

ARTICLE

Disease Ecology

Phenology underlies inconsistent urbanization effects on avian malaria (*Leucocytozoon* sp.) in juvenile songbirdsDavide M. Dominoni¹  | Bedur Faleh Ali Albalawi² | Magdalena Mladenova^{1,3} | Barbara Helm^{1,4} | Francesco Baldini^{1,5}¹School of Biodiversity, One Health and Veterinary Medicine, University of Glasgow, Glasgow, UK²Department of Biology, University of Tabuk, Tabuk, Saudi Arabia³Veeva Systems, Pleasanton, California, USA⁴Bird Migration Unit, Swiss Ornithological Institute, Sempach, Switzerland⁵Ifakara Health Institute, Environmental Health and Ecological Sciences Department, Morogoro, United Republic of Tanzania

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Funding information

European Molecular Biology

Organization, Grant/Award Number:

43-2014; European Commission,

Grant/Award Number: 618578; The

Academy of Medical Sciences,

Grant/Award Number: SBF007\100094;

AXA Research Fund, Grant/Award

Number: 14-AXA-PDOC-130; Medical

Research Council, Grant/Award Number:

MR/P025501/1

Handling Editor: Whitney C. Preisser

Abstract

Urbanization can modify species interactions, including those between parasites and their hosts. In birds, urbanization can either increase or decrease avian malaria (haemosporidian) infection, depending on host species, parasite, or study location. However, temporal coordination between parasites and hosts, which may impact infection outcomes, has not been studied in urban ecology. To fill this gap, we collected blood samples from wild blue tit nestlings (*Cyanistes caeruleus*) in urban and forest habitats to examine how their hatch dates affected the prevalence and intensity of haemosporidian infection. To separate parasites and quantify parasite load, we newly developed a species-specific quantitative polymerase chain reaction (qPCR) assay. We found that *Leucocytozoon* prevalence was driven by nestling hatch date: nestlings that hatched earlier in the spring had a lower probability of being infected, independent of habitat type. In the few heavily infected nestlings, intensity of infection was associated with low body mass, suggesting fitness risks of infection. These results highlight the importance of breeding early to avoid early-life infection with malaria parasites. Underappreciated phenological effects may underpin other ecological impacts of urbanization.

KEYWORDS

avian malaria, birds, Black fly, blue tit, *Leucocytozoon*, qPCR, urbanization

Davide M Dominoni and Bedur Faleh Ali Albalawi contributed equally to the work reported here.

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INTRODUCTION

As human populations continue to grow and cities expand into previously undeveloped areas, wildlife encounters with humans and human activities become more frequent (Gaynor et al., 2018; Soulsbury et al., 2015). This often leads to a restructuring of species composition and biodiversity via changes in several ecological processes, including species interactions (Shochat et al., 2010). An example of species interactions that may be affected by urbanization is those between wildlife and their pathogens (Becker et al., 2015; Bradley & Altizer, 2007; Delgado-V. & French, 2012; Hassell et al., 2017), and specifically avian haemosporidian malaria parasites.

Avian malaria parasites have been of central interest in the ecological literature of host–parasite interactions and vector-borne diseases over recent decades (Bensch et al., 2009). Identified in bird species around the world, it can pose significant threats to avian biodiversity, particularly for species in isolated island ecosystems that have not previously been exposed to the disease (Woodworth et al., 2005). Its wide distribution also enables comparative studies across geographical areas and ecological contexts, including urban environments (Beadell et al., 2006; Bensch et al., 2009). Avian malaria is caused by haemosporidian parasites of the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*, which are transmitted through the bite of insect vectors (Valkiunas, 2004). These vectors are usually Culicidae mosquitoes in the case of *Plasmodium* species, *Culicoides* biting midges for *Haemoproteus* species and *Leucocytozoon caulleryi*, and *Simulium* blackflies for other *Leucocytozoon* species (Valkiunas & Atkinson, 2020). These parasites infect host blood cells (mainly red cells, but also the white blood cells), tissues, and other major organs such as the liver, brain, heart, and lungs (Valkiunas, 2004).

The effects of haemosporidian infections on their host are complex as symptoms in birds can range from undetectable or minor illness to severe disease and death. In many cases, infected birds may show no visible symptoms but can still spread the disease through their vectors (Valkiunas, 2004); yet, infections can also affect various host traits. Examples include parental effort (Knowles et al., 2010; Mukhin et al., 2016), body condition, and body mass (Merino et al., 2000; Valkiunas, Žičkus, et al., 2006), global gene expression (Videvall et al., 2015) as well as specific physiological processes such as iron balance (Palinauskas et al., 2008) and telomere loss (Asghar et al., 2015; Sudyka et al., 2019). In turn, these changes have been suggested to negatively affect fitness-related traits, such as reproductive success (Marzal et al., 2005; Ots & Hōrak, 1998; Sudyka et al., 2019) and survival (Asghar et al., 2015; Dawson & Bortolotti, 2000; Ilgūnas et al., 2016), potentially leading to population decline

(Dadam et al., 2019). Conversely, several studies have pointed to opposite or absent links between malaria infection and fitness (Marzal et al., 2008). Effects of malaria often depend on the parasite strain (Lachish et al., 2011a), on the species, population, age, sex, or nutritional condition of the host (Emmenegger et al., 2020, 2023; Sudyka et al., 2019) and on environmental conditions (e.g., food availability) during both growth and adulthood (Isaksson et al., 2013; Lachish et al., 2013). Several studies have also suggested that evolution of resistance in response to malaria infection may be more common than currently appreciated (Kilpatrick, 2006; Woodworth et al., 2005).

