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Like mother like nest: similarity in microbial communities of adult female Pied Flycatchers and their nests

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Microbial relationships between birds and nesting environments are complex and remain poorly understood. Past studies have focused on between-nest variation in egg/chick bacterial profiles with little attention given to the microbial relationships between adult birds and their nests. Moreover, very little microbial research has included mycology despite fungi being prevalent in nesting environments and important correlates of fitness in chicks. In this study, we identified microbes associated with feathers, skin and nests of Pied Flycatchers *Ficedula hypoleuca*, an internationally-declining migrant songbird. From 75 samples, we isolated 50 bacterial OTUs (Operational Taxonomic Units; dominated by *Enterococcus*, *Sanguibacter*, *Pseduomonas*) and 63 fungal OTUs (dominated by *Penicillium*, *Aspergillus*), many of which had not previously been isolated from birds. Although females had significantly higher non-haemolytic bacterial OTU richness and males significantly higher fungal OTU richness, there was considerable diversity in actual OTUs isolated and thus there was no "typical" female, male, or nest microbial profile. Interestingly though, we show for the first time that the microflora of individual females is significantly more similar the microflora of her own nest than the site-level average of all nests. This suggests microbes are shared within female-nest pairs such that microbial communities start to converge. This is probably a two-way interaction as gut/skin microbes were isolated from nests and plant/soil microbes were isolated from females. Convergence was not seen for males, which probably reflects the role of the female as sole nest builder and egg incubator in this species. We discuss these findings in relation to microbial transfer pathways and avian nesting behaviour.

Keywords: avian microbiology, bird microbes, plumage bacteria, *Ficedula hypoleuca*, nestboxes, wild passerines

Zusammenfassung

Ähnlichkeit der Mikrobiome von adulten weiblichen Trauerschnäppern und ihren Nestern

Mikrobielle Zusammenhänge zwischen Vögeln und Brutumgebungen sind komplex und bis heute schlecht verstanden. Vergangene Untersuchungen waren konzentriert auf Unterschiede in bakteriellen Profilen von Eiern und Küken zwischen Nestern, mit nur geringer Aufmerksamkeit auf mikrobielle Zusammenhänge zwischen adulten Vögeln und ihren Nestern. Darüber hinaus erstreckten sich die mikrobiologischen Untersuchungen nur selten auf Pilze, obwohl diese in Brutumgebungen häufig anzutreffen sind und wichtige Korrelate darstellen für die Fitness der Küken. In dieser Untersuchung identifizierten wir Mikroorganismen von Federn, Haut und aus Nestern des Trauerschnäppers *Ficedula hypoleuca*, einem Singvogel, der international abnimmt. Aus 75 Proben isolierten wir 50 bakterielle OTUs (Operational Taxonomic Units; hauptsächlich *Enterococccus*, *Sanguibacter*, *Pseudomonas*) und 63 Pilz-OTUs (hauptsächlich *Penicillium*, *Aspergillus*), von denen viele bislang noch nicht bei Vögeln isoliert wurden. Obwohl Weibchen einen signifikant höheren nicht-hämolytisch bakteriellen OTU-Reichtum aufwiesen und Männchen einen signifikant höheren OTU-Reichtum an Pilzen, gab es wesentliche Unterschiede zwischen einzelnen OTUs, so dass man nicht von „typisch" weiblichen, männlichen oder Nest-OTUs sprechen kann. Interessanterweise können wir zum ersten Mal zeigen, dass die Mikroflora eines einzelnen Weibchens signifikant ähnlicher der Mikroflora ihres eigenen Nestes ist als dem Durchschnitt aller Nester im Untersuchungsgebiet. Dies legt nahe, dass Mikroorganismen zwischen Weibchen und Nest ausgetauscht werden, so dass die Mikrobiome anfangen zu konvergieren. Dabei handelt es sich wahrscheinlich um eine bidirektionale Interaktion, da Mikroorganismen aus dem Darm und von der Haut aus dem Nest isoliert wurden und Pflanzen- und Bodenmikroorganismen von den Vögeln. Eine Konvergenz der Mikrobiome konnte für Männchen nicht gezeigt werden, was vermutlich die Rolle der Weibchen dieser Art wiederspiegelt, allein für Nestbau und Brüten verantwortlich zu sein. Wir diskutieren diese Ergebnisse in Relation zu Übertragungswegen von Mikroorganismen und Brutverhalten von Vögeln.

<u>Introduction</u>

Research into host-microbe ecosystems is revolutionising the way we view avian biology (McFall-Ngai et al, 2013; Solar et al 2015). Studies that focus on identifying microbes associated with focal avian species or quantifying the structure of microbiomes with which birds interact are vital for understanding physiological processes, disease and immune responses, bird behaviour and socio-biology, population dynamics, community change, and links between the biotic and abiotic environments (Archie and Theis 2011; Bordenstein and Theis 2015; Moyers et al. 2015; Lewis et al. 2016). As well as being important from a scientific perspective, such knowledge can be vital in informing effective avian management and conservation initiatives (Dille et al. 2016).

Several studies have shown that the avian nesting environment microbiome is a complex one (Singleton and Harper 1998; Berger et al. 2003; Goodenough and Stallwood 2010). Microbes within the nesting environment can come from a variety of sources, including the birds themselves (feathers, skin, cloacal contact, faecal material) and the local environment (nest material including vegetation and mammal hair, cavity walls for cavity-nesting birds, and nest-dwelling ectoparasites) (Peralta-Sanchez et al. 2010; Ruiz-de-Castañeda et al. 2011a). The avian origins of some nest microbes, and species-specific differences in material used to construct nests, means that it is not surprising that there is considerable interspecific variation in nest microflora. This has been demonstrated in direct comparative studies by Goodenough and Stallwood (2010) and Peralta-Sánchez et al (2012), and is also evident by comparing single-species studies by different authors (e.g. the importance of *Pseudomonas* for House Wrens *Troglodytes aedon* (Singleton and Harper 1998) but not Starlings *Sturnus vulgaris* (Berger et al. 2003)). Differences in uropygial oils and the size of the uropygial gland can also have an effect (Soler et al. 2008, 2012). In addition, spatial variability in environmental factors including nesting material gives rise to substantial intraspecific variation in nest microflora. Microclimate might also have a role – for example, Goodenough and Stallwood (2012) showed substantial differences in nestbox microbial community relative to orientation (and thus temperature and humidity).

