Nestbox use is linked to increased paternity loss in infected blue tits, but not great tits

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Abstract

Host-parasite interactions and reproductive strategies are critical aspects of avian life history, yet knowledge of their interplay in the context of nesting site microhabitat, in particular between natural and artificial nesting sites, is lacking. The choice of nesting site is critical for successful breeding, by ensuring protection from predators and a suitable environment for incubation and chick development. Furthermore, an optimal nesting site could mitigate the adverse effects of infection and other stressors on the resulting mating success and fitness of breeding birds. In this study, we investigated the prevalence of haemosporidian parasites and their relationship with paternity loss in blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*) breeding in natural cavities and nestboxes. High parasite prevalence was observed in both species, with 72% of blue tits and 67% of great tits infected, mostly with *Plasmodium* spp. In blue tits, paternity loss by a social father, defined as the proportion of extra-pair offspring per nest, was influenced by an interaction between infection status and cavity type. Infected blue tit males breeding in nestboxes experienced higher paternity loss compared to those in natural cavities, suggesting that properties of nesting sites might alleviate or exacerbate the negative effects of infection on mating success. This effect was species-specific, as it was not observed in great tits. These results highlight how microhabitat, individual health, and reproductive strategies interact, and showcase the potential evolutionary implications of breeding in nesting sites provided by humans.

Introduction

Many species rely on tree hollows for shelter, protection from predators, roosting and nesting (Gibbons & Lindenmayer, 2002; Martin et al., 2004; Mccomb & Noble, 1982; Von Haartman, 1957). Tree hollows (hereafter natural cavities) vary widely in terms of height from the ground, internal size, internal depth, shape and size of the opening (Van Balen et al., 1982). In urban areas and secondary or managed forests, natural cavities are scarce and nestboxes serve as artificial substitutes (López-Baucells et al., 2016; Remm & Lõhmus, 2011). Nestboxes have become a key tool in ecological research, especially for studying aspects of the life-history, breeding biology and ecology of passerine birds (Huhta & Jokimäki, 2001; Lambrechts et al., 2010; Minot, 1981; Møller, 1989; Newton, 1994; Van Balen et al., 1982; Von Haartman, 1957). They are also used in conservation efforts to provide additional nesting sites when natural habitats are lost (Arlettaz et al., 2010; Hamerstrom et al., 1973; Newton, 1994). However, nestboxes differ from natural cavities in their construction materials, internal and external size and shape, microclimate, height, location (trunk versus branch) and spatial distribution (Alatalo et al., 1988; Maziarz et al., 2017; Sudyka, Di Lecce, & Szulkin, 2022; Von Haartman, 1971). Numerous comparative studies have shown differences between nestboxes and natural cavities in terms of breeding density, laying date, clutch size, predation rate, nest failure, ectoparasite loads, reproductive success and phenotypic traits (Alatalo et al., 1988; Czeszczewik & Walankiewicz, 2003; East & Perrins, 1988; Evans et al., 2002; Janas et al., 2024; Johnson & Kermott, 1994; Llambías & Fernández, 2008; Miller, 2002; Mitrus, 2003; Nilsson, 1984a, 1984b; Purcell et al., 1997; Robertson & Rendell, 1990; Sudyka, Di Lecce, Wojas, et al., 2022; Wesołowski & Stańska, 2001). Accumulated evidence has therefore raised concerns about the validity of conclusions drawn from nestbox data (Lambrechts et al., 2010; Møller, 1989; Sudyka, Di Lecce, & Szulkin, 2022; Van Balen et al., 1982; Wesołowski, 2011; Wesołowski & Stańska, 2001).

Several ecological correlates of breeding in natural and artificial cavities still require further exploration. One notable gap in our understanding is the frequency of occurrence (i.e. prevalence) of endoparasitic infections, particularly those caused by haemosporidians (Sporozoa: Haemosporida). Haemosporidian parasites, including the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*, are vector-transmitted apicomplexan organisms commonly infecting birds, and causing avian malaria and malaria-like diseases (Valkiunas, 2004). In many avian populations, whether naïve or endemic to these infections, their negative effects on host morphology, physiology, behaviour, and ultimately reproductive success and survival have been well-documented (Asghar et al., 2015; Gilman et al., 2007; Knowles et al., 2010; Martínez-de la Puente et al., 2009; Marzal et al., 2004; Merino et al., 2000; Remacha et al., 2023; Spencer et al., 2005). For instance, infected birds may reduce rates of food delivery to nestlings, and this in turn may affect nestling condition and fledging success (Schoepf et al., 2022). In addition, because haemosporidian infections may affect host traits that act as secondary sexual ornaments, such as plumage or song characteristics, they may be indirectly linked to host mate choice (Badás et al., 2018; Borgia & Collis, 1989; Zuk et al., 1990).

