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Goodenough, Anne E ORCID: 0000-0002-7662-6670 and Stallwood, Bethan (2011) Differences in Culturable Microbial Communities in Bird Nestboxes According to Orientation and Influences on Offspring Quality in Great Tits (Parus major). Microbial Ecology, 63 (4). pp. 986-995. doi:10.1007/s00248-011-9992-7

Official URL: http://dx.doi.org/10.1007/s00248-011-9992-7
DOI: http://dx.doi.org/10.1007/s00248-011-9992-7
EPrint URI: https://eprints.glos.ac.uk/id/eprint/3336

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Published in Microbial Ecology, and available online at:

http://link.springer.com/article/10.1007%2Fs00248-011-9992-7

We recommend you cite the published (post-print) version.

The URL for the published version is http://dx.doi.org/10.1007/s00248-011-9992-7

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Differences in culturable microbial communities in bird nestboxes according to orientation and influences on offspring quality in great tits (*Parus major*)

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Suggested Running Title: Bird nestbox microbes and offspring quality

28 Abstract

Although bird-microbial interactions have become a topic of increasing research, the influence of nest-site characteristics, such as cavity orientation, on nest microbial communities in free-living passerines has not, to our knowledge, been investigated. This is despite the possibility of microbial

32 differences explaining non-random patterns in nest-site selection and offspring quality, such as those exhibited by great tits (*Parus major*). We swabbed great tit nestboxes that faced either south-southwest (180-269°) or north-northeast (0-89°). Overall, 28 bacterial species and 11 fungal species were isolated, but the culturable microbial community differed substantially between

36 different orientations - indeed nestboxes could be classified to their orientation group with high accuracy using microbial data. Nestboxes facing south-southwest had a significantly higher fungal load (typically double) than those facing north-northeast due to a higher abundance of two species, *Epicoccum purpurascens* and *Cladosporium cladosporioides*. There was no relationship between

40 total bacterial load and orientation, although the abundance of one species, *Pseudomonas veronii*, was significantly lower in south-southwest boxes. The abundance of the allergen *E. purpurascens* explained almost 20% of the variation in offspring quality, being significantly and inversely related to chick size (high loads associated with small, poor quality, chicks). Our results provide

44 empirical evidence for a correlation between nestbox orientation and culturable microbial load, and a further correlation between abundance of one species, *E. purpurascens*, and offspring quality. Thus, microbial load, which is itself influenced by nest cavity parameters, could be the proximate factor that influences nest-site choice through its effect on offspring quality (and thus overall fecundity).

48

Keywords

Avian breeding biology; Bird-microbe interactions; Chick growth; *Epicoccum purpurascens;*

52 Nest-site selection

The interactions between birds and micro-organisms is a topic of increasing study [36]. Recent research has shown that birds have distinctive plumage bacteria that can influence plumage colour and quality [10, 11, 59] and gut bacteria that vary according to diet both within and between
56 species [6, 18]. The importance, however, of microbial species on avian reproductive success, offspring condition, and life-history traits is still poorly understood [10, 35, 36]. In particular, little research has been conducted on the microbial species associated with the nesting environment of free-living passerines [3, 42]. This is despite preliminary studies demonstrating diverse microbial
60 communities, which are highly host specific [19, 20].

The influence of nesting environment on the cloacal bacteria of nestlings has been demonstrated using a partial cross-fostering experiment [36]. However, the factors responsible for the
64 differences in microbial assemblage have not been explored. Moreover, although microbial species, particularly pathogens such as *Enterobacter cloacae* and *Staphylococcus hyicus*, can have a significant influence on avian offspring survival at embryonic and nestling stages [13, 46, 52], something that may be partially countered by use of aromatic plant material [41], little is known
68 about the effects of microbes of offspring quality at fledging [11, 42]. This is despite: (1) offspring quality often being affected by nesting environment; (2) condition at fledging being a fundamental influence on offspring survival and fecundity [26, 34, 43, 45, 50]; (3) bird-associated bacteria occurring at increased loads in environments with little direct sunlight, such as nest cavities [55];
72 and (4) plumage bacterial load being known to correlate with condition of adult birds during nesting [25, 54]. If offspring quality is related to the presence or abundance of certain microbial species in a nesting environment this could act as a selection pressure on nest-site choice, especially if microbial differences are consistently associated with abiotic differences in nest-site characteristics.
76

