

Habitat-specific variation in bacterial loads of wooden nest boxes in the pre-breeding period

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Abstract

Among environmental factors affecting life - history traits of birds breeding in nest boxes, an influence of microbial communities is relatively poorly understood. In this study, nest boxes used for breeding by great tit (*Parus major*) and blue tit (*Cyanistes caeruleus*) were sampled before the start of the breeding season to assess the bacterial loads of the nest box. Samples from the entrance hole and the interior of the nest box were taken at two different study sites: an urban parkland and a natural forest. Nest boxes were sampled to check if their bacterial loads differed between habitats. The second objective of this study was to check whether the occupancy of the nest boxes during the previous season would influence the bacterial load of the nest box. To verify this prediction, two categories of nest boxes were sampled at both study sites: nest boxes occupied by any of the two tit species in the previous season for breeding and nest boxes that had remained empty that year. The bacterial load of the nest box was significantly higher in the forest study area in both the occupied and unoccupied nest boxes. The nest boxes used for breeding in the previous season had significantly higher bacterial loads, but only in the forest area. Our results suggest that the bacterial load of the nest box can vary between habitats and may be positively related to the presence of the nests in the previous breeding season.

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ABSTRACT

Among environmental factors affecting life - history traits of birds breeding in nest boxes, an influence of microbial communities is relatively poorly understood. In this study, nest boxes used for breeding by great tit (*Parus major*) and blue tit (*Cyanistes caeruleus*) were sampled before the start of the breeding season to assess the bacterial loads of the nest box. Samples from the entrance hole and the interior of the nest box were taken at two different study sites: an urban parkland and a natural forest. Nest boxes were sampled to check if their bacterial loads differed between habitats. The second objective of this study was to check whether the occupancy of the nest boxes during the previous season would influence the bacterial load of the nest box. To verify this prediction, two categories of nest boxes were sampled at both study sites: nest boxes occupied by any of the two tit species in the previous season for breeding and nest boxes that had

remained empty that year. The bacterial load of the nest box was significantly higher in the forest study area in both the occupied and unoccupied nest boxes. The nest boxes used for breeding in the previous season had significantly higher bacterial loads, but only in the forest area. Our results suggest that the bacterial load of the nest box can vary between habitats and may be positively related to the presence of the nests in the previous breeding season.

Keywords: nest boxes, bacterial load, great tit, blue tit

COVER LETTER

On behalf of all coauthors, I am submitting a manuscript titled ‘**Habitat-specific variation in bacterial loads of wooden nest boxes in the pre-breeding period**’ for possible publication in Ecology and Evolution. The manuscript is new, approved by all coauthors, and is not considered for publication elsewhere. This study is a research article and is a continuation of our previous work (Zabłotni, A., Kaliński, A., Bańbura, M., Gładalski, M., Markowski, M., Skwarska, J., Wawrzyniak, J., & Bańbura, J. (2020). Experimental nest replacement suggests that the bacterial load of nests may mediate nestling physiological condition in cavity nesting Great Tits (*Parus major*). *Journal of Ornithology*, 161, 819-828) on the influence of bacterial loads on hole-nesting passerines. This study presents observed differences in bacterial loads in nest boxes located in two distinct habitats: urban parkland and natural forest. We showed that the bacterial load was on average higher in the nest boxes at the forest study site. We assume that this result is related to differences between habitats, including the composition of plant species in parkland and forest. We also showed that the presence of the nest in the nest box in the previous season positively affected the bacterial load, but only in the forest area. The results presented are relatively novel and supplement our understanding of the factors that potentially can affect the breeding performance of birds using artificial nest boxes with a new dimension related to microorganism-host interactions. Therefore, we consider Ecology and Evolution to be the most appropriate journal to publish the findings.