To date, most avian nest microbial studies have focused on the effect of specific microbes on egg viability (Cook et al. 2003; Ruiz-de-Castañeda et al. 2011a; Grizard et al. 2014), incubation behaviour (Cook et al. 2005), hatching success (Soler et al. 2012, 2015), and fledging success (Mills et al. 1999; Moreno et al. 2003; Goodenough and Stallwood 2012). Nest-specific temporal patterns have also been investigated. For example, Mills et al. (1999) tracked individual Tree Swallows *Tachycineta bicolor* and showed an increase in both microbial load and OTU richness between hatching and fledging whereas González-Braojos et al. (2012; 2015) found that nestling Pied Flycatchers *Ficedula hypoleuca* raised in nests containing material from previous nesting attempts had higher microbial loads but that this did not change with nestling age for individual chicks. The potential for there to be strong nest-level effects on chick microbial community has been demonstrated by Lucas and Heeb (2005) using a partial cross-fostering experiment that showed non-related chicks raised in the same nest (nest-siblings) exhibited greater microflora similarity than related chicks raised in different nests (genetic-siblings).

Although we know a lot more about nest-microbe interactions than when Burtt (1999) first encouraged ornithological researchers to "think small", there is still much that remains a mystery. In particular, very little

research has been conducted on the relationship between adult birds and their nesting environments. This is despite adult birds being both a source of microbes and the recipient of microbes originating from the nesting environment that could have a potential role in shaping avian life histories (Stewart and Rambo 2000). In one of the only studies on this topic, Saag et al. (2011) showed plumage bacterial load of adult female great tits was higher in the nest-building period than when females were provisioning for chicks, possibly due to increased contact with nesting material. Additional support for this was offered by a follow-up study (Kilgas et al. 2012), which showed that plumage bacterial load increased during the nest-building process. Both studies focussed on bacterial load and did not identify bacterial Operational Taxonomic Units (OTUs). Moreover, despite fungi being prevalent on birds and in nesting environments (Hubálek 1976; 1978 Stewart and Rambo 2000), and being important correlates of body condition (Goodenough and Stallwood 2012), fungal OTUs were not considered.

Here, we identify the bacteria and fungi associated with adult breeding Pied Flycatchers. This extends work undertaken previously on Pied Flycatcher microbial load (González-Braojos et al. 2012; 2015). Our aim is to establish whether there are any systematic differences in microbial communities between nests and the feathers and skin of adult birds. We then consider microbial communities in more detail to compare the microflora of each bird relative to its own nest and other nests at the same site. This has not seemingly been examined previously for free-living passerines of any species but Brandl et al. (2014) profiled the microbial communities of reed warbler *Acrocephalus scirpaceus* eggs and nestlings from the same nest and found that they were more similar than expected by chance. We hypothesise that there will be considerable variation in the microbial community of different nests and the microbial community of different individual birds because of the range of factors, both avian and environmental, that can affect the microbiomes involved. We expect to see differences between birds and nests, with the former being dominated by avian symbionts, including plumage microbes, and the latter being dominated by environmental isolates. However, we also expect to see some convergence in bird-nest microbial communities such that there is a greater level of similarity between birds and their own nest compared to the site-level average.

Methods

Study site

Our study site was woodland in Herefordshire and Powys (U.K.) containing around 100 artificial wooden nestboxes affixed to mature trees. All nestboxes were sited at approximately the same height (~2.5 m above the ground) and constructed from the same material (5-ply wood).

In 2012, around 25% of nestboxes were used by were used by Pied Flycatchers, an internationally-declining migrant that winters in sub-Saharan Africa and breeds in woodlands throughout Europe. The remaining nestboxes were used predominantly by blue tits *Cyanistes caeruleus* and great tit *Parus major*, with occasional use by Eurasian nuthatch *Sitta europaea* and common redstart *Phoenicurus phoenicurus*. Pied flycatcher nests were typically made from dead leaves and woody debris. Given the influence of old nesting material on parasite and microbial loads (Singleton and Harper 1998; González-Braojos et al. 2012), old nests had been removed at

the end of the preceding breeding season. Nest monitoring was undertaken weekly (by co-author DGC) between May 2012 and July 2012 using standard British Trust for Ornithology (BTO) Nest Record Scheme protocols.

Collecting swabs

We collected microbial samples from nests used by Pied Flycatchers using sterile rayon-tipped swabs pre-moistened with phosphate buffer at pH 7.1±0.1 (Steriswab™, Medical Wire and Equipment Company, UK) as per Ruiz-de-Castañeda et al. (2011a). All bird handling and swabbing was undertaken by an experienced ringer (co-author DGC) under licence from the British Trust for Ornithology with an approved endorsement to swab Pied Flycatchers and their nests for the purposes of this study. This licence met all legal requirements and the conditions of the licence were respected at all times.