Previous research has found that the frequency of nestbox occupation by great tits (*Parus major*) correlates with orientation, with nestboxes facing south-southwest being used less frequently than boxes facing other directions [21]. Further analysis revealed that great tit chicks from south-southwest
80 oriented boxes were of significantly lower quality than chicks from boxes facing other directions [22]. This suggests that avoidance of boxes associated with low chick quality is due to the responsiveness of parent birds to offspring-condition selection pressures that relate to orientation. However, it is not clear what mechanism(s) are responsible for reduced offspring quality in nestboxes facing south-
84 southwest, and thus what drives parental responsiveness in nest-site selection.

We hypothesise that the microbial load in nestboxes facing south-southwest might be higher than that in boxes facing other directions, possibly because of differences in the nestbox microclimate. Nestboxes used by great tits are known to be microbially-diverse and both bacterial and fungal loads

88 are extremely high [20]. A systematic difference in microbial load (generally, or for specific species) according to orientation would constitute an effect of nest-site characteristics on nest microbial abundance and might also explain why nestboxes facing south-southwest are associated with lower offspring quality in great tits. In this study we aim to: (1) identify and quantify culturable

92 bacteria and fungi from nestboxes occupied by great tits (*Parus major*); (2) establish any differences in microbial species diversity or abundance between boxes according to orientation using species-level (univariate) and community-level (multivariate) analyses; and (3) determine whether any nestbox-occurring bacterial or fungal species influence avian offspring quality as determined by

96 an widely-used proxy of chick growth and condition (wing length) immediately prior to fledging. Our results provide empirical evidence for a correlation between nestbox orientation and microbial load and a further correlation between microbial load and offspring condition, which suggests that microbial species can become nest-site selection pressures.

100 Materials and Methods

Study site

This study was undertaken at Nagshead Nature Reserve (Gloucestershire, UK), which covers 308 ha centred on 2°34’0”W, 51°47’0”N. The reserve has a nationally-important diversity of breeding birds,
104 with many birds nesting in one of the site’s 400 wooden nestboxes, which are equally-sized (internal dimensions: 110mm width, 170mm depth, 210mm mid-point height) and spaced at about 30m intervals. The nestboxes are routinely monitored by the Royal Society for the Protection of Birds, who manage the site. Previous research demonstrated that nestboxes facing south-southwest at this
108 study site are warmer by an average 1°C than boxes facing other directions: A. Goodenough, unpublished data. These differences are relatively small and the temperatures of all nestboxes are below those that are likely to cause thermal stress to nestlings (such that they are unlikely to be directly responsible for non-directionally uniform patterns of nestbox selection and success), but
112 small differences could modify microbial communities and thus have an indirect influence.

Study design

In order to avoid known or potential variables confounding analyses, a paired research design was
116 used [as per 20]. All the *P. major* nests in nestboxes facing south-southwest (180-269°) in 2006 that contained two or more live young at day 15 post-hatching (n = 10) were studied. Each nest was paired with a *P. major* nest in a nestbox facing, as nearly as possible, the diametrically opposite direction (i.e. a nest facing 200° was paired with one facing as near 20° as possible). This
120 gave 20 nests in 10 pairs. Given that bacterial loading of nests has previously been found to correlate with timing in the breeding season with late nests having a higher abundance of bacteria [3], and could potentially correlate with chick density, nests were paired according to the number of chicks (at day 15 post-hatching) and hatching date (± one day) to ensure that neither of these
124 factors confounded analysis. Nests were also paired according to woodland type (sheep grazed or ungrazed). All boxes were located within a relatively shaded woodland environment.

Avian offspring quality

128 Biometrics of chicks (N = 93) from each nest (N = 20) were taken 15 days after hatching under licence from English Nature (Licence Number: 20060590; Licensee: AEG). This was as near to fledging as possible while mitigating the risk of disturbance-induced premature fledging. Size and condition were quantified by measuring right wing length (the distance between the carpal joint
132 and the tip of the longest primary wing feather) using a stopped ruler to the nearest 1 mm [23].