Empirical quantification of infection status depending on breeding cavity type is highly relevant to uncovering the evolutionary consequence of breeding in artificial nesting sites. This is because infection status with avian malaria and malaria-like parasites can impact extra-pair matings, an important aspect of the mating behaviour of socially monogamous birds with biparental care (Westneat et al., 1990). Copulations outside the pair bond occur commonly, leading to multiple paternity within a brood, with high variation among species, populations and individuals (Brouwer & Griffith, 2019; Griffith et al., 2002; Westneat & Stewart, 2003). Among the underlying determinants of levels of extra-pair paternity, ecological factors such as breeding density, breeding synchrony and habitat characteristics have been identified, together with individual factors such as propensity for extra-pair behaviour and individual quality (Di Lecce et al., 2024; Forstmeier, 2007; Kempenaers & Schlicht, 2010; Petrie & Kempenaers, 1998). In general, it has been suggested that females soliciting or engaging in extra-pair copulations might be seeking parasite resistance genes to improve the genetic quality of their offspring (Beltran-Bech & Richard, 2014; Schmoll, 2011). To date, patterns of social pairing in monogamous birds in relation to haemosporidian infections have been poorly studied, and even less is known about how infections with these parasites affect extra-pair matings (Pigeault et al., 2019; Podmokła et al., 2015). For example, infected males were more likely to be cuckolded than uninfected ones in purple martins (*Progne subis*) (Wagner et al., 1997) and blue tits (*Cyanistes caeruleus*) (Podmokła et al., 2015), but not in western bluebirds (*Sialia mexicana*) (Jacobs et al., 2014). Interestingly, in blue tits, paternity loss was dependent on the infection status of the social female, with males paired with uninfected females being more likely to be cuckolded (Podmokła et al., 2015).

Currently, virtually nothing is known about how natural and artificial cavities relate to the infection status of their occupants, and whether infection-related paternity loss differs between the two cavity types. Divergent responses between artificial and natural cavities are likely because properties of nesting sites can considerably affect reproductive success of breeding birds (Broughton et al., 2011; Czeszczewik & Walankiewicz, 2003; Maziarz et al., 2015; Wesołowski, 2002; Wesołowski & Rowiński, 2004, 2012), potentially weighing on costs and benefits of extra-pair mating. The association between cavity type and haemosporidian prevalence in nest occupants may arise either through the non-random selection of nesting sites by infected and uninfected individuals, varying exposure to vectors in nests with differing attributes, or an interplay between these two mechanisms. Nesting site selection is a non-random, active process aimed at minimizing predation risk to the incubating adult and its clutch (Gómez-Serrano & López-López, 2014), while also ensuring an appropriate microclimate for incubation and chick development (Ardia et al., 2006). Given the impact of haemosporidian infections on host body condition and behavior, it may be expected that infected individuals occupy second-choice nesting sites, as only birds of high phenotypic quality can secure nesting sites with preferred attributes. As for the mechanism of varying exposure to haemosporidian vectors, available data indicates that even between nestboxes hung at the same height, significant variation in the abundance of vectors exists (Martínez-de la Puente et al., 2009; Žiegytė et al., 2021), mostly driven by nest temperature (Martínez-de la Puente et al., 2010; but see García-del Río et al., 2025). Populations in which individuals have access to a matrix of nesting sites differing in attributes, such as artificial versus natural cavities, can thus create a unique opportunity to explore this phenomenon.

In this study, we used a comparative framework to examine the relationship between paternity loss, the prevalence of haemosporidian infections, and cavity type (natural or artificial). We used blue tits and great tits – two passerine species with a socially monogamous mating system – breeding over two consecutive seasons in natural cavities and nestboxes located in an urban forest in Warsaw, Poland. We did not expect differences in terms of infection status of birds between the two cavity types, based on previous findings in this study system, where no difference in parental quality in terms of plumage coloration was detected between individuals nesting in natural and artificial cavities (Janas et al., 2024). However, we expected a different outcome for paternity loss in natural cavities and nestboxes depending on the infection status of breeding birds, because previous evidence showed that nestboxes are worse nesting sites than natural cavities for blue tits in terms of the number of fledged young (Sudyka, Di Lecce, Wojas, et al., 2022). It is therefore more likely that females breeding in nestboxes would seek or engage in extra-pair copulations to increase their reproductive success when their social partner is infected, leading males to pay a higher price for being infected in the lower-quality nesting site (nestboxes) compared to natural cavities.