1. INTRODUCTION

Microorganisms are ubiquitous in a variety of habitats, and they inhabit all available spaces there. One of these habitats, avian nests, constitutes a unique environment for a variety of them (Gonzalez-Braojos et al., 2012; 2015; Goodenough and Stalwood, 2010; Zabłotni et al. 2020). Bacteria colonizing avian nests and spaces, which birds can potentially choose to breed, affect their avian hosts in both positive and negative ways (Goodenough and Stalwood, 2010; Singleton and Harper, 2010; Benskin et al., 2009). Some of the bacteria that colonize avian nests are commensals that feed on nest components consisting mainly of organic matter, but many bacterial species, including pathogenic strains, can exert a negative impact on birds (see Benskin et al., 2009 for the review). Recent research has shown that the nest microbiota plays an important role in mediating the life histories of birds. In particular, microorganisms shape the microbiome of their hosts and play a key role in the digestion of food (see Grond et al., 2009 for the review), pathogen defence (Soler et al., 2008; 2010), as well as influence plumage quality (Gunderson, 2008; Al Rubaiee et al., 2021). There is also a growing body of evidence showing the importance of microbial species for traits of avian condition and, consequently, for their reproductive success (Gonzales-Braojos et al., 2015; Goodenough and Stalwood, 2010; Zabłotni et al. 2020). However, relationships between nest bacterial load and life - history traits of birds are relatively poorly understood, mainly because the majority of studies on animal-associated microbiota are conducted on captive animals (Carina-Audisio et al., 2000). Little is still known about the complex relationships between different species of birds and their microbiota under particular environmental conditions in the wild. Given the ubiquity of bacterial communities and the complexity of host-microbiota interactions, it is important to document both the differences in abundance and diversity of microorganisms in different habitats (Horner-Devine et al., 2004; Levin et al., 2021).

Among a wide variety of locations where different species of birds breed, nests located in cavities are unique, as cavities constitute a particularly suitable environment for different types of microorganism (Gonzalez-Braojos et al., 2012; Goodenough and Stalwood, 2010; Berger et al., 2003; Goodenough and Stalwood, 2012). The special characteristics of nest boxes (and other cavities, including natural ones) result from

maintaining relatively stable physical conditions, such as humidity and temperature inside them, which make them suitable for microbial colonization and growth (Gonzalez-Braojos et al., 2015; Devaynes et al., 2018). Additionally, nest boxes are frequently used for breeding in several consecutive seasons, ensuring steady delivery of organic matter (feathers, peeled fragments of the epidermis, or excreted feces) essential for microbial growth. It should also be mentioned that outside the breeding season, several species of birds use nest boxes as roosting places during an autumn-winter period (Mainwaring, 2011). Furthermore, available data suggest that the prevalent practice of cleaning nest boxes after the breeding season does not remove all litter, leaving enough residues for bacterial growth (Zabłotni et al., 2020). Furthermore, it is highly probable that the bacterial load of the nest box itself depends on a variety of environmental characteristics of a particular habitat. The physical properties of habitats include several traits such as humidity, thermal conditions, chemical properties of soil, and probably many other characteristics that influence the assemblages of bacteria within different spatial scales (see Horner-Devine et al., 2004 for a review). Furthermore, the richness of plant species in a particular habitat can also play an important role (Saag et al., 2011). Since the available studies on natural microbial diversity in nest boxes placed in different habitats are rather scant (Burt and Ichida, 1999), there is a need to focus on microorganisms colonizing nest boxes which are used not only for breeding but also as roosting sites of many avian species.

For the above reasons and given that in our previous study we had demonstrated a negative influence of bacteria on the physiological condition of wild birds (Zabłotni et al., 2020), we conducted the study in two sets of wooden nest boxes that are used by the two secondary cavity nesting bird species, the great tit *Parus major* and the blue tit *Cyanistes caeruleus* in two different habitats: urban parkland and a deciduous forest. Both these tit species interchangeably use nest boxes for breeding in consecutive breeding seasons; however, in a particular year some of the nest boxes may remain unoccupied throughout the breeding season. This may potentially lead to a variation in the abundance of bacteria in nest boxes. The second potential source of variation in bacterial loads in nest boxes may be due to habitat differences between the two study sites. Probably, the different physical conditions that prevail at either site (temperature, humidity, soil characteristics, and similar characteristics) can shape the microbial communities in nest boxes. Since the nest boxes itself offer diverse conditions for bacterial communities, we decided to sample both the nest box interior and the nest box entrance hole. Therefore, we estimated the interior bacterial loads of the nest box and the entrance hole of the nest box to (i) check if there was a significant difference between the two habitats and (ii) test the following prediction: the nest boxes used for breeding during the preceding year had higher bacterial loads than the nest boxes that had remained empty that year.

