a changing world

Abstract

We discuss current knowledge regarding the impact of haemosporidian parasites on avian host communities, in relation to global climate change and how climate can alter diversity and distribution patterns. Avian haemosporidian parasites are globally distributed, highly diverse, intracellular blood parasites, known to infect a wide range of host species (Bensch et al. 2009). Although over 10,000 unique parasite lineages have been recovered, there are significant biases in research effort that consequently has left many host species and populations yet to be screened (Bensch et al. 2009). The majority of avian haemosporidian studies published to date have sampled Passerine species across Africa and Europe, leaving further host orders on other continents lacking in prevalence data. This research bias means that knowledge of global distribution is patchy, and the key factors which drive prevalence patterns remain a subject of debate. Haemosporidian parasites rely on diptheran insects to complete their life cycle and for transmission to new hosts, making them sensitive to external climate factors which then influence their survival and reproduction (Seghal, 2015). It is therefore surprising so few studies to date have screened vector and vertebrate host communities simultaneously, or recorded habitat and climate data in synchrony. As specific climate changes vary across space and time, we ask how diversity and distribution of avian haemosporidian parasites translates into fitness consequences for the hosts, and whether these can be predicted. Several studies have identified key fitness trade-offs between host reproductive performance and haemosporidian infection intensity. There are also examples where no significant costs associated with chronic infections were identified. We introduce a study which attempts to use four populations of breeding blue tits (Cyanistes caeruleus) and great tits (Parus major) as a model system to understand how climate gradients influence the outcome of haemosporidian infections on host communities. Simultaneous sampling of populations that differ climatically will help inform predictions of avian haemosporidian parasite responses to climate changes, and could help to identify which host populations are likely to experience increasing pressure from haemosporidian infections as climate changes continue.
Keywords: Avian haemosporidian parasite, host, vector, model system, climate change

Introduction

Altered community compositions that result from species movements, of which habitat degradation and climate change are key drivers, also leads to altered parasite communities and species interactions, a factor often overlooked in species conservation debates (LaPointe, Atkinson & Samuel, 2012, Bonneaud et al. 2009, Pérez-Rodríguez et al. 2014). Studies have revealed that even as underlying chronic infections, haemosporidian parasites can have important fitness consequences for hosts (Marzal, 2012). As a complex disease system, avian haemosporidian parasites require substantial research effort to fully understand the processes and driving mechanisms behind their global success (Paaijmans et al. 2010). Here we discuss what is currently known about avian haemosporidian parasites, focusing on how different parasite species and genera influence host populations in relation to habitat and climate characteristics. Furthermore, we introduce a study which utilises two popular model host species, in order to assess haemosporidian prevalence and diversity, in tandem with fitness consequences to the hosts along an elevation gradient. We use a correlational approach, assessing fitness characteristics in relation to prevalence of the different parasite genera. The elevation gradient is designed to replicate climate changes that occur along latitudinal gradients, whilst minimising additional sources of variation between host populations.

Insect populations are predicted to shift more rapidly and intensely than vertebrates in response to accelerating climate changes, due to their exothermic physiology, short generation times, and high potential reproductive output (IPCC 2014, Paaijmans et al. 2010, Bayoh & Lindsay 2004, Travis, 2003). Many invertebrate species represent important vectors of both anthropogenic and wildlife diseases (Medlock et al. 2015, Wood, 2006, Paul, Ariey & Robert, 2003). Ongoing environmental and climate changes will have significant consequences for parasite diversity and prevalence within natural communities, mostly through effects on vector populations (Seghal 2015, Kovats et al. 2001). While some areas globally are predicted to become more favourable for insect survival and reproduction, others are expected to become less favourable. Notwithstanding, these conditions can also vary at fine spatial scales...
making overall effects difficult to predict. Intergovernmental Panel on Climate Change (IPCC) figures indicate that while arid and semi-arid areas have been becoming increasingly drier, other areas, particularly mid-to-high latitudes are becoming wetter (IPCC, 2014, Kovats et al. 2001). Additionally, in areas where precipitation has increased, there have been disproportionate increases in the frequency of the most extreme precipitation events (Kovats et al. 2001). A number of publications predict the frequency and severity of disease epidemics is likely to increase in the future through insect range shifts and population increases (Medlock et al. 2015, Lafferty, 2009, Patz et al. 2004).

Avian haemosporidian parasites represent a highly prevalent and diverse group of minute intracellular protozoan parasites within the phylum Apicomplexa (Bensch et al. 2000). Research has primarily focused on parasites belonging to the genera *Haemoproteus*, *Leucocytozoon* and *Plasmodium*. The latter being a genus which includes the causative parasites of malaria (Sehgal, 2015, Bensch et al. 2009). As these parasites rely on alternating cycles of asexual and sexual reproduction within both vertebrate hosts and insect vectors to complete their life cycles, external factors such as temperature, rainfall and habitat characteristics can become influential during replication within the insect vector stage, and subsequently can drive prevalence and distribution of the parasites (Sehgal, 2015, LaPointe, Atkinson & Samuel, 2012, Garamszegi, 2011, Martens et al. 1995). Additionally, parasites within the avian haemosporidia comprise both widespread generalists and highly specialist lineages that exhibit very different preferences and external optima, such that the outcomes of climate change may also depend on which parasites are present within a given community (Perez-Rodriguez et al. 2014). Due to the widespread occurrence of these parasites, both geographically and across avian hosts, and their sensitivity to climate change, they represent a useful study system to improve the functional understanding of future outcomes of climate change for vector-mediated human diseases.

Model systems have long been a popular research tool to understand climate change impacts on vector-mediated diseases (Perez-Rodriguez et al. 2013, Knowles, Palinauskas & Sheldon, 2010, Lafferty, 2009, Kovats et al. 2001). Modern models can be assessed not solely globally, but also at both local habitat and population scales, thus simplifying the understanding of the epidemiological system and permitting influential characteristics within it to be amplified and investigated further (LaPointe,
Atkinson & Samuel, 2012, Wood, 2006, Beadell et al. 2006). Altitudinal gradient is one such factor. It shares many similarities with those that occur along latitudinal scales (Baillie & Brunton, 2011, Zamora-Vilchis, Williams & Johnson, 2012), but it can also allow monitoring of populations of parasites across similar habitats, day lengths, and geographical distance (Atkinson et al. 2014, van Rooyen et al. 2013b, Mills, Gage & Khan, 2010). This is advantageous to include in a model that may also have to consider populations with similar species composition, but that also exhibit interplay of migration of parasites between sites (Dufva, 1996). Through modelling, less effort is then required to capture a representative proportion of smaller study populations. Subsequently, the sample data that is collected can be more intensively acquired with re-captures over several years solidifying the dataset and enabling long-term individual life-history and infection data to be recorded (Wilkinson et al. 2016). The key factors driving variation within a population and its functioning system can be more easily identified when there are fewer variables to consider (Lachish et al. 2011). Differences in parasite diversity or prevalence patterns, and associated fitness costs to hosts between study populations, can help identify the size and direction of system responses that arise under different climate conditions (Perez-Rodriguez et al. 2014, Bensch et al. 2007). As well as a useful model for assessing climate change influences on vector-mediated disease systems (Garamszegi, 2011), avian haemosporidian parasites have become an increasingly popular system to investigate host life-history trade-offs (Knowles et al. 2009, Isaksson et al. 2013) and host-parasite co-evolutionary interactions (Jenkins & Owens, 2011, Ricklefs, Fallon & Bermingham, 2004).

Diversity of avian haemosporidian parasites

The use of avian hosts as model systems has greatly advanced malaria studies (Marzal, 2012). That these parasites are transmitted by insect vectors was first confirmed by experimentally exposing caged birds to infectious Culex mosquito bites. It was also using this system that the full Plasmodium life cycle was first observed (Marzal, 2012). Initial screening of infection was carried out by scanning multiple fields of Giemsa solution stained blood smears under a light microscope, to look for the presence of infected erythrocytes (Marzal, 2012). Development of modern polymerase chain reaction (PCR) techniques, allowed for quick, relatively easy and reliable amplification of large numbers of parasite DNA, and revolutionised molecular
biological research, including the study of avian haemosporidia (Sehgal, 2015, Kamau et al. 2014, Bensch et al. 2009, Hellgren et al. 2004, Waldenstrom et al. 2004, Cosgrove et al. 2006). Application of PCR techniques to screen for haemosporidian infection revealed a much higher diversity and prevalence of these parasites than had previously been recorded (Sehgal, 2015, Palinauskas, 2009, Bensch et al. 2009). There are currently around 200 haemosporidian species as distinguished by morphological characteristics (Marzal, 2012, Bensch et al. 2009). Since the widespread application of molecular techniques to screen for parasite infections, a new estimate of 10,000 species lineages, equal to that of the total avian species diversity has been proposed (Wood et al. 2006, Bensch et al. 2009). This is based on genetic sequencing of avian parasite DNA at the mitochondrial cytochrome b gene, which functions as part of the electron transport chain, driving production of adenosine triphosphate (ATP), the cell’s energy source (Esposti et al. 1993).

There are a number of public depositories that have been set up in order to catalogue sampled biodiversity. Bensch et al. (2009), introduced MalAvi, a public database dedicated to recording haemosporidian parasites recovered from avian hosts. The database is constructed on amplified parasite cytochrome b gene fragments. These fragments have accumulated a wealth of data, however recent analysis using several alternative parasite genes, chitinase, ama-1, trap and msp-1, for example, have revealed even greater diversity than recovered from the cytochrome b gene (Sehgal, 2015). The result of this, and the range of questions that can be asked about the system, is that no universal protocol has yet been adopted to study haemosporidian parasites in natural populations (see Bensch et al. 2009). Additionally, there has been some issues with sampling bias within the avian haemosporidian disease system, with Passerine birds dominating as source hosts, indicating a considerable proportion of avian haemosporidian diversity is still to be detected in many species (see Table 1 in Appendix). Passeriformes have been reported as a particularly susceptible avian order (Valkiunas, 2005), however this may be influenced by their dominance in species diversity (see Table 1 in Appendix) as an artefact of sampling bias, particularly as they include many useful model species that are studied frequently. Continued research effort, and the potential for future wide-spread application of full-genome and next-generation sequencing techniques, could help to resolve some current issues defining species lineages (Sehgal, 2015). As more species and populations are screened for
haemosporidian parasites, sampling bias may be reduced and it could be possible in
the future to identify global prevalence and distribution trends among key parasite
species lineages.

**Current knowledge of distribution patterns**

Considering only about 50% of all avian species have been screened for avian
haemosporidian parasites, *Plasmodium* infections have been documented in all avian
orders, excluding only ostriches (Struthioniformes), mousebirds (Coliiformes) and
trogons and quetzals (Trogoniformes) (LaPointe et al. 2012). *Plasmodium relictum* and
*Plasmodium circumflexum* parasites, of which there are several distinct lineages, are
thought to have the broadest host range and geographical distribution of
However, *Plasmodium* parasites have been much more intensely studied than either
*Haemoproteus* or *Leucocytozoon* species lineages, so this conclusion is potentially
the result of research bias (Santiago-Alarcon et al. 2014, Perez-Rodriguez et al. 2013,
Loiseau et al. 2013, Synek et al. 2013). *Plasmodium* parasites appear to be highly
prevalent in tropical climates, where transmission can occur all year round, and
habitats generally experience higher annual rainfall and warmer temperatures (Norte
reportedly the most commonly encountered haemosporidian genera among birds
sampled in Europe (Norte et al. 2009, Ricklefs, Fallon & Bermingham, 2004), although
there are many exceptions. Among studies that have sampled coastal bird species
and populations at high altitudes and cold latitudes, *Leucocytozoon* appears to
frequently be the more prevalent parasite genera (Soares et al. 2016, Zagalska &
Bensch, 2015, Cornet et al. 2014, van Rooyen et al. 2013). These differences may be
largely attributable to differences in abundance of the vector species responsible for
transmitting the different haemosporidian parasite genera (Perez-Rodriguez et al.
2014).

A brief analysis of records deposited on the MalAvi database (Bensch, 2009), returned
3,556 records that included data on recorded prevalence of haemosporidia. 6 avian
groups with the most records were chosen to compare prevalence patterns and
parasite distribution between groups of varying relatedness (Figure 1 & Figure 3).
Where records were available, we calculated the proportion of lineage diversity for
each avian group represented by the three parasite genera; *Leucocytozoon*, *Haemoproteus* and *Plasmodium* (Figure 1). Average recorded prevalence was then calculated for each avian group, on each continent (Figure 3). There was significant variation in terms of most prevalent parasite genus, and distribution of lineage diversity between groups. Parasite composition and lineage diversity differed between even closely related avian groups such as the Corvidae and Passeridae, both families within the order Passeriformes (Figure 1). While *Haemoproteus* parasites represented 29% of haemosporidian diversity for both Corvidae and Passeridae, *Plasmodium* and *Leucocytozoon* lineage diversity differed significantly. There was significant variation both between and within locations, and avian groups in average reported prevalence (Figure 3). By location, Africa showed the greatest variation in average recorded

![Figure 1: Pie charts showing the proportion of haemosporidian lineage diversity represented by each parasite genus; *Plasmodium* (Plas), *Haemoproteus* (Haem) and *Leucocytozoon* (Leuc), across 6 avian groups, used later in the text. Avian groups chosen as best represented from MalAvi records, and include both closely and distantly related groups.](image)
prevalence between groups, and Europe the least (Figure 3). The Falconiformes and Corvidae showed the highest and second highest average recorded prevalence across all continents, with 51.15% (N=710) and 36.55% (N=114) infected individuals respectively. N refers to total number of sampled individuals. The two avian groups with the lowest average recorded prevalence were the Anatidae and Charadriiformes with 13.94% (N=1091) and 8.65% (N=558) infected individuals respectively. These results demonstrate the variation in distribution and prevalence patterns that occurs between haemoporidian parasites.

Global prevalence of avian haemosporidian infection is reported to have increased since the 1940s, particularly in Africa and Europe, which represent major bird migratory corridors (Garamszegi, 2011), although this may also be a result of geographical sampling bias (Figure 2). This is in parallel with rising temperatures and rainfall, increasing insect abundance and significantly expanding vector species’ ranges (Marzal, 2012, Loiseau et al. 2012, Garamszegi, 2011). A number of avian populations have recently revealed presence of haemosporidian parasites in habitats previously thought too harsh to permit vector survival and reproduction, where avian hosts were considered unlikely to carry infections (Zagalska-Neubauer & Bensch, 2015, Vansreels et al. 2015, Palinauskas et al. 2009). These habitats containing the new parasite populations are typically coastal, or those at latitudinal extremes or high altitudes. Additionally, the rate at which research on avian haemosporidian parasites has been published, since the widespread application of molecular techniques to screen for these parasites, has dramatically increased (Marzal, 2012).

Due to biases in research toward certain species and locations, as well as a recent shift from predominantly microscopic to molecular screening methods, historical prevalence and distribution trends still remain poorly understood (Njabo et al. 2011,
Knowles, Nakagawa & Sheldon, 2009). Future research should focus on sampling data-deficient host species and populations, and improving our understanding of parasite-vector associations, and vector host feeding preferences (Loaiza & Miller, 2013, Santiago-Alarcon, Palinauskas & Schaefer, 2012, Hellgren, Bensch & Malmqvist, 2008). Repeated sampling of individuals over time, and knowledge of the functional relationships between species and lineages would greatly improve the power of the avian-haemosporidian model system to inform predictions of vector-mediated disease responses to ongoing climate change (Sehgal, 2015, Pérez-Rodríguez et al. 2014).
**Figure 3:** Global Map showing average recorded prevalence (y-axis, mean prevalence reported for all hosts within each avian group harbouring infection from *Plasmodium, Haemoproteus* or *Leucocytozoon* parasites), on each continent where records were available, for 6 different avian groups (x-axis). These included, (abbreviations given in brackets) the family Columbidae (Colu), order Falconiformes (Falc), family Anatidae (Anat), order Charadriiformes (Char) and two avian families within the order Passeriformes; Corvidae (Corv) and Passeridae (Pass). The data was obtained from all records which provided prevalence data on the MalAvi database, which aims to collate available sequence data for all sampled avian haemosporidian parasites and document the diversity within this vast parasite group (Bensch et al. 2009). Families were chosen when there was sufficient sampling of the group to provide data for each continent. When sampling pools were limited it was necessary to use avian order to give the maximum average prevalence figures. 100% prevalence values are likely an artefact of sample size here as only one individual was reported screened in each case.
Life cycle of avian haemosporidian parasites

Transmission of avian haemosporidia occurs via hematophagous dipteran vectors. Typically only females transmit infections between vertebrate hosts, as the majority require a blood-meal to reproduce and lay eggs (Kimura, Darbro & Harrington, 2010). Life-history tactics employed by the different vector species that transmit avian haemosporidian parasites are highly variable. As *Plasmodium* parasite life cycles are by far the best documented, their typical life cycle is outlined below as a general description (Santiago-Alarcon, Palinauskas & Schaefer, 2012). Infections usually begin during a blood meal with the transfer of parasites to a new avian host, as infectious sporozoites within the saliva of a female vector (Paul, Ariey & Robert, 2003). Sporozoites complete an initial cycle of pre-erythrocytic asexual reproduction, producing merozoites immediately following inoculation of the new host (Marzal, 2012). This is followed by multiple cycles of multiplication within connective tissue cells (fibroblasts) and white blood cells (macrophages). This eventually leads to gametogony, the production of gametocytes in the hosts circulating blood (Paul, Ariey & Robert, 2003, Marzal. 2012). Merozoites can infect several host tissue cells including; liver, lungs, spleen, brain and kidneys, where they continue to replicate until they cause invaded cells to rupture (Valkiunas, 2005).