This is a highly replicable measurement and the single best correlate of relative body mass [24]; it has also been used previously in great tit fledgling-survival research [17].

136 Swabbing procedure

Immediately post-fledging, nests were removed from nestboxes under licence and each box was swabbed using a sterile rayon-tipped swab pre-moistened with phosphate buffer at pH 7.1 ± 0.1 (Steriswab™, Medical Wire and Equipment Company, UK). As the swab effectively became the
140 unit of study, the swabbing procedure was carefully standardised as to time and area swabbed: boxes were swabbed for 30 seconds during which time 3.5m of nestbox was swabbed in a standardised order (base followed by sides from the base to the top). As an experimental control, two unoccupied nestboxes were swabbed as per the above method. As a procedural control, two air
144 swabs (exposed to the air for the same time as the swabbing procedure) were taken [35]. Swabs were kept on ice in the field and thereafter at 4°C for a maximum of 2 days before processing [42, 62].

Culturing microbes

148 In the laboratory, swabs were thoroughly washed in 10ml of sterile 1.3% (w/v) nutrient broth (Oxoid, Cambridge, UK), which was subsequently regarded as the undiluted (10^0) concentration. Decimal serial dilutions were undertaken down to 10^{-9}. To establish the two most appropriate concentrations for each individual sample for subsequent culturing, three 10µl drops of each
152 dilution were cultured on 2.8% (w/v) nutrient agar at pH 7.4(Oxoid CM0003) and incubated for 48 hours incubation at 28°C, during which time the serial dilutions themselves were stored in the fridge at 4°C. Plates were inspected to determine the two most suitable dilution factors (that with around 30 colonies per drop and the dilution immediately below this: typically 10^{-8} and 10^{-9} for
156 nestbox swabs and 10^0-10^{-2} for the control swabs) for each sample. Of the two most suitable dilution factors for each sample, 100µl was then cultured on separate plates containing nutrient agar to encourage bacterial growth and 3.9% (w/v) potato dextrose agar (PDA) at pH 5.6 (Oxoid CM0139) to encourage fungal growth. Use of comparatively high dilution factors reduced bias
160 towards fast-growing species by reducing inter-isolate competition [20]. Plates were incubated for 7 days at 28°C before Colony Forming Units (CFUs) were counted, to avoid bias towards fast-growing species. The number of CFUs of each bacterial species on the original swab was calculated by averaging the count of the two nutrient agar plates (after correcting for different serial dilutions)
164 and counting back to give number per 100µl of H^0. This was then multiplied by 100 to give the number per 10ml of H^0 and thus per nestbox swab. This process was repeated for fungal species using the two PDA plates.

Identification of isolates

Identification of fungi was undertaken taxonomically using standard keys [14, 33, 61] using colony morphology, colour and production of defuse pigment, together with hyphae and conidia arrangement on stained heat-fixed slides at 400x magnification. For some slow-growing isolates, identification was not possible after 7 days incubation, and in these cases identification was undertaken following a further incubation period or 14 to 28 days. Identification of two isolates was verified by a specialist mycologist at CABI Bioscience (Nomica, Egham, Surrey, UK). In both cases, the specialist identifications, which were undertaken on a blind basis, matched the initial identification made by the authors.