Methods

Study site and field methods

Detailed information on the study site and field methods can be found in Sudyka, Di Lecce, Wojas, et al. (2022). Briefly, in 2018 and 2019 we collected data from wild blue tits and great tits breeding in natural cavities and nestboxes in Bielany Forest (52°17'37.0"N 20°57'22.6"E), an old-growth natural reserve within Warsaw, Poland, protected under the Natura 2000 scheme. We monitored naturally occurring cavities (formed by decay) and excavated cavities, mainly located in hornbeams (*Carpinus betulus*) and common oaks (*Quercus robur*), in a 50 ha area in the SE part of the reserve. In January 2018, we set up 65 woodcrete Schwegler 1b nestboxes in an overall area of 15 ha in the NW part of the forest. Schwegler 1b nestboxes had an entrance hole of 32 mm of diameter, an internal diameter of 12 cm, and a distance between the lower edge of the entrance hole and the nestbox floor of 15 cm (Sudyka, Di Lecce, & Szulkin, 2022). Nestboxes were set up 50 meters from each other, based on an average nearest neighbour distance of ca. 50 m (Krebs, 1971). We hung nestboxes at approximately 2.91 m high above ground level (with the nestbox entrance at around 2.84 m), with a random orientation and without any protective devices against predation (Sudyka, Di Lecce, Wojas, et al., 2022). To avoid a non-random distribution of breeding birds due to inter- and intra-specific competition, the natural cavity and nestbox plots were separated by a minimum distance of 200 m. The plots shared the same environment and were functionally homogeneous in terms of ambient temperature, humidity, sound pollution, air pollution (measured as PM 2.5 concentration) and, importantly, food availability assessed in both study years by frassfall collection (Sudyka, Di Lecce, Wojas, et al., 2022; Sudyka, Di Lecce, & Szulkin, 2022; Wesołowski & Rowiński, 2014). Moreover, predation pressure is unlikely to vary at this scale, as a distance of 200 m is a territory of one individual/pair in case of martens –

a major predator in the forest (Zalewski & Jędrzejewski, 2006). Nestboxes were cleaned in late October/early November 2018 and in February/early March 2019, to remove nesting material from the previous breeding season and/or winter roosts. This step was unnecessary in natural cavities as nest material naturally degrades there (Hebda et al., 2013; Sudyka, Di Lecce, Wojas, et al., 2022; Wesołowski, 2000), ensuring similar baseline conditions between the two types of cavities at the start of each breeding season. We located natural cavities with intense nest searches performed from the end of March/beginning of April during the nest building stage, followed by individual monitoring of nests. Nest searches were effective, as we were able to locate most nests at the nest building stage: 81% of nests were found at nest building in 2018 and 80% in 2019. In later nesting stages (i.e. after hatching), it becomes easier to spot even very high nests since nestlings can be loud and parental feeding activity becomes very frequent, especially close to fledging. In May we resumed nest searches to locate replacement nests, that is a new nesting attempt after a first failed one. When nests were inaccessible from the ground, we accessed cavities with ladders or, if above 6-7 m, by climbing on trees with special spikes. We inspected cavities using portable led lights, mirrors and an NTS200 Digital Inspection Camera (NovoTech Industries Ltd.), equipped with a 8.2 mm camera head at the end of a 5 m long probe. We inspected nestboxes weekly from the start of April, and monitored individual nests from the onset of incubation. Several variables were recorded for each nest in both natural cavities and nestboxes: laying date, clutch size, hatching day (day 0), number of hatched and fledged nestlings (which was assessed by inspecting the nest for dead nestlings every day after 17 days from hatching). Adults breeding in natural cavities were caught when nestlings were at least 14 days old (hatching day = Day 0) with mist-nets (Ecotone, Gdynia, Poland), positioned in front or in proximity of the nest. Adults breeding in nestboxes were mostly caught with self-releasing traps installed inside the nestbox, when nestlings were around 11 days old or older. Each bird was ringed, sexed by the presence (female) or absence (male) of a brood patch, and aged (one year old or older) by comparing great and primary wing coverts (Svensson, 1992). Blood was collected from nestlings 14 days after hatching and from adult birds by puncturing the brachial vein with a sterile needle into heparin-free capillary tubes and preserved in 99% ethanol at +4 °C until DNA isolation.

Avian malaria detection

Genomic DNA was extracted from 1112 blood samples using the Blood Mini kit with overnight incubation at 37 °C and from 71 feather and tissue samples using the Genomic Mini kit (A&A Biotechnology, Gdynia, Poland). Before performing the molecular screening for haemosporidian parasites in 235 blood samples collected from adult birds, DNA extracts were diluted to a concentration of approximately 25 ng/µl. We screened samples using the method described by Ciloglu et al. (2018). This multiplex assay allows for the simultaneous detection and discrimination of parasites of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* in a single reaction, by using three primer sets which target either a fragment between the 5 end of cytochrome b and a non-coding region of mtDNA (*Haemoproteus*), a fragment of a non-coding region of the mtDNA (*Plasmodium*), or a fragment of the cytochrome c oxidase subunit 1 (COX1) gene of mtDNA (*Leucocytozoon*). The three genera are then discriminated based on the size of the PCR product: 525–533 bp in the case of *Haemoproteus* parasites, 377–379 bp in the case of *Plasmodium*, and 218 bp in the case of *Leucocytozoon*. In contrast to the most commonly used screening protocol, which rests on the use of nested PCR (Hellgren et al., 2004), the protocol by Ciloglu et al. (2018) allows for the discrimination between *Haemoproteus* and *Plasmodium* without the need to sequence the PCR products. Moreover, in comparison with the nested PCR protocol, the multiplex protocol performs better in terms of detection of multiple infections (Ciloglu et al., 2018). The reactions were set up using 2 × Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany), and each reaction contained approximately 50 ng of total genomic DNA. Each PCR run contained one negative control (ddH2O) every 22 reactions and a positive control. Amplification products (6 µl) were run on 2% agarose gel stained with SimplySafe (Eurx, Gdańsk, Poland) and visualized under UV light. Samples identified as negative were screened a second time to ensure the repeatability of results, and subsequently checked for DNA quality using a protocol for sex identification (Griffiths et al., 1998).