This stage of the parasite life cycle is associated with the acute stage of infection. Infected birds often suffer anaemia because of red blood cell destruction and the relatively sudden influx of parasites into the host bloodstream (Marzal, 2012). Host parasitaemia rises steadily during the acute phase, up to a crisis point where a complex interplay between environmental, genetic and physiological factors, determines the course and severity of infection (LaPointe, Atkinson & Samuel, 2012). *Plasmodium*, and *Haemoproteus* parasites are also known to produce an insoluble pigment called hemozoin through digestion of the oxygen-carrying, iron rich proteins, haemoglobin, in the hosts’ red blood cells (Valkiunas, 2005). Hemozoin is deposited in the host liver and spleen as part of the host immune response, but results in blood becoming thin and watery, and the liver and spleen increasingly darkening in post-mortem examinations (Atkinson, Dusek & Lease, 2001). There are also direct detrimental effects on host energetic and nutritional reserves through depletion of haemoglobin molecules (van de Crommenacker et al. 2012, Palinauskas et al. 2008). Infected hosts are immune to re-infection with the same haemosporidian parasite.
lineage. However, there is some evidence to suggest initial infections may increase host susceptibility to secondary infections from other pathogens and parasites (Farias et al. 2012, Jarvi, Schultz & Atkinson, 2002). Progression of the disease and the clinical signs that occur have been shown to closely parallel the number of haemosporidian parasites present in the reticuloendothelial system and the peripheral blood circulation (hereafter referred to as parasitaemia) (LaPointe, Atkinson & Samuel, 2012). Individuals who survive the acute stage of infection may carry a chronic infection for extended periods, possibly for the rest of their lives, or may be able to clear the infection through activation of the acquired immune response system (Westerdahl et al. 2005, Atkinson, 2005).

Figure 4

*Image courtesy of Atkinson C.T. (1999-2001) Caption: The complex life cycle of haemosporidian parasites begins with (A) an infected insect biting a susceptible bird. Separate infectious and developmental stages occur in (B), the bird host, and (C), the insect vectors.*
The parasite completes its life cycle when gametocytes circulating in the host blood are then taken up by another blood-feed from a competent vector (Valkiūnas, 2005). Only once gametocytes reach the vector midgut do they continue their development, transforming via gametogenesis into true gametes, and fusing to form zygotes (Schrader et al. 2003, LaPointe, Atkinson & Samuel, 2003). A motile zygote is eventually able to penetrate the midgut wall following which parasites begin development as oocysts (da Costa Lim-Junior & Pratt-Riccio, 2016). Oocysts produce thousands of sporozoites via a process of asexual reproduction called sporogony (Valkiūnas, 2005). Once oocysts reach a certain size, they rupture, releasing sporozoites which eventually gain access to the insects’ salivary ducts, via the salivary glands (LaPointe, Atkinson & Samuel, 2012). Here they penetrate glandular cells, and can transmit sporozoites to a new host during subsequent blood feeding, thus completing one full life cycle (LaPointe, Atkinson & Samuel, 2012, Michel & Kafatos, 2005). The variation in species specificity of avian haemosporidian parasites seems partially dependent on vector immune responses (Václav et al. 2016). Studies have shown that while the initial stages of haemosporidian parasite invasion of the vector appears to exhibit little or no species-specificity (Aliota et al. 2011), evidence of immunity and blocked development has been reported for the later stages of infection, particularly invasion of the midgut epithelium (LaPointe, Atkinson & Samuel, 2012, Aliota et al. 2011, Michel & Kafatos, 2005).

**Parasite-vector associations**

There are significant differences in habitat requirements of the different haemosporidian parasite vectors during reproduction, which determines species abundance and composition within natural communities (Pérez-Rodríguez et al. 2014). This is largely as each avian haemosporidian parasite genera relies on different invertebrate orders that primarily transmit the parasite between hosts (Silva-Iturriza, Ketmaier & Tiedemann, 2012, Paul, Diallo & Brey, 2004). Culicidae mosquitoes (important vectors of *Plasmodium* parasites) lay their eggs on the surface of standing, or very slow moving water, and/or above the waterline when areas are prone to flooding (Wood, 2007). Larvae are aquatic, developing through 4 larval instars before pupating. Adults emerge directly from the pupa at the water’s surface (Bayoh & Lindsay, 2004). Simuliidae blackflies, thought to be the primary vector of *Leucocytozoon* parasites, complete development in fast flowing water (which ranges
according to the species) from large bodies of turbulent water to streams and intermittent surface run-off (Silva-Iturriza, Ketmaier & Tiedemann, 2012). Ceratopogonidae vectors that include species that transmit a number of important diseases and arboviruses among humans, as well as parasitic nematodes, are the primary vectors of *Haemoproteus* parasites. These vectors are also known to transmit *Leucocytozoon* species lineages. Some of the species in this group are autogenous, meaning they can mature an initial batch of eggs before taking a blood meal, but require a blood meal for subsequent egg production (Santiago-Alarcon, Palinauskas & Schaefer, 2012). These vectors lay eggs in wet soil or other semi-aquatic habitat types. Unlike most other vectors of avian haemosporidian parasites, Hippoboscidae (louse-flies) (important vectors of *Haemoproteus* parasites) reproduce without large water bodies for developmental stages. Hippoboscidae larvae develop one at a time, inside the mother, and emerge after several larval instars, as a pre-pupa. This pre-pupa then hardens into a puparium, in which the vector completes development, emerging as an adult (Valkiunas, 2005, Atkinson, 1991).

Among the competent avian haemosporidian vectors that have been identified, considerable variation in habitat requirements and environmental tolerances, have been reported (Sehgal, 2015, Perez-Rodriguez, et al. 2013). Vectors show varying degrees of specificity in host feeding preferences. Generalist feeding is reported in a number of vector species and is thought to have been highly significant in aiding haemosporidian transmission between host species (LaPointe, Atkinson & Samuel, 2012, Njabo et al. 2011, Hellgren, Perez-Tris & Bensch, 2009). The environment plays an important role determining the abundance and diversity of parasites within a given natural community, by determining the distribution of different vector species (Sehgal, 2015). The species interactions and environmental conditions that occur within a given community are therefore considered key determinants of parasite spatiotemporal distribution patterns, and community composition (Baille et al. 2012, Knowles et al. 2011, Wood, 2006). There have been very few papers published on avian haemosporidian-vector associations, and simultaneous sampling of vectors and vertebrate hosts is unusual. Despite this, variation in the prevalence and distribution of avian haemosporidian parasites in a community is predicted to be primarily the result of parasite-vector interactions, and environmental conditions during developmental stages within the vector (Boothe et al. 2015, LaPointe, Atkinson &
Samuel, 2012). Indeed, parasites present in the insect community are thought to give a more accurate account of local parasite transmission within a habitat, as birds, particularly migratory species, are likely to have acquired infections from various locations, potentially very far from its initial sample point (Sehgal, 2015).

Natural vector populations are logistically difficult to study (Samuel et al. 2015). Vectors occupy specific and usually more localised ecological niches within a given habitat than birds, some vectors have even been shown to vary in their preferred heights in the canopy and this is thought to relate to feeding preferences (Ferraguti et al. 2013). These specific requirements and behaviours make it very difficult to design and subsequently place traps that target broad insect species groups (Lühken et al. 2014, Farajollahi et al. 2009, Noyes, 1989). Sampling and screening methods can have profound effects on the diversity and prevalence of vectors and subsequently also haemosporidian parasites that are identified in a given community (Carlson et al. 2015). Among sampled insects, only a small fraction are considered to have carried avian haemosporidian parasites in contrast to the vast majority of sampled birds being found to actually carry chronic infections (Caillouët et al. 2012). Only reproductive female vectors blood feed (da Costa Lima-Junior & Pratt-Riccio, 2016), and of these, females will feed on a range of host species, including humans, other birds, mammals and reptiles. Additionally, the parasite will often not be able to complete its’ life cycle across every species that it encounters, and thus only a subset will be competent vectors (Njabo et al. 2011, Paul, Ariey & Robet, 2003). It is necessary therefore to conduct additional analyses, to identify source hosts and vector competencies, to enable identification of which species are primarily responsible for transmission of the different parasite lineages (Sehgal, 2015). Confirming competent vectors generally requires successful transmission or inoculation of the infection to a susceptible host, although presence of the parasites in the salivary glands may help identify likely vectors (Santiago-Alarcon, Palinauskas & Schaefer, 2012).

**Stages of infection**

Avian malaria infections are characterised by severe fluctuations in blood parasitaemia, a measure of infection intensity (Knowles, Nakagawa & Sheldon, 2009). The rate of destruction of red blood cells, the hosts’ oxygen carrying agent, increases with parasitaemia. Excessive loss of red blood cells results in characteristic acute
stage anaemia and reduced mobility, as the parasite competes with the host over metabolic resources (Allander & Bennett, 1995). Following this, parasitaemia usually stabilises at low levels (known as the chronic phase) with some species of *Plasmodium* being able to disappear from the blood circulation entirely, hiding in a dormant form during exoerythrocytic asexual stages (Cornet et al. 2014). Reappearance of parasites circulating in the blood, known as relapse, can occur months or up to years after initial infection (Cornet et al. 2014, Huff, 1968). Cycles of high replication, or relapse, followed by prolonged latency, coupled with the diversity and persistence of haemosporidian parasites, means that the costs of infection vary considerably across the organism’s lifetime (van Rooyen et al. 2013, van de Crommenacker et al. 2012). Relapse is often associated with periods of elevated stress levels, such as under conditions of food shortage, or when undertaking energetically costly activities, such as breeding and migration, during which time host immune system functioning is often suppressed (Cornet et al. 2014). Periods of dormancy among avian haemosporidian parasites have been hypothesised as a coping mechanism by which parasites can survive periods of low vector activity. This can happen when transmission opportunities are very limited, or when parasite prevalence is very high and availability of susceptible hosts becomes limited (Cornet et al. 2014). In such instances, parasites must be able to persist for extended periods of time in the current host, without causing death of the host, as that would also prevent further transmission.

A recent study carried out on great tits by van Rooyen et al. (2013) showed that there are consistent differences between different parasite genera in terms of persistence within the host. Infection with *Plasmodium* and *Haemoproteus* parasite lineages were found to be highly persistent across years, whilst infections with *Leucocytozoon* parasite species were more transient (van Rooyen et al. 2013). *Leucocytozoon* infected individuals were found to frequently change infection status, appearing either uninfected, or found to carry parasites of a different species lineage during subsequent sampling attempts (van Rooyen et al. 2013). Furthermore, among *Plasmodium* and *Haemoproteus* parasite infections, blood parasitaemia remained relatively constant across sampling attempts, whereas *Leucocytozoon* parasite infections regularly fluctuated significantly within host individuals across this period (van Rooyen et al. 2013). Given this level of variation even in cellular processes, long-term datasets that
attempt to identify the proportion of time that hosts spend at these various stages of infection are necessary in order to fully understand the relative impact different parasite lineages impose on their hosts (Wilkinson et al. 2016, Fallon et al. 2003). The potentially life-long persistence of haemosporidian parasites within avian hosts indicates that even if costs are low or insignificant over a single breeding attempt, these could accumulate across the individuals’ lifespan to reduce lifetime reproductive success. This could result in a population-level effect if the reproductive potential of an entire population becomes limited. This potential reproductive influence may place endangered species’ survival prospects at risk (Rigaud, Perrot-Minnot & Brown, 2010).

**Fitness costs associated with avian haemosporidian infection**

Haemosporidian parasites have shown considerable variation in how infections manifest within hosts (Isaksson et al. 2013, Lachish et al. 2011). A study carried out on blackbirds (*Turdus merula*), reported significant differences in parasitaemias associated with different parasite lineages (Bentz et al. 2006). These differences were thought to result from variation in parasite life-histories, as well as in host resistance mechanisms. Another study, which screened 5 common European avian host species reported significant differences in host susceptibility and infection patterns, even reporting resistance in starlings (*Sturnus vulgaris*) (Palinauskas et al. 2008). While we are still to fully understand the precise mechanisms and qualities that infers resistance among avian hosts, certain major histocompatibility complex (MHC) supertypes appear to offer qualitative and quantitative resistance to a number of haemosporidian parasite lineages (Sepil et al. 2013, Bonneaud et al. 2006, Westerdahl et al. 2005). The MHC are cell surface proteins that constitute part of the specific immune response system and are important in recognizing, and binding to, foreign antigens. Hosts with certain MHC supertypes are able to mount a targeted immune response to invasion by haemosporidian parasites, completely clearing infections from the peripheral blood before significant trade-offs occur (Westerdahl et al. 2005, Bensch & Akesson, 2003). The majority of avian hosts sampled in the wild, however, appear to carry persistent chronic infections, the significance of which have been the subject of debate (Asghar, Hasselquist & Bensch, 2011). Due to reproduction being a central measure of fitness, and associated with suppressed immune system functioning, most avian haemosporidian studies assessing the costs of infection have sampled during host breeding periods (Dawson & Bortolotti, 2000).
In extreme cases, avian haemosporidian parasites can result in severe acute infections and high mortalities among host populations, but primarily only when they share a short evolutionary association (Marzal, 2012, Atkinson, Dusek & Lease, 2001). The mechanism by which epidemics occur is likely due to some hosts being naïve to haemosporidian parasites and having not experienced them before, and/or that specific species have not had time to develop an effective specific immune response to infection (Tomás et al. 2007, Hellgren, Pérez-Tris & Bensch, 2009). Severe detrimental effects reported among some birds is evidence such parasites can represent an important selection pressure acting on wild populations (Marzal, 2012). One of the most famous and devastating examples is that of Hawaiian avifauna following the introduction of a single species lineage; *Plasmodium relictum* pSGS1, along with a competent vector, the southern house mosquito (*Culex quinquefasciatus*) (Samuel et al. 2015, van Riper et al. 1986). This synergistic infection led to a widespread population decline and extinction of over half the Islands’ native honeycreeper species (LaPointe, Goff & Atkinson, 2010). Birds in private collections and aviaries often suffer high mortalities when exposed to haemosporidian infection, and as exotic species, they are unlikely to have had much exposure to native parasite lineages (Ellis, Kunkel & Ricklefs, 2014, Olias et al. 2011). Domestic birds are considered at risk to disease outbreak when kept at very high densities, which has been linked to immune suppression, and is known to enhance parasite transmission between birds (Hasson, 2015, Morii, 1992). Furthermore, injured and ill birds taken to rehabilitation, and birds relocated during large-scale conservation efforts, are expected to be exposed to very different parasite communities, against which they may have very little immunity or tolerance (Vanstreels et al. 2015, Xavier, 2015, Vanstreels et al. 2014). These instances could have significant implications for conservation efforts if high mortalities are reported (Vanstreels et al. 2015, Woodworth et al. 2005).

There is a wealth of literature that has used correlative approaches to assess fitness trade-offs associated with infection to avian host individuals and populations (Isaksson et al. 2013, Johnson & Hoverman, 2012, Siikamaki et al. 1997). Although significant trade-offs associated with haemosporidian infection have been reported across the reproductive attempt, effects appear to be somewhat species, and population, specific (Hellgren, Pérez-Tris, & Bensch, 2009, Szymanski & Lovette, 2005). Delays in the
onset of breeding can have significant consequences for overall reproductive success. A study which microscopically screened female great tit blood smears for haematozoan parasites, identified 10 parasite taxa within 7 genera and reported an overall prevalence of 84%. The authors reported significantly delayed onset of reproduction among parasitized females, with longer delays among those with mixed parasite infections (Allander & Bennett, 1995). Infected females may take longer to gain sufficient weight to initiate egg formation, when parasites compete with the host over nutritional and energetic reserves (Allander & Bennett, 1995). Delayed onset of breeding, leading to significant reductions in clutch size and fledging success, has been recorded among house martins (Delichon urbica), harbouring Haemoproteus and Plasmodium infections (Marzal et al. 2013). Here, infected individuals also had lower recorded feather growth rates. Individuals that gained infections in their African wintering grounds, could suffer delayed migration to summer breeding grounds, and subsequently delayed reproduction (Marzal et al. 2013). Delays in reproduction can result in rearing periods being out of synchrony with peak food abundance, that in turn leads to severe reductions in offspring body condition and survival (Nooker, Dunn & Whittingham, 2005).

Terminal investment hypothesis predicts that individuals will invest more in current reproduction if the likelihood of surviving until future reproductive events is low (Bonneaud et al. 2004). In support of this hypothesis, several studies have reported significantly higher clutch sizes among individuals harbouring harmful or intense haemosporidian parasite infections. Great tits (Parus major) infected with Plasmodium parasites laid significantly larger clutches than uninfected females in a study in Sweden (Oppliger, Christe & Richner, 1997). Similarly, Fargallo & Merino (2004) reported a positive relationship between female clutch size and species richness of four protozoan parasites (Haemoproteus majoris, Leucocytozoon majoris, Hepatozoon parus and Trypanosoma avium) among female blue tits (Cyanistes caeruleus) sampled in central Spain (Fargallo & Merino, 2004). However, Haemosporidian parasites are typically encountered as underlying chronic infections, and reports of significant mortalities among hosts are unusual (Marzal, 2012, Merino et al. 2000). Haemosporidian blood parasites are expected to compete with the host over metabolic resources and parents may be able to compensate for this by lowering initial investment in reproduction (Agnew, Koella & Michalakis, 2000). Reducing clutch
size lowers initial reproductive costs, particularly for females who invest less in egg production (Agnew, Koella & Michalakis, 2000, Korpimaki et al. 1993). If this allows parents to maintain energetic reserves, provisioning rates may be higher, or at least conserved, per offspring thus maximising the likelihood of offspring survival among infected parents (Knowles, Nakagawa & Sheldon, 2009). There are several studies that support this alternative reproductive tactic. In a study in Portugal, reduced clutch size and increased egg volume was reported among female great tits (Parus major) harbouring Plasmodium infections compared to uninfected birds, or those harbouring Haemoproteus or Leucocytozoon parasite infections (Norte et al. 2009). Great tits (Parus major) in central Sweden had reduced egg volume and clutch size in response to low food availability and intense infections by Trypanosoma blood parasites, which are related to the Haemosporida (Dufva, 1996). Similarly, female Tengmalm’s owls (Aegolius funereus) reduced clutch size in response to intense Leucocytozoon ziemanni infection (Korpimaki et al. 1993). Prevalence was over 90%, and the reduced clutch size did not translate to lower fledging success, suggesting this may be a plastic response to parasitism, allowing parents to maximise offspring survival and reduce detrimental costs of reproduction to parents by hatching fewer offspring initially (Korpimaki et al. 1993). Differences in reproductive investment may therefore help interpret how intense a selection pressure haemosporidian parasites are exerting on different host individuals.

