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Bacterial species richness at three stages of the breeding season in *Cyanistes caeruleus* (blue tit)

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ABSTRACT

Blue tits are exposed to a vast array of bacteria throughout their life cycle and are particularly exposed during a breeding attempt. Any pathogenic bacteria within their microbiome can have a detrimental effect on their fitness and that of the nestlings they are raising. This study aims to identify the bacterial species richness that birds of this species are exposed to during three key stages of the breeding cycle: nest build, clutch completion and immediately post fledging. Nests were swabbed at these time points across four deciduous woodland sites in the United Kingdom and genomic DNA extracted prior to T-RFLP analysis. This is the first known instance of this technique being used to assess the nest microbiome and the first culture independent assessment of nest microbiome within this species. This revealed 103 distinct OTUs (Operational Taxonomic Units) across all sites and stages with an increase in taxa richness at each stage. There were differences in the microbiomes of each nest across breeding stage and site with evidence suggesting the nest microbiome is largely determined by the local environment.

1. Introduction

Bacteria form a large part of Earth's biomass, outnumbering animal and plant cells and have adapted to be able to survive and thrive in all known conditions (Jacob et al., 2014). Symbiotic relationships between animals and bacteria are omnipresent and can play a significant role in animal evolution (Van Veelen et al., 2017). Bacteria can be hugely beneficial and in some cases essential in processes such as digestion, nutrient synthesis and protection from pathogen colonisation (Jacob et al., 2014). There is increasing evidence over the importance of host – microbiome relationships to the survival of species (Glasl et al., 2016) with indications that many have co-evolved (Goodrich et al., 2016). Conversely, some bacteria are pathogenic, with the potential to cause a reduction in fitness or death to an individual, as such they impose strong selective pressures on host life-history traits (Clayton and Moore, 1997). It is therefore of upmost importance to obtain knowledge of an individual's microbiome and the challenges it faces when exposed to new sources of bacteria and any associated pathogenicity (Burtt and Ichida, 1999).

Plumage microbiomes have been the main focus of bird microbiome studies (i.e. Kilgas et al., 2012, Shawkey et al., 2005, Burtt and Ichida, 1999) with Kilgas et al. (2012) identifying wild bird's plumage play host to a vast microbiome with evidence suggesting it influences the host behaviour and life histories. Van Veelen et al. (2017) investigated both plumage and nest microbiomes, finding bird and nest-associated bacteria showed substantial OTU (Operational Taxonomic Units) co-occurrences sharing dominant taxonomic groups within *Lullula arboea* (woodlarks) and *Alauda arvensis* (skylarks). Similarly, Goodenough et al. (2017) discovered the skin and feather microbiome of individual female *Ficedula hypoleuca* (pied flycatchers) were closest to their nest microbiome. This convergence is likely due to bi-directional transfer of bacteria between the nest and plumage given the close contact between the female and nest during the breeding attempt. It is thus reasonable to assume that studies relating to only plumage microbiome or nest microbiome offer a fair comparison to each other.

All organisms have competing energy demands and this is especially true for female birds during the breeding season (Monclus et al., 2017). One energy demand exercised by the birds is to control plumage bacteria by self-preening. During preening, uropygial oil is secreted which contains beneficial, symbiotic bacteria, antibacterial compounds (Shawkey et al., 2003) and wax esters believed to be released as a food source for beneficial bacteria within the microbiome (Jacob et al., 2014), all of which can control the load of pathogenic, feather-degrading species (Soler et al., 2008; Martin-Vivaldi et al., 2009). Preening is however a time-consuming process and during the breeding season there is a trade-off with parental effort (Lucas et al., 2005).

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Initially this includes nest building, followed by egg incubation and then nest sanitation and collecting food for the chicks once hatched. This activity leads to increased free-living bacteria abundance within the plumage (Alt et al., 2015). Bacteria within the plumage microbiome can be a problem as certain species are capable of degrading β-keratin which constitutes more than 90% of feathers (Alt et al., 2015). Feather degrading bacteria can reduce fitness through thermoregulation problems (Ichida et al., 2001) and lower flight efficiency (Moller et al., 2012) while any alteration to plumage colour can affect feather based communication (Kilgas et al., 2012). Altricial nestlings are prone to such reductions in fitness which can affect their long term survival. However the birds may not be passive recipients of such increase in bacteria. Experimentally altering the bacterial density that *Parus major* (great tits) were exposed to resulted in an increase in the size of their uropygial gland and quantity of secretions (Jacob et al., 2014). They do this despite the already increased energy demand of the breeding effort and an increased probability of olfactory detection by predators, illustrating the importance that birds place upon actively influencing their microbiome.