Parentage assignment

Detailed information can be found in Di Lecce et al. (2023), and Di Lecce et al. (2024). DNA sequencing was performed by Diversity Arrays Technology Pty, Ltd (Canberra, AU) with DArTseqLD, a high-throughput genotyping-by-sequencing method employing genomic complexity reduction with restriction enzyme pairs, on 621 individuals and 11,665 binary SNPs in blue tits and 522 individuals and 8,105 binary SNPs in great tits. All subsequent analyses were performed in R (R Core Team, 2023) separately for each species. After filtering out individuals and loci with call rate below 70% using *dartR* (Gruber et al., 2018), we retained 616 genotypes and 8,646 binary SNPs in blue tits, and 521 genotypes and 6,545 binary SNPs in great tits. A total of 4,656 loci was used in blue tits and a total of 3,727 loci was used in great tits to compute a Genomewide Relatedness Matrix (GRM) for individuals from both the natural cavity and the nestbox plot, using the function snpgdsGRM with the method GCTA (Yang et al., 2011), implemented in *SNPRelate* (Zheng et al., 2012). The GRM was then compared to a social pedigree based on field data, which included all ringed individuals, and extra-pair paternity (i.e. cuckolded fathers and extra-pair fathers) was identified based on discrepancies between the GRM and the social pedigree (Di Lecce et al., 2023, 2024; Perrier et al., 2018).

Statistical analysis

In the analyses described below, infection status was coded in two ways: (i) binary variable (0/1) indicating absence or presence of haemosporidian parasites and therefore uninfected or infected individuals, and (ii) categorical variable with 3 levels: (a) no infection; (b) single infection (when only one genus was detected) and (c) co-infection (when two or three parasite genera were detected). Due to power limitations, we were not able to test for genus-specific effects. The general expectation is that co-infections are more harmful to the host than single infections (Marzal et al., 2008; Pigeault et al., 2018) and that the type of co-infection, defined by the genera of co-occurring parasites, may differentially affect the host. However, sample size for each type of co-infection in our study was too low for analysing them separately. Therefore, the category "co-infection" included all combinations of co-infections occurring in our study site (see Figure 1). Furthermore, due to the limited number of single infection occurrences (11/76 in blue tits and 6/66 in great tits), single infections of *Haemoproteus* and *Leucocytozoon* in both species were included when infection status was coded as a binary variable, but excluded from the "single infection" category when infection status was coded as a categorical variable, as different genera are known to differ in their average pathogenicity (Valkiunas, 2004). When individuals were sampled in both years (9 blue tits and 12 great tits), we kept the first instance of sampling (except for three individuals whose infection status was not available). We tested whether infection status was associated with cavity type (natural cavity or nestbox), sex, age and year, with a generalized linear mixed model with a binomial error distribution and a logit link function (with infection status coded as a binary response and nest id included as a random effect) and a multinomial logistic regression (with infection status coded as categorical response with 3 levels: no infection, single infection and co-infection). In blue tits, we removed the random effect of nest id because of model convergence problems. Parental sex and age were included in the models, as evidence suggests that there are differences in haemosporidian prevalence in relation to host sex and age (Deviche et al., 2005; Valdebenito et al., 2024; Van Oers et al., 2009). Furthermore, we explored whether loss of paternity in males (number of extra-pair nestlings over total number of nestlings in the clutch) was related to cavity type and infection status. Unfortunately, we were not able to investigate paternity gain of individual males, i.e. extra-pair offspring sired in other nests, given that only 7 blue tit males and 4 great tit males acted both as social fathers and extra-pair fathers in our sampled populations, suggesting that males might have sired extra-pair offspring in unsampled nests. Models with paternity loss fitted as response variable included cavity type, infection status, age and year. Age was included, as there is evidence that younger males may lose more paternity than older ones (Moreno et al., 2010; Tarof et al., 2011; but see a meta-analysis reporting no evidence for an association between male age and within-pair paternity: Cleasby & Nakagawa, 2012). Models were checked for dispersion and zero-inflation in *DHARMa* (Hartig, 2022) and for multicollinearity (VIF scores in each model never exceeded 2). We used the package *emmeans* v 1.10.1 to compute posthoc contrasts (Lenth, 2025). All statistical analyses were performed in R 4.3.2 (R Core Team, 2023).