Significant associations between infection and reproductive effort have been reported, and support the notion that high reproductive investment limits the ability of hosts to control chronic infections, and that this may increase susceptibility and exposure to other infection types (Knowles, Wood & Sheldon, 2010, Knowles, Nakagawa & Sheldon, 2009, Nordling et al. 1998). Norte et al. (2009) reported higher prevalence of Haemoproteus parasites among male great tits (Parus major) with higher fledging success. Another study on great tits, reported that parents with enlarged broods experienced increased prevalence and intensity of H. majoris parasites (Allander, 1997). Larger clutches were also associated with higher parasitaemias among blue tits (Cyanistes caeruleus) harbouring Plasmodium relictum and P. circumflexum infections (Knowles et al. 2011). This was also observed among pied flycatchers (Ficedula hypoleuca), however, the parasite lineage reported for this species responded differently between the sexes (Siikamaki et al. 1997). Among females, H.
*pallidus* infection intensity tended to increase, and among males, *H. balmorali* infection intensity tended to increase as brood sizes were artificially enlarged (Siikamaki et al. 1997).

There have been some studies that have not identified any negative consequences of haemosporidian infections on sampled hosts (Bensch et al. 2007, Kilpatrik et al. 2006, Dufva, 1996). Ortego et al. (2008) reported no significant trade-offs in clutch size, hatching success, or fledging success among lesser kestrels (*Falco naumanni*) with *Haemoproteus* or *Plasmodium* parasite infections, but with one exception. Males infected with *Plasmodium* parasites fledged fewer offspring than those with other infection types (Ortego et al. 2008). Significant effects were only identified when comparing infections by different parasite lineages individually. Cloutier et al. (2011) reported no significant association between *Plasmodium* sp. RBG1 infection and reproductive performance among red-billed gulls (*Larus scopulinus*) although infected individuals tended to have lower residual body condition than uninfected birds.

Complex outcomes of infection have been reported among studies assessing mixed parasite infections, even among co-infecting endoparasite and ectoparasite species. For example, a significant negative association between *Haemoproteus* infection intensity, and abundance of adult fleas carried by blue tits (*Cyanistes caeruleus*) has been recorded (Tomàs et al. 2007). In many cases, it is predicted that co-infecting parasites will amplify detrimental effects to the host through increased parasite intensity and competition over limited resources. Evans & Otter (1998) showed a lethal effect of combined infection with *Haemoproteus noctuae* and *Leucocytozoon ziemanni* parasites in juvenile snowy owls (*Nyctea scandiaca*), despite neither parasite being considered pathogenic as single infections. Another two parasite lineages, *Plasmodium relictum* and *Plasmodium ashfordi*, both highly virulent strains, have been shown to act synergistically when co-infecting some avian species (Palinauskas et al. 2011). To accurately interpret biological systems, it is necessary to identify mixed parasite infections, as interactions between co-infecting parasites can be important determinants of how infection manifests within the host, as well as the strength of the selection pressure they impose (Pérez-Rodríguez et al. 2014, Choisy & de Roode, 2010, Rigaud, Perrot-Minnot & Brown, 2010).

In addition to correlational studies, natural population sampling and contrasting individual animals with different infection types, two experimental approaches have
been applied to assess the costs of haemosporidian infection on hosts (Marzal, 2012). The first is a technique that inoculates uninfected individuals with a given parasite lineage, the second, is to medically treat infected individuals and compare their performance with respective uninfected control groups (Marzal, 2012, Zehlindjiev et al. 2008). A caveat to the use of anti-malarial compounds on birds, despite a number having been synthesised using the avian model (Marzal, 2012), is that a full medical assessment of these drugs has not been performed and thus the appropriate dose and potential side-effects for treated birds remains unknown (Knowles, Palinauskas & Sheldon, 2010, Tomas et al. 2007, Stoskopf & Beier, 1979). Additionally, the human and avian parasite-host system differs greatly in diversity and there has been some variation in the drugs’ efficacy at clearing different parasite lineages from host blood (Marzal, 2012). Detrimental effects of medication have been reported in some studies, such as increased likelihood of nest abandonment, and alterations in female thermoregulation (Knowles, Palinauskas & Sheldon, 2010, Sanz et al. 2001), which likely conflicts with positive effects associated with overcoming haemosporidian infections. The advantage of experimental studies is that they can reduce additional sources of variation which could mask the costs imposed by parasitic infection. They could also provide quantitative support for effects of parasitic infection identified in correlational studies (Marzal, 2012, Knowles, Nakagawa & Sheldon, 2009). Although, few studies using experimental methods to investigate avian haemosporidian infections have been published, results indicate that trade-offs associated with infection could be much higher than detected by correlational studies (Knowles, Palinauskas & Sheldon, 2010, Tomas et al. 2007, Marzal et al. 2005, Merino et al. 2000).

A study which treated female blue tits (Cyanistes caeruleus) with Malarone™ (an antimalarial drug that can significantly reduce and clear Plasmodium parasites from the blood) found treated female blue tits had significantly higher hatching and fledging success than untreated birds (Knowles et al. 2010). Plasmodium infection was associated with reduced provisioning rates and greater within-brood variation in offspring mass which likely produced these reported differences in fledging success between groups (Knowles et al. 2010). Similarly, blue tits medically treated with Primaquine™ (a malarial relapse prevention drug that significantly reduces Haemoproteus majoris and Leucocytozoon majoris infection intensities) displayed
significantly higher fledging success than control females (Merino et al. 2000). Control females also displayed a significant negative association in the study between *Haemoproteus majoris* infection intensity at fledging and body mass (Merino et al. 2000). This is despite no significant difference between medicated and control groups in terms of clutch size, offspring mass or tarsus length being detected. Lower fledging success among infected individuals appears to therefore be the result of reduced provisioning rates or parental working capacity during the rearing period. Thomas et al. (2007) showed that an observed decrease in immunoglobulin level following either a high or low dose treatment with Primaquine™ resulted in significantly increased provisioning rates among blue tits. Marzal et al (2005) also used Primaquine™ and a saline control to randomly treat *Haemoproteus prognei* infections in house martins (*Delichon urbica*) and reported that treated individuals had significantly higher clutch sizes, and greater success in both hatching and fledging.

The costs of infection are expected to vary across an organism’s lifetime (Knowles, Palinauskas & Sheldon, 2010). Transmission of infection to pre-fledgling chicks could impair healthy growth and development, lowering an individuals’ phenotypic quality and limiting both likelihood of surviving and securing a mate (Quillfeldt et al. 2014, Krams et al. 2013, Pérez-Rodríguez et al. 2013). Young birds are often thought to suffer more severe infection than adults because hosts take time to develop a specific immune response (Allander & Bennett, 1995). However, a recent experimental study that infected nestling rock pigeons (*Columba livia*) with *Haemoproteus columbae* parasites reported no significant difference in chick body mass, age at fledging, fledging success or even post-fledging survival, of infected chicks compared with control birds. This was despite long-term studies of older birds revealing significant fitness consequences associated with chronic *H. columbae* infections (Knutie, Waite & Clayton, 2012). This style of investigation highlights the advantages of long-term studies on host populations. By gaining a full understanding of the severity of infection, and the proportion of time individuals spend suffering different intensities of infection across their lifetime, we may gain a better perspective of the relative selection pressure posed by different parasite lineages (Samuel et al. 2015, Lachish et al. 2013). A potential bias that arises from primarily assessing the influence of haemosporidian infection on reproductive performance is that infection may also influence the likelihood of securing a mate or reproducing period (Arriero & Moller, 2008). This could
occur if infected individuals suffered increased predation, altered behaviour, or changes in secondary sexual traits that influence female mate choice. This concept is supported by Hunt et al. (1998), who showed that female blue tits (*Cyanistes caeruleus*) preferred mates with brighter crests, and are sensitive to UV reflectance. *Haemoproteus* parasite infection was later shown to reduce intensity of plumage colouration, and subsequently survival among great tits (*Parus major*) (Dufva & Allander, 2014, Hunt et al. 1999).

Several studies have demonstrated behavioural changes among avian hosts harbouring haemosporidian parasite infections (Marinov et al. 2015, Dunn et al. 2011, Gilman et al. 2007, Moller & Nielsen, 2007). Gilman et al. (2007) reported significant associations between haemosporidian infection and white-crowned sparrow (*Zonotrichia leucophrys orianta*) singing behaviour. While a study in northeast Bulgaria, reported wild-caught nightingales (*Luscinia megarhynchos*) harbouring haemosporidian parasite infections displayed a significant positive correlation between risk-taking behaviour and infection intensity (Marinov et al. 2015). Furthermore, Dunn et al. (2011) compared performance between great tits (*Parus major*) testing positive for *Plasmodium* and *Leucocytozoon* parasites and individuals who did not appear to carry haemosporidian infection, and between males and females across three behavioural traits. Among infected birds, males showed improved, while females showed significantly impaired problem-solving abilities, compared to uninfected control groups (Dunn et al. 2011). However, both infected and uninfected females showed greater problem-solving abilities than males. Infected females were more explorative than uninfected females, while among males no significant difference was detected (Dunn et al. 2011). Lastly, the authors reported infected males were more risk-averse than uninfected males, while among females no significant difference was detected (Dunn et al. 2011). Variation in haemosporidian prevalence could occur due to some behaviours offering an interactive protection to an individual bird or behavioural change that results in avoiding potential infection (Perez-Rodriguez et al. 2013, Lachish et al. 2011, Wiersch et al. 2007). Behavioural traits such as boldness and exploration are correlated with dispersal and individuals regularly exploring different habitats are more likely to be exposed to increasingly diverse parasite assemblages (van Rooyen et al. 2014, Hellgren, Pérez-Tris & Bensch, 2009, Hellgren et al. 2007). Haemosporidian parasites could therefore
influence reproductive success indirectly through complex associations between infection and behaviour, such as higher predation rates among infected birds displaying increased risk-taking behaviour, or reduced foraging success among infected birds with impaired problem-solving abilities (Moller & Nielsen 2007). Studies that do not consider the influence of haemosporidian infection on host behaviour and secondary-sexual characteristics (a factor that determines mate acquisition) are therefore likely to underestimate the full influence of these infections across host populations (Dunn et al. 2011).

Furthermore, there is a lack of consistency in research protocol among studies on avian haemosporidian parasites. Papers that report only prevalence (presence/absence) of haemosporidia frequently report weaker effect sizes, or no effect, whereas studies measuring parasite intensity (blood parasitaemia) often report more significant results (Bentz et al. 2006, Knowles et al. 2011). This situation is possibly due to parasitaemia representing a more responsive and dynamic measure than infection status alone (Fallon et al. 2004, Paul, Diallo & Brey, 2004). Bentz et al. (2006) noted studies based on microscopic examination of blood smears could be reinterpreted in terms of parasitaemia, rather than prevalence, and allow for improved comparison of results between studies. Examples of how choice of research method can influence results can be found in two studies carried out on great reed warblers (Acrocephalus arundinaceus) sampled in Sweden (Ashgar et al. 2011, Bensch et al. 2007). Both studies recovered a combined prevalence of 31% for Haemoproteus and Plasmodium parasites. Ashgar et al. (2011), used quantitative amplification (qPCR) to assess intensity of Plasmodium ashfordi, Plasmodium relictum and Haemoproteus payevskyi infections. Bensch et al. (2007) used PCR techniques that screened more holistically for Haemoproteus and Plasmodium parasite infections, however, the three most prevalent lineages were the same as those deployed by Ashgar et al. (2011). Ashgar et al. (2011) noted that only infections resulting in high parasitaemias were associated with significant fitness trade-offs, and that intense Haemoproteus payevskyi infections resulted in significant reduction of the number of fledged offspring. There was a marginal delay in reproduction among birds harbouring intense Haemoproteus payevskyi infections and a marginal reduction in offspring recruitment success among great reed warblers with intense Plasmodium ashfordi infections (Ashgar et al. 2011). In disagreement, Bensch et al. (2007) reported no significant differences in body
condition or reproductive performance between parasitized and un-parasitized individuals. Although the effect was non-significant, the authors reported a tendency for parasitized individuals to produce a higher number of recruited offspring (offspring that survived to maturity and were recaptured during subsequent sampling attempts) than uninfected birds (Bensch et al. 2007).

In summary, we have shown that haemosporidian parasites can constitute important selection pressures on host populations (Knowles, Palinauskas & Sheldon, 2010). However, there is considerable variation in the costs to hosts reported for haemosporidian parasites, which appears to partly arise from differences across research methods (Sehgal, 2015, Fallon et al. 2003, Dufva, 1996). Significant effects of infection are most frequently reported among individuals harbouring simultaneous infections by multiple parasite lineages, (Marzal, 2012, Rigaud, Perrot-Minnot & Brown, 2010). This is thought to be due to parasites competing over host resources, which is expected to amplify effects to the host (Choisy & de Roode, 2010, Chasar et al. 2009, Zehtindjiev et al. 2008). However, there are numerous examples of mixed infections having complex, non-linear outcomes on host performance (Xavier, 2015, Ortego et al. 2008). A comprehensive study of over 260 bird species in the Western Palearctic revealed a significant negative association between adult survival rate and recorded haemosporidian diversity found infecting a host species (Arriero & Moller, 2008). As prevalence increases with climate warming, the success of different avian host populations at developing acquired immune responses to emerging parasite lineages could be an important determinant of host population persistence. Improving the understanding of when and why these parasites cause significant fitness consequences to their hosts will require substantial and ongoing research effort that assesses long-term datasets from different hosts and parasite species and populations.

**Environmental and climate change effects**

Although species composition and species interactions within a community ultimately determine prevalence and diversity of haemosporidian parasites, environmental and climate factors are thought to be key determinants of parasite transmission (Njabo et al. 2011, Chasar et al. 2009, Wood, 2006). As ectothermic organisms, insect vectors are highly sensitive to their external environment, and studies have shown positive
associations between climate factors such as temperature and precipitation as well as relations with insect abundance and diversity (Pérez-Rodríguez et al. 2014, Garamszegi, 2011, Poncon et al. 2007). Insect abundance can enhance parasite transmission by increasing encounter rates with susceptible hosts (Pérez-Rodríguez et al. 2014, Pérez-Rodríguez et al. 2013, Mills, Gage & Khan, 2010, Keesing, Holt & Ostfeld, 2006). There are also direct and indirect consequences of habitat characteristics (Sehgal, 2015) such as vegetation cover, human disturbance, and distance from suitable vector breeding grounds, on subsequent vector and parasite abundance within natural communities (Chasar et al. 2009). Krama et al. (2015) found that prevalence of haemosporidian parasites in mixed-species tit flocks decreased as distance from water bodies increased. It is generally accepted that higher availability of vector breeding sites will enhance parasite transmission by increasing the abundance and diversity of vectors to transmit infections (LaPointe, Atkinson & Samuel, 2012). Habitat fragmentation and development also leads to more open spaces, which has been shown to enhance vector dispersal (LaPointe, Atkinson & Samuel, 2012).

Rainfall can have both direct and indirect consequences for haemosporidian parasites by influencing vector development time and the availability of suitable habitat for vector reproduction (Pérez-Rodríguez et al. 2014, Okanga, Cumming & Hockey, 2013, Garamszegi, 2011). A recent study by Okanga et al. (2013) sampled Ploceidae birds (bishops, weavers and allies) and their vectors in the Western Cape, South Africa, over a two-year period. They also recorded a number of environmental and climate characters. Okanga et al. (2013) found that the majority of mosquito species captured belonged to the Culex culex species complex, which are thought to be the predominant vector species for avian Plasmodium parasite transmission (Haemoproteus and Leucocytozoon parasites were excluded from the study). Okanga et al. (2013) results found Plasmodium infection prevalence showed a significant positive relationship with rainfall two months prior to the sampling date, and mosquito prevalence patterns varied closely with rainfall. Water salinity was also identified as a key determinant of infection prevalence. Prevalence of both mosquitoes and Plasmodium parasites showed seasonal fluctuations. Given the two-month delay in parasite response to rainfall, the authors concluded that an asynchronous co-variation between Plasmodium prevalence and mosquito abundance could be attributable to a lag in
response times to changing rainfall patterns (Okanga et al. 2013). Vectors show variation in sensitivity to external conditions and those that are more sensitive may be less prevalent in more arid environments, or in areas where there are strong seasonal fluctuations in climate, with subsequent consequences for parasite distribution.

Temperature is also considered a major limiting factor for parasite development and survival within the vector (Paaijmans, Cator & Thomas, 2013, Bayoh & Lindsay, 2011, Gething et al. 2011) and is an important determinant of parasite and vector metabolic rates (Mordecai et al. 2013, Kovats et al. 2001). One major factor limiting transmission is the extrinsic incubation period; The time it takes for the parasite to reproduce sexually within the dipteran vector and travel to the salivary glands (Atkinson et al. 2014, Cator et al. 2014, Blanford et al. 2013). This process can take 14 days and exhibits significant variation in response to environmental temperature, which can also influence transmission success (Paaijmans, Cator & Thomas, 2013, Gething et al. 2011, LaPointe, Goff & Atkinson, 2010, Paaijmans et al. 2010). As there is a very high risk the vector could die during this time, the parasite may find it advantageous to manipulate vector behaviour in order to maximise chances of a successful transmission (Cator et al. 2014). Haemosporidiain parasites have been shown to decrease predation risk during the external incubation period by suppressing the vectors appetite after taking an initial infected blood meal (Cator et al. 2014). This reduces the risk of competition with other parasite lineages during this stage and maximises the likelihood of the vector surviving, and transmitting, the parasite to a new host individual. Additionally, parasites may actively increase their potential to infect a new host. A recent study by Cornet et al. (2014) showed both uninfected and infected vectors exhibit feeding preferences for infected hosts.