2. MATERIALS AND METHODS

2.1. Study sites

This study was carried out in 2020 in two different habitats: an urban parkland and a deciduous forest. The study sites are located ~ 10 km apart and are separated by the city of Łódź. The urban parkland study site (51°45'N; 19°24'E) consists of Łódź Botanical Garden, which covers a total area of approximately 67 ha. It is mainly of anthropogenic origin and has fragmented tree and bush cover with very few remnants of natural stands including birches (*Betula pendula*), beeches (*Fagus sylvatica*) and numerous alien species planted intentionally by garden administrators (Gładalski et al., 2016). The forest study site (51°50' N; 19°29' E) is an area of approximately 145 ha located in the interior of a rich, mature mixed deciduous forest called the Łagiewniki Forest (1250 ha in total). Oaks (*Quercus robur* and *Q. petraea*) are the dominant tree species in the forest. Both study sites were supplied with standard wooden nest boxes, each made of pinewood (200 in the parkland area and 300 in the forest area). After each breeding season in mid-October, each nest box was cleaned with a wire brush, so there were no visible nest remnants.

2.2 Bacterial sampling

In the first half of March 2020, 40 nest boxes (20 at each study site) were randomly chosen for bacterial sampling. About half of the nest boxes at each study site had been occupied by one of the two tit species (great tit or blue tit) in the previous year for breeding, while the other half had remained empty that year.

(5 occupied by blue tits, 6 by great tits and 4 by blue tits, 6 by great tits in the parkland and in the forest, respectively). Using disinfected latex gloves, an entrance hole of each nest box was swabbed with circular movement for 30 s with a sterile cotton swab previously moistened with sterile phosphate-buffered saline (PBS, pH 7.2; Adlab, Poland). Subsequently, a restricted area of ca. 2.5 cm² of an internal rear wall of every nest box in the middle of its height was swabbed in the same manner. A sample per entrance hole and another sample per inner wall of each nest box were taken (in a standardized manner with respect to time and area).

2.3. Lab procedures

Once in the laboratory, bacteria from the swabs were transferred to the solution in the manner used in our previous study on the bacterial load (see Zablotni et al., 2020 for technical details). The bacteria were cultivated and incubated on Tryptic Soy Agar medium (TSA) for 48 h at 37±1°C, then for an additional 48 h at 25±1°C and the colony forming units (CFU) were counted. The results were expressed as CFU/ml.

2.4. Statistical analyzes

The bacterial load of the nest box entrance hole and the nest box interior variables were normalized by ln transformation prior to analyses. A two-way ANOVA was used to test whether the study area and the occupancy of the nest box affected the nest box entrance hole bacterial load in the study year; the interaction term was also calculated. This approach was repeated for nest box interior bacterial load. Interactions, both significant and non-significant were included in the models. Analyses were performed using Statistica ver. 12 software (StatSoft Inc., 2014).

3. RESULTS

The mean, minimum and maximum ln transformed values of the bacterial loads in the entrance hole and the interior bacterial loads of the nest box in the parkland and forest, occupied for breeding and empty in the previous year, are given in Tables 1 and 2.

The mean entrance hole bacterial load differed significantly between the study areas and was higher in the forest study area (Table 1, Table 3, Fig.1). Similarly, the interior bacterial load of the nest box differed significantly between the nest boxes occupied and unoccupied in the previous year (which was higher in the nest boxes occupied in the previous season), but the difference was significant only in the forest study area (Table 2, Table 3, Fig.2).

The correlation between the entrance bacterial load and the interior bacterial load was non-significant in the parkland study area and in the forest study area ($r = 0.41$, $p = 0.075$ and $r = -0.43$, $p = 0.129$, respectively).

FIGURE 1. Mean ($\pm$ standard errors) entrance hole bacterial load in the nest boxes occupied and unoccupied in the previous season in two study areas

FIGURE 2. Mean ($\pm$ standard errors) nest box interior bacterial load in the nest boxes occupied and unoccupied in the previous season in two study areas

4. DISCUSSION

We found that both the bacterial load of the entrance hole and the bacterial load of the nest box were significantly higher in the forest than the bacterial loads in the parkland, both in the nest boxes occupied and unoccupied in the previous season. We also found that the interior bacterial load of the nest box was higher in the nest boxes used for breeding in the previous season, but the difference was significant only at the forest site.

We revealed a clear difference in the interior bacterial load of the nest box between deciduous forest and urban parkland. This result suggests that the bacterial loads in nest boxes are strongly habitat dependent. The interior of the nest box is probably readily colonized by a variety of microorganisms shortly after placing the nest box in a particular environment. Birds play the main role of transferring microorganisms to nest boxes when visiting them, as well as to other various cavities as places potentially suitable for breeding or