In total, we took 75 swab samples. These were sub-divided into: nest swabs (n = 21); feather and abdominal skin swabs of adult females (n = 40; 21 feather swabs and 19 skin swabs) and feathers and abdominal skin of adult males (n = 14; equally split between feather and skin swabs). This gave 21 nest-female feather swab parings, with additional skins swabs in 19 out of the possible 21 cases. Swabs of most female birds were made during the second half of incubation, after lifting the birds while they were sitting on the eggs. Skin swabs were not taken in two cases because the hens were caught after the young had hatched, so intimate contact of the brood patch with incubating eggs had ceased for a period prior to swabbing. The sample size for nest and female swabs was the maximum possible given the population size and localised distribution of this declining migrant species (all nesting attempts at the study site being studied for the one year of the licence endorsement).

Swabs of male birds were only possible during the nestling feeding period when males could be temporarily captured in the box using a manually-operated nestbox trap. This comprised one half of a ping-pong ball placed in the nest box and tied to a length of fishing line that was passed through the entrance hole so that the fieldworker stationed ~50 m away could raise the ball to block the entrance after the male bird entered. Owing to logistical issues, including some male Pied Flycatchers being polygamous and not assisting with chick rearing for some nests, male swabbing was only possible in one third of cases. Although the male swabs were taken at a different stage of the nesting process relative to the female swabs, the actual difference in time was slight as female swabs were taken late in the incubation period and male swabs were taken very soon after hatching.

As the swab was the unit of study, we carefully standardised the swabbing procedure. Nests were swabbed for 10 s in a standardised pattern, with the swab being passed along the edges and then across the base in a cross formation. Bird swabs were also 10 s in duration and carefully standardised. For the feather swabs, each wing in turn was raised slightly and the swab was passed over the breast plumage on that side in the direction from the upper body towards the tail. For the skin swab the breast feathers were parted (as if assessing brood patch presence/condition). Breast feathers were parted carefully by hand with the bird handler wearing single-use, sterile, disposable gloves and the swab was then passed over the skin under the breast feathers, down each side of the chest and belly. Air swabs were taken by exposing an unused swab to the air for 10 s (the same time as for swabbing) and processing and plating as data swabs as per Lomardo et al. (1996), Stewart and Rambo

(2000), Goodenough and Stallwood (2010) to highlight any contamination problems. Post swabbing, all swabs were refrigerated at 4°C for a maximum of 7 days before processing as per Saag et al. (2011).

Culturing Microbes

In the laboratory, we washed swabs in 5 ml of sterile phosphate buffer (Fisher Scientific, Leistershire, UK) by placing on an orbital shaker for 24 hr. A 100 μl aliquot of swab supernatant was then cultured on three different media: (1) 2.8% (w/v) nutrient agar at pH 7.4 (Oxoid CM0003) to encourage bacterial growth; (2) 4% (w/v) horse blood base number 2 agar at pH 7.4 for haemolytic bacterial isolates (Oxoid CM0271); and (3) 6.5% (w/v) sabouraud dextrose agar at pH 5.6 (Oxoid CM0041) with 0.1% (v/v) of chloramphenicol (Oxoid SR00780) to encourage fungal growth while inhibiting bacteria. The rationale for using blood agar was to be able to identify likely pathogenic bacterial isolates through the presence of alpha or beta haemolysis. This method has been used previously in avian disease research to identify pathogenic OTUs, including those in the genera *Enterococci, Escherichia, Gallibacterium, Staphylococci,* and *Streptococci* from wild and captive birds (Bojesen, et al. 2003; Roy et al. 2006; Poeta et al. 2007; Smyth and McNamee 2008). This approach was also used in bacterial profiling of reed warbler nests to identify potential pathogens (Brandl et al. 2014). The presence of haemolysis was taken as an indicative result only before identification as not all pathogenic bacteria produce a haemolytic reaction and haemolytic reactions can occur with some non-pathogenic bacteria. All plates were incubated for 72 hrs (fungal plates at 28°C; bacterial plates at 35°C) before being assessed to determine the number of culturable OTUs. Each putative OTU was sub-cultured to ensure colony purity. All lab work was undertaken using full aseptic technique within an ethanol-sterilized class 100 Laminar Flow Hood (Labcaire VLF6, Clevedon, UK), which provided a BS5726-accredited class A sterile environment.

In this study, we used culture-based methods rather than culture-independent techniques such as next-generation sequencing or Ribosomal Intergenic Spacer Analysis with subsequent band sequencing. It is important to note that the microbial community found using these techniques will likely be a subset of the overall community since OTUs that could not be cultured in aerobic conditions would be excluded. Despite this limitation, culture-based techniques are still appropriate for testing microbial ecology hypotheses in birds (e.g. Ruiz-de-Castañeda et al. 2011a; Ruiz-de-Castañeda et al. 2011b; González-Braojos et al. 2012). This is especially true when studies are comparative (when multiple samples are processed using identical techniques so that the limitations are consistent between samples; as here) or where money, equipment or resources are limiting factors.

Identifying microbes

We identified OTUs biochemically using a GEN III MicroStation (BIOLOG, Hayward, California, USA). This is a 96-well plate system that combines 71 sole-carbon substrate utilization assays with 23 chemical sensitivity assays. Utilisation and sensitivity are indicated by reduction of tetrazolium violet. This is an accurate method for identification of microbial isolates (Klingler et al. 1992; Konopka et al. 1998 and references therein). Bacterial isolates were identified using GEN III Microplates, while fungal isolates were identified using GEN II FF MicroPlates for Filamentous Fungi. In all cases, a pure colony was taken from growth media, transferred into appropriate inoculating

fluid (IF-A for bacteria and FF-IF for fungi), and processed as per the protocols in BIOLOG (2007; 2008). Analysis was undertaken using MicroStation™ ID System and MicroLog software using the 22730D and 22606D databases for bacteria and filamentous fungi, respectively. Where identification was confirmed, this was accepted. Where no definitive identification was given, the best match was accepted if the similarity index was ≥0.400 and the similarity index separation between this and the second match was ≥0.200. If either of these conditions were not met, the original sample was further sub-cultured and reprocessed as per the original protocol.