During a breeding attempt nest-building birds are exposed to an ever-changing and highly variable community of bacteria. For instance, *P. major* were at their most 'dirty' during the nest building phase with an increase in microbial density (Kilgas et al., 2012). It is hypothesised that this is a result of increased contact with the ground and nest materials, introducing new and potentially pathogenic bacteria to the bird. Upon laying, bacteria from the cloacal cavity is released on to the nest (Sanders et al., 2005), and what may have been a commensal organism within the gut could have a pathogenic effect on skin or feathers, an occurrence commonly experienced within vertebrate species were gut commensals such as *Escherichia coli* acting pathogenically in extra-intestinal infections (Tenaillon et al., 2010). Following hatching food items are constantly being brought in to the nest as well as the chicks defecating which gives further avenues for bacteria to be introduced (Benskin et al., 2015). We have previously identified *C. caeruleus* are subjected to an increasing number of bacteria during the breeding attempt (Devaynes et al., 2018) supporting the introduction of additional bacterial taxa.

Many of the studies which identify the microbiome within the nest are restricted by culturable bias with only 0.1–10% of microbes able to be cultured under lab conditions (Grizard et al., 2014), whilst this is acceptable for comparisons within a study it does not give a true indication of the total bacterial species richness the birds are exposed to. Those using culture-independent techniques are few, with only Jacob et al. (2014) finding 180 OTUs across 52 *P. major* nests present within the literature. This study was limited as they sampled only once during the breeding attempt so no information was gathered on the development of the nest microbiome over this period. Kilgas et al. (2012) found the density of plumage bacteria changed rapidly during nest building, although here they only assessed bacterial density and not community composition.

This study aims to address this gap in the literature offering an additional non-culture bias comparison and investigating the bacterial species richness of *C. caeruleus* nests at three time points; nest build, clutch completion and immediately post fledging using Terminal – Restriction Fragment length Polymorphisms (T-RFLP) analysis. This will allow true comparisons on how the bacterial community composition progresses during a breeding attempt. Additionally given that Goodenough et al. (2017) found the nest microbiome to be dependent upon the local environment, samples will be collected across four sites to discover any intra-site difference between nests and inter-site difference between the relative local environments.

Cyanistes caeruleus is a small passerine bird in the family Paridae, with a distribution throughout temperate and Mediterranean Europe and western Asia, residing mainly within deciduous woodland. Their abundance, with an estimated 20–44 million breeding pairs in the UK (Ichida et al., 2001) and their affinity for occupying nest boxes makes them an ideal study species. *Cyanistes caeruleus* assess breeding sites and choose a mate over winter with nesting building beginning early April to coincide with the emergence of caterpillars, the key food item for their chicks. Eggs are laid one a day from late April with a clutch size of between six and thirteen, the number dependent upon food availability. Incubation is performed exclusively by the female and lasts fourteen to sixteen days before hatching. The altricial chicks require constant feeding which is predominantly undertaken by the male as the female is needed to keep her featherless chicks warm. Chicks spend sixteen to eighteen days in the nest prior to fledging (BTO, 2018). *Cyanistes caeruleus* breeding phenology thus offers various opportunities for the development of the nest microbiome.

T-RFLP analysis has been shown to be a robust and reproducible methodology for rapid exploration of microbial community structure with a higher resolution for detecting less abundant species than other microbial profiling techniques (Torok et al., 2008) and used in the fields of; agriculture (Kaur et al., 2017; Zhu et al., 2017), marine biology (Tada et al., 2017) and soil community studies (Gschwendtner et al., 2016; Fry et al., 2016). It does have its vulnerabilities owed to primer choice, template DNA concentration and number of PCR cycles (Zhu et al., 2002) but this is true of all PCR-based technologies. This study will determine how the bacterial community of the nest progresses throughout the breeding attempt whilst utilising a novel method within this field, assessing its efficacy.

2. Methods

2.1. Study sites

The study was performed in the breeding season of 2016 across four sites in England to incorporate local and broader scale geographical differences. Three sites were located in Lancashire, and a fourth site was located within the Forest of Dean, Gloucestershire (refer to Table 1).

2.2. Field sampling

Nest boxes were observed to determine breeding activity and bacterial samples taken under Natural England licence (2014/SCI/0288) at

Table 1
Study site location, description and nest box status.

Site	Map reference	Vegetation type	Nest boxes	Nest box orientation	No. of nests sampled
Ruff Wood	53°33′36.27″N, 002°51′59.43″W	Mixed deciduous woodland.	None present, 20 placed for this study.	North to East.	11
Scutchers Acres	53°35′17.68″N, 002°49′23.79″W	Mixed deciduous and coniferous woodland.	None present, 20 placed for this study.	North to East.	10
Mere Sands Wood Nature Reserve	53°38′05.59″N, 002°50′16.06″W	Mixed deciduous woodland.	Present, some monitoring from local volunteer groups.	Random covering all aspects.	10
Nagshead Nature Reserve	51°46′25.59″N, 002°34′20.76″W	Oak dominated deciduous woodland.	400 present, managed by RSPB.	North to East & North to West.	6