A recent report by Mordecai et al. (2013) stated that although temperature has long been known to influence haemosporidian development, most models assume an unrealistic, constant linear response will be observed in parasite and mosquito life-history traits in response to temperature change. Mordecai et al. (2013) constructed a model based on field and laboratory data from Africa and found that parasite transmission fell sharply above 28°C. These findings were in contrast to most models having predicted optimal transmission occurring at 31°C. The authors therefore proposed an alternative optimal temperature for parasite transmission to occur at 25°C, which lead to very different predictions of parasite distribution and prevalence.
under ongoing climate change trends. Other effects relating to temperature that have been identified include altered seasons and disturbed seasonal climate changes in temperate regions, including daily temperature fluctuations. Localised changes to these temperatures could promote insect metabolism, allow longer vector breeding seasons, and a higher number of reproductive events per season, thus significantly increasing vector abundance (Loiseau et al. 2012b, Garamszegi, 2011, Chasar et al. 2009, Paul, Diallo & Brey, 2004). Thorough understanding of vector and parasite responses to external conditions, and more detailed climate and habitat data, can improve the accuracy of predictive maps for haemosporidian and malaria spatial and temporal distribution patterns (Blandford, 2013).

In temperate regions, parasite abundance often fluctuates with seasonal climate and host reproductive cycles (Beaudoin et al. 1970). Over winter months when vectors are inactive, the majority of birds will carry underlying chronic infections with either low parasitaemias, or absence of parasites from the blood, as infections can lie dormant in organs such as the liver and spleen (Kim & Tsuda, 2010, Norte et al. 2009, Fallon et al. 2004, Beaudoin et al. 1970). As temperatures increase, vectors emerge and become highly abundant, and avian hosts begin to breed, there is a sharp rise in parasite prevalence and intensity, as new individuals become infected, primarily naïve juveniles previously unexposed to the disease (Beaudoin et al. 1970). At the same time, reproductive activities, which are energetically costly and have been shown repeatedly to lead to energetic trade-offs with immune function, can trigger chronic infections to relapse (Beudoin et al. 1970). Thus, seasonal fluctuations and habitat type have been shown to influence the haemosporidian parasite community (Sehgal, 2015). In tropical habitats where climatic fluctuations are generally reduced compared to temperate ones, climate may be permissible for insect reproduction and parasite transmission to occur year-round. This could enhance the prevalence and diversity of haemosporidian parasites that occur in tropical habitats compared to temperate ones (Sehgal, 2015). A study comparing host specificity of Plasmodium parasites in three African habitats, two that experienced significant seasonal fluctuations in climate, and one lowland forest that experienced little fluctuation, parasites tended to be more generalist species in the two more variable habitats (Loiseau et al. 2012). Host generalist haemosporidian parasite species readily infect a wide range of hosts and have the potential to spread quickly through naïve populations through a process of
host-switching (Sehgal, 2015, Szymanski & Lovette, 2005, Ricklefs, Fallon & Bermingham, 2004, Ricklefs & Fallon, 2002). This suggests that climate change, which includes altered and more severe seasonal fluctuations in temperature and precipitation (Lafferty, 2009) may lead to higher prevalence of parasites across communities.

A suite of additional unknown factors can influence the epidemiology of infection, which can be influenced by climate variables. These factors can also determine the rate at which parasites spread within a host community. The frequency vectors need to blood-feed will help determine transmission rates (Kim & Tsuda, 2010, Paiijmans et al. 2010, Hellgren, Perez-Tris & Bensch, 2009), while the relative abundance of competent vectors, susceptible hosts and community composition can determine encounter rates (Okanga et al. 2015, Samuel et al. 2015). Increased parasite abundance and diversity leads to increased competition between parasites and this can lead to host community instability, or their extinction, many of which can carry species-specific parasite lineages (Fallon et al. 2004). Overall, we expect to see heterogeneous changes in both environmental conditions, and prevalence and diversity patterns of vectors, and their associated haemosporidian parasites as some areas become more favourable for vector survival and reproduction, but others reduce favourability (Pérez-Rodríguez et al. 2014, Bonneaua et al. 2009, Minakawa et al. 2002).

**Assessing climate change impacts on avian haemosporidian infections**

One method to predict the likely outcomes climate change will have on avian haemosporidian disease systems is to simultaneously sample and compare host populations which are situated along a climate or altitude gradient. Choosing populations with proximate geographic distances minimises variation between a population’s broad habitat characters and species composition (Zamora-vilchis, Williams & Johnson, 2012). Ideally sites should also be comparable in levels of human disturbance and habitat fragmentation (Schroder & Schmidt, 2014, Loiseau et al. 2012, Chasar et al. 2009). Contrasting results from different studies can be difficult as they frequently vary in sampling period (year or season), and the specific protocols or measures that were used to assess the parasite community and host impact. These differences between studies can produce significant differences in results that are
independent of climate effects or the focus of the study (Bentz et al. 2006, Waldenström et al. 2004, Fallon et al. 2003). In the following chapter, I present a study of avian haemosporidian prevalence and diversity, as well as an analysis of significant fitness trade-offs associated with infection by different parasite genera (Leucocytozoon, Haemoproteus, and Plasmodium) at four sites with different altitudes. I use two host species, which have both been extensively used as model systems for avian malaria research, blue tits (Cyanistes caeruleus) and great tits (Parus major). Results are compared with a similar study conducted on great tits, and differences between the two systems are assessed (van Rooyen et al. 2013a). The purpose of this study is to assess probable implications of ongoing climate change trends on host populations with which haemosporidian parasites likely share a long evolutionary association, and whereby the majority of individuals’ also harbour underlying chronic infection.

Conclusions and further research opportunities

Haemosporidian parasites can constitute a strong selection pressure acting on host populations (Sepil et al. 2013 Arriero, & Møller, 2008), and as such, are thought to have been important in shaping host life history tactics and evolution (Agnew, Koella & Michalakis, 2000, Sheldon & Verhulst, 1996). As a popular model system in vector-mediated disease research, avian haemosporidian studies have focused on a wide range of different subjects including disease epidemiology, behaviour and co-evolutionary interactions (Knowles et al. 2011, Knowles, Palinauskas & Sheldon, 2010, Delhey & Kempenaers, 2006). Although much research has already been carried out on this globally distributed, highly diverse disease system, there has been consistent bias towards studying the parasites in a subset of avian host species and geographic locations (Bensch et al. 2009). Knowledge of parasite-vector interactions between Leucocytozoon and Haemoproteus parasites and their associated vectors is still very limited (Lalubin et al. 2013, Njabo et al. 2011). Considerable variation between haemosporidian parasite studies likely stems from a lack of consistency in research protocols, making it difficult to directly compare results or identify broad trends in prevalence and fitness costs among hosts and vectors (Bentz et al. 2006, Waldenström et al. 2004). As climate change and habitat fragmentation trends accelerate, global biodiversity, which has been falling at its fastest rate since the last major extinction event, is expected to continue to decline (Brook, Sodhi & Bradshaw,
2008). Many avian species are already showing alarming population declines, and increasing prevalence of haemosporidian parasites would be expected to amplify selection pressure imposed on remaining host populations (Atkinson et al. 2014, Mills, Gage & Khan, 2010, Martens et al. 1995).

To build our understanding of the processes and mechanisms that drive fitness consequences, and prevalence patterns, future studies will focus on assessing the system as a whole (Pérez-Rodríguez et al. 2014, Njabo et al. 2011, Rigaud, Perrot-Minnot & Brown, 2010). Better screening for mixed parasite infections will also improve our understanding of the relative selection pressures imposed by different parasite lineages, or combinations of lineages, and the outcomes of parasite interactions (Synek et al. 2013, Rigaud, Perrot-Minnot & Brown, 2010, Bentz et al. 2006). Sampling and experimentation across the parasites entire life cycle could give valuable insight about how these parasites influence their hosts, and allow spatiotemporal predictions about the impact of the disease under climate change to be made. Long-term studies will also reveal trends in prevalence and across communities where parasite prevalence and composition are known (Wilkinson et al. 2016, Imura et al. 2012, Bensch et al. 2007). Experimentation such as treatment or inoculation will likely continue to help identify the cellular and metabolic processes and interactions that drive this system (Keesing, Holt & Ostfeld, 2006, Kovats et al. 2001). Improved recording of environmental parameters, such as climate data and habitat features, will build a more accurate picture of how predicted climate changes will influence this complex host-parasite-vector disease system (Schaffner et al. 2013).
## Appendix 1
### Table 1
Proportion of total avian diversity, MalAvi sampling effort, and average haemosporidian parasite prevalence recorded for each bird order

<table>
<thead>
<tr>
<th>Host Order</th>
<th>Common names</th>
<th>% avian species diversity within order</th>
<th>% total MalAvi records (with prev. data)</th>
<th>No. studies</th>
<th>No. host spp.</th>
<th>No. individuals</th>
<th>No. Countries sampling carried out in</th>
<th>No. Parasite Lineages recovered</th>
<th>Average recorded prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anseriformes</td>
<td>Swans, geese and ducks</td>
<td>1.68</td>
<td>0.87</td>
<td>6</td>
<td>9</td>
<td>588</td>
<td>6</td>
<td>20</td>
<td>9.88%</td>
</tr>
<tr>
<td>Apterygiformes (Struthioniformes, Rheiformes, Casuariformes)</td>
<td>Flightless ground birds including kiwi's</td>
<td>0.10</td>
<td>0.08</td>
<td>2</td>
<td>2</td>
<td>83</td>
<td>1</td>
<td>2</td>
<td>48.24%</td>
</tr>
<tr>
<td>Bucerotiformes</td>
<td>Hornbills</td>
<td>0.56</td>
<td>0.08</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>66.67%</td>
</tr>
<tr>
<td>Charadriiformes</td>
<td>Oystercatchers, avocets, stilts, thick-knees, pratincoles, coursers, plovers, sandpipers and relatives, phalaropes, skuas, terns and auks</td>
<td>3.47</td>
<td>0.76</td>
<td>10</td>
<td>13</td>
<td>1091</td>
<td>10</td>
<td>17</td>
<td>13.43%</td>
</tr>
<tr>
<td>Ciconiiformes (Gaviiformes, Podicipediformes)</td>
<td>Herons, storks, ibises, flamingoes, (divers and grebes)</td>
<td>1.46</td>
<td>0.67</td>
<td>5</td>
<td>8</td>
<td>321</td>
<td>5</td>
<td>17</td>
<td>22.74%</td>
</tr>
<tr>
<td>Columbiformes</td>
<td>Pigeons and doves</td>
<td>3.14</td>
<td>1.32</td>
<td>13</td>
<td>16</td>
<td>140</td>
<td>14</td>
<td>40</td>
<td>37.73%</td>
</tr>
<tr>
<td>Coraciiformes</td>
<td>Kingfishers, bee-eaters, rollers</td>
<td>2.09</td>
<td>0.53</td>
<td>5</td>
<td>9</td>
<td>109</td>
<td>5</td>
<td>18</td>
<td>31.31%</td>
</tr>
<tr>
<td>Cuculiformes</td>
<td>Cuckoos</td>
<td>1.67</td>
<td>0.08</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>66.67%</td>
</tr>
<tr>
<td>Falconiformes (Accipitriformes)</td>
<td>Falcons, Caracaras, osprey, hawks and eagles</td>
<td>3.05</td>
<td>0.93</td>
<td>7</td>
<td>10</td>
<td>1015</td>
<td>6</td>
<td>23</td>
<td>18.36%</td>
</tr>
<tr>
<td>Galbuliformes</td>
<td>Jacamars and Puffbirds</td>
<td>0.18</td>
<td>0.03</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>50.00%</td>
</tr>
<tr>
<td>Host Order</td>
<td>Common names</td>
<td>% avian species diversity within order</td>
<td>% total MalAvi records (with prev. data)</td>
<td>No. studies</td>
<td>No. host spp.</td>
<td>No. individuals</td>
<td>No. Countries sampling carried out in</td>
<td>No. Parasite Lineages recovered</td>
<td>Average recorded prevalence</td>
</tr>
<tr>
<td>---------------------------</td>
<td>----------------------------------------------------------------</td>
<td>--------------------------------------</td>
<td>----------------------------------------</td>
<td>-------------</td>
<td>---------------</td>
<td>-----------------</td>
<td>--------------------------------------</td>
<td>-------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Gruiformes</td>
<td>Bustards, cranes, rails, crakes and gallinules</td>
<td>2.06</td>
<td>0.62</td>
<td>3</td>
<td>4</td>
<td>22</td>
<td>8</td>
<td>14</td>
<td>10.56%</td>
</tr>
<tr>
<td>Musophagiformes</td>
<td>Turacos</td>
<td>0.23</td>
<td>0.03</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>50.00%</td>
</tr>
<tr>
<td>Passeriformes</td>
<td>Small perching birds</td>
<td>59.92</td>
<td>89.88</td>
<td>114</td>
<td>485</td>
<td>50805</td>
<td>68</td>
<td>931</td>
<td>22.11%</td>
</tr>
<tr>
<td>Pelecaniformes</td>
<td>Pelicans, tropicbirds, gannets, cormorants, frigatebirds</td>
<td>0.66</td>
<td>0.39</td>
<td>5</td>
<td>5</td>
<td>137</td>
<td>4</td>
<td>8</td>
<td>20.07%</td>
</tr>
<tr>
<td>Piciformes</td>
<td>Woodpeckers</td>
<td>4.09</td>
<td>0.42</td>
<td>5</td>
<td>13</td>
<td>31</td>
<td>6</td>
<td>14</td>
<td>53.56%</td>
</tr>
<tr>
<td>Procellariiformes</td>
<td>Albatross, shearwaters, storm-petrels</td>
<td>1.27</td>
<td>0.03</td>
<td>1</td>
<td>1</td>
<td>28</td>
<td>1</td>
<td>1</td>
<td>3.57%</td>
</tr>
<tr>
<td>Psittaciformes</td>
<td>Parrots</td>
<td>3.62</td>
<td>0.14</td>
<td>2</td>
<td>4</td>
<td>7</td>
<td>3</td>
<td>4</td>
<td>100.00%</td>
</tr>
<tr>
<td>Sphenisciformes</td>
<td>Penguins</td>
<td>0.17</td>
<td>0.22</td>
<td>4</td>
<td>3</td>
<td>348</td>
<td>3</td>
<td>8</td>
<td>49.49%</td>
</tr>
<tr>
<td>Strigiformes</td>
<td>Owls, barn owls and grass owls</td>
<td>2.08</td>
<td>0.56</td>
<td>5</td>
<td>5</td>
<td>193</td>
<td>5</td>
<td>14</td>
<td>53.39%</td>
</tr>
<tr>
<td>Trochiliformes (Apodiformes)</td>
<td>Hummingbirds and swifts</td>
<td>4.31</td>
<td>1.63</td>
<td>7</td>
<td>27</td>
<td>398</td>
<td>3</td>
<td>16</td>
<td>46.61%</td>
</tr>
<tr>
<td>Trogoniformes</td>
<td>Trogons and Quetzals</td>
<td>0.38</td>
<td>0.11</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>43.75%</td>
</tr>
<tr>
<td>Upupiformes</td>
<td>Hoopoes</td>
<td>0.01</td>
<td>0.06</td>
<td>2</td>
<td>1</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>33.33%</td>
</tr>
</tbody>
</table>

Order names in brackets represent orders for which MalAvi has not recognised due to disagreements between various avian taxonomies, but which can be paired through information given on the British Trust for Ornithology website.

Orders missing from Malavi database that could not be paired with another order: Tinamiformes (Tinamous) and Caprimulgiformes (nightjars) comprise the remaining total avian diversity missing from the column titled ‘% total avian species diversity’.

**Bold** font to highlight avian orders where fewer than 10 individuals have been screened and results deposited on the MalAvi database. **Bold italic** used to highlight dominant avian order on the MalAvi database, the Passeriformes.

*Taxonomic data obtained from BTO website, which uses a taxonomy based on Perrins (2003).*

*Prevalence data obtained from the MalAvi database, Staffan Bensch, Martin Egerhill & Bjorn Cranbeck, Lund University, Department of Biology, Version 2.3.0., downloaded; 02/28/2017. Supported by CANMove and MalariaRCN.*
Data Chapter

An epidemiological study of haemosporidian infections in blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*) along an elevation gradient.

*Jessica Lewis, Louis Salle, Aisha Bruendl, Andy Russell, Alexis Chaine and Camille Bonneaud*

**Abstract**

Climate characteristics are expected to play a decisive role in determining vector-mediated disease epidemiology (Mills et al. 2010). However, clear predictions on the responses of parasites to climatic changes are lacking (Seghal, 2015). Here I investigate differences in prevalence, diversity and costs associated with haemosporidian infections in breeding populations of blue tits (*Cyanistes caeruleus*), and great tits (*Parus major*) situated along a significant altitude gradient (430m-1530m). Avian haemosporidian parasites were detected in 92.7% blue tits and 91.5% great tits, representing 44 unique mitochondrial haplotypes. In total we recovered 11 verified *Leucocytozoon* parasite lineages, 3 *Haemoproteus* and 1 *Plasmodium relictum*. Mixed infections were reported in 17.7% blue tits and 21.3% great tits. While distribution of the detected parasite lineages differed between hosts and sites, we found no significant effect of altitude on infection prevalence, or parasite lineage richness. Furthermore, there was no detected effect of altitude on the cost of infection, measured either as individual body condition or reproductive success. Blue tits infected with *Plasmodium* lineages, and great tits infected with *Haemoproteus* lineages were significantly heavier than uninfected conspecifics or those carrying other infection types. Blue tits infected with either *Haemoproteus* or *Leucocytozoon* parasites exhibited significantly delayed hatching. The cost of infection on reproductive success was significant among birds carrying mixed infections only. Great tits infected with more than one parasite lineage showed significantly reduced hatching success, while blue tits carrying mixed infections had reduced fledging success. The results suggest the impacts of climatic differences on infections were either negligible, or that connectivity of vectors and hosts between sites prevented detection of any variation that occurred as a result of climate differences. The variation in haemosporidian parasite prevalence and diversity
between sites and species is supportive of previous studies that suggest small-scale habitat heterogeneity influences vector community composition and individual host movements which may be important in determining host infection patterns (Sehgal, 2015, Perez-Rodriquez et al. 2013). Better detection of mixed parasite infections (van Rooyen et al. 2013) and further long-term sampling of natural populations, will help inform predictions of how vector-mediated diseases will respond to climate change (Martens et al. 1995, Garamszegi, 2011).