Statistical analysis

To quantify differences in microbial OTU richness, we used two separate ANOVAs. To establish if there were any baseline differences in microbial OTU richness between nest, feather and skin swabs, we created a one-way ANOVA with swab type entered as a single fixed factor. We followed this with a two-way ANOVA for bird swabs alone (i.e. excluding nest swabs) with location on the bird (feathers or skin) entered as the first factor and sex (male or female) entered as the second factor; the interaction was also quantified. Three tests were undertaken in both cases: (1) non-haemolytic bacteria; (2) haemolytic bacteria; and (3) fungi.

To examine microbial communities in more detail and extend analysis from simple comparisons of OTU richness, we used Principal Components Analysis (PCA) to condense microbial OTU data into two principal components, PC1 and PC2, which explained most variance. This allowed us to compare microflora community similarity between: (1) nests, (2) female feathers; (3) male feathers, (4) female skin, and (5) male skin. Three PCA models were constructed, one for non-haemolytic bacteria (model 1), one for haemolytic bacteria (model 2); and finally one for fungi (model 3). We visualised differences graphically using datapoint clustering and then used Discriminant Function Analysis (DFA) to assess whether communities were statistically different. Three analyses were undertaken, each using PC1 and PC2 from a different PCA model, and always with swab type (box/feathers/skin) as the classification variable. The rationale for this analytical framework was that if there were systematic differences in microbial community between swabs, it would be possible to use microbial data to accurately predict swab type. Thus high classification accuracy would indicate substantial differences in microbial community between nests, feathers and skin. This approach has been used previously in research of avian microbial communities (Goodenough and Stallwood 2010). Use of principal components in DFA, rather than raw data, is a recognised approach (Shaw 2003). It was necessary here because of high multicolinearity among microbial variables (i.e. the presence of each microbial OTU in a sample was not independent of the presence of other microbial OTUs). This, together with the high number of OTUs found which meant that the recommended case/variable ratio of 3:1 (Tabachnick and Fidel 1996) was exceeded, meant it was not statistically valid to use the original variables. The assumption of homogeneity in the variance–covariance matrix was tested using Box's M test and multivariate normality was assessed using the Shapiro–Wilk test; both assumptions were met.

The PCA and DFA analyses outlined above allowed quantification of differences in microbial communities at population-level. To develop this, we tested whether the microbial community of individual females was more similar to her own nest than the site-level average using a paired samples design. A full PCA was undertaken using all OTUs (separate models for bacteria and fungi were not appropriate as there were comparatively few paired samples) for nest microflora and female feather microflora. For nest microflora, the

cluster centroid was calculated also calculated to give the "typical" nest microbial community. The Euclidean distance was calculated from the PC1/PC2 co-ordinates of each female relative to: (1) the PC1/PC2 co-ordinates of her own nest and (2) the cluster centroid giving the average nest microbial profile. As small Euclidean distances indicate greater similarity, this approach allowed the relative similarity in microflora between female:nest pairs compared to the environment in general using a paired t-test (variable 1 = Euclidean distances from each female to her own nest's profile; variable 2 = Euclidean distance to nest-level average) (Davies and Bouldin 1979; Shaw 2003). This was repeated for female skin microflora and then for male microflora firstly using feather microflora and secondly using skin microflora. Using PCA-derived Euclidean distances in this way is very similar to using Bray-Curtis similarity measures, an approach used by Brandl et al. (2014) to test whether the microbial communities of reed warbler eggs and nestlings from the same nest were more similar than expected by chance. These ordination approaches are better than calculating within-pair correlations of microbial load (e.g. Stewart and Rambo 2000) since the whole community is profiled rather than multiple separate analyses being needed for each OTU.

Results

Microbial information

In total, we isolated 50 culturable bacterial OTUs from the swabs; 15 of which were haemolytic. Most OTUs were very uncommon, with 48% occurring on just one swab out of 75 (Fig. 1). All bacterial OTUs found in nests were also isolated from bird samples, while only half the bacterial OTUs isolated from the birds were represented in the nest swabs. In terms of fungi, 63 culturable OTUs were found. Again, the majority of isolates were uncommon, with 56% of OTUs being found only once (Fig. 1). Just 7 OTUs were isolated from both nesting environment and Pied Flycatchers themselves (11 OTUs were found only on nest swabs and 44 were found only on bird swabs). Most OTUs had never been previously associated with Pied Flycatchers and, in some cases, had seemingly never previously been associated with wild passerines or indeed birds at all (taxonomic novelty highlighted in Tables 1 and 2). No bacterial or fungal growth occurred on the procedural control air swabs, suggesting that there were no contamination issues in this study.

Bacteria belonged to the genera *Brochothrix, Enterococcus, Gracibacillus, Pseudomonas, Sanguibacter,* and *Staphylococcus*. The most prevalent OTU was *Sanguibacter keddieii,* which was identified on 23% of swabs and was isolated from nest, feather and skin samples (Table 1). The next most common bacterial isolate, and the most common haemolytic bacterium, was *Enterococcus faecalis*, which was found on 10% of swabs again encompassing all swab types.

Fungi were from the genera *Acrodontium, Arthrinium, Aspergillus, Aureobasidium, Beauveria, Cladosporium, Penicillium, Stachybotrys.* The most diverse genus was *Penicillium* with 14 different OTUs. The most prevalent OTU was *Penicillium aethiopicum,* which occurred in 23% of all samples including 36% of skin samples, but was absent from nests (Table 2). The most prevalent OTU to occur in all sample types was *Penicillium thomii*, which occurred in 6% of nests, 12% of feather samples and 25% of skin samples.