Studies of the impact of urbanization on the prevalence of haemosporidian parasites showed mixed results. The relationship between urbanization and malaria infection is context-specific and associates with several factors (Bobby Bailly et al., 2016; Bichet et al., 2020; Caizergues et al., 2023; Fokidis et al., 2008; Jiménez-Peñuela et al., 2021; Santiago-Alarcon et al., 2013; reviewed in Sehgal, 2015), including effects of year of study, latitude, city, and habitat type of surrounding rural areas (e.g., desert vs. tropical rainforest). An overlooked factor that may modulate the impact of urbanization on malaria parasite prevalence is phenology. Phenology, which affects disease patterns via fluctuations in abundance and condition of hosts, vectors, and parasites (Cosgrove et al., 2008; Martinez-Bakker & Helm, 2015; Stevenson et al., 2015), can differ between habitats (Dominoni et al., 2020; French-Constant et al., 2016; Hajdasz et al., 2019). The emergence and transmission of avian haemosporidian in wild populations are strongly seasonal (García et al., 2021; Han et al., 2023; Hernández-Lara et al., 2017; Kim & Tsuda, 2010), partly due to seasonal changes in composition and abundance of vectors (Cornet et al., 2014; Han et al., 2023; Woodford et al., 2018) and to seasonal relapse of parasites in birds (Deviche et al., 2001); additionally, year-to-year variation in malaria prevalence is common (Bensch et al., 2007; Lachish et al., 2011b). Mosquitoes and other malaria vectors, such as midges and black flies, breed in stagnant or flowing water, and their populations can fluctuate depending on seasonal and yearly environmental conditions, such as temperature and rainfall (Ferraguti et al., 2024; Montarsi et al., 2015). During rainy seasons, when river flows and stagnant water reserves expand, vector populations can increase, thus potentially escalating the transmission of avian haemosporidian. Conversely, in dry seasons or cold winters when vector populations decrease, the transmission of avian haemosporidian is usually low (Montarsi et al., 2015). In turn, aspects of phenology may differ between urban and non-urban habitats. One example are urban heat islands, areas in the city where temperatures are higher than in surrounding rural areas

(Kolokotroni & Giridharan, 2008). Higher urban temperature may increase the winter survival of vectors and advance both vector and bird phenology (Capilla-Lasheras et al., 2022; Ishtiaq et al., 2017), further extending the transmission season of the disease (Townroe & Callaghan, 2014). The relative seasonal timing of host life history stages and vector emergence may be affected by urbanization, too, for example, because species differ in response to ambient temperature (Thackeray et al., 2010, 2016). Thus, urbanization can potentially alter the seasonal patterns of avian haemosporidian transmission and modulate its impact on urban bird populations.

Importantly, effects of many diseases also differ depending on host condition, physiology, and the timing of host, vector, and parasite life cycle stages in relation to each other (Martinez-Bakker & Helm, 2015; Stevenson et al., 2015). For example, reproductive stages can be particularly sensitive to pathogens due to seasonal relapse in adults and developmental processes in offspring (Farzan & Yang, 2018; McDevitt-Galles et al., 2020). Phenological contributions to the prevalence of patent avian haemosporidian infection (when parasites can be detected circulating in the blood rather than dormant in tissues) can be particularly well studied in nestlings, whose time of exposure had only started with hatching. Being naïve to infections, the nestlings' host response is driven by innate immune responses directly linked to the physiological effects of the parasite (e.g., anemia and tissue damage), rather than being shaped by acquired immune responses as in adults. Moreover, parasite intensity in nestlings can serve as a useful proxy for estimating the time since infection, as it reflects the progression of the acute phase (Khan & Fallis, 1970). Immediate, intensity-dependent effects—such as reductions in body mass—can therefore provide a clear window into the health costs of infection. Additionally, nestling body mass is a commonly used predictor for a nestling's future fitness (Both et al., 1999).

Thus, the aim of the present study was to disentangle the effects of phenology and urbanization on avian haemosporidian malaria in nestling songbirds. We study prevalence and intensity of avian haemosporidian in early postnatal life of blue tits (*Cyanistes caeruleus*), a species that has served as a major study system in urban and disease ecology (Bambini et al., 2019; Branston et al., 2021; Chatelain et al., 2021; Jarrett et al., 2020). The blue tit is found across Europe in a variety of habitats, including urban, suburban, and rural environments, where they showed distinct responses to urbanization (Capilla-Lasheras et al., 2017; McGlade et al., 2023). Seasonal patterns of avian haemosporidian have been described in both the blue tit and its arthropod vectors (Capilla-Lasheras et al., 2017; Cosgrove et al., 2008;

Woodford et al., 2018). In Scotland and northern England, malaria vector emergence usually occurs in mid-late May, with a peak abundance in July (Sanders et al., 2019; Searle et al., 2013; White et al., 2017; Woodford et al., 2018). Conversely, in the same areas blue tit nestlings hatch on average in early-mid-May. In Scotland, blue tit nestlings have been found to be infected with both *Haemoproteus* and *Leucocytozoon* parasites, but not *Plasmodium* (Capilla-Lasheras et al., 2017; Woodford et al., 2018). The blue tits' well-described breeding biology and physiology, including in our study populations, can aid in the interpretation of data on avian haemosporidian and its impact. Thus, we here examine how the prevalence and intensity of infection with malaria parasites varied between forest and urban blue tit nestlings in Scotland, while explicitly considering the birds' breeding phenology.