**Keywords:** Avian haemosporidian parasite, blue tit (Cyanistes caeruleus), great tit (Parus major), vector-mediated disease, fitness consequences, altitude gradient.

**Introduction**

Host-parasite relationships are increasingly being disturbed by changing environments (Khasnis & Nettleman, 2005, Patz et al. 2004). Vector-mediated diseases such as malaria and yellow fever are particularly sensitive to environmental conditions as they depend on insects to complete their life cycle and move to a new host (Loiseau et al. 2012, LaPointe et al. 2010, Martens et al. 1997). Increasing temperatures have been demonstrated to significantly increase insect and parasite metabolic rates, speeding up their rates of development (Blanford et al. 2013, Paaijmans et al. 2013, Bayoh & Lindsay, 2004). Warmer climates have been predicted to benefit insects directly by extending the length of the reproductive season and the geographical range of insects (Schroder & Schmidt, 2014, Mills et al. 2010, Kovats et al. 2001). Several insect species have already shown temperature-driven population prevalence and distribution shifts (Samuel et al. 2015, Zamora-vilchis et al. 2012, Paaijmans et al. 2010, Martens et al. 1995). An extensive body of research has shown positive associations between environmental factors such as rainfall frequency and insect abundance and activity (Shaman & Day, 2007, Minakawa et al. 2002). Insect densities can also fluctuate rapidly in response to climate changes through high reproductive outputs and reproductive synchronisation, significantly increasing the potential for vector-mediated disease transmission between hosts (Zamora-vilchis et al. 2012).

Avian haemosporidian parasites represent an incredibly diverse, globally prevalent group of vector-mediated, protozoan blood parasites, thought to include
over 10,000 species lineages, primarily within the genera *Leucocytozoon*, *Haemoproteus* and *Plasmodium* (Bensch et al. 2004). The different parasite genera have been shown to associate with different vector species that primarily transmit them between hosts. Vector species have specific habitat requirements for reproduction, and environmental preferences which can significantly influence local parasite community composition (Sehgal, 2015, Cator et al. 2014, van Rooyen et al. 2013, Valkiunas 2005). *Plasmodium* parasites are widely associated with (family) Culicidae mosquitoes that lay eggs on the surface of standing water bodies (Njabo et al. 2011, Marzal et al. 2011, Njabo et al. 2009), while *Leucocytozoon* are primarily transmitted by blackflies (*Simulium* spp.), which develop in fast-flowing streams (Seghal, 2015, Cornuault et al. 2013, Bunbury et al. 2006, Malmqvist et al. 2004). *Haemoproteus* parasites by contrast are primarily transmitted by louse flies (family Hippoboscidae), which reproduce without large water bodies and instead develop larvae within the mother, completing development externally in a hardened puparium. Biting midges (family Ceratopogonidae) which lay eggs in wet soil and other semi-aquatic habitats, are also key vectors for *Haemoproteus* parasites (van Rooyen et al. 2013, Marzal et al. 2011, Valkiunas 2005). Therefore, community species compositions and environmental characteristics, play a decisive role in determining the prevalence and distribution of parasites present, and subsequently the impact they have on susceptible hosts (Perez-Rodriguez et al. 2014, Perez-Rodriguez et al. 2013, Cornuault et al. 2013).

Avian haemosporidian parasites are highly prevalent in wild birds, however they are typically encountered as underlying chronic infections characterised by very low levels of parasitaemia (Knowles, Palinauskas & Sheldon, 2010). Infected birds often lack any physical signs of infection, (Ashgar et al. 2011, Ortego et al. 2008), however, a number of studies have demonstrated significant costs associated with haemosporidian infections across the host reproductive attempt (Knowles, Palinauskas & Sheldon, 2010, Marzal et al. 2005, Merino et al. 2000). Significant costs associated with haemosporidian infections suggests these parasites could represent an important selection pressure acting on natural host populations (Knowles, Palinauskas & Sheldon, 2010). Studies indicate costs associated with haemosporidian infection are greatest during times of elevated stress, such as during breeding and migration. Such costs include energetic
trade-offs with immune system functioning (Baille et al. 2012, Knowles et al. 2009, Ilmonen et al. 1999, Nordling et al. 1998, Korpimaki et al. 1993). Delays in the initiation of breeding activities have been reported among a number of species, including great tits (Allander & Bennett, 1995), house martins (Delichon urbica) (Marzal et al. 2013), tree swallows (Tachycineta bicolor) (Nooker, Dunn & Whittingham, 2005) and great reed warblers (Acrocephalus arundinaceus) (Allander & Bennett, 1995, Asghar et al. 2011). Infection has been associated with reductions in female nutritional reserves, supported by reductions in clutch sizes among infected blue tits (Cyanistes caeruleus) (Merila & Andersson, 1999), house martins (Marzal et al. 2005), great tits (Isaksson et al. 2013), and boreal owls (Aegolius funereus funereus), (Korpimaki et al. 1993). Sanz et al. (2001) reported altered female thermoregulation among infected pied flycatchers (Ficedula hypoleuca), that had lower hatching success than uninfected conspecifics.

Later in the reproductive attempt, haemosporidian infections have been shown to limit the ability of blue tit parents to care for their offspring (Tomas et al. 2007). Higher provisioning rates were recorded among birds treated with Primaquine™ for infections by primarily Haemoproteus majoris and Leucocytozoon majoris parasites (Tomas et al. 2007). Effects of haemosporidian infection on fledgling condition have been reported repeatedly among blue tits (Knowles, Palinauskas & Sheldon, 2010, Merila & Andersson, 1999). Similarly, significant reductions in the number of fledged offspring have been reported for great reed warblers (Acrocephalus arundinaceus) (Asghar et al. 2011). Haemosporidian parasites are also highly persistent within individuals, which results in build-up of parasites within host organs and tissues over time, and exposes hosts to extended periods of elevated energetic demands on nutritional, and energetic resources (Perez-Rodriguez et al. 2014, Baille et al. 2012, Marzal 2012). These parasites could create population level effects by reducing the rate at which host populations are able to recover following disease epidemics, habitat destruction or environmental disaster (LaPointe et al. 2010, Rigaud et al. 2010, Brook et al. 2008). Despite the various costs that have been identified, considerable variation exists in host susceptibility to infection, even among closely related and sympatric host species (Palinauskas et al. 2008), which could represent examples of host adaptation or tolerance to infection (Atkinson et al. 2013, Sepil et al. 2013).
In summary, avian haemosporidian parasites are a globally distributed and highly diverse group of parasites which have been studied for centuries, and which have been shown to impose significant fitness costs among some hosts (Bensch et al. 2009, Merino et al. 2000). Vector abundance and haemosporidian prevalence appears to have shown increasing trends in recent years, particularly in Africa and Europe, although small scale variation in prevalence patterns have been frequently reported (Medlock et al. 2015, Sehgal 2015, Marzal 2012, Garamszegi 2011). Prevalence changes have been shown to correlate with temperature and rainfall patterns but are also considered to be largely a result of increasing vector abundance and species range shifts that alter community composition (Marzal, 2012). There is recent evidence to suggest that parasite optimal transmission temperatures may be dramatically lower than many current transmission models predict (Mordecai et al. 2013). Although this has only been shown for very few haemosporidian lineages, and genera have been shown to vary in thermal optima (Perez-Rodriguez et al. 2013, Blanford et al. 2013, Sehgal 2015), it could still result in strikingly different prevalence and distribution patterns than previously predicted. Even subtle changes in climate could have important consequences for highly vulnerable host populations that may be restricted to habitats where temperature currently limits vector activity, and haemosporidian transmission (LaPointe et al. 2010, Atkinson et al. 2014). Consequences of increasing parasite prevalence to hosts where haemosporidia are already prevalent and established are less clear but will likely add pressure to populations already experiencing ongoing habitat fragmentation, and species decline (Ashgar et al. 2011, Garamszegi 2011, Brook et al. 2008, Woodworth et al. 2005). Understanding the implications of vector-mediated diseases on their hosts, and responses to environmental change, could improve prediction and mitigation of epidemics (Mills et al. 2010). Such research could also inform species conservation and population monitoring efforts that build on continuing trends of global climate change and habitat fragmentation (Sehgal, 2015, Atkinson et al. 2014, Woodworth et al. 2005).

Study aims and predictions

We assessed how climate influences prevalence, diversity and reproductive costs associated with infection by different haemosporidian parasites, across an elevation gradient. We tested this using two popular model host species; Blue tits
(Cyanistes caeruleus) and great tits (Parus major). Low temperatures have been previously shown to limit vector activity and reproduction, reducing insect abundance (Paaijmans et al. 2010, Bayoh & Lindsay, 2004). Additionally, low temperatures indirectly reduce transmission rates by slowing the rate of extrinsic sporogonic development of haemosporidian parasites (Atkinson et al. 2014, LaPointe, Goff & Atkinson, 2010, Paaijmans et al. 2010). Several studies have also reported significantly lower prevalence and diversity of haemosporidian parasites, particularly Plasmodium species lineages, in high altitude host populations (van Rooyen et al. 2013b, Imura et al. 2012, Atkinson, 2005, Khasnis & Nettleman, 2005). In support of the literature, we expected high prevalence and diversity of haemosporidian parasites among sampled hosts with a negative association between parasite prevalence, diversity and altitude (Blanford et al. 2013, Zamora-vilchis et al. 2012, Paaijmans et al. 2010, LaPointe et al. 2010). Additionally, some parasite lineages were predicted to be more sensitive to climate gradients than others, producing differences in parasite community between high and low elevation sites (van Rooyen et al. 2013b).

As haemosporidian parasite infections incur energetic costs to hosts, and have been previously demonstrated to significantly reduce host fitness, we expected negative associations between reproductive success and haemosporidian infection in the present study (Knowles et al. 2010, Tomas et al. 2007, Marzal et al. 2005, Merino et al. 2000). However, specific effects are expected to differ between parasite species and genera, and between blue tits and great tits (van Rooyen et al. 2013, Sepil et al. 2013, Ortego et al. 2008, Bensch et al. 2000). Parasite diversity has been positively associated with costs to hosts (Perez-Rodriguez et al. 2014, Pedersen & Fenton, 2007, Keesing, Holt & Ostfeld 2006). This is likely due to hosts encountering a more diverse parasite assemblage and increasing the likelihood of infection by a parasite species to which they are more susceptible, or have not developed specific immunity against (Keesing, Holt & Ostfeld, 2006). More diverse parasite communities often include more virulent strains (Johnson & Hoverman 2012, Rigaud, Perrot-Minnot & Brown, 2010). Higher prevalence and diversity of parasites in a community will likely also increase the occurrence of simultaneous infection by more than one parasite species, or mixed infections (Perez-Rodriguez et al. 2014, Synek et al. 2013). Mixed infections have been associated with increased costs to hosts through

Materials and methods

Ethics statement

Animal care permits were obtained under A. S. Chaine from the French bird ringing office (CRBPO; n°13619), the state of Ariège (Préfecture de l’Ariège, Protection des Populations, n°A09-4) and the Région Midi-Pyrénées (DIREN, n°2012-07).

Study system and field methods

We captured blue tits (Cyanistes caeruleus; N = 68) and great tits (Parus major; N=47) from 4 nest box populations of the French Pyrenees (Ariège) between 5th May and 22nd June 2015. Birds were captured using traps temporarily positioned at nest box entrances and placed under constant supervision for a maximum of 90 minutes. The 4 sites were situated 4-16 km apart, and spanned an altitudinal gradient of 1100m (Moulis; 42°57'90"N-42°58'36"N, 01°05'31"E-01°05'73"E, 430-593m, Cescau; 42°55'34"N-42°56'46"N, 01°02'47"E-01°03'47"E 555-923m, Galey; 42°56'64"N-42°57'24"N, 00°54'13"E-00°55'30"E, 944-1193m Castera; 42°53'74N-42°55'07"N, 01°05'40"E-01°03'43"E, 1058-1530m). Sites comprised a mixture of habitat types; Primarily deciduous woodland and small patches of coniferous forest, with some low intensity pastoral farmland and small deforested areas with human settlements and buildings. Each site contained areas of standing water (either natural or water provided for farm animals) and small natural brooks and streams, that potentially provided suitable habitat for deposition of vector eggs and larvae. Moulis and Galey were located on southeastern facing slopes, whereas Cescau and Castera were on northwestern facing.
At the start of the breeding season, nest boxes were monitored periodically for nesting building activity. Nest building phases were categorised into 6 groups to document progression: ‘None’, ‘Few strands’, ‘¼ cup’ (more material but incomplete nest-cup), ‘½ cup’ (the centre of the nest-cup is missing), ‘Full cup unlined’ (nest-cup is complete but has no, or very little lining material), and ‘Full nest’ (nest cup is covered with fur and other, softer material). Nests below ‘½ cup’ were visited every 3 days, and from then on nest height was recorded each visit using callipers (accurate to 0.1mm). Nests classed as ‘½ cup’ or above were visited daily to confirm date of first egg laying, after which they were visited daily from the 5th laying day to confirm the start of incubation. The species occupying each nest box was identified by observing individuals entering the nest with material in their beak, or by making video recordings (30 minutes to 1 hour) of the nest box entrance for visitation. Once incubation had been confirmed, nests were left undisturbed for 11 days for blue tits and for 12 days for great tits, after which they were then visited daily to confirm hatching date. Nest boxes where not all eggs had hatched were visited for 6 subsequent days to check for delayed hatching. Chicks were weighed when they were 6 and 15 days old. Adults were captured on the nest when the chicks were either 10 (blue tits) or 16 (blue and great tits) days old.

We collected ~50-100μl blood from all adults via brachial venipuncture and immediately stored in ~1ml (1x) Phosphate Buffered Saline (PBS) and (0.5M) Ethylene Diamine Tetra Acetic acid (EDTA) solution. Samples were stored at 4°C. Morphological measurements (head width, tarsus, wing chord length, weight, sex, age and fat content) were recorded and each individual was given a 7-digit metal identification ring and coloured bands for field identification and so parents were distinguishable on parental care videos. Head width and tarsus length were measured using callipers accurate to 0.1mm, wing chord was measured with a ruler accurate to 1mm, sex was determined based on presence (female) or absence (male) of a brood patch and intensity of plumage characteristics. Plumage was also used to assess the age of individuals and defined as either yearling or after second year, adult, based on the pigmentation and colour intensity of the primary coverts (Svensson, 1992). Fat was determined by deposits visible on the chest when feathers were gently blown out of the way and scored as: 0- no fat; 1- barely visible; 2- clearly visible; or 3- thick layer visible.
under the skin. Chicks were banded with 2 colour bands along with a 7-digit metal band for post-fledging identification when they were either 12 (blue tits) or 15 (great tits) days old. Chick weight, wing chord length and tarsus lengths were also recorded.

For 20 blue tit nests, temporary brood manipulations were carried out. These involved pairing two nest boxes at similar locations and altitudes, and for which chicks had hatched on the same day or one day apart. This experiment assessed the effect of brood size manipulations on provisioning rates. Two chicks were temporarily swapped between nests so each experienced their natural brood, plus 2, and minus 2, chicks for consecutive days starting from day 12 of the rearing phase. Parental care videos were also recorded for 3 hours on each day during this time. This provided an additional source of disruption and disturbance for the populations and will is addressed in the analysis and discussion.

**Screening for haemosporidian infections**

We extracted DNA from the blood samples using the DNeasy Qiagen DNA extraction kit. Prior to carrying out extraction, all samples were digested at 56°C overnight after adding: 1μl Tris-HCl (1M), 0.5μl NaCl (5M), and 10μl Proteinase K (20mg/μl) per 100μl of blood/buffer mix. The quality and concentration of extracted DNA was analysed on a spectrophotometer (Trinean Dropsense). DNA samples were then screened for the presence of haemosporidian parasites using a nested PCR (Hellgren et al. 2004) that amplifies fragments of the parasite cytochrome b gene and allows for simultaneous screening of the 3 parasite genera: *Leucocytozoon*, *Plasmodium* and *Haemoproteus*. The first PCR amplification used primers that amplify a fragment of the cytochrome b gene that is highly conserved across all three parasite genera; HaemNFI ([I] – universal base; inosine, 5’-CATATATTTAAGAGAAATATGGAG-3’) and HaemNR3 (5’-ATAGAAAGATAAG-3’). It was performed in 25μl reaction volumes containing approximately 25ng genomic DNA per reaction, 14.525μl milliQ water, 2.5μl (10X) PCR Buffer, 0.75μl (1.5mM) MgCl2, 1μl (10mM) dNTPs, 1.5μl (0.6μM) of both the forward and reverse primer, and 0.1μl (0.5U) DreamTaq DNA polymerase (Thermo Fisher Scientific). The second round of PCR amplified either *Haemoproteus* and *Plasmodium* or *Leucocytozoon*. To amplify *Haemoproteus/Plasmodium*, the following primers were used: HAEMF (5’-ATGTTGCTTTTGATATG-3’) and HAEMR2 (5’-
GCATTATCTGGATGTGATAATGGT-3') (Bensch et al. 2000). To amplify Leucocytozoon, we used: HaemFL (5’-ATGGTGTTTTAGATACTTACATT-3’) and HaemR2L (5’-CATTATCTGGATGATAATGGIGC-3’). Thermal profiles consisted of: 94°C for 3 minutes followed by 20 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 45 seconds, and a final elongation step at 72°C for 10 minutes. Thermal profiles were identical for PCRs 1 and 2, with the exception that the second amplification was carried out in 35 cycles instead of 20. All PCR reactions were carried out in Applied Biosystems Veriti™ Thermal Cycler. Positive (individual known to be infected) and negative (ddH₂O) controls were added to each PCR. All PCR products were loaded onto 1.5% agarose gels, stained with RedSafe™ Nucleic Acid Staining Solution (20,000x) (iNtRON Biotechnology inc.) and run at 100V for 45 minutes before visualising under UV to look for presence/ absence of a 500bp PCR fragment. We used a 1000-100bp DNA ladder (exACTgeneTM by Fisher BioReagents) as a reference. Negative samples were confirmed by repeated PCR.