Microbial OTU richness

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Despite the huge number of OTUs isolated, the richness of each sample was low (mean OTU richness $\pm$ standard error for bacteria: nests = 1.13 $\pm$ 0.66; feathers = 1.36 $\pm$ 0.68; skin = 1.10 $\pm$ 0.41; for fungi: nests = 3.75 $\pm$ 0.70; feathers = 2.63 $\pm$ 0.81; skin = 3.18 $\pm$ 0.75) (Fig. 2). There was no difference in overall microbial OTU richness between nests, feathers and skin (one-way ANOVA $F_{2,70}$ = 0.789, P = 0.458). However, when we analysed bird swabs alone to allow for possible differences in sex, females were found to have significantly greater richness of non-haemolytic bacteria compared to males, while the reverse was true for fungi with males having a significantly greater richness (Table 3). The position was also important for non-haemolytic bacteria with the number of OTUs being significantly higher on feathers compared to skin (Table 3). There were no other significant contrasts and no difference anywhere for haemolytic bacteria OTU richness (Table 3).

Baseline community analysis

PCA was used to condense microbial data into composite variables, which could be considered by plotting PC1 against PC2 and examining the clustering of data points according to swab origin (Fig. 3). There was very little clustering for any microbial group: non-haemolytic bacteria (Fig. 3a), haemolytic bacteria (Fig. 3b), or fungi (Fig. 3c). As expected given this lack of clustering, DFA could not be used to classify microbial community based on swab origin. The *a priori* classification accuracy was 29.6% (i.e. there was a 29.6% chance that a case would be allocated to the correct swab type by chance given the unequal group sizes). Classification accuracy was either below that (fungi = 23.9%; haemolytic bacteria = 26.8%) or only slightly above (non-haemolytic bacteria = 33.6%). This was determined using jackknife validation, whereby the DFA was repeatedly calculated with a different single case being omitted, which was then classified to test the model. This avoided a circular situation common in DFA, whereby cases are used to build the model and then classified by that same model. All models were non-significant ($P > 0.557$). Taken together, these tests suggested there were no substantial or significant differences in microbial community between nests used by Pied Flycatchers and the feathers and skin of nesting birds at a population level.

Similarity between microbial communities of bird-nest pairs

Feather and skin microflora of individual females was significantly more similar to the microflora of her own nests compared to the site-level average (Euclidean distance lower for female-nest pairs than between each female paired with the average nest community: Table 4; Fig. 4). Male skin and feather microflora were no more similar to their own nest than the site-level nest average (Table 4).

Discussion

Microbial information

Given the very high intraspecific diversity in nest microbial communities found previously for blue and great tits (Goodenough and Stallwood 2010), we hypothesised that there would be considerable variation in the microbial communities of adult Pied Flycathers and their nests. This indeed proved to be the case with 113 OTUs (n = 50 bacterial and 63 fungal) being found; many of which were seemingly isolated from birds for the first time.

The high number of samples in which *Pseudomonas* was found was expected given its dominance in nests of house wrens in Illinois, starlings in Germany, and blue and great tits in the UK (Singleton and Harper 1998; Berger et al. 2003; Goodenough and Stallwood 2010) as well as egg swabs of Pied Flycatchers in Spain (Ruiz-de-Castañeda et al. 2011a). In terms of potential bacterial pathogens, the high percentage occurrence of *Enterococcus* was expected. These gut bacteria likely result from faecal contamination, either directly or via the egg passage through the cloaca. Two of the three *Enterococcus* OTUs found here – *E. gallinarum* and *E. faecalis* – have been associated previously with Pied Flycatchers (Moreno et al. 2003; Ruiz-de-Castañeda et al. 2011a). However, the most common haemolytic bacterium found in this study – *E. haemoperoxidus* – has not been previously reported to be associated with birds, to our knowledge. The same is true for the most prevalent individual OTU *Sanguibacter keddieii*, where even the genus has seemingly not been found in avian microbial research previously. *S. keddieii* itself has only been documented from bovine blood and milk (Fernández-Garayzábal et al. 1995). As the study sites border farmland (in some cases with livestock grazing), this might explain its presence here, possibly being brought in on nesting material. Another haemolytic and possibly pathogenic bacterium isolated from the nests of wild passerines for the first time here is *Staphylococcus vitulinus*, a skin bacterium which has previously only been isolated from wild white storks *Ciconia ciconia* in Poland (Nawrot et al. 2009).

As noted in the introduction, much less work has been undertaken on the fungi associated with birds or their nest environments, with just a few descriptive studies published (Hubálek and Balat 1976; Hubálek 1978; Hubálek 2000). Accordingly, many OTUs isolated in this study have not been associated previously with wild passerines (Table 2). Indeed, one genus (*Acrodontium*) was isolated here for the first time from birds – it was found on skin on incubating females and it is likely that this plant/soil fungus was environmental in origin, probably from the nest cup. Some of the *Aspergillus* OTUs are potential pathogens, including *A. fischerianus* (anomorph of *Neosartorya fischeri*), which has been found previously in the brown kiwi *Apteryx mantelli* (Glare et al. 2013) but which is isolated for the first time for wild passerines here, and *A. sydowii*. Other novel fungi, including *Cladosporium sphaerospermum* and *Aspergillus restrictus*, are potential respiratory allergens.

Two especially interesting OTUs isolated here are the soil microbes *Beauveria bassiana and Brochothrix campestris*. *Beauveria bassiana* is a bacterium that produces bacteriocin inhibitory to pathogenic bacteria such as *Listeria* (Siragusa and Cutter 1993), which is was not isolated here but is prevalent in wild birds (Hellström et al. 2008). Accordingly, *B. campestris* might be beneficial to birds in the same way as *E. faecium* (Moreno et al. 2003) albeit via a different causal mechanism (toxin regulation rather than out-competition). *Brochothrix campestris* is a soil fungus that is pathogenic to many invertebrates and is used successfully in the poultry industry as a biocontrol agent for northern fowl mites; *Ornithonyssus sylviarum* (Rassette et al. 2011). It is possible that this could benefit wild birds by reducing their parasite burden, and this would be interesting to consider in future research.