roosting (Saag et al., 2011; Burt and Ichida, 1999). Bacteria are found on avian beaks and toes, however, the communities in the plumage are most important in this context, since bacteria are both most numerous and most diverse in feathers (Burt and Ichida, 1999; Bison et al., 2007). In our study system, the nest boxes were used mainly by the blue tit and the great tit. Both species forage mainly on leaves and twigs. Plants, as a source of bacteria, are suggested to play an important role in the transmission of these organisms to plumage (Bison et al., 2007). This impact of the plant host on the bacterial microbiota was shown between predators and prey in the trophic networks of blue tit (Dion-Phenix et al., 2021). Given that our study sites differ markedly floristically, with deciduous forests being richer in plant species than urban parkland, it seems very likely that the forest site may maintain richer bacterial communities. Saag et al. (2011) showed such a pattern of habitat-related differences in bacterial density and species richness. They revealed that although the number of phylotypes per bird was higher in coniferous habitat, bacterial densities were higher in deciduous habitat. It is also supported by the results presented by Broughton and Gross (2000) who suggested that microbial activity was positively correlated with plant productivity. Similarly, Bisson et al. (2007) found that microorganisms from particular groups were more abundant in plumage sampled from American redstart *Setophaga ruticilla* in wet versus dry habitats. It is in line with our results, since rich, mature forests retain more moisture than urban parkland with its many open areas with no or little tree cover and, therefore, open to direct sunlight. However, the richness of plant species is not the only distinction between the two sites. The Botanical Garden is a place where various agrotechnological works are conducted throughout the year (own observations). These procedures include the application of different chemicals, such as insecticides, which probably affect different organisms, including bacteria that are present not only in plants but also in soil. For example, in 2013 and 2014, a large-scale molluscicide treatment was applied in the Garden to eradicate the invasive Spanish slug *Arion vulgaris* (see Bańbura et al. 2019 for details). Presumably, regular use of chemicals in parkland, contrary to the forest area, can disturb microbial communities, including the sheer number of bacteria in different ways. In addition, other factors, such as the presence of bird aggregations influence soil properties and microbial community in the soil (Wang et al., 2020), which may be important in the garden area where during the autumn-winter period, wintering birds gather in relatively large quantities (own observations). For the above reasons, the microbial profile of the soil itself may play an important role in the acquisition of plumage bacterial (Bison et al., 2007; Lucas et al., 2003; Llado et al., 2017). It may be important in the context of this study, since the great tit relatively frequently forages on the ground (Gosler, 1993; own observations), which means that the birds acquire bacteria directly from the soil and then carry them into nest boxes.

In addition to basic habitat characteristics including plant species composition and soil characteristics, there are probably other factors that can contribute to bacterial loads in nest boxes. It is known that different taxa of animals visit nest boxes for a variety of purposes. Many invertebrates (snails, spiders, or insects) are opportunistic species that use nest boxes year-round as shelters, roosting places, or to build the nests and raise their offspring (i.e. wasps) (McComb and Noble, 1982; Broughton et al., 2015; own observations). Invertebrate species colonizing nest boxes carry their bacteria acquired from the environment. Since the forest area is a more diverse habitat than the parkland area in terms of plant species composition, it is likely that the invertebrate assemblies are also richer in the forest. The next potential factor is the presence of particular mammal species, which visit nest boxes for two main reasons. In both our study areas, brown long-eared bats (*Plecotus auratus*) and noctules (*Nyctalus noctule*) occasionally use empty nest boxes for breeding and roosting during the summer / autumn period, and they are more frequent in the forest (own observations). The pine marten (*Martes martes*), in turn, is a predatory species that tries to reach the nest in the nest box and grab an adult bird or nestlings with its paws as a prey. In some breeding seasons, the predation rate was very high in the forest (Kaliński et al., 2014), but not in the parkland where marten predation occurred only exceptionally (own observations). Both bats and martens carry their bacterial flora which is transferred to nest boxes; however, in the case of martens, this transfer is restricted mainly to the entrance hole.

We also tested whether the occupancy of the nest box in the previous year had affected the bacterial load of the nest box. Our results are ambiguous on that point since this effect was significant in the case of the nest

box interior but not in the case of the nest box entrance and only in the forest study site. It is not clear why this effect was found only at one study site. A possible explanation may be related to the intensity of using nest boxes as roosting sites during an autumn-winter period. It is well known that wintering tits spend the nights in nest boxes (Mainwaring, 2011; own observations) and thus probably transfer bacteria there. In the parkland, contrary to the forest area, both tit species are more abundant during winter (own observations) and probably use nest boxes as roosting sites more intensely. If this assumption is true, it should at least partially eliminate the expected difference in bacterial load between nest boxes occupied and unoccupied for breeding. However, it seems that this effect is not strong enough to nullify the striking difference between the study sites. Yet, we do not have any quantitative data on the intensity of using nest boxes as roosting sites out of the breeding season and, therefore, we cannot make any plausible conclusion on that issue.