Positive PCR products were cleaned by removing excess primers and unincorporated dNTPs using an ExoSAP (Exonuclease I – Shrimp Alkaline Phosphatase) mix containing; 2 units of Exonuclease (Thermo Fisher Scientific Inc.), 3 units of Antarctic phosphatase, 1μl Antarctic Phosphatase Buffer (both New England BioLabs) and 1.3μl MilliQ water per reaction, with a total volume of 3μl ExoSAP mixture added to each sample that consisted of 20μl PCR product. The thermal profile for the ExoSAP reaction was as follows; 37°C for 40 minutes, 80°C for 15 minutes, 4°C thereafter. The sequencing reaction was run in 10μl reaction volumes consisting of; 5μl ExoSAP product, 2μl 5x Buffer, 1μl ABI Big Dye, 1μl Milli Q water and 1μl forward or reverse primer. The thermal profile consisted of 25 cycles of: 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes, with an initial denaturation step of 96°C for 1 minute and followed by cooling to 4°C after the reaction. Samples were then filtered using Sephadex® gel columns (Sigma-Aldrich®) and sequenced on an ABI capillary sequencer (Applied Biosystems).

Phylogenetic and statistical analyses

Full sequences cropped to include only high quality fragments (clean electropherogram peaks without noise or overlapping curves) were aligned in BioEdit v7.0.5.3 (Hall, 1999) and compared to published sequences using the
Basic Local Alignment Search Tool on the National Center for Biotechnology Information (NCBI BLAST) webpage. Mixed infections were determined by double peaks in electropherogram results. Only sequences with 436bp of high quality fragment were included in phylogenetic analysis carried out using Molecular Evolutionary Genetic Analysis software (MEGA) version 6 (Tamura, Stecher, Peterson, Filipski & Kumar, 2013). When a sufficient length fragment of high quality DNA could not successfully be sequenced (resulting in messy or failed sequencing reactions), PCR amplification and sequencing was repeated, electropherogram results were checked to look for shorter fragments of high quality sequence, and these were checked with the same NCBI BLAST searches to identify published parasite lineages containing identical fragments. We constructed a phylogenetic tree (Figure 1) using a Maximum Likelihood Jukes-Cantor model with 1000 bootstrap replications, and a fragment of the human malaria parasite *Plasmodium falciparum* (isolate 3D7; GenBank Accession no. AY282930) to root the tree.

All statistical analyses were conducted in RStudio v 0.99.491 (RStudio Team (2015) RStudio; Integrated Development for R. RStudio Inc., Boston, MA). We first tested for differences in prevalence of infection as a function of altitude by running 3 logistic regression models (logit function) with infection status with either of the 3 parasite genera (infected or not) as dependent variables and with altitude, species, sex and age as explanatory terms. A similar model was also run with mixed infection status (infected with one versus multiple parasite lineages) as the response variable. To investigate site differences in prevalence, we combined the two highest altitude sites (Galey and Castera) into a pooled model and ran 3 logistic regression models with infection status with either of the 3 parasite genera (infected or not) as dependent variables. Both site and species were retained as explanatory terms. A similar model was run with mixed infection status (infected with one versus multiple parasite lineages) as the explanatory term. We compared the diversity of parasite lineages at the two sites in which the highest number of individuals were caught (low altitude Moulis and intermediate altitude Cescau) using Fisher’s Exact tests. We also compared Simpson’s Diversity Index (SDI) of parasite lineages with, and without, non-verified lineages between Moulis and Cescau from Fisher’s Exact tests.
The low prevalence of *Plasmodium* lineages precluded their inclusion in any of the statistical models assessing costs of infection on the reproductive performance described below. We tested the effect of infection on body mass using ANOVAs with body mass as the dependent variable and with infection status (with either *Leucocytozoon* or *Haemoproteus*). Tarsus length was retained as an explanatory term. Models were run separately for each host species. Similar models were also run for each of the host species with mixed infection status (i.e. infected with one versus multiple parasite lineages), and tarsus length as an explanatory term. Dates recorded for reproductive events including incubation start date, hatch date and fledge date were significantly correlated with one another (Welch two sample t test; \( t > 16.64 \) and correlation coefficient \( r > 0.85 \) for all comparisons, all \( p < 0.001 \)). Therefore, for each of the two host species, we tested for an effect of infection on timing of breeding by running generalised linear models with hatch date as the dependent variable. Infection status with either *Leucocytozoon* or *Haemoproteus* parasites and altitude were retained as explanatory variables. Similar models were run for each host species with mixed infection status and altitude as explanatory terms.

To investigate the effects of infection on reproductive investment for each of the host species, we categorised hatching success as either 100% or <100% of eggs hatched. We then conducted generalised linear models with a binomial error structure with hatching success as the dependent variable and infection status (*Leucocytozoon* and *Haemoproteus*), altitude and clutch size as explanatory terms. The same models were run for each host species, with mixed infection status, instead of having infection with *Leucocytozoon/Haemoproteus* as an explanatory term. Reproductive investment was also measured on the average mass of all chicks in a nest when chicks were 6 days old. To test for an effect of infection in each of the two host species independently, we ran general linear models with average chick mass at day 6 as the dependent variable, and altitude, infection status with *Leucocytozoon* or *Haemoproteus*, and brood size as explanatory terms. The same models were repeated with mixed infection status instead of infection with *Leucocytozoon* or *Haemoproteus*, as an explanatory term. Finally, to test for an effect of infection on reproductive success, we categorised fledging success as a binary term (100% fledging and <100% fledging). We then conducted generalised linear models with a binomial error
structure with fledging success as the dependent variable and with infection status (*Leucocytozoon* or *Haemoproteus*), altitude, and clutch size as explanatory terms. The same models were run for each host species, with mixed infection status instead of infection with *Leucocytozoon* or *Haemoproteus* as explanatory terms.

**Results**

**Haemosporidian prevalence and diversity**

<table>
<thead>
<tr>
<th>Verified sequences</th>
<th>Parasite genus</th>
<th>Identical sequences (Accession number)</th>
<th>Blue tits</th>
<th>Great tits</th>
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</thead>
<tbody>
<tr>
<td>PAMA01</td>
<td><em>Leucocytozoon</em></td>
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</table>
In total, avian haemosporidian parasites were detected in 92% (106/115) of sampled individuals. Prevalence for each of the 3 haemosporidian parasite genus ranged from 11% to 66% in great tits and from 7% to 76% in blue tits (Figure 1). Mixed infections with 2 parasite genera always included *Leucocytozoon* lineages and reached 19% of individuals in both host species (Figure 1). In total, we detected 44 unique mitochondrial lineages (342bp minimum length), 15 of which were verified and found infecting 1 to 23 individuals (Table 1). Of those, 11 lineages were found to be identical to published *Leucocytozoon* lineages, 3 to *Haemoproteus* lineages and 1 to *P. relictum* lineage pSGS1 (Table 1, Figure 2). The 2 remaining lineages belonged to the genus *Leucocytozoon*, and had not previously been identified in the literature (Table 1, Figure 2).

![Figure 1](image)

**Figure 1:** Prevalence (% individuals infected) of each of the 3 haemosporidian parasite genera (*Leucocytozoon*, *Haemoproteus* and *Plasmodium*), as well as prevalence of mixed infection with *Leucocytozoon* and either *Haemoproteus* or *Plasmodium* lineages (mixed), and total recorded prevalence of haemosporidian infections (All/ All infections). Shown in (a) blue tits and great tits separately, for all sites combined and (b) overall infection prevalence in both species, recorded from Moulis and Cescau sites.
Effect of altitude on prevalence and diversity

We found no significant effect of altitude, host species, sex or age on the probability of infection with parasite lineages of the genera *Leucocytozoon* and *Plasmodium*, or on the probability of simultaneous infection with more than one parasite species lineage (mixed infection) (Table 2). Similarly, there was no significant effect of altitude, host species, or sex on the probability of harbouring infection with *Haemoproteus* parasite lineages, but older individuals were significantly more likely to harbour infection with *Haemoproteus* parasites than yearlings (Table 2, Figure 3).

**Figure 2:** Maximum likelihood phylogenetic tree of verified sequences computed using a 436bp fragment of amplified parasite cytochrome b gene (Maximum likelihood Jukes-Cantor substitution model with uniform rates). Numbers above nodes represent support values based on 1000 bootstrap replications. The denomination “PARU” indicates lineages that were found in both blue and great tits, while “CYCA” were found in blue tits only and “PAMA” in great tits only. *Plasmodium falciparum* (in bold) was used to root the tree and fragments of published sequences were added for comparison (in bold; *Leucocytozoon* lineages: Rooyen et al. 2013, *Plasmodium* lineages; Glaziot et al. (2012), *Haemoproteus* payevskyi and *H. pallidus*: Hellgren et al. (2007) and *Haemoproteus* isolate TURDUS3; Peres-Tris et al. 2007). Accession numbers of all sequences are indicated.
Prevalence of infection with *Haemoproteus* parasites was also marginally different between sites (Logistic regression: $X^2=4.63$, $p=0.10$). Prevalence of infection with *Leucocytozoon* or *Plasmodium* species lineages, or carrying mixed infection, however, did not differ between sites (Logistic regressions: *Leucocytozoon*: $X^2=0.28$, $p=0.87$, *Plasmodium*: $X^2=0.42$, $p=0.81$, Mixed infection: $X^2=1.83$, $p=0.40$).

**Table 2:** Logistic regression models with infection status with each parasite genus (*Leucocytozoon*, *Haemoproteus* or *Plasmodium*) or with a combination of lineages (Mixed infection) as the response variables and with altitude, species, sex and age as explanatory terms. * denotes significant effect ($p < 0.05$).

<table>
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<th>Explanatory term</th>
<th>Est.</th>
<th>St. error</th>
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<th>$P$</th>
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<td></td>
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<td><strong>Haemoproteus</strong></td>
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<tr>
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<tr>
<td>Sex</td>
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<td>0.34</td>
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<tr>
<td><strong>Plasmodium</strong></td>
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</tr>
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<tr>
<td><strong>Mixed Infection</strong></td>
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<tr>
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<td>0.25</td>
<td>0.47</td>
<td>0.27</td>
<td>0.60</td>
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</table>

**Figure 3:** Proportion of sampled individuals from each age category; yearling or after second year, found to be infected with *Haemoproteus* parasites. Statistical analysis showed a significant positive relationship between age and prevalence of *Haemoproteus* parasite infections in either a) blue tits or b) great tits.

Prevalence of infection with *Haemoproteus* parasites was also marginally different between sites (Logistic regression: $X^2=4.63$, $p=0.10$). Prevalence of infection with *Leucocytozoon* or *Plasmodium* species lineages, or carrying mixed infection, however, did not differ between sites (Logistic regressions: *Leucocytozoon*: $X^2=0.28$, $p=0.87$, *Plasmodium*: $X^2=0.42$, $p=0.81$, Mixed infection: $X^2=1.83$, $p=0.40$).

**Table 2:** Logistic regression models with infection status with each parasite genus (*Leucocytozoon*, *Haemoproteus* or *Plasmodium*) or with a combination of lineages (Mixed infection) as the response variables and with altitude, species, sex and age as explanatory terms. * denotes significant effect ($p < 0.05$).

<table>
<thead>
<tr>
<th>Explanatory term</th>
<th>Est.</th>
<th>St. error</th>
<th>$X^2$</th>
<th>$P$</th>
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<td>0.72</td>
<td>0.40</td>
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<tr>
<td><strong>Haemoproteus</strong></td>
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<td>0.98</td>
</tr>
<tr>
<td>Age</td>
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<td>0.48</td>
<td>5.90</td>
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<td>0.34</td>
<td>0.47</td>
<td>0.52</td>
<td>0.47</td>
</tr>
<tr>
<td><strong>Plasmodium</strong></td>
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<td></td>
</tr>
<tr>
<td>Altitude</td>
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<td>0.002</td>
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<tr>
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<td>0.68</td>
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<tr>
<td>Age</td>
<td>-0.07</td>
<td>0.71</td>
<td>0.009</td>
<td>0.92</td>
</tr>
<tr>
<td>Host species</td>
<td>0.44</td>
<td>0.70</td>
<td>0.40</td>
<td>0.53</td>
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<tr>
<td><strong>Mixed Infection</strong></td>
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<tr>
<td>Altitude</td>
<td>-0.0009</td>
<td>0.001</td>
<td>0.48</td>
<td>0.49</td>
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<tr>
<td>Sex</td>
<td>-0.41</td>
<td>0.46</td>
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<tr>
<td>Host species</td>
<td>0.25</td>
<td>0.47</td>
<td>0.27</td>
<td>0.60</td>
</tr>
</tbody>
</table>
There was a significant difference in parasite lineage richness between the 2 sites in which the highest numbers of individuals were caught (Moulis and Cescau) (Table 3, Figure 4). This was significant when considering blue tits and great tits together (Fisher’s Exact test: p=0.042 excluding unverified parasite species lineages, and p=0.0007 including unverified parasite species lineages), great tits only (Fisher’s Exact test: p = 0.03 excluding unverified parasite species lineages, and p = 0.01 including unverified parasite species lineages), and blue tits only, but only when including unverified sequences (Fisher’s Exact test: p=0.027 including unverified lineages). When excluding unverified parasite sequences, however, there was no significant difference in parasite lineage richness between sites among blue tits (Fisher’s Exact test: p = 0.25). Moulis exhibited greater diversity of lineages in all cases, with one exception. When including all recorded parasite species lineages among great tits, Cescau had greater parasite lineage richness than Moulis (Table 3, Figure 4). We found no differences in the diversity (Fisher’s Exact test: p = 0.25) of the parasite communities between blue and great tits in Moulis and Cescau, when considering verified parasite species lineages only. However, great tits harboured a significantly higher parasite diversity relative to blue tits when including unverified parasite species lineages (Fisher’s Exact test: p = 0.05) (Table 3, Figure 4).

<table>
<thead>
<tr>
<th></th>
<th>Blue tits</th>
<th>Great tits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Moulis</td>
<td>Cescau</td>
</tr>
<tr>
<td>Including unverified lineages</td>
<td>6.5</td>
<td>4.12</td>
</tr>
<tr>
<td>Excluding unverified sequences</td>
<td>2.33</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 3: **Simpson’s Diversity Index scores for parasite lineage richness recorded from the two lowest altitude sites; Moulis and Cescau, and SDI scores for the two sites combined (Mo + Ce). Sample sizes for Galey and Castera disabled their use in statistical analyses. Figures calculated for blue tits and great tits separately.

**Effect of infection on body mass**

Great tits infected with *Haemoproteus* were significantly heavier than those with other infection types and non-infected individuals (ANOVA, *Haemoproteus*: $F_{(1,42)} = 7.86$, $p=0.008$), but this was not true of individuals infected either with
Leucocytozoon or Plasmodium lineages (Leucocytozoon: $F_{(1,44)}=0.50$, $p=0.48$; Plasmodium: $F_{(1,42)}=0.25$, $p=0.62$; tarsus: $F_{(1,40)}=14.96$, $p=0.0004$). Among blue tits, individuals infected with Plasmodium were significantly heavier than individuals with other infection types, or individuals who did not appear to be infected (ANOVA, Plasmodium: $F_{(1,61)}=5.38$, $p=0.02$). The mass of blue tits with either Leucocytozoon or Haemoproteus parasite infection was not significantly different from those with other infection types, or uninfected individuals (Leucocytozoon: $F_{(1,65)}=1.03$, $p=0.31$, Haemoproteus: $F_{(1,61)}=0.02$, $p=0.88$, tarsus: $F_{(1,65)}=22.87$, $p<0.0001$). Similarly, the mass of both great tits and blue tits carrying mixed infections with different parasite lineages, was not significantly different from that of individuals infected with only one parasite lineage ($p>0.05$).

**Effect of infection on reproduction**

We found no association between the timing of reproductive initiation and infection status in great tits, however in blue tits, Leucocytozoon and Haemoproteus infections resulted in a significant delay in hatching (Table 4). We found that great tits initiated breeding an average of 4 days later than blue tits (Welch two sample t-test: $t_{56}=2.8$, $p=0.007$; mean for blue and great tits respectively; 26th and 30th April). However, for both species, altitude significantly influenced hatch date, with great tits at high altitude hatching chicks an average of 5.39 days later than at lower altitude (Table 4). Among blue tits hatch date occurred an average of 4.64 days later at higher
elevations. Blue tits carrying mixed parasite infections did not display significantly different hatch dates relative to those infected with just one parasite lineage (Table 4).

We found no significant association between investment in reproduction and infection status in either host species. Only among blue tits did we discover a significant association between clutch size and both altitude and age; Individuals reproducing at lower altitudes and older individuals laid significantly larger clutches (Table 4). There was also a marginal effect of mixed infection on blue tit clutch size with individuals harbouring 2 parasite lineages tending to lay smaller clutches than individuals infected with only a single lineage (p=0.08, Table 4). However, there was no effect of either *Leucocytozoon* or *Haemoproteus* parasite infections on blue tit clutch size. Among great tits, we found no significant association between clutch size and infection status, including mixed parasite infections, age or altitude (Table 4). The proportion of clutch that hatched was marginally negatively associated with altitude and mixed infection in blue tits, however there was no significant effect of infection with either *Leucocytozoon* or *Haemoproteus* parasites (Table 4). Among great tits, there was a significant negative effect of mixed parasite infections on the proportion of the clutch that hatched, however, there was no association with altitude or infection with either *Leucocytozoon* or *Haemoproteus* parasites (Table 4 Figure 5a).