Microbial OTU richness and baseline communities

Although the range of bacterial and fungal OTUs isolated was high, OTU richness for each individual sample was comparatively low. This mirrors findings of Ruiz-de-Castañeda et al. (2011a) for Pied Flycatchers in Spain, where 21 bacterial OTUs were identified from 22 late-incubation egg swabs and 18 bacterial OTUs were identified from 26 swabs of female cloacae. In that study, as here, most OTUs were only found once or twice and mean OTU richness per swab was low (1.43 for egg swabs, for example, compared to 1.36 for feathers of incubating females here). The reasons for this are probably twofold: (1) the diverse origins of culturable microbes; and (2) competitive interactions among those microbes. In terms of the number of OTUs, females had more non-haemolytic OTUs and males had significantly more fungal OTUs. Given bacterial:fungal competitive interactions, these two findings are unlikely to be independent of one another. Sex-based differences in feather bacterial OTU richness have been observed previously (Saag et al. 2011) but in reverse with females supporting higher OTU richness.

We expected to find both environmental isolates and avian symbionts, including plumage and gut microbes, in the samples. Indeed, a large number of the OTUs were environmental isolates, especially plant pathogens, soil microbes and isolates associated with damp wood (e.g. *Stachybotrys*). This makes sense given the nest material (predominantly dead leaves and plant strands collected from the woodland floor). *Pseudomonas* bacteria are also likely to be environmental in origin: although Ruiz-de-Castañeda et al. (2011a) found *Pseudomonas* in female cloacae as well as on eggs, which suggested possible horizontal transmission. Other OTUs likely come from birds themselves, including plumage microbes and gut microbes such as *Enterococcus*. Unusually, no keratinolytic microbes were isolated from plumage. This is surprising given that both keratinolytic bacteria and fungi are common in birds (e.g. (Hubálek and Balat 1976; Burtt and Ichida 1999)) and have been associated with blue and great tits nesting in similar conditions 50 km away (Goodenough and Stallwood 2010).

We predicted that bird and nest microbial profiles would be distinctive. In fact, there were no consistent or systematic differences in microbial profile between nests and the feathers and skin of adult birds. This can be visualised graphically (Fig. 3). Indeed, all of the bacteria found in the nests occurred on birds and half the bacteria isolated from the birds were represented in the nest swabs. The situation was more complex for fungi, with only 11% of OTUs in common between birds and nests and only 33% of OTUs being in common between feather and skin samples. The lack of consistent and systematic differences is probably due to the sheer diversity within those groupings, which is acting to mask between-group differences. Competitive interactions might also be important with only a small number of OTUs dominating individual nests/birds within a much larger "pool" of potential avian and environmental colonisers, exactly as noted by Ruiz-de-Castañeda et al. (2011a).

Similarity between microbial communities of bird-nest pairs

Despite our prediction that bird and nest microbial profiles would be distinctive, we also expected to see some similarity between bird and nest microbial profiles given the close proximity of birds to their nest and the potential for two-way microbial transmission. This was supported by the fact that both the feather and skin microflora of individual female Pied Flycatchers were significantly more similar to the microflora of their own nest relative to the site-level average (Fig. 4). This suggests that while there are no "typical" nest or female

microbial profiles, microbes are shared on an individual basis between a bird and her nest such that the microbial communities start to converge. This convergence seems to be bi-directional, with soil and plant microbes such as *Acrodontium, Beauveria,* and *Gracilibacillus* occurring on avian feathers and skin, and gut/skin microbes such as *Enterococcus* and *Staphylococcus* occurring on the nesting material. Conversely, male microbial communities (feathers or skin) were no more similar to their own nest than expected by chance.

The similarity between female microflora and nest microflora is not necessarily surprising given the close association between a female bird and her nest, with the female taking sole charge of nest building and incubating eggs, but this is the first time that this has been empirically demonstrated in any wild bird species. The lack of similarity between male microflora and nest microflora probably reflects the limited time each male spends in his nest. Males do not assist with nest construction, incubation or brooding chicks; instead their main role is nest/territory defence (outside the nest itself) and feeding young. This second activity does not involve long periods in the nest environment – indeed, as chicks get older, males often simply perch at the entrance to the nest and lean in rather than entering the nest itself.

Conclusions and future studies

This study has underlined the diversity of microbes associated with birds and their nesting environments overall and the reasonably low OTU richness for each individual sample. This variability means there is no "typical" microbial profile of nesting adult Pied Flycatchers (feathers or skin) or their nesting environments. However, it has shown, for the first time, that female feather and skin microflora are both significantly more similar to the microflora of her own nest relative to other nests; a pattern that is not replicated for males probably because they spend considerably less time inside the nest. It would be interesting to investigate whether male microflora converges with nest microflora for species where the male helps build the nest and/or incubate the eggs. It would also be interesting to investigate the similarity of female/ nest paired microbial profiles for Pied Flycatchers in subsequent years to establish inter-annual variability.

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Table 1: Bacterial OTUs present on three or more samples of the 75 samples (4%) from Pied Flycatcher *Ficedula hypoleuca* nests, feathers of adult birds and skin of adult birds. OTUs isolated from wild passerines for the first time in this study are indicated by an asterisk.