Results

Avian malaria

Overall, 76/105 (72%) adult blue tits and 66/98 (67%) adult great tits were infected with haemosporidian parasites. In both species, individuals were mostly infected with *Plasmodium* spp. (45% in blue tits and 46% in great tits), while 2% blue tits and 5% great tits were infected with *Haemoproteus*, and 9% blue tits and 1% great tits with *Leucocytozoon*. Co-infections were detected in 17% of blue tits and 15% of great tits. In both species, these infections were dominated by *Plasmodium-Leucocytozoon* co-infections, followed by *Plasmodium-Haemoproteus* co-infections. Figure

1 presents the community composition and respective frequencies of haemosporidian parasites infecting blue tits and great tits in relation to the cavity type they occupied. Interestingly, there were no single infections with *Haemoproteus* in blue tits occupying natural cavities, and no single infections with *Leucocytozoon* in great tits occupying nestboxes. Co-infections with *Leucocytozoon* and *Haemoproteus* were the least common and were entirely absent in great tits and in blue tits occupying nestboxes. Infection status was not associated with cavity type or sex, but tended to be positively associated with age in both species (p=0.068 in blue tits; p=0.051 in great tits; Table S1). A positive association with age was apparent when infection status was coded as categorical variable accounting for infections with single and multiple parasite genera (in blue tits, single infection: p=0.013; co-infection: p=0.025; in great tits, single infection: p=0.015; Table S2).

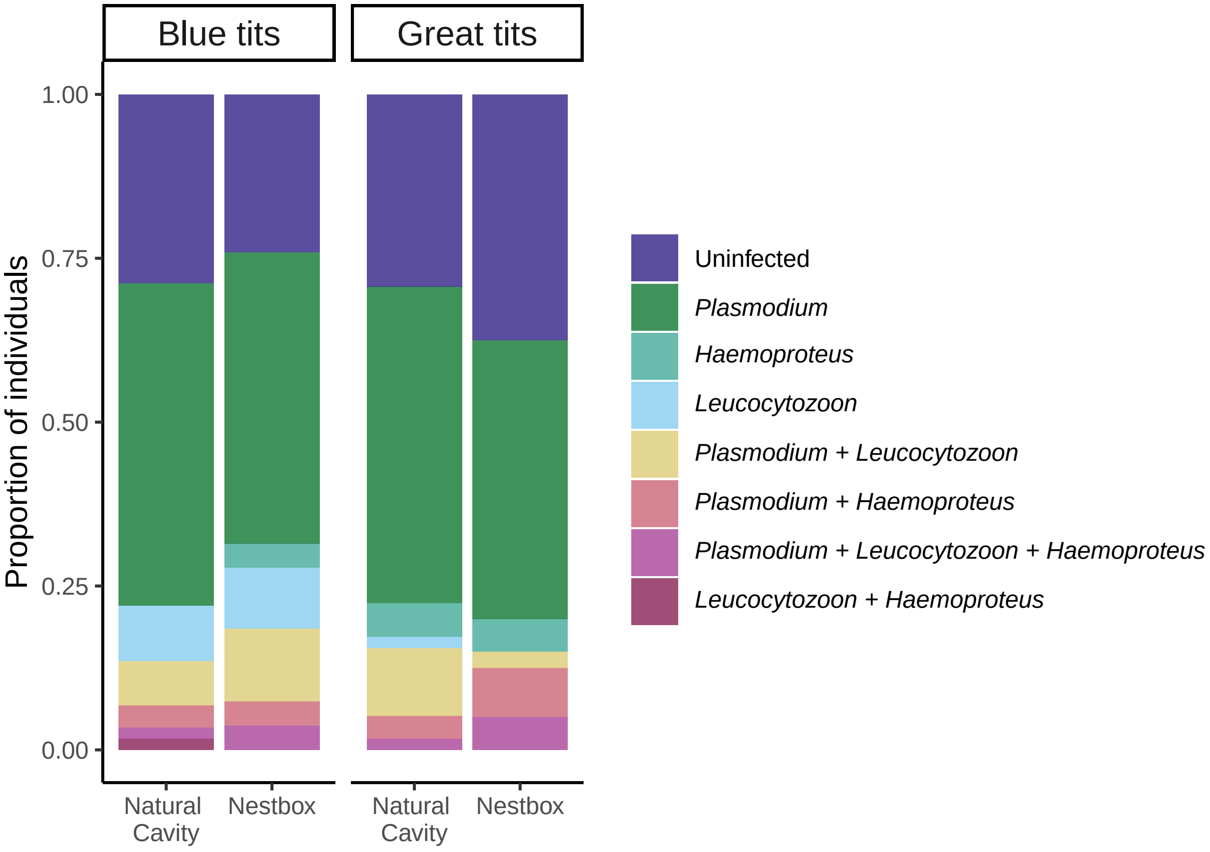

Figure 1. Proportion of individuals uninfected and infected with haemosporidian parasites (taking into account the infection type: single vs co-infection) by host species (blue tits or great tits) and cavity type (natural cavity or nestbox).