Neither infection with *Leucocytozoon*, *Haemoproteus*, multiple lineages, or altitude affected chick mass at day 6 in great tits. However adult mass was positively associated with chick mass. Heavier parents reared heavier chicks at day 6 (Table 4). While infection status did not affect chick mass at day 6 in blue tits either, there was a significant effect of altitude, with chicks at day 6 being heavier at higher altitudes. Adult mass was not associated with chick mass in blue tits (Table 4). Among blue tits only there was a significant negative association between mixed parasite infection and range in within-brood chick mass at day 6 (Table 4).
There was no association with mixed infections in great tits, and for both species, there was no association between range in chick mass and infection with either *Leucocytozoon* or *Haemoproteus* parasites, or altitude. Among great tits there was no effect of infection status or altitude on reproductive success, measured as either the proportion of the brood that fledged, or number of fledged offspring (Table 4, Figure 5b). Similarly, there was no effect of altitude or infection with either *Leucocytozoon* or *Haemoproteus* parasites on blue tit reproductive success. However, mixed parasite infections were associated with a significant reduction in both the proportion and number of fledged offspring compared with single infections or uninfected individuals (Table 4, Figure 5c). Among blue tits only there was also a significant effect of age, with older individuals fledging more offspring than yearlings (Table 4).

**Figure 5:** Significant fitness trade-offs. (a) Great tits with mixed infections tended to lay smaller clutches than those with single infections or no detected infection (b) Great tits with mixed infections tended to fledge fewer offspring, than those with single infections, or no detected infection, however the effect was only marginal (c) Blue tits with mixed infections fledged significantly fewer offspring than those with single infections, or without any detected infection.

<table>
<thead>
<tr>
<th>Infection Type</th>
<th>Mean Great Tit Clutch Size</th>
<th>Mean Great Tit Fledging Success</th>
<th>Mean Blue Tit Fledging Success</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed Yes</td>
<td>6.90 ± 0.26</td>
<td>5.00 ± 0.22</td>
<td>3.83 ± 0.26</td>
</tr>
<tr>
<td>Mixed No</td>
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<td>5.89 ± 0.22</td>
<td>5.30 ± 0.23</td>
</tr>
<tr>
<td>Haem Only</td>
<td>7.57 ± 0.22</td>
<td>6.25 ± 0.23</td>
<td>5.43 ± 0.23</td>
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<tr>
<td>Leuc Only</td>
<td>7.60 ± 0.23</td>
<td>5.81 ± 0.23</td>
<td>5.32 ± 0.23</td>
</tr>
</tbody>
</table>

(a) Great tit clutch size

(b) Great tit fledging success

(c) Blue tit fledging success
Table 4 (following 2 pages): General and generalised linear models testing the effect of infection status on timing of reproduction, on reproductive effort and success. Infection status is determined as the presence/absence of *Leucocytozoon* or *Haemoproteus* lineages, as well as the presence/absence of multiple parasite lineages (Mixed infection). * denotes significant effect (p < 0.05).

<table>
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<th>X^2</th>
<th>P</th>
<th>Est. ± St. Error</th>
<th>X^2</th>
<th>P</th>
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<td>&lt;0.0001*</td>
<td>0.02 ± 0.005</td>
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<tr>
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<td>2.34 ± 0.87</td>
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<td>0.007*</td>
<td>1.80 ± 2.96</td>
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<td>0.54</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Weight</td>
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<td>0.73</td>
<td>-1.65 ± 1.30</td>
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<tr>
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<td>Age</td>
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<td>-0.63 ± 2.38</td>
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<td>0.79</td>
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</tr>
<tr>
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<td>&lt;0.0001*</td>
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<td>&lt;0.0001*</td>
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<td>1.85 ± 2.57</td>
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<tr>
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<td>Weight</td>
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<td>-0.11 ± 0.63</td>
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<tr>
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<td>Weight</td>
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<td>0.35</td>
<td>0.35 ± 0.24</td>
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<tr>
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<tr>
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<td>0.0</td>
<td>0.96</td>
<td>-0.10 ± 0.06</td>
<td>3.2</td>
<td>0.08(*)</td>
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<tr>
<td></td>
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<td>0.07(*)</td>
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<tr>
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<tr>
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<tr>
<td>Mean chick mass day 6</td>
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<td>0.001 ± 0.0006</td>
<td>6.23</td>
<td>0.01*</td>
<td>-0.001 ± 0.001</td>
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<td>0.21</td>
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<td></td>
</tr>
<tr>
<td></td>
<td><em>Haemoproteus</em></td>
<td>0.08 ± 0.23</td>
<td>0.11</td>
<td>0.74</td>
<td>0.36 ± 0.58</td>
<td>0.34</td>
<td>0.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brood Size</td>
<td>-0.03 ± 0.06</td>
<td>0.27</td>
<td>0.60</td>
<td>0.12 ± 0.16</td>
<td>0.65</td>
<td>0.42</td>
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<tr>
<td>Model</td>
<td>Explanatory Term</td>
<td>Blue tits</td>
<td>Great tits</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
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<td>Est. ± St. Error</td>
<td>X²</td>
<td>P</td>
<td>Est. ± St. Error</td>
<td>X²</td>
<td>P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Chick mass day 6</td>
<td>Altitude</td>
<td>0.001 ± 0.0006</td>
<td>6.74</td>
<td>0.009*</td>
<td>-0.001 ± 0.001</td>
<td>2.15</td>
<td>0.14</td>
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<td></td>
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<tr>
<td></td>
<td>Mixed Infection</td>
<td>0.32 ± 0.21</td>
<td>2.30</td>
<td>0.13</td>
<td>0.63 ± 0.56</td>
<td>1.27</td>
<td>0.26</td>
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<tr>
<td></td>
<td>Brood Size</td>
<td>-0.05 ± 0.06</td>
<td>0.65</td>
<td>0.42</td>
<td>0.17 ± 0.15</td>
<td>1.14</td>
<td>0.29</td>
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<tr>
<td>Range Chick mass day 6</td>
<td>Altitude</td>
<td>-0.0003 ± 0.0007</td>
<td>0.14</td>
<td>0.71</td>
<td>-0.001 ± 0.0007</td>
<td>2.41</td>
<td>0.12</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Leucocytozoon</td>
<td>-0.02 ± 0.29</td>
<td>0.004</td>
<td>0.95</td>
<td>0.37 ± 0.42</td>
<td>0.80</td>
<td>0.37</td>
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</tr>
<tr>
<td></td>
<td>Haemoproteus</td>
<td>-0.43 ± 0.28</td>
<td>2.39</td>
<td>0.12</td>
<td>0.25 ± 0.42</td>
<td>0.35</td>
<td>0.55</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Brood Size</td>
<td>0.22 ± 0.08</td>
<td>8.76</td>
<td>0.003*</td>
<td>0.32 ± 0.11</td>
<td>8.22</td>
<td>0.004*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range Chick mass day 6</td>
<td>Altitude</td>
<td>-0.0003 ± 0.0007</td>
<td>0.23</td>
<td>0.63</td>
<td>-0.001 ± 0.0007</td>
<td>3.41</td>
<td>0.06(*)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Mixed Infection</td>
<td>-0.41 ± 0.26</td>
<td>2.56</td>
<td>0.11</td>
<td>0.15 ± 0.41</td>
<td>0.14</td>
<td>0.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brood Size</td>
<td>0.23 ± 0.07</td>
<td>10.25</td>
<td>0.001*</td>
<td>0.29 ± 0.11</td>
<td>6.51</td>
<td>0.01*</td>
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<tr>
<td>Proportion of Brood Fledged</td>
<td>Altitude</td>
<td>0.0001 ± 0.0002</td>
<td>0.28</td>
<td>0.60</td>
<td>-0.00005 ± 0.0002</td>
<td>0.09</td>
<td>0.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leucocytozoon</td>
<td>0.005 ± 0.09</td>
<td>0.00</td>
<td>0.96</td>
<td>0.01 ± 0.09</td>
<td>0.02</td>
<td>0.89</td>
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<tr>
<td></td>
<td>Haemoproteus</td>
<td>-0.12 ± 0.09</td>
<td>1.65</td>
<td>0.20</td>
<td>0.04 ± 0.10</td>
<td>0.16</td>
<td>0.69</td>
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<tr>
<td></td>
<td>Weight</td>
<td>-0.12 ± 0.07</td>
<td>2.77</td>
<td>0.10</td>
<td>0.02 ± 0.04</td>
<td>0.16</td>
<td>0.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>-0.16 ± 0.08</td>
<td>4.48</td>
<td>0.03*</td>
<td>-0.04 ± 0.08</td>
<td>0.24</td>
<td>0.62</td>
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</tr>
<tr>
<td></td>
<td>Brood Size</td>
<td>-1.13 ± 0.03</td>
<td>1974.10</td>
<td>&lt;0.0001*</td>
<td>-1.03 ± 0.02</td>
<td>1886.03</td>
<td>&lt;0.0001*</td>
<td></td>
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</tr>
<tr>
<td>Proportion of Brood Fledged</td>
<td>Altitude</td>
<td>0.00008 ± 0.0002</td>
<td>0.13</td>
<td>0.71</td>
<td>-0.00005 ± 0.0001</td>
<td>0.12</td>
<td>0.73</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Mixed Infection</td>
<td>-0.21 ± 0.08</td>
<td>6.62</td>
<td>0.01*</td>
<td>0.03 ± 0.08</td>
<td>0.11</td>
<td>0.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weight</td>
<td>-0.11 ± 0.07</td>
<td>2.44</td>
<td>0.11</td>
<td>0.02 ± 0.04</td>
<td>0.45</td>
<td>0.50</td>
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<tr>
<td></td>
<td>Age</td>
<td>0.12 ± 0.07</td>
<td>2.61</td>
<td>0.11</td>
<td>0.02 ± 0.03</td>
<td>0.30</td>
<td>0.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brood Size</td>
<td>-1.14 ± 0.02</td>
<td>2172.98</td>
<td>&lt;0.0001*</td>
<td>-1.03 ± 0.02</td>
<td>2059.83</td>
<td>&lt;0.0001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number Fledged</td>
<td>Altitude</td>
<td>0.0004 ± 0.0004</td>
<td>0.86</td>
<td>0.35</td>
<td>-0.000001 ± 0.0003</td>
<td>0.00</td>
<td>0.997</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leucocytozoon</td>
<td>0.02 ± 0.16</td>
<td>0.02</td>
<td>0.90</td>
<td>-0.02 ± 0.15</td>
<td>0.01</td>
<td>0.92</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Haemoproteus</td>
<td>-0.05 ± 0.15</td>
<td>0.11</td>
<td>0.74</td>
<td>0.004 ± 0.15</td>
<td>0.00</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brood Size</td>
<td>-1.00 ± 0.04</td>
<td>524.79</td>
<td>&lt;0.0001*</td>
<td>-0.87 ± 0.04</td>
<td>324.87</td>
<td>&lt;0.0001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number Fledged</td>
<td>Altitude</td>
<td>0.0002 ± 0.0004</td>
<td>0.44</td>
<td>0.51</td>
<td>0.00001 ± 0.0003</td>
<td>0.00</td>
<td>0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mixed Infection</td>
<td>-0.32 ± 0.15</td>
<td>4.79</td>
<td>0.03*</td>
<td>0.02 ± 0.15</td>
<td>0.02</td>
<td>0.88</td>
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</tr>
<tr>
<td></td>
<td>Brood Size</td>
<td>-1.02 ± 0.04</td>
<td>621.11</td>
<td>&lt;0.0001*</td>
<td>-0.86 ± 0.04</td>
<td>344.11</td>
<td>&lt;0.0001*</td>
<td></td>
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</tr>
</tbody>
</table>
Host communities across sites

Group sizes reflect the variation that occurred in observed occupancy of the nest-boxes between sites, with blue tits occupying increasingly fewer nest-boxes at higher sites. Great tits followed a similar trend however, the second lowest site, Cescau was their highest occupied site, and also the highest occupied site when comparing overall occupancy of nestboxes by all species. There was also an increasing number of nest-boxes occupied by non-focal species as sites increased in altitude, with marsh tits (*Poecile palustris*) more frequent at lower altitude sites and coal tits (*Periparus ater*) being more commonly recorded at higher altitude sites. Occasionally nuthatches (*Sitta europaea*) and crested tits (*Lophophanes cristatus*) were also recorded using the nest-boxes provided (Table 5).

<table>
<thead>
<tr>
<th>Site</th>
<th>GT Occ.</th>
<th>BT Occ.</th>
<th>Other</th>
<th>Additional species</th>
<th>Unoccupied</th>
<th>Sample size</th>
<th>Prop. Occ.</th>
<th>Prop. GT Occ.</th>
<th>Prop. BT Occ.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moulis</td>
<td>27</td>
<td>47</td>
<td>5</td>
<td>MT</td>
<td>78</td>
<td>157</td>
<td>50.32%</td>
<td>17.20%</td>
<td>29.94%</td>
</tr>
<tr>
<td>Cescau</td>
<td>38</td>
<td>42</td>
<td>14</td>
<td>NH, MT, CoT</td>
<td>52</td>
<td>146</td>
<td>64.38%</td>
<td>26.03%</td>
<td>28.77%</td>
</tr>
<tr>
<td>Galey</td>
<td>16</td>
<td>11</td>
<td>20</td>
<td>CoT</td>
<td>108</td>
<td>155</td>
<td>30.32%</td>
<td>10.32%</td>
<td>7.10%</td>
</tr>
<tr>
<td>Castera</td>
<td>15</td>
<td>1</td>
<td>23</td>
<td>CoT, CrT, NH</td>
<td>116</td>
<td>155</td>
<td>25.16%</td>
<td>9.68%</td>
<td>0.65%</td>
</tr>
</tbody>
</table>

Extended discussion

Main findings

Here we report on the prevalence and distribution of haemosporidian parasites in a mixed natural host community. Consistent with the literature, we detected a diverse and highly prevalent parasite assemblage. The majority of detected infections were represented by *Leucocytozoon* parasite lineages, however, a number of widespread generalist lineages belonging to *Plasmodium* and *Haemoproteus* parasite genera’ were also present. Results suggest that diversity of haemosporidian parasites was higher at lower altitudes (Fisher’s Exact test of Simpson’s Diversity Indices: p=0.042 excluding unverified parasite species lineages, and p=0.0007 including unverified parasite species lineages for host
species combined). While no significant effect of altitude on parasite prevalence was detected (Table 2: Logistic regression model results for prevalence of: *Leucocytoon*: $X^2 = 0.04$, $p = 0.84$, *Haemoproteus*: $X^2 = 1.01$, $p = 0.32$, *Plasmodium*: $X^2 = 0.008$, $p = 0.93$ or mixed parasite infections: $X^2 = 0.48$, $p = 0.49$) there appeared to be lineage-specific variation in prevalence patterns between sites. Some association between altitude and reproduction was detected, with both host species initiating reproduction later at higher altitudes (Table 4: Generalised linear model results for association between hatch date and altitude in blue tits: $X^2 = 59.64$, $p<0.0001$, and great tits; $X^2 = 15.08$, $p=0.0001$). Among blue tits only, individuals reproducing at higher altitudes also showed reduced initial reproductive investment, and had significantly smaller clutches than those at lower altitudes ($X^2 = 4.35$, $p = 0.04$). On day 6 of the rearing phase, blue tit chicks reared at higher altitudes weighed significantly less than those reared at lower altitudes ($X^2 = 6.74$, $p = 0.009$). However, overall reproductive success showed no significant association with altitude in either host species (total number of fledged offspring among blue tits: $X^2 = 0.86$, $p = 0.35$ and great tits: $X^2 = 0.00$, $p = 0.97$, and proportion of the brood that fledged among blue tits: $X^2 = 0.28$, $p = 0.60$ and great tits: $X^2 = 0.09$, $p = 0.76$).

There was a positive relationship between infection and body mass in both species but this was genus-specific. Blue tits infected with *Plasmodium* parasites and great tits carrying *Haemoproteus* infections were significantly heavier than uninfected conspecifics, or those with other infection types (ANOVA, great tits with *Haemoproteus* infections: $F_{1,42} = 7.86$, $p = 0.008$; ANOVA, blue tits with *Plasmodium* infections: $F_{1,61} = 5.38$, $p = 0.02$). Infection with either *Haemoproteus* or *Leucocytozoon* parasites resulted in a significant delay in the onset of breeding among blue tits only (generalised linear model with *Leucocytozoon*: $X^2 = 10.15$, $p = 0.001$, and *Haemoproteus*: $X^2 = 7.25$, $p = 0.007$ as explanatory terms). Costs associated with haemosporidian infection were mixed. Only simultaneous infection by more than one parasite genera resulted in significant reductions in reproductive success, and the specific measures differed between host species. Our results are supportive of energetic trade-offs associated with haemosporidian infection (Thomas et al. 2007, Ilmonen et al. 1999, Allander, 1997), as well as of altered female thermo-regulatory abilities (Allander & Bennett, 1995) through the reduced hatching success of great tits carrying mixed parasite infections.
(generalised linear model: $X^2 = 4.4$, $p=0.04$). The lack of significant reductions in overall fledging success among great tits with any haemosporidian infection type suggests they may be able to compensate for lower hatching success during the rearing phase (Sanz et al. 2001). By contrast blue tits appear to suffer greater energetic costs associated with infection, limiting their potential reproductive output, and leading to significant reductions in fledging success among individuals with mixed parasite infections (generalised linear model for number of fledged offspring: $X^2 = 4.79$, $p= 0.03$). Significant costs detected over a single reproductive attempt among individuals carrying mixed haemosporidian parasite infections, will likely result in significantly lower lifetime reproductive output. These reductions could potentially then lead to population declines for blue tits living at high elevations, or in less favourable environments.