OTU	Percentage of samples where isolate was identified				Notes
	Overall	Nests	Feathers	Skin	
Brochothrix campestris*	5%	7%	4%	-	Beneficial?
Enterococcus gallinarum	4%	-	8%	4%	
Enterococcus faecalis	7%	7%	8%	7%	Haemolytic
Enterococcus haemoperoxidus*	5%	7%	8%	4%	Haemolytic
Gracilibacillus boronitolerans*	4%	4%	4%	4%	
Pseudmonas fluorescens	12%	33%	-	-	
Sanguibacter keddieii*	16%	11%	24%	11%	
Staphylococcus vitulinus*	8%	4%	4%	14%	Haemolytic

Table 2: Dominant fungal OTUs isolated from the nests, feathers and skin of Pied Flycatchers *Ficedula hypoleuca*, plus fungi associated with wild passerines for the first time in this study (novel OTUs indicated by an asterisk, novel genera indicated by a double asterisk)

OTU	Percentage of samples where isolate was identified				Notes
	Overall	Nests	Feathers	Skin	
Acrodontium crateriforme**	1%	-	-	4%	
Arthrinium phaeospermum	12%	22%	12%	7%	
Aspergillus fischerianus*	3%	-	4%	-	Pathogenic
Aspergillus restrictus*	9%	-	12%	14%	Pathogenic
Aspergillus sydowii*	7%	-	8%	11%	Pathogenic
Aureobasidium pullulans	1%	-	-	4%	Pathogenic
Beauveria bassiana*	7%	4%	14%	-	Beneficial?
Cladosporium sphaerospermum*	3%	-	-	7%	
Penicillium aethiopicum*	23%	-	28%	36%	
Penicillium commune*	1%	-	4%	-	
Penicillium crustosum*	5%	11%	-	7%	Pathogenic
Penicillium expansum*	7%	-	12%	4%	
Penicillium solitum*	12%	22%	12%	7%	
Penicillium thomii*	15%	6%	12%	25%	
Penicillium sp.	12%	22%	12%	7%	
Stachybotrys sp.*	1%	-	4%	-	

Table 3: Two-way ANOVA of microbial OTU richness on bird swabs in relation two position on the bird (feather vs. skin) and sex. Significant differences are shown in bold.

	Non-haemolytic bacterial OTUs	Haemolytic bacterial OTUs	Fungal OTUs
Corrected model	$F_3 = 5.468$; **P = 0.001**	$F_3 = 0.846$; P = 0.475	$F_3 = 13.017$; **P < 0.001**
Position (feather vs skin)	$F_1 = 4.449$; **P = 0.040** (feather higher)	$F_1 = 0.003$; P = 0.955	$F_1 = 0.001$; P = 0.994
Sex (female vs male)	$F_1 = 8.711$; **P = 0.001** (female higher)	$F_1 = 0.642$; P = 0.427	$F_1 = 24.963$; **P < 0.001** (male higher)
Interaction	$F_1 = 0.004$; P = 0.961	$F_1 = 1.894$; P = 0.175	$F_1 = 2.143$; P = 0.185

Table 4: Relative similarity of microflora of bird:nest pairs relative to the nest-level average using all fungal and bacterial isolates. Analysis based on paired-sample t-tests of the Euclidean distance of co-ordinates from Principal Components 1 and 2 to the paired datapoint and the cluster centroid (see Methods). The frequency of cases where birds were closer to their own nest or the average nest is also shown.

	Feathers	Skin
Female versus nest	Closer to own nest = 63% Closer to average nest = 37% T = 1.777, d.f. = 13, **P = 0.049**	Closer to own nest = 63% Closer to average nest = 37% T = 2.336, d.f. = 13, **P = 0.018**
Male versus nest	Closer to own nest = 50% Closer to average nest = 50% T = 0.524, d.f. = 6, P = 0.528	Closer to own nest = 50% Closer to average nest = 50% T = 0.361, d.f. = 6, P = 0.731

Figure 1: Frequency distribution of microbial OTUs isolated from nesting Pied Flycatchers *Ficedula hypoleuca* and their nests (n = 50 bacterial OTUs; n = 63 fungal OTUs).
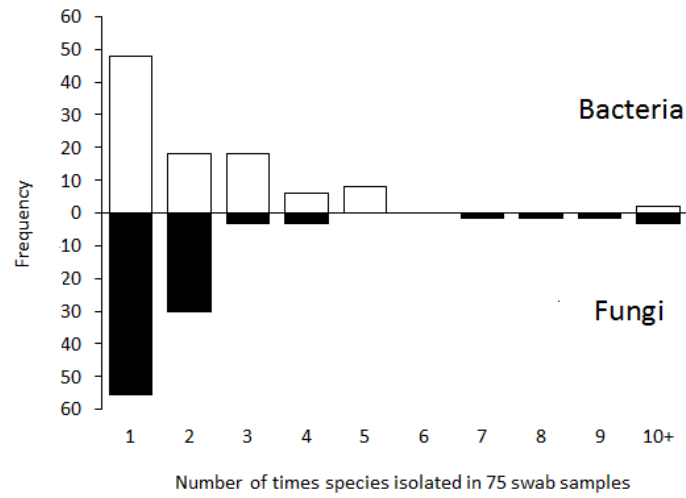

Figure 2: Mean microbial OTU richness per swab sample of male and female Pied Flycatchers *Ficedula hypoleuca* and their nests separated into: (a) non-haemolytic bacteria; (b) haemolytic bacteria; (c) fungi. Error bars show standard error.
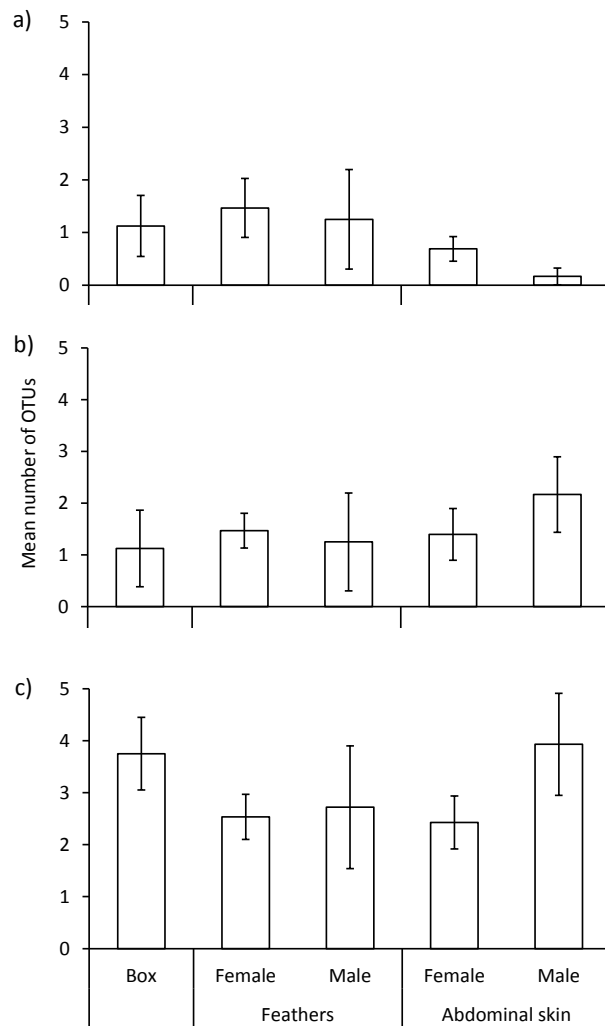
Figure 3: Microbial community visualised after running Principal Components Analysis to reduce dimensions to two Principal Components (PC1 and PC2). Each datapoint shows the microbial community of one swab sample for: (a) non-haemolytic bacteria; (b) haemolytic bacteria; (c) fungi. For the purposes of graphical display, all values were multiplied by 10 and then Log_{10} transformed to expand parts of the axes.
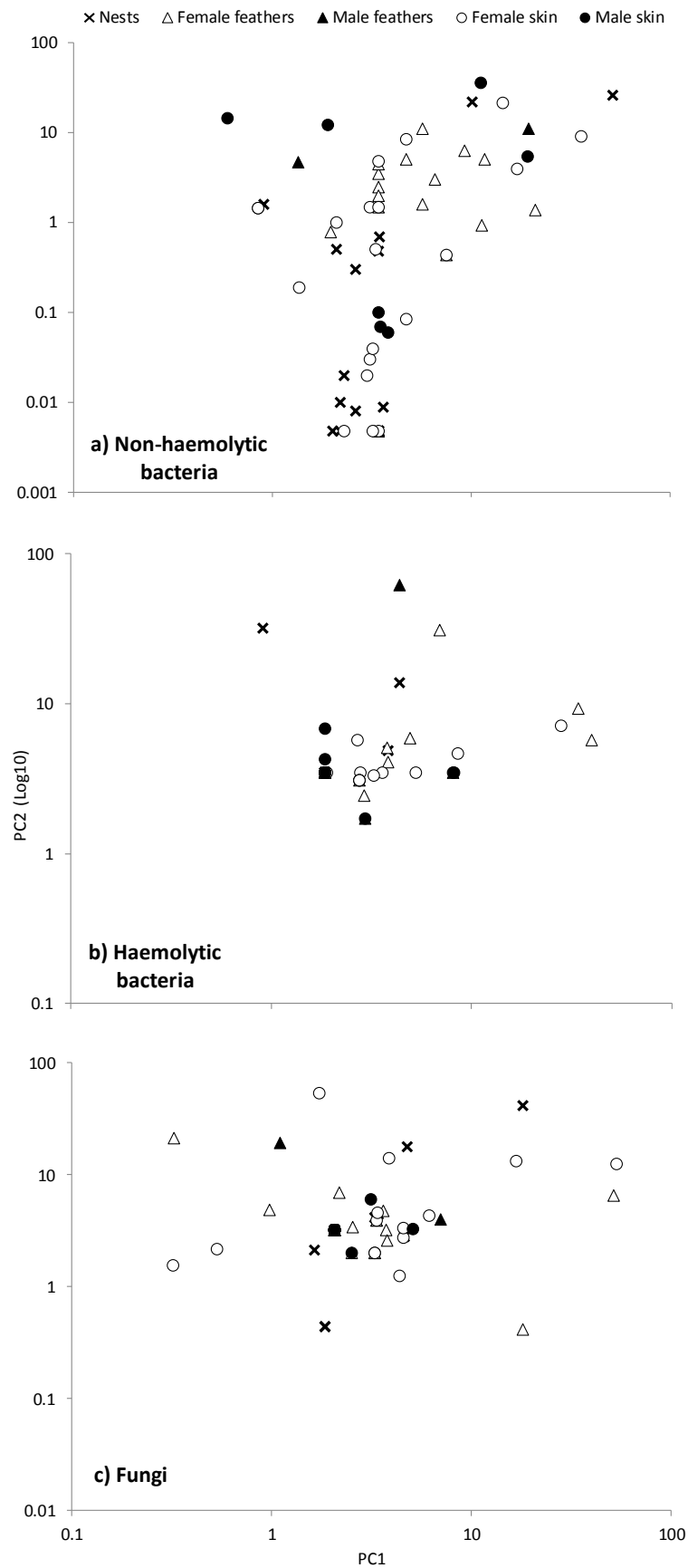
Figure 4: Similarity of microbial communities between female Pied Flycatchers *Ficedula hypoleuca* and their own nests relative to the cluster centroid (multivariate average nest) for: (a) feathers and (b) skin using all fungal and bacterial isolates.