5. CONCLUSIONS

Our study demonstrates that the observed difference in the bacterial load of the nest box is probably related to habitat-related differences between the two study sites. The most important factors are probably associated with the richness of plant species and the physical properties of the soil, with additional factors resulting from the specificity of the two study sites. However, taking into account the complex and yet relatively weakly understood relations between microorganisms, their hosts, and the environment, probably other factors play their role and we are still far from understanding this issue. Furthermore, we should treat our results with care since we do not have data on the richness of bacterial species at both study sites. However, our study suggests that the bacterial load may be a potentially important factor for the life-history traits of secondary cavity nesting birds, since it may vary considerably between habitats. Therefore, since studies on cavity nesting birds with the use of artificial wooden nest boxes are widely conducted across a geographical range and a variety of habitats, and given that bacterial load may affect host fitness components, we consider our study perspective as a promising field for future research.

AUTHOR CONTRIBUTION

Agnieszka Zabłotni: laboratory procedures, writing – review and editing. **Adam Kaliński:** investigation, conceptualization, fieldwork, writing – original draft, statistical analyses. **Mirosława Bańbura:** fieldwork, writing – review and editing. **Michał Gładalski:** fieldwork, writing – review and editing. **Marcin Markowski:** writing – review and editing. **Joanna Skwarska:** writing – review and editing. **Jarosław Wawrzyniak:** writing – review and editing. **Jerzy Bańbura:** conceptualization, writing – review and editing.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

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ETHICAL STATEMENT

All procedures were approved by the Local Ethical Committee in Łódź (Lokalna Komisja Etyczna do Spraw Doświadczeń na Zwierzętach w Łodzi, Uniwersytet Medyczny w Łodzi – Zakład Farmakodynamiki, ul. Muszyńskiego 1, 90-151 Łódź). All the methods were carried out under Polish law.

DATA AVAILABILITY STATEMENT

The dataset generated and analysed during the current study is available in the figshare repository, <https://figshare.com/s/8cf266bd98ae89b5f394>.

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TABLE 1 . The mean, minimum, and maximum values of the entrance hole bacterial load (CFU/ml) in the parkland and forest study areas in both nest categories (occupied and unoccupied in the previous year). Values are ln-transformed. SD values are given in parentheses.

	Parkland	Parkland	Parkland	Forest	Forest	Forest
	Mean	Min	Max	Mean	Min	Max
Occupied	7.54 (± 2.01)	5.30	10.37	13.56 (± 1.91)	10.40	15.40
Unoccupied	6.86 (± 0.65)	5.99	7.78	14.98 (± 2.08)	12.82	17.97

TABLE 2 . The mean, minimum, and maximum values of the nest box interior bacterial load (CFU/ml) in the parkland and forest study areas in both nest categories (occupied and unoccupied in the previous year). Values are ln-transformed. SD values are given in parentheses.

	Parkland	Parkland	Parkland	Forest	Forest	Forest
	Mean	Min	Max	Mean	Min	Max
Occupied	8.08 (± 2.01)	5.30	12.47	14.35 (± 1.85)	10.82	16.07
Unoccupied	8.58 (± 1.16)	6.68	10.82	11.35 (± 1.98)	8.99	15.40

TABLE 3 . Summary of the bacterial load in the two-way ANOVA of the entrance hole bacterial load (top) and the bacterial load in the nest box (bottom). The effects of the study area, nest box occupancy in the previous season, and the interaction between these factors are given.

Factor	Df	F	p
Entrance hole bacterial load Intercept	1; 31	1550.001	<0.001
Study area	1; 31	168.089	<0.001
Previous season occupancy	1; 31	0.465	0.500
Study area* Previous season occupancy	1; 30	3.865	0.064
Nest box interior bacterial load Intercept	1; 31	1199.654	<0.001
Study area	1; 31	54.575	<0.001
Previous season occupancy	1; 31	4.165	0.049

Factor	Df	F	<i>p</i>
Study area* Previous season occupancy	1; 30	8.207	0.007

FIGURE CAPTIONS

FIGURE 1. Mean ($\pm$ standard errors) entrance hole bacterial load in the nest boxes occupied and unoccupied in the previous season in two study areas

FIGURE 2. Mean ($\pm$ standard errors) nest box interior bacterial load in the nest boxes occupied and unoccupied in the previous season in two study areas