To disentangle factors that might blur associations, we also introduce methodological improvements. Although detection of haemosporidian parasites in host blood is relatively easy using molecular techniques and/or microscopy (Godfrey Jr. et al., 1987; Hellgren et al., 2004), it imposes challenges. The nested PCR protocol (nPCR) developed by Bensch et al. (2000), and modified later (Hellgren et al., 2004), has been widely used to detect the three genera of *Haemosporidia* and to assess the reliability and sensitivity of newly developed protocols (Ishtiaq et al., 2017). Nevertheless, the nPCR and other PCR-based protocols often overestimate mixed infection because of cross-reactivity of genus-specific primers (Valkiūnas, Bensch, et al., 2006). Indeed, in a previous comparison of urban–non-urban blue tits, in nine out of 12 samples that were positive for both *Leucocytozoon* and *Haemoproteus/Plasmodium* by nPCR, sequencing revealed that only one of the two parasites—either *Leucocytozoon* or *Haemoproteus*—was present (Capilla-Lasheras et al., 2017). Thus, the field is in demand of a new sensitive and accurate molecular technique to quantify the intensity of, and more importantly distinguish between, parasite genera in avian blood. Here, we will apply a novel quantitative PCR approach that can distinguish and quantify *Leucocytozoon* and *Haemoproteus* with high sensitivity and specificity, which is particularly important when assessing parasite prevalence and intensity in nestlings when parasite development in peripheral blood is at its early stages.

With this approach, we compare malaria infection in blue tit nestlings from multiple urban and rural populations. We previously found a tentative suggestion that urbanization can affect malaria prevalence in nestling blue tits, based on 1 year and few data (Capilla-Lasheras et al., 2017). Since there are also differences in nestling hatch timing between years at our sites (Branston et al., 2021), we conducted urban–rural comparisons over 4 years, during which the birds' breeding phenology varied

distinctly. We tested for interactive effects between urbanization and hatching phenology, specifically addressing the following questions:

Is the prevalence of malaria parasites affected by urbanization and spring phenology?

We hypothesize that urban and forest blue tit nestlings differ in the prevalence of malaria parasites. We further predict effects of phenology of host reproduction on prevalence. In arthropods, spring emergence typically responds stronger to temperature than vertebrate reproduction, but patterns differ between terrestrial and aquatic species, and can be more variable between years in birds than in insects (Both et al., 2009; Burgess et al., 2018; Sanders et al., 2019). We hypothesize that in Scotland, where blue tits can hatch before vector emergence, low overlap between nestling growth and vector presence will reduce the risk of malaria infection. Thus, early-hatched nestlings should show lower prevalence than late-hatched nestlings. Whether these effects are similar in urban and rural blue tits depends on how urbanization affects host and vector phenology. Thus, if blue tits from both habitats breed on average early, we expect little malaria prevalence in both populations. However, if urban and rural birds differ in reproductive timing, inter-annual variation in host phenology could modulate differences in their malaria prevalence, assuming that vector phenology does not vary in synchrony.

Does malaria infection intensity impact nestling body mass and fledging success?

We hypothesize that malaria infection will impact nestling condition and survival prospects. Specifically, we predict that malaria infection will reduce body mass before fledging and the probability of a chick's fledging, independently of hatching habitat, but depending on time since infection as estimated from malaria intensity.

MATERIALS AND METHODS

Fieldwork

The study took place in three forest sites and two urban sites in Scotland where ~500 nest boxes were monitored for research. The forest sites were: (1) the Scottish Centre for Ecology and the Natural Environment (SCENE) (56° 7' N, 4° 36' W), (2) the Salloch campsite (56° 7' N, 4° 36' W), and (3) Cashel farm (56° 6' N, 4° 34' W). The urban

sites were in Glasgow: (1) Kelvingrove Park (55° 52' N, 4° 17' W) and (2) the Garscube Sports Complex (55° 54' N, 4° 18' W). Descriptions of study sites and sample sizes for each site can be found in Appendix S1: Table S1. We worked at these sites between April and June for four consecutive years, 2016–2019. At the beginning of April, boxes were visited once a week to monitor nest building, laying date, clutch size, and the start of incubation. After the tenth day of incubation (first day of incubation was defined as when the last egg was laid), nests were visited every other day to estimate hatch date. When nestlings were found in a nest, we estimated whether these had hatched the same day (Day 0) or the day before (Day 1) based on their appearance, and thus a hatch date was assigned to every nest. On Day 13 after the first egg hatched (referred to as sampling date), we randomly selected three to four nestlings per nest, weighed them, and collected a blood sample (30–50 µL) by puncturing their brachial vein. Nest boxes were checked after fledging to search for any dead nestling. Each nestling was assigned a fledging success of 0 if it died before leaving the nest, or 1 if it successfully fledged.

Quantitative polymerase chain reaction (qPCR)-based quantification of malaria infection

DNA extraction

DNA was extracted from blood samples using a commercial kit (DNeasy whole-blood extraction kit, Qiagen) and eluted with 80 µL of water. Successful DNA extraction was confirmed using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE) and tested against the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) bird reference gene, following the protocol by (Atema et al., 2013), using primer pair: forward (5'-TGTGATTTC AATGGTGACAGC-3') and reverse (5'-AGCTTGACAAAATGGTCGTTC-3') and the following thermal cycling profile: 95°C for 10 min, 40 cycles at 95°C for 1 min, 1 min at 60°C, and 1 min at 72°C, and then a final extension for 5 min at 72°C. The PCR products were then run on a 1% agarose gel stained with SYBRTM Safe DNA Gel Stain (ThermoFisher Scientific, California, USA) and visualized under ultraviolet light.

Sequencing *Leucocytozoon* and *Haemoproteus* mitochondrial genes

To develop a qPCR that could quantify either *Leucocytozoon* or *Haemoproteus* parasites in bird DNA without cross-reactivity, we designed genus-specific primers.