Table 1. Generalized linear model investigating paternity loss (quantified as the proportion of extra-pair offspring over the total number of offspring in the nest) in relation to cavity type, infection status (coded as uninfected or infected), age and year. Natural cavity was the reference level for cavity type, Uninfected for infection status, Younger for age and 2018 for year. Significant differences ($p < 0.05$) are marked in bold.

	Blue tits (n=46)				Great tits (n=45)			
	Estimate	Std error	z value	p	Estimate	Std error	z value	p
(Intercept)	-1.782	0.370	-4.811	0	-2.245	0.504	-4.451	0
Cavity type (Nestbox)	-0.615	0.582	-1.056	0.291	0.292	0.635	0.460	0.646
Infection status (Infected)	-0.865	0.565	-1.532	0.126	0.717	0.556	1.289	0.197
Age (Older)	-0.522	0.449	-1.163	0.245	-0.910	0.381	-2.388	**0.017**
Year (2019)	0.114	0.372	0.305	0.76	0.018	0.389	0.047	0.962
Cavity type (Nestbox) x Infection status (Infected)	1.739	0.771	2.257	**0.024**	0.257	0.762	0.337	0.736

Paternity

We obtained genotypes for 118 adults and 497 nestlings in blue tits and 114 adults and 402 nestlings in great tits. The social male was cuckolded in 25/59 nests (42%) in blue tits and in 25/54 nests (46%) in great tits. Further details on paternity in the population can be found in Di Lecce et al. (2023). In blue tits, paternity loss (quantified as the proportion of extra-pair offspring over the total number of offspring in the nest) was driven by an interaction between infection status and cavity type (Table 1; Figure 2): paternity loss was higher in infected males from nestboxes compared to infected males in natural cavities (pairwise comparison of estimated marginal means: z-ratio = -2.134, p = 0.033). We found no evidence for this relationship when infection status was coded as a categorical variable with 3 levels (no infection, single infection and co-infection; Table S3).

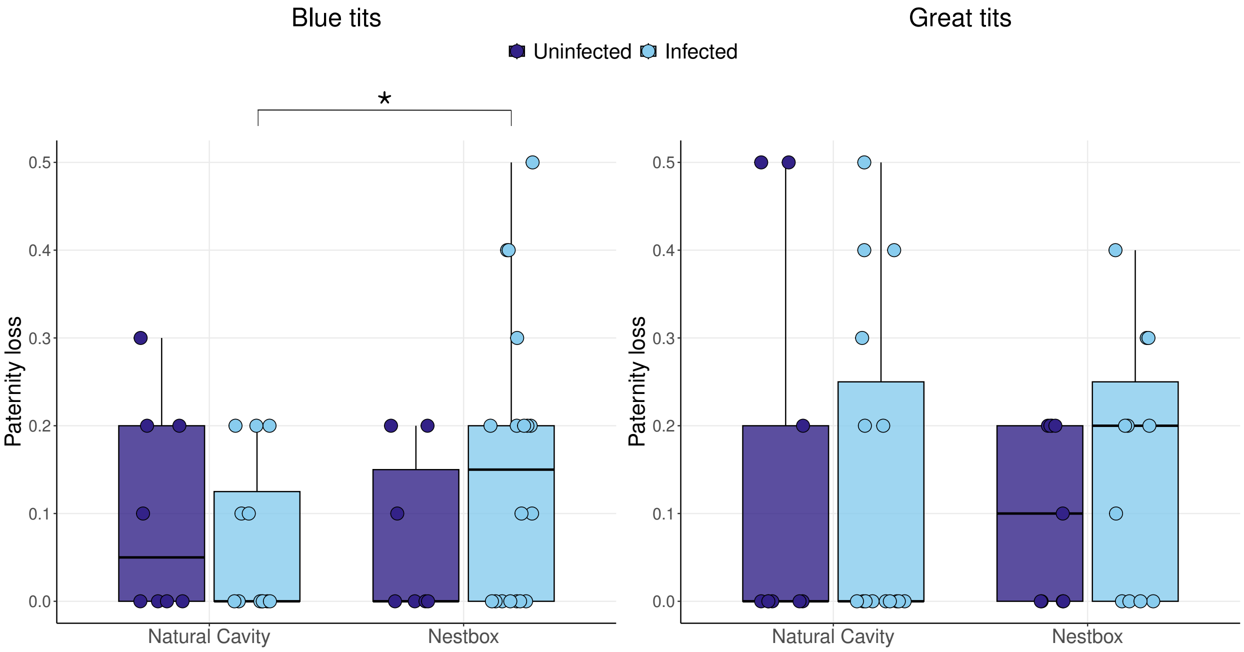

Figure 2. Paternity loss (quantified as proportion of extra-pair offspring over the total number of offspring in the nest) by cavity type and infection status. Raw data is plotted together with boxplots: horizontal bold lines show data median, boxes represent the inter-quartile range (IQR) and whiskers represent 1.5 * IQR. The asterisk denotes the significant pairwise difference according to a posthoc test.