**Detected diversity and distribution of haemosporidian parasites**

Species lineage richness and presence of particular parasite lineages varied between sites. Overall, we recovered a high diversity of *Leucocytozoon* parasites and comparatively lower diversity of *Haemoproteus* and *Plasmodium* parasites. These were largely represented by widespread generalist species which have been previously sampled from multiple host species and locations. These results are consistent with findings from van Rooyen et al (2013a), who recovered a total of three *Plasmodium*, two *Haemoproteus* and 13 *Leucocytozoon* species lineages from great tits sampled over three years in Canton of Vaud, western Switzerland. *Haemoproteus* and *Plasmodium* parasites were found to be highly persistent across years, even showing similar levels of parasitaemia within individuals. Infection with *Leucocytozoon* species lineages was consistently more transient (van Rooyen et al. 2013a). The two most prevalent parasite species lineages, were identical to sequence fragments from the current study (*Leucocytozoon* H022 and *Plasmodium relictum* pSGS1). Overall prevalence for birds recaptured and sampled across at least two years was 98.2% (van Rooyen et al. 2013a), similar to 92% combined infection prevalence in the current study. In contrast, van Rooyen et al. (2013a) reported *Plasmodium* was the most prevalent parasite genus, and recovered higher prevalence of mixed parasite infections; 82% compared to 19% of birds sampled in the current study. Given that both studies applied the same PCR screening protocol, the differences in prevalence of the three genera are potentially the result of differences in habitat
suitability for different vector species. The study location used by van Rooyen et al. (2013a), Canton of Vaud in western Switzerland, was near a number of large standing water bodies and thus would be expected to contain ideal breeding sites for vectors typically associated with transmission of *Plasmodium* parasites (Atkinson, 2005). In contrast water in our sites consisted primarily of fast-flowing, snow-melt streams, which are considered suitable breeding sites for vectors of *Leucocytozoon* parasites, although, there were some water containers for livestock.

Another study by Fargallo and Merino (1999), microscopically screened blue tits from central Spain for *Haemoproteus majoris* and *Leucocytozoon majoris* parasites, recording a prevalence of 91.7% and 43.8% respectively. *Plasmodium* parasites were not reported. Considering both studies appeared to be carried out in similar habitats (within 700km, at very similar latitudes, and at 1200m elevation), the contrasting dominance of *Haemoproteus* parasites lineages could be a result of differences in screening methodologies between the studies (Sehgal, 2015). However, is also supportive of small-scale environmental and habitat factors driving local parasite community composition (Lachish et al. 2012, Knowles et al. 2011, Bensch et al. 2007). Furthermore, these observations are consistent with host movements at the population and species levels, having historically determined which parasite lineages host populations encountered (Perez-Rodriguez et al. 2013a, Perez-Rodriguez et al. 2013b, Loaiza & Miller 2013). A study which screened 12 sympatric species of Passerine birds (Bensch et al. 2000) using a PCR protocol also used in the present study (Waldenstrom et al. 2004), reported 17 mitochondrial haplotypes within the genera *Haemoproteus* and *Plasmodium*, but only one parasite lineage was recorded in multiple host species. Both blue tits and great tits were found to be infected with a *Haemoproteus majoris* lineage, indicating host-switching may occur regularly between these species, and vector host preferences may be similar for both host species (Bensch et al. 2000).

**Lack of detected altitude effect on parasite prevalence and diversity**

The elevation range included in our study did not appear to significantly limit development, or restrict transmission of avian haemosporidian parasites at the highest sites. This is suggested by detection of *Plasmodium relictum*. *Plasmodium* is generally regarded as the least thermo-tolerant of the three
genera (Sehgal, 2015) and was detected in an individual sampled at 1414m above sea level. Although it is possible that this individual acquired the infection at a lower elevation. This could indicate that haemosporidian parasites are more tolerant of lower temperatures than previously thought. There have been recent publications to suggest previous predictions of Plasmodium parasites responding to climate change that overestimated optimal developmental temperatures, or predicted unrealistic simple positive linear responses between parasite prevalence and temperature (Sehgal, 2015, Mordecai et al. 2013, Gething et al. 2011, Craig et al. 1999). Alternatively, there are several publications recognising seasonal fluctuations and patterns of infection among avian hosts harbouring haemosporidian parasites (Beaudoin et al. 1970). This suggests many of the infections detected in the present study could have been acquired the previous season, and notwithstanding may have been acquired some distance from the capture location, therefore not necessarily being indicative of a locally transmitted parasite population (Sehgal et al. 2015, Vanstreels et al. 2014, Dawson & Bortolotti 1999). Some movement of hosts between study sites was observed in the current study, and too much emigration and immigration between sites could be masking any altitudinal effect.
In a further study by van Rooyen et al. (2013b) the effects of altitude were assessed on great tits sampled between 2009-2011, at three sites of different altitudes (380m, 668m and 1000m above sea level). Sites differed in vegetation and habitat characteristics, which potentially influenced observed variation in prevalence and distribution of the different parasite lineages (van Rooyen et al. 2013b). In this study, all sites contained similar vegetation types, and similar vector breeding habitats; Streams marshy patches and some artificial water containers for livestock. Figures for the altitudinal distribution of parasites were variable in relation to the present study (Figure 6). Van Rooyen et al. (2013b) reported *Plasmodium relictum pSGS1* as the most prevalent lineage, occurring in 95.9% of low elevation birds, and with an overall prevalence of 70.6% compared to just 10.6% of great tits and 4.4% of blue tits respectively in the present study. By lineage richness *Leucocytozoon* was the most prevalent parasite genus in both studies, and the most prevalent parasite genus at high altitude sites. However, van Rooyen et al. (2013b) reported mixed associations between altitude and prevalence of the different *Leucocytozoon* lineages. The most prevalent *Leucocytozoon* lineage, PARUS4 declined in

![Figure 6](image-url)
prevalence from low to mid-altitude, but increased again at the high altitude site. Another *Leucocytozoon* lineage, PARUS 22 showed equal prevalence at the low and intermediate sites but increased in prevalence at high altitude. A single *Leucocytozoon* lineage, PARUS 19, was exclusively sampled at low altitude, with one exception (van Rooyen et al. 2013b).

Distribution of the second most prevalent lineage sampled by van Rooyen et al. (2013b), *P. polare* was recorded exclusively at the mid-altitude site. We also report highly localised distribution of the second most prevalent parasite lineage, *Haemoproteus majoris* H022, which was recorded in 17.0% great tits and 11.8% blue tits. This parasite lineage was reported almost exclusively from the Cescau site, although there was one exception. Finally, mixed infections were detected at a much lower rate in the present study, occurring in 17.65% sampled blue tits and 21.28% sampled great tits, compared to 60.6% of all sampled birds harbouring mixed parasite infections reported by van Rooyen et al. (2013b). Cloning and amplification of co-infecting parasites, which was carried out by van Rooyen et al. (2013b) would be expected to improve detection and identification of mixed infections from the present study. This is potentially due to the observation that *Plasmodium* parasites can occur at very low parasitaemias. This may also potentially be due to the observation that *Plasmodium* parasites can occur at much lower parasitaemias than *Haemoproteus* or *Leucocytozoon* parasites (Cornet et al. 2014). In some cases, *Plasmodium* parasites even disappear from the peripheral blood circulation during exoerythrocytic asexual reproductive stages (Cornet et al. 2014), and therefore may not achieve detection when co-occurring in the same host with parasites from other genera.

**Fitness consequences**

We report a significant delay in the onset of breeding, measured as hatch date, among individuals reproducing at higher altitudes, which is consistent with observations of later budburst at higher sites. Blue tits and not great tits, infected with either *Haemoproteus* or *Leucocytozoon* parasites, initiated breeding an average of 2 (n= 13) and 3 (n= 52) days later respectively than *Plasmodium* infected blue tits (n= 5) or those who did not appear to carry any type of haemosporidian infection (n= 5). Birds in temperate regions have been shown to co-ordinate reproductive activities with phenological events. Their reproduction often commences with seasonal changes in nature, such as increasing
temperatures and budburst that correlates with emergence of and increasing abundance of insect prey (Beaudoin et al. 1970). Delayed reproduction, when recorded at higher altitudinal sites, may suggest the beginnings of phenotypic plasticity where birds might be attempting to co-ordinate reproduction with peak food abundance. Such an alteration in annual breeding schedule due to parasitic infection can be delayed at higher altitudes where spring and summer arrive slightly later (Nager & van Noordwijk, 1995). This is supportive of the expectation that our altitude range is sufficient to produce a meaningful climate gradient across the sites, based on an adiabatic lapse rate (the rate at which atmospheric temperature drops with increasing altitude of 6.5°C/1000m) (Giambelluca & Schroeder, 1998). Infection has also been previously shown to correlate with parental effort, with higher prevalence and parasitaemias reported among parents with larger clutches or enlarged broods (Allander, 1997, Knowles et al. 2010).

In this study, great tits harbouring mixed parasite infections hatched significantly fewer offspring than those without detected infections, or those with single parasite infections. This result is supportive of recent studies indicating that haemosporidian parasite infection can influence female thermo-regulation too, thus potentially limiting their ability to incubate eggs as effectively (Sanz et al. 2001, Oppliger et al. 1997 Knowles et al. 2010). Significant costs associated with infection were reported later in breeding attempts by blue tits. Among broods with at least one parent carrying a mixed infection, a significantly lower proportion of chicks survived to fledging than among broods with parents who appeared uninfected, or those carrying single infections. In contrast, several experimental studies have reported highly significant reductions in both clutch size and hatching success (Knowles, Palinauskas & Sheldon, 2009, Marzal et al. 2005). These studies have shown that early reductions can result in highly significant consequences for overall reproductive output. In an experimental study carried out in Spain, an 18% higher clutch size was reported among house martins (Delichon urbica) medically treated with primaquine for Haemoproteus infections (Marzal, 2005). This result increased to 39% larger broods (number of hatched offspring) and 42% higher fledging success among treated individuals compared with infected control birds (Marzal 2005). Knowles et al. (2010) also reported higher clutch sizes and hatching success among female blue tits treated for
*Plasmodium relictum* infections in a study near Oxford, UK. They found medicated females had significantly higher provisioning rates than untreated infected females, which translated into reduced within-brood variation in offspring mass, and improved body condition of the smallest offspring. This was in spite of there being no significant effect on the number of fledged offspring. A study carried out at 1200m elevation in central Spain on a population of pied flycatchers (*Ficedula hypoleuca*) reported significantly lower hatching success yet no effect on fledgling condition, or overall fledging success among parents harbouring *Haemoproteus balmorali* parasites (Sanz et al. 2001). Similarly, we report a lack of any significant reduction in fledging success for mixed parasite infected great tits, despite significant reductions in the proportion of the clutch that hatched.

Our results showed some positive associations between haemosporidian prevalence and age, which has also been reported across several studies (Knowles et al. 2011, Norris, Ots & Horak, 1998, Anwar & Read, 1994). Knowles et al. (2011) reported a significant increase in parasite prevalence and parasitaemia among older individuals in a long-term study on blue tits. This association is likely attributable to the ability of many haemosporidian parasites to persist within the host individual for extended periods, (van Rooyen et al. 2013, Synek et al. 2013) and due to the chance of encountering an infective vector increasing with time (Merila & Andersson 1999, LaPointe et al. 2012). There were also significant associations with age in a number of blue tit reproductive performance parameters, with older individuals producing more offspring than younger individuals. This trend supports theories of older individuals having developed an acquired immunity, resulting in better immune response or better tolerance to haemosporidian infections (Isaksson et al. 2013, Ots & Horak, 1998, Norris, Anwar & Read, 1994). The mechanism behind lower reproductive success among younger individuals here may be due to higher energetic costs of infection among young birds. This is supportive of conclusions from Wood et al. (2007) whom suggested that the costs of infection vary across an organism’s life-time, which makes the demographic distribution of host populations an important consideration for conservation plans for critically endangered birds. However, parent experience, or number of previous reproductive events has also been linked to current reproductive success (Lachish et al. 2011). Our results support the parent experience theory as older parents raised more offspring to
independence than yearlings. Among blue tits in this study, older individuals invested earlier in the reproductive attempts, laying larger clutches than younger parents.

**Altitude effect on cost of reproduction**

In this study, reproductive success was not significantly different between individuals breeding at different altitudes (Table 4). There was a significant negative association between altitude and clutch size, but among blue tits only. This relationship is thought to relate to lower food abundance at higher altitudes, which has previously been linked to reduced clutch sizes in a number of species (Klomp, 1970, Nooker et al. 2005). Egg formation is a costly investment for females and condition and nutritional status are thought to be key determinants of the time required for egg production to occur. Blue tits, which have very few energetic reserves, rely heavily on food availability directly before the reproductive attempt in order to initiate egg formation (Allander & Bennett, 1995). Mean chick mass measured on day 6 was positively associated with altitude, but only among blue tits. This association indicates that reproducing at higher altitudes is costly for blue tits, and parents at high altitudes may compensate by raising fewer offspring of higher quality, in order to maximise chick survival (Lu, 2005). The observation that offspring at higher altitudes tended to be heavier in this study, is supported by another recent study that found blackbirds (*Turdus merula maximus*) breeding at high altitudes in the Lhasa mountains, Tibet. In Lu, (2005) increased egg size and decreased clutch size was detected compared to lower altitude birds. This strategy is thought to improve breeding success by investing more energy on fewer offspring, in response to harsher climates and lower food abundance which constrain time and resources. This strategy appears to compensate any altitude-associated costs among blue tits in this study, as neither the proportion of the brood that fledged, or total number of fledged offspring showed any significant association with altitude (Table 4: Proportion of the brood that fledged: Blue tits: $X^2 = 0.28$, $p = 0.6$, great tits: $X^2 = 0.09$, $p = 0.76$, and number of fledged offspring: Blue tits: $X^2 = 0.86$, $p = 0.35$, great tits: $X^2 = 0.00$, $p = 0.97$).

**Study limitations**
Other potential explanations for the low detected costs of infection can also be suggested from this study. The most severely infected individuals were potentially excluded from this study altogether due to the costs and physiological consequences of infection limiting their potential to reproduce (Valkiūnas, 2005). Severe acute infections have been associated with significantly reduced activity levels, leading to a worsening of the body condition, thus resulting in increasingly moribund and emaciated individuals, and eventually mortality for some hosts (Palinauskas et al. 2008, Atkinson, Dusek & Lease, 2001). For obvious reasons these individuals were unlikely to have been able to, or attempted to reproduce. There are also potential effects which may reduce reproductive success through delaying or reducing the likelihood of an infected individual making a reproductive attempt, but which may not directly limit the ability of the individual to successfully raise offspring (Dufva & Allander, 2014). Factors such as female preference could help explain lower recorded costs than expected, or than reported from experimental studies. For example, carotenoid-based plumage coloration has been frequently cited in sexual-selection theories due to it presenting an honest indication of individual phenotypic quality (Dufva & Allander, 2014, Shawkey, Pillai & Hill, 2009, Horak et al. 2001). Plumage characteristics are thought to relate to quality in terms of foraging efficiency and health status, as carotenoids are exclusively obtained from the diet (Dufva & Allander, 2014, Horak et al. 2001, Hunt et al. 1998). Trade-offs between the use of carotenoids for plumage colour strength and intensity, and use in immune system functioning and detoxification, have been previously demonstrated (Dufva & Allander, 2014, Horak et al. 2001). Research indicates that although blue tit plumage appears monochromatic, avian vision differs from human vision in a number of important ways, giving birds’ sensitivity to a broader spectrum of light than humans, including ultraviolet (Hunt et al. 1998). Subsequently examination of blue tit plumage coloration and intensity under different light filters, has revealed blue tits to be sexually dichromatic in several areas. This detected variation beyond human sensitivity has been shown to carry fitness consequences. In choice trials, blue tit females prefer males with the brightest crest feathers (Hunt et al. 1998). Furthermore, haemosporidian infection has been associated with reduced UV reflectance of plumage feathers in a number of bird species (Horak et al. 2001). The consequences of this fact are that males that carry parasite infections may suffer severe reproductive costs through female preference for uninfected males with
brighter plumage (Horak et al. 2001, Hunt et al. 1998). Individuals that did not reproduce were excluded from this study, resulting in a potential under-estimation of the true costs associated with haemosporidian infection within host populations.

**Conclusions**

This study showed that haemosporidian parasites are distributed unevenly across habitats and host populations, and that the costs of infection can differ between closely related host species. We did not detect any significant altitude effect on prevalence, or on fitness costs associated with haemosporidian infection. This could be due to movement of hosts between sites, masking any altitude effect, or because a larger elevation gradient was required. Costs associated with infection were primarily detected among individuals harbouring mixed parasite infections. While single infections with *Haemoproteus* and *Leucocytozoon* parasite lineages resulted in significantly delayed hatch date among blue tits, only mixed parasite infections were associated with significant reductions in overall reproductive success. Contrastingly, there was a significant reduction in the proportion of the clutch that hatched among great tits with mixed parasite infections, but this did not translate into lower reproductive success. Overall, blue tits appeared to suffer greater costs associated with haemosporidian infection than great tits, over a single reproductive attempt. Our results are supportive of energetic costs associated with haemosporidian infections (Merila & Andersson, 1999, Norris, Anwar & Read, 1994). Reproductive effort has been shown to trade-off with specific immune response and parasite defence, and this has been linked to increased intensity of haemosporidian infections during reproduction (Nordling et al. 1998). If haemosporidian parasites impose additional costs to parents, they could influence the life-history costs of reproduction, lowering adult survival or reducing the number of reproductive attempts, and eventually limiting lifetime reproductive output among individuals with persistent, mixed infections (Nordling et al. 1998).

Future studies will benefit from better screening and identification of mixed parasite infections among sampled hosts, and simultaneous sampling and screening of vector communities (Perez-Rodriguez et al. 2014, Synek et al. 2013, Choisy & Roode, 2010). Given that different lineages vary in costs imposed to hosts, knowledge of what factors favour certain virulent strains, would enable
effective monitoring programmes to be set up for particularly susceptible host populations. Establishing widely used protocols that include climate and vector data will allow future studies to be more easily compared, and results combined, building a detailed picture of the relationships between climate factors and haemosporidian disease (Sehgal, 2015). Long-term studies which resample individuals across seasons and years, will help researchers build lineage-specific distribution maps, improving our knowledge of the impact of different lineages on host population dynamics. Only with a better knowledge of the diversity and driving mechanisms of this globally distributed, vector-mediated disease system, can we accurately predict the outcomes of ongoing climate changes.

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