First, we detected *Leucocytozoon* or *Haemoproteus*-infected birds using the PCR protocol by Fallon et al. (2003) using the primer set 292F/631R, with some minor modifications (30 s instead of one minute for the denaturation, annealing, and elongation steps) on a subset of samples. Then, we sequenced the amplified conserved region of the mitochondrial genome corresponding to mitochondrial SSU and LSU rRNA genes (Fallon et al., 2003). Briefly, PCRs were performed in a 20- μ L reaction mixture using 1xGoTaq G2 hot start green master mix (Promega, USA), 0.32 μ M of each primer, and two μ L of DNA. Cycling conditions were as follows: 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 52°C and 30 s at 72°C, and a final elongation step at 72°C for 10 min. Amplification was evaluated on a 1% agarose gel, purified, and sequenced on both strands (Eurofins Genomics).

Designing haemosporidia genus-specific qPCR primers

Obtained sequences (GenBank submission ID 3014995) were aligned using Clustal Omega (Sievers et al., 2011) to reference intergenetic space between *cytB* and *cox1* genes from several haemosporidians, including *Leucocytozoon fringilinarum* (KY653765.1), *Leucocytozoon majoris* (FJ168563.1), *Leucocytozoon dubreuilii* (KY653795.1), *Haemoproteus coatneyi* (KT698210.1), *Haemoproteus sacharovi* (KY653811.1), *Haemoproteus tartakovskyi* (KY653810.1), *Haemoproteus* sp. (KY653805.1), *Haemoproteus belopolskyi* (KY653790.1), *Plasmodium relictum* (KY653773.1), *Plasmodium elongatum* (KY653801.1), *Plasmodium lutzi* (KY653816.1), *Plasmodium unalis* (KY653814.1), and *Plasmodium vaughani* (KY653792.1). Based on the alignment, we selected primer sequences that were different between genera, but that were conserved within each genus (Appendix S1: Figure S1 and Table 1).

Testing haemosporidia genus-specific qPCR primers

To test the efficiency and specificity of the primers, amplicons were cloned (Invitrogen TA cloning kit) and

qPCR tested on different numbers of plasmids from each target gene and two negative controls: uninfected bird sample (to ensure that primers are not amplifying bird genes) and distilled nuclease-free water. Samples were run in two technical replicates (accepted only if no more than 0.5 cycle threshold (Ct) difference between the replicates). Best primer concentration for L146F/L146R was 300 nM each with a primer efficiency of 94.1%, and for H160F/H160R, it was 600 nM each with a primer efficiency of 106.6%. When a primer set was tested against a different genus, only the most concentrated standard (10^7 gene copies) showed cross-reactivity, but as this was observed ~20 cycles after the expected Ct for the specific target, it suggested high specificity of each primer set (Appendix S1: Figure S2).

qPCR quantification of parasite infections

Leucocytozoon and *Haemoproteus* infections were quantified by qPCR using our genus-specific primers against *Leucocytozoon* or *Haemoproteus* in individual reactions (Table 1), and normalized against the bird reference gene using GAPDH-Forward 5'-TGTGATTTCATGTTGACAGC-3' and GAPDH-Reverse 5'-AGCTTGACAAAATGGTCGTTC-3' at 100 nM final concentration following (Atema et al., 2013). The total volume of each reaction was 15 μ L and included 5 μ L of DNA diluted to 12 ng/ μ L, primers (see Table 1), 7.5 μ L of SYBR Select Master Mix (Applied Biosystems), and nuclease-free water volumes. Reactions were run in MicroAmp Optical 96-well plates (Applied Biosystems) on Stratagene Mx3000 (ThermoFisher). Cycling conditions were as follows: 15 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at specific annealing temperature (57°C for *Leucocytozoon*, 58°C for *Haemoproteus*, 60°C for GAPDH), followed by a melting curve. Quality checks were performed on the data and any duplicates from individual birds whose differences exceeded >0.5 cycle threshold (Ct) value were repeated. Additionally, samples that showed low quantities of the bird GAPDH reference gene, that is, amplified after 29 Ct, were excluded from the analysis as potential false negatives following observations that target parasite genes in low parasitemic samples tend to

TABLE 1 Haemosporidia genus-specific quantitative PCR primers.

Target genus	Primer name	5'-3' sequence	Product size (bp)	Annealing temperature (°C)	Final concentration (nM)
<i>Leucocytozoon</i>	L146F	GCTTCTATCGGTGAACCTCTCGA	146	58	300
	L146R	TTATTCTTTGCCTGGAGGTAATG			300
<i>Haemoproteus</i>	H160F	AGCTCACGCATCGCTTCTAA	160	57	600
	H160R	ATTTCTTTGCTGGAGGTTAC			600

amplify 10 Ct after the reference. Finally, the relative infection intensity of malaria parasites was calculated for each sample as: $x = 2^{-(\text{parasite Ct} - \text{GAPDH Ct})}$.

Statistical analyses

General approach

All analyses were conducted in R, version 4.1.2 (R Development Core Team, 2015). All models were linear or generalized linear mixed models (LMM or GLMM), which we ran using the R packages *lme4* (Bates et al., 2015) or *glmm.tmb* (Brooks et al., 2017). Nestbox identity was always included as a random factor to account for variation explained by the same nestbox hosting different broods in different years, and to account for family effects (siblings sampled in the same nest). We checked that models adhered to assumptions of normality and homoskedasticity of residuals using the R package *performance* (Lüdtke et al., 2023). We used the same package to check for collinearity of predictors based on variance inflation factor (VIF). All models adhered to assumptions and collinearity was an issue only for year and hatch date (VIF for all other predictors <2). Models therefore included hatch date, the target predictor in our predictions. We explored the additional explanatory power of year by including it as a random factor, but since its explanatory power was negligible, we removed it from the models. Among the remaining factors, model selection was conducted only when testing the significance of interactions, using likelihood ratio tests. If an interaction was not significant, it was removed from the model, but we left all main effects in the model as they were all crucial to test our hypotheses. All explanatory variables were scaled and mean-centered (across years) to improve the interpretability and comparability of model coefficients (Schielzeth, 2010). For example, when hatch date was used as an explanatory covariate, we calculated mean-centered hatch date as the deviation from the grand mean hatch date of all years, which was Day 137 (May 17), and then scaled it.

Prevalence: We analyzed the variation in malaria prevalence as a function of hatch date and urbanization with a binomial GLMM. Habitat, nestling hatch date (mean-centered across years), and brood size were modeled as fixed effects. We also included the interaction between habitat and hatch date to specifically test the hypothesis that the relationship between urbanization and infection prevalence might depend on the phenological timing of the birds.

Offspring fitness: To test for possible effects of malaria infection on nestlings, we first analyzed the relationship

between infection intensity and nestling mass. We used infection intensity rather than prevalence because higher intensity should be positively correlated to the number of days the animal has been infected for, and thereby the likelihood that it would cause effects on physiological state and condition (Valkiunas, 2004). Indeed, while for adult birds low infection could simply be a result of a chronic condition, in newly exposed chicks, infection intensity can be used as a proxy for time since infection because the infection was constrained by the nestlings' time of hatching. For the nestling mass, we used a linear mixed model (LMM) with a Gaussian error structure, using the function *lmer*. Nestling mass was modeled as the response variable, while infection intensity, nestling hatch date (mean-centered across years), and brood size were included as fixed effects. We also included the two-way interactions between intensity and habitat, intensity and hatch date, and hatch date and habitat. We repeated this same model using only birds that were heavily infected, which we defined as the upper 75% quantile ($N = 11$; Appendix S1: Figure S3). We used a similar approach to analyze the relationship between malaria infection and a chick's fledging success, but using a binomial GLMM with the probability of fledging (0 = dead, 1 = fledged) as the response variable. However, because models that included infection intensity as a predictor did not converge, we decided to use infection prevalence instead.

RESULTS

Is the prevalence of malaria parasites affected by urbanization and spring phenology?

Haemoproteus prevalence was very low in our dataset, as only 6 out of the 320 nestlings screened were infected with this parasite (1.8%), equally found in urban ($N = 3$) and forest sites ($N = 3$). Conversely, *Leucocytozoon* prevalence was moderately high: 32.5% of nestlings screened were infected with this parasite. In only two instances, we detected co-infection. As *Haemoproteus* infection was very low and likely not playing a strong role in our system, we decided to focus only on *Leucocytozoon* for all the following analyses.

As predicted, we found that *Leucocytozoon* prevalence did not differ significantly between urban and forest sites, but was positively associated with hatch date (Figure 1; Appendix S1: Table S2). All other variables included in our model did not affect *Leucocytozoon* prevalence (Appendix S1: Table S2).

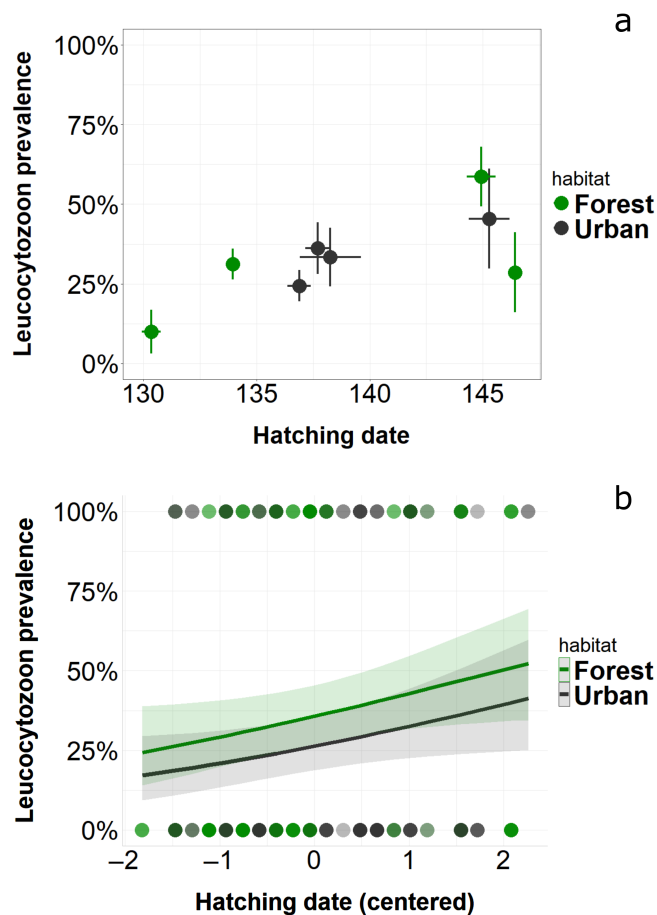


FIGURE 1 Variation in *Leucocytozoon* prevalence in nestling blue tits is positively related to the date of hatching. Nestlings hatched later in the spring have a higher probability to be infected with *Leucocytozoon*. This effect was independent of habitat. (a) Raw means and standard errors calculated separately by year and habitat. (b) Model predictions. Lines represent estimated means from Binomial generalized linear mixed model (GLMM), and shaded area depicts CIs. Points represent individual birds (different tones of gray and green distinguish between multiple birds having the same value).

Does malaria infection intensity impact nestling body mass and fledging success?

We found a strong and significant effect of hatch date on body mass, independent of habitat: both forest and urban nestlings were heavier later than earlier in the season (Figure 2a; Appendix S1: Table S3). Moreover, forest nestlings were heavier than urban nestlings, but nestling body mass was unrelated to brood size (Appendix S1: Table S3). Across all infected nestlings, body mass was not affected by *Leucocytozoon* infection intensity (Appendix S1: Table S3). However, infection intensity was heavily skewed, with most birds showing intensity close to 0 and few birds with much higher intensity levels, which were all forest birds (Appendix S1:

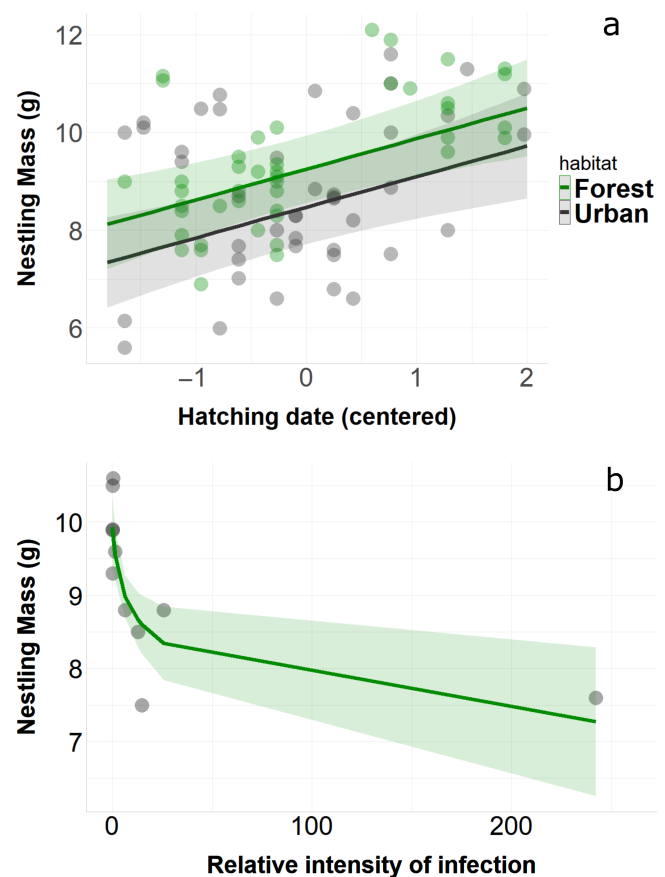


FIGURE 2 (a) Nestling mass is affected by season but not habitat. In both environments, nestling mass increases over the spring. (b) In the 11 highly infected individuals (which were all forest birds), nestling body mass is negatively related to *Leucocytozoon* infection intensity (parasite gene quantity relative to bird housekeeping gene). In both panels, lines are estimated means from Gaussian linear mixed model (LMM), while shaded areas represent CIs and dots represent raw data (different tones of gray and green distinguish between multiple birds having the same value).

Figure S3). As these low-intensity nestlings were likely only recently infected, we ran a subsequent model using only highly infected individuals to predict nestling mass. We defined highly infected individuals as those with relative level of infection above the 75% quantile of the data. Using this subset of 11 birds, our model showed that nestling mass was significantly negatively related to infection intensity: as infection intensity increases, nestling mass decreases (Figure 2b; Appendix S1: Table S4).

Across all nestlings, fledging success was significantly lower in the city than in the forest (Figure 3; Appendix S1: Table S5), and positively associated with nestling body mass (Appendix S1: Table S5). *Leucocytozoon* prevalence, brood size, and hatch date were not related to fledging success (Appendix S1: Table S5).

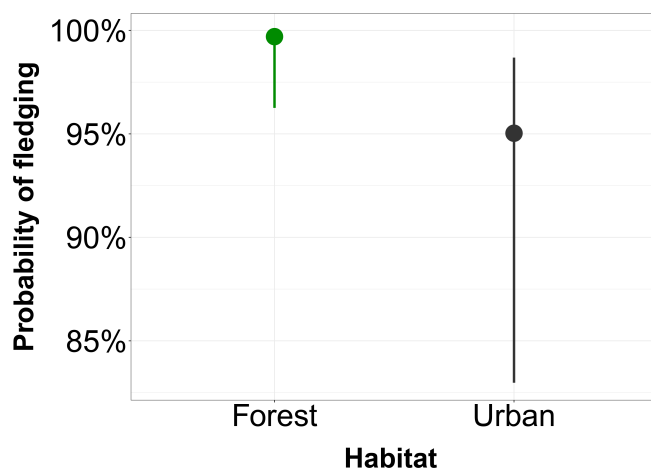


FIGURE 3 Urban nestlings have a lower probability of successfully fledging than forest nestlings. Points are estimated means over the four study years from a binomial generalized linear mixed model (GLMM), while error bars depict CIs.

DISCUSSION

This is the first study, to our knowledge, that has explicitly investigated the interplay between phenology and urbanization in determining avian haemosporidian malaria infection status. Our results provide evidence that the timing of hatching, rather than urbanization, can explain variation in prevalence of a haemosporidian parasite, *Leucocytozoon*, in blue tit nestlings. In both urban and forest habitats, infection prevalence varied between years, but in no consistent manner. On average, nestlings that hatched earlier in the spring had a lower probability of being infected. This effect was quite strong: Early in spring, the probability of infection was as low as 20%, but later in the spring, it rose to 60%. The phenology of hatching also influenced nestlings' body mass, independently of habitat type. Nestlings born earlier in the spring were lighter than those that hatched later in the season, in contrast to previous studies in woodland passerines (Askenmo, 1973; Womack et al., 2022) and the widespread evidence that being early has fitness advantages (Verboven & Visser, 1998). Heavier nestlings were also much more likely to fledge. Low *Leucocytozoon* infection intensity had no detectable effects on body mass of 13-day-old nestlings. In the few nestlings that were found to be heavily infected, which were all forest birds, the intensity of infection was negatively related to body mass, suggesting that *Leucocytozoon* infection could potentially affect the health and fitness of blue tit nestlings. However, infection prevalence did not explain variation in fledging success across urban and forest nestlings.

Among the concise literature on avian haemosporidian in the context of urbanization, studies published to date

have provided mixed results (Sehgal, 2015). While generally avian haemosporidian prevalence has been found to be lower in urban than in non-urban birds of the same species, this pattern can vary depending on the species, life stage, and local environmental conditions. For example, in house sparrows (*Passer domesticus*), studies conducted in Spain and USA found higher prevalence of *Leucocytozoon* in populations inhabiting rural habitats compared to urbanized areas (Jiménez-Peñuela et al., 2019; Santiago-Alarcon et al., 2020), but no urban–rural differences were found in France (Bichet et al., 2020). In France, malaria infection in adult and nestling great tits (*Parus major*) was higher in urban than in rural populations in Montpellier, but lower in urban adults than in rural adults of the same species in Besancon and Dijon (Bailly et al., 2016; Caizergues et al., 2024). A study on five songbird species in an arid region of the southwest USA showed that adult birds generally exhibited lower *Haemoproteus* parasite prevalence in urban compared to rural populations, but species and sex-specific differences were found (Fokidis et al., 2008). Our study confirms the mixed evidence. In some years, *Leucocytozoon* prevalence was higher in forest than in urban nestlings, and in other years, the pattern was reversed. Our analysis suggests that this pattern is likely explained by the fact that the time of hatching of nestlings in the two populations differed between the years, and that hatching time, rather than habitat type, drives differences in prevalence between urban and forest birds. We thus assume that early hatching nestlings do not overlap much with the emergence of *Leucocytozoon* vectors, thereby escaping infection.

Passerine bird phenology in temperate areas can vary considerably in time and space (Burgess et al., 2018) and can be governed by both large-scale environmental variables, such as average spring temperatures (Phillimore et al., 2016; Visser & Holleman, 2001), as well as by microenvironmental factors, such as the availability of insect prey (Noordwijk et al., 1995). Similarly, the phenology of the vectors that transmit haemosporidian parasites can change across different years and over relatively small spatial scales. Thus, the mixed relationships observed between avian haemosporidia prevalence and urbanization may reflect variation in both reproductive phenology and vector emergence. Like bird reproduction, vector emergence is influenced by ambient temperature, but organisms vary in response to spring temperature. Thus, while terrestrial invertebrates generally advance phenology faster than vertebrates in warmer springs, these relationships are reversed in freshwater habitats, where the dipteran vectors develop (Thackeray et al., 2010, 2016). Furthermore, different vector species might respond in distinct ways (Ferraguti et al., 2024). For instance, in our system, avian haemosporidians are

transmitted by blackflies, which breed in fast-flowing streams—habitats that may be less sensitive to temperature fluctuations and urbanization than the small, temporary water bodies used by mosquito vectors. Such different developmental habitats can influence phenological patterns of vectors in an urbanization context. Urban environments often provide artificial water reservoirs such as ornamental ponds, fountains, gutters, and containers, which can serve as potential mosquito vector-breeding grounds (Townroe & Callaghan, 2014). These microhabitats can sustain vector populations beyond the normal breeding season, potentially leading to more persistent transmission of avian malaria throughout the year in urban areas (Kolimenakis et al., 2021; Snow & Medlock, 2006; Townroe & Callaghan, 2014). However, pollution and vector control activities in urban areas can considerably reduce larval numbers (Stockwell et al., 2006), and flowing waters—crucial for blackfly larval development—can be scarce in urban areas but more abundant in rural and agricultural areas. Future studies should investigate how urbanization and seasonality may affect the abundance and phenology of different vectors.

In addition to hatching and vector phenology, seasonal changes of haemosporidian parasitemia within blue tit hosts could also contribute to phenological effects on nestling malaria prevalence. After infection, birds enter the acute phase after which parasitemia decreases drastically, known as the chronic phase (or latent stage). The chronic phase is mostly not infectious, as the majority of parasites only persist in internal organs and not in peripheral blood. However, when birds in temperate regions start breeding, an increase in parasitemia is commonly observed. This relapse is induced by changes in corticosterone and gonadal hormones that are believed to directly trigger parasites in tissues (Applegate, 1970; Valkiūnas et al., 2004). Evidence of parasite relapse in *Haemoproteus* and *Trypanosoma* in blackcaps following increases in day lengths further suggests a suppressive role of melatonin, reducing relapses when nights are long (Valkiūnas et al., 2004). Urban-related environmental factors known to affect phenology, such as artificial light at night, may thus also affect seasonal relapse of malaria parasites, for example, via effects on melatonin or on the immune system (Becker et al., 2020). In a system where most of the hosts have chronic infections, seasonal relapses that are timed with the vector's peak in abundance are considered an adaptive strategy of the parasite to ensure transmission to the new-born susceptible birds (Allan & Mahrt, 1989). Thus, synchrony between vector emergence and avian reproduction is key for parasite persistence across seasons (Martinez-Bakker & Helm, 2015), and these interactions could be facilitated, or reduced, by combinations of environmental change in urban habitats.

We could not measure vector abundance and phenology at our sites, nor could we disentangle the effects of artificial light at night and other urban factors, which could have helped to explain our results. Future studies should integrate bird, vector, and environmental data to gain a holistic understanding of how avian malaria infection varies in space and time with respect to urbanization.

Our analyses also revealed a negative relationship between the intensity of *Leucocytozoon* infection and the nestlings' body mass before fledging (i.e., when they were 2 weeks old), at least in those individuals that were heavily infected. High infection levels should reflect the time from first infection. Given that *Leucocytozoon* intrinsic incubation period (the time between infection and the appearance of parasites in the blood) is approximately 1 week, it is likely that these nestlings were infected soon after hatching. However, *Leucocytozoon* infection did not affect fledging success, which is more likely to be a function of poor diet leading to early nest mortality, especially in urban environments (Pollock et al., 2017; Seress et al., 2020). Nevertheless, since body mass before fledging is one of the most important predictors of future recruitment probability in small woodland passerines (Both et al., 1999; Linden et al., 1992), our results point to potential long-term fitness consequences of early-life infection with haemosporidian parasites in these species, as suggested for great reed warblers (*Acrocephalus arundinaceus*) (Asghar et al., 2015).

Molecular tools have significantly advanced avian haemosporidian research by offering greater sensitivity and specificity than microscopy. Nested PCR (nPCR) has been instrumental in detecting *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* (Bensch et al., 2000; Hellgren et al., 2004), but it has limitations, namely underestimating or overestimating mixed infections (Capilla-Lasheras et al., 2017; Valkiūnas, Bensch, et al., 2006) and its inability to quantify parasite load. To address these issues, we developed an SYBR-based qPCR assay using genus-specific primers for *Leucocytozoon* and *Haemoproteus*. This method requires two separate reactions, avoids the need for probes, and enables sensitive, cost-effective quantification. While validated only in Scottish samples, in silico analyses suggest broad applicability due to conserved primer regions across lineages, although field validation on other lineages is required. Compared to nPCR, our assay improves specificity by distinguishing single from mixed infections, detects down to 10^{-5} parasite gene copies per blood cell, and allows quantification. Other recent methods have tackled similar challenges: a genus-specific nPCR improves detection of mixed infections and phylogenetic resolution (Musa et al., 2024), and a multiplex PCR enables simultaneous detection of all three genera (Ciloglu et al., 2019), though neither quantifies parasite load. Quantitative methods like a universal qPCR (Bell et al., 2015) and digital

droplet PCR (ddPCR) (Huang et al., 2020) offer absolute quantification but cannot distinguish genera or detect mixed infections. A comparative study highlighted differences in detection rates across microscopy, multiplex, and nPCR, suggesting that combining methods may be optimal (Musa et al., 2022). Future work should compare our qPCR with these approaches to evaluate its strengths and best-use scenarios.

Our study has some limitations. First, we sampled five populations in a small geographical area in and around Glasgow. Thus, it is unclear if our results can be generalized elsewhere. Second, as mentioned above, we do not have information on vector abundance and phenology in the different years and habitats, which does not allow us to verify our hypothesis that nestlings avoid overlapping with malaria vectors in years when they hatch earlier than average. This also prevents us from understanding how environmental conditions during spring, particularly temperature, can influence vector phenology, which might be particularly relevant in the context of climate warming, and how this might affect host–parasite interactions. Third, even though we sampled hundreds of birds over 4 years, *Leucocytozoon* prevalence was relatively low, especially in some years, which resulted in a small sample size for the fitness analyses. Moreover, *Haemoproteus* prevalence was extremely low and *Plasmodium* infection was never detected. The exact reasons still need to be clarified. For example, low or absent *Plasmodium* transmission might be a consequence of low abundance of *Culex* mosquito vectors; however, this is less plausible for *Haemoproteus*, whose *Culicoides* biting midge vectors are highly abundant in the study sites. Alternatively, as the latent period of *Leucocytozoon* is much shorter (~3 days) than in *Plasmodium* and *Haemoproteus* (~10–14 days), early sampling of nestlings at Day 13 after hatching might have selectively favored the detection of the fastest parasite genus.

In summary, our study revealed previously unappreciated relationships between passerine reproductive timing, haemosporidian infection, and urbanization. Our results highlight the importance of breeding early to avoid early-life infection with malaria parasites, at least in blue tits, and support recent findings that infection can have a detectable impact on fitness-related traits. This has potential implications for climate warming, which is already leading many passerines to breed earlier. Whether insect vectors are also responding in the same direction, and to the same extent, is largely unknown, as long-term time series of vector phenology in relation to local environmental conditions are scarce. There is a clear research gap at the interface between bird and vector phenology, especially in an urbanization context. Thus, we anticipate future studies to answer exciting questions on this topic.

ACKNOWLEDGMENTS

We thank Robyn Womack, Luke Woodford, Georgia Kirby, Heather Ferguson, and many master and undergraduate students who were involved in field sampling for this project. We thank the staff of SCENE, particularly Matt Newton and Hannele Honkanen, for hosting us and supporting our work. We thank Glasgow City Council and the Loch Lomond and the Trossachs National Park for giving us access to the urban and forest field sites, respectively. This research was funded by the University of Glasgow and by funding through a Marie-Curie Career Integration Grant to BH (EC CIG [618578] Wildclocks). BAA was funded by the Saudi Ministry of Education and by the University of Tabuk. FB was funded by an AXA RF fellowship (14-AXA-PDOC-130), an EMBO LT fellowship (43-2014), the Medical Research Council GCRF Infections Foundation Awards (MR/P025501/1), and The Academy of Medical Sciences Springboard Award (ref:SBF007\100094).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data and code (Dominoni, 2026) are available from Zenodo: <https://doi.org/10.5281/zenodo.18198666>.

ETHICS STATEMENT

Permission for bird trapping and ringing was granted by the British Trust for Ornithology with licenses to Davide M. Dominoni (permit number: C/6822). All blood samples were collected under the Home Office project licenses P6859F36E and PP2193834, issued to Davide M. Dominoni. Permission to disturb nests was given by NatureScot (formerly Scottish Natural Heritage), with license 52463.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Dominoni, Davide M., Bedur Faleh Ali Albalawi, Magdalena Mladenova, Barbara Helm, and Francesco Baldini. 2026.

"Phenology Underlies Inconsistent Urbanization Effects on Avian Malaria (*Leucocytozoon* Sp.) in Juvenile Songbirds." *Ecosphere* 17(2): e70545.

<https://doi.org/10.1002/ecs2.70545>