Discussion

In this study, we explored the relationship between breeding cavity type (natural cavity versus nestbox), haemosporidian infections, and paternity loss in blue tits and great tits. We found that cavity type interacted with individual infection status to drive paternity loss in one of the study species. Specifically, paternity loss was higher in infected blue tit males breeding in nestboxes compared to those in natural cavities, suggesting that blue tit males incurred a greater reproductive cost due to infection in artificial cavities than in natural ones.

Higher rates of paternity loss in infected males breeding in nestboxes may suggest that artificial nesting sites exacerbate the negative effects of infection on reproductive strategies. In general, infected males may lose paternity either because they are unable to effectively guard their mates or defend their territory, and/or because their mates are more likely to actively seek extra-pair copulations or to copulate with extra-pair males when they visit their nest. Mate guarding is energetically costly and time consuming, as it entails an increase in vigilance and deterrence activities, and imposes constraints on foraging activities (Askenmo et al., 1992; Cuthill & MacDonald, 1990; Komdeur, 2001). Previously, it was shown that extra-pair paternity in blue tits may be primarily driven by extraterritorial forays of males, while females visit foreign territories much less commonly (Schlicht et al., 2015). Based on these findings, we suggest that infected blue tit males occupying nestboxes in our population suffer increased paternity loss because they are either unable to guard their mates and ward off forays of neighboring males, or because their mates are more likely to accept copulations from extra-pair males visiting nestboxes. Compared to natural cavities, nestboxes in Bielany Forest have been shown to be nesting sites of lower quality in blue tits, with lower reproductive success and longer nestling periods, which entail prolonged parental care and increased risk of predation (Sudyka, Di Lecce, Wojas, et al., 2022). If females expect their current reproductive success to be low because their mate is infected, it is likely that they optimize their reproductive decisions by engaging in extra-pair copulations.

We did not observe a similar effect in great tits, which may reflect species-specific differences in the degree to which cavity type influences breeding conditions, or in the vulnerability of individuals, especially ill or in poor state, to these conditions. Previous evidence in Bielany Forest showed that reproductive outcome in great tits was not affected by cavity type (Sudyka, Di Lecce, Wojas, et al., 2022), suggesting that great tit males breeding in nestboxes may not be hindered in mate guarding and warding off forays of neighboring males, or that great tit females are not more likely to accept copulations from extra-pair males visiting nestboxes. Larger sample sizes and data on paternity gain (i.e. the number of extra-pair offspring sired in other nests), which was not available in this study, would allow us to get a more robust and comprehensive picture, given that extra-pair fertilizations contribute to the total fertilization success of each male (Raj Pant et al., 2022 and references within; Whittingham & Dunn, 2004) and that within-pair and extra-pair reproductive success do not always co-vary (Kleven et al., 2005; Raj Pant et al., 2022; Vedder et al., 2010; Whittingham & Dunn, 2004).

We observed a high prevalence of *Plasmodium* spp., and a low prevalence of *Haemoproteus* and *Leucocytozoon* spp., with similar levels of infection across cavity types (Figure 1; Table S1, S2). The similar levels of infection between cavity types seem to suggest that infection status with haemosporidian parasites does not strongly mediate nesting site choice in these populations. However, since birds were sampled a few weeks after the nesting site was chosen, we cannot exclude the possibility that new infections acquired over the course of the nesting cycle obscure such an association. It was previously reported that great tits preferred nestboxes over natural cavities in secondary forests (Lõhmus & Remm, 2005). Such preferences may stem from the shortage of suitable quality natural cavities available for the birds in such environments. Another study in

Bielany Forest found no evidence of a preference towards a cavity type over the other by more or less ornamented parents (Janas et al., 2024). To our knowledge, no study has systematically assessed nesting site preference in tits in environments with superabundant natural cavities (i.e., old-growth or primaeval forests). On the other hand, this lack of evidence might be due to the study design of spatial separation of cavity types, aimed at disentangling parental quality from the impact of cavity type. Because we did not monitor exposure to vectors, it is not possible to conclude whether cavity type (natural cavity versus nestbox) is associated with different probabilities of infection with haemosporidians. Studies on the spatial occurrence of arthropods, such as *Plasmodium*-transmitting mosquitoes (Culicidae), and biting midges (Ceratopogonidae), which are vectors of *Haemoproteus* and *Leucocytozoon* (Santiago-Alarcon et al., 2012), show vertical structuring of these organisms (Černý et al., 2011). This suggests that even nests located close to each other horizontally may differ in exposure to vectors if situated at different heights, which is the case for natural cavities. Additional evidence showing variation in the abundance of vectors between nestboxes hung at the same height (Martínez-de la Puente et al., 2009; Žiegytė et al., 2021) further suggests that potential differences in vector exposure exist between natural cavities and nestboxes. Given the length of the prepatent period of haemosporidian parasites, sampling nestlings to monitor vector exposure in the two types of cavities, especially in the case of small passerines, would not shed light on the topic. We therefore encourage future studies to investigate haemosporidian infections in their arthropod vectors and monitor the spatial occurrence of these organisms in both natural cavities and nestboxes.