Bacteria were identified using fatty acid methyl ester (FAME) analysis using gas-liquid chromatography to profile the type and concentrations of fatty acids in each isolate and compare them to over 200,000 profiles of known species [32]. This was undertaken through the Sherlock® Microbial Identification System (MIDI Inc., Newark, Delaware, version 4.5) using the Sherlock® Rapid Methods technique and the RTSB50 (environmental isolates) reference library. The use of the FAME and Sherlock® systems to identify isolates has been widely used and is an accurate and validated identification method [31, 47, 48, 65]. Cells from each isolate were harvested from 24 hr old sub-culture on 4% (w/v) Trypticase Soy Broth agar at pH 7.3 (Oxoid CM0131) following incubation at 28°C to ensure a typical FAME profile [47] and the fatty acids were extracted by saponification using a sodium hydroxide/methanol solution. Where similarity indices (SI) of ≥0.500 was given between an isolate and reference (an excellent species-level match: [67]), FAME identification was accepted. When FAME profiles indicated a close match to two (or more) species, the closest match was accepted provided the separation between this and the next closest match was ≥0.200 (double the minimum recommended separation: [32]). For the five isolates that did not have an SI ≥0.500, confirmation of the FAME identification was provided by DNA sequencing. DNA was extracted using a commercial kit (DNeasy® kit, Qiagen, Sussex, UK) and the 16S rRNA gene was amplified using polymerase chain reaction (PCR) in a 50-µl reaction containing 25 µl Taq Master Mix (Qiagen), 21 µl deionized water, and 2 µl each of two oligonucleotide primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 530r (5′-ATTACCGCGGCTGCTGGC-3′); MWG Operon, Cologne, Germany). The PCR conditions comprised initial denaturing for 15 min at 95°C, followed by 30 cycles of 1 min denature at 95°C, 1 min anneal at 56°C, and 1.5 min extension at 72°C [66]. This was followed by a final extension step of 10 min at 72°C. The amplified 16S rRNA gene was separated from total DNA using

200 submerged horizontal gel electrophoresis using an 0.7% (w/v) agarose gel pre-stained with ethidium bromide (Fisher Scientific, Loughborough, U.K) at 100 V for 45 min with a TBE running buffer (Eppendorf, Cambridge, UK). The PCR bands were cut from the gel and purified using a commercial gel extraction kit (QIAquick® Gel Extraction Kit: Qiagen) and 100%
204 isopropanol according to the manufacturer's protocols. Identifications of fluorescent *Pseudomonas* spp. by FAME were verified by growth at high (42°) and low (3°C) temperatures and gelatin hydrolysis reactions [7, 27, 67] as atypical strains can occasionally be confused [47, 68]. This represents a polyphasic approach to identification combining phenotypic (FAME), genotypic
208 (DNA), and biochemical techniques [32, 64].

Statistical analyses

Statistical analyses used microbial abundance data (number of CFUs for each microbial species)
212 in all cases. Analysis was undertaken using SPSS for windows version 16.1.

To quantify any differences in the microbial loading of the nestboxes according to orientation, total bacterial and fungal loads were compared between nestbox pairs using paired sample *t*-tests
216 after count data had been log (*ln*+1) transformed to normalise them. Paired t-tests were also used to establish whether differences in the abundance of individual species between boxes of different orientations, observed graphically, were significant. Tests were not undertaken without *a priori* reason to avoid pseudo-significance [16].
220

To consider differences in the overall culturable microbial community on the basis of nestbox orientation, multivariate approaches were used. Principal Components Analysis (PCA) was undertaken to condense the extensive microbial data into two principal components, PC1 and PC2,
224 which explained most variance. Three PCA models were constructed using bacteria (model 1), fungi (model 2), and, finally, all microbial isolates (model 3). Verimax orthogonal rotation was undertaken in all cases. Scatter plots were then constructed using PC1 and PC2 of each model, with data points coded according to the nestbox orientation group (north-northeast or south-
228 southwest). These plots were examined to ascertain whether there were notable visual differences in microbial communities, according to orientation, based on the clustering of data points. Then, to establish objectively whether microbial community could be used as an accurate predictor of nestbox orientation, Discriminant Function Analysis (DFA) was undertaken. Three analyses were
232 undertaken, each using PC1 and PC2 of one of the PCA analyses (such that there was one analysis

for the bacterial community, one for fungal community and one for complete microbial community). In all cases orientation category was used as the classification variable. The rationale for this process was that if there were important differences in microbial community according to
236 orientation, it would be possible to use microbial data to predict orientation group with a high degree of accuracy; in other words, DFA was used to ascertain how important an influence orientation was upon microbial community. Use of principal components rather than raw data is a recognised approach [57] and was necessary here for two reasons: (1) the minimum recommended
240 case:variable ratio of 3:1 [63] was exceeded due to the high number of microbial species (variables) and the relatively small number of nestboxes (cases); and (2) there was high multicolinearity in the microbial dataset (abundances of different microbes correlated with one another), which needed circumventing [57]. The classification accuracy, or power, of each DFA was ascertained
244 using a jackknife validation procedure that involved repeatedly calculating the DFA, each time having omitted a different single case which was then classified [57]. All DFAs were accompanied by a MANOVA to establish whether differences between groups were statistically significant [39]. The assumption of homogeneity in the variance-covariance matrix was tested using Box's
248 *M*-test, while multivariate normality was assessed using the Shapiro-Wilk test.

To examine the influence of microbial species on offspring quality asymmetry, the microbial species which occurred in ≥40% of nestboxes were regressed against chick wing length. All-subsets
252 regression was used, such that separate analyses were constructed for all qualifying variables individually, and then all possible variable combinations were analysed until the full model had been constructed. Models were compared post-hoc using AIC, which combines model fit and parsimony [1]. Given inconsistency in stepwise algorithms, this was considered superior to
256 undertaking a single stepwise analysis. Isolates that occurred in <40% of boxes (i.e. fewer than 8 samples) were never included as: (1) this contravenes the recommended sample size for regression using a specific variable; and (2) adding in too many variables could violate the case: variable ratio of 3:1, which could have invalidated statistical rigour [63]. In terms of the dependent variable,
260 wing length, it was only statistically-valid to use measurements of one chick per brood in analysis to introducing pseudoreplication by dint of having multiple samples (chicks) per experimental unit (nestbox) [29, 57] and which could not be allowed for by entering nestbox as a random factor into the analysis given that it was the nestbox itself (or, more correctly, its microbial community)
264 that was the object of study. Accordingly, a representative chick was selected from each brood (the median chick according to weight [22]) to allow analysis of the "average" chick per nest [54].

Results

Microbial species

268 In total, 28 culturable bacterial species and 11 culturable fungal species were found in the nestboxes (Table 1). The majority of these were comparatively rare, with one third of species occurring in 10% of boxes or fewer. Several potential pathogens were found (bacteria: *Aeromonas hydrophila*, *Enterobacter cloacae, Staphylococcus hyicus* and; fungi: *Aspergillus flavus*, *Candida albicans,* and
272 *Microsporum gallinae*).

Microbial loads

Overall, the bacterial and fungal loads of the nestboxes were very high (Table 2). Swabs of
276 unoccupied boxes showed a range of species including *Pseudomonas* spp. and *Cladosporium* spp. but in very low numbers (abundance = 0.0001% of a typical nest (i.e. for every one CFU isolated from an unoccupied nestbox, nearly 1 million were isolated from an occupied box)). This suggests that the recent presence of birds in the box is the major influence on microbial loads, either directly
280 (plumage and gut microbes) or indirectly (through increased internal temperatures or introduction of nesting material). The air swabs taken as procedural controls had an extremely low abundance of microorganisms (abundance = 0.000000005% of a typical nestbox (i.e. for every one CFU isolated from the air, 20 billion were isolated from a typical occupied nestbox)).

284

Relationships between microbial loading and orientation

Nestboxes facing south-southwest had, on average, double the fungal load of boxes facing north-northeast, a difference that was highly significant (paired t-test: t = 3.111, d.f. = 9, P =
288 0.013; Table 2). This difference was due to a higher abundance of two individual fungal species, *Epicoccum purpurascens* and *Cladosporium cladosporioides* (t = 2.423, d.f. = 9, P = 0.038 and t = 2.502, d.f. = 9, P = 0.034, respectively). The differences in fungal load with respect to orientation became non-significant when the values for *E. purpurascens* and *C. cladosporioides* were
292 removed from the dataset (Table 2). There was no difference in total bacterial load according to orientation (Table 2). However, the abundance of *P. veronii* was significantly lower in boxes facing south-southwest than those facing north-northeast (t = -2.561, d.f. = 9, P = 0.031).

296 Principal Components Analysis (PCA) was used to condense the species information into composite variables in order to synthesise the microbial community. Community-level differences were established visually by plotting PC1 against PC2 and examining the clustering of data points

according to orientation (Fig. 1a-c). The best clustering was provided when fungal species alone
300 were entered into the PCA