Conclusions

The interaction between infection status and cavity type on paternity loss observed in this study highlights the importance of considering both intrinsic (infection status) and extrinsic (nesting site) factors when studying reproductive success. Human intervention in breeding habitats, such as the provision of artificial breeding sites, may influence reproductive outcomes, interacting with other factors that shape species-specific breeding strategies. Artificial nestboxes are widely used in avian research and conservation, however this study shows that breeding cavity might interact with host-parasite dynamics, which have the potential to influence fitness and evolutionary trajectories in cavity-nesting birds.

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Supplementary

Nestbox use is linked to increased paternity loss in infected blue tits, but not great tits

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Table S1. Generalized linear model in blue tits and generalized linear mixed model in great tits, with presence/absence of haemosporidian parasites (1/0) as the dependent variable. Cavity type, sex, age and year were included as predictors. Nest identity was introduced as random effect in the model for great tits. Natural cavity was the reference level for cavity type, Female for sex, Younger for age and 2018 for year. Significant differences ($p < 0.05$) are marked in bold.

	Blue tits (n=105)				Great tits (n=98)			
	Estimate	Std error	z value	p	Estimate	Std error	z value	p
(Intercept)	0.650	0.548	1.185	0.236	0.683	0.497	1.374	0.169
Cavity type (Nestbox)	0.406	0.478	0.849	0.396	-0.511	0.569	-0.898	0.369
Sex (Male)	-0.675	0.467	-1.446	0.148	-1.007	0.526	-1.917	0.06
Age (Older)	0.975	0.534	1.825	0.068	1.226	0.627	1.955	0.051
Year (2019)	0.355	0.490	0.726	0.468	1.326	0.630	2.104	**0.035**
Random effects					Variance			
Nest id					0.56			

Table S2. Multinomial logistic regression with infection status as the dependent variable coded as a categorical variable with three levels: no infection, single infection (when only one genus was detected) or co-infection (when two or three parasite genera were detected). Cavity type, sex, age and year were included as predictors. Nest identity was introduced as random effect. Natural cavity was the reference level for cavity type, Female for sex, Younger for age and 2018 for year. Significant differences ($P < 0.05$) are marked in bold.

	Blue tits (n=105)						Great tits (n=98)					
	Single infection			Co-infection			Single infection			Co-infection		
	Estimate	Std error	p	Estimate	Std error	p	Estimate	Std error	p	Estimate	Std error	p
(Intercept)	-0.254	0.481	0.598	-1.373	0.654	0.036	-0.845	0.532	0.112	-1.232	0.678	0.069
Cavity type (Nestbox)	-0.223	0.484	0.645	-0.133	0.611	0.828	0.465	0.500	0.352	0.082	0.654	0.9
Sex (Male)	-0.717	0.470	0.127	0.206	0.604	0.733	-0.156	0.468	0.738	-0.583	0.637	0.36
Age (Older)	1.305	0.525	**0.013**	1.436	0.642	**0.025**	1.242	0.511	**0.015**	0.073	0.721	0.92
Year (2019)	0.929	0.501	0.064	0.095	0.646	0.883	0.970	0.512	0.058	1.339	0.652	**0.04**

Table S3. Generalized linear model investigating paternity loss (quantified as the proportion of extra-pair offspring over the total number of offspring in the nest) in relation to cavity type, age, year and infection status, coded as categorical variable with three levels: no infection, single infection (when only one genus was detected) or co-infection (when two or three parasite genera were detected). Natural cavity was the reference level for cavity type, Uninfected for infection status and Younger for age. Significant differences ($p < 0.05$) are marked in bold.

	Blue tits (n=46)				Great tits (n=45)			
	Estimate	Std error	z value	p	Estimate	Std error	z value	p
(Intercept)	-2.354	0.477	-4.940	<0.001	-2.140	0.488	-4.391	<0.001
Cavity type (Nestbox)	0.319	0.568	0.561	0.575	0.620	0.581	1.064	0.287
Infection status (Single infection)	0.147	0.527	0.279	0.78	0.296	0.539	0.549	0.583
Infection status (Co-infection)	-0.501	0.833	-0.601	0.548	-0.371	0.860	-0.432	0.666
Infection status (Single infection) x Cavity type (Nestbox)	-0.329	0.691	-0.476	0.634	-0.087	0.761	-0.114	0.909
Infection status (Co-infection) x Cavity type (Nestbox)	1.163	0.970	1.200	0.23	0.294	1.141	0.258	0.796
Age (Older)	0.026	0.312	0.083	0.934	-0.757	0.362	-2.092	**0.036**
Year (2019)	-0.033	0.332	-0.099	0.921	0.320	0.386	0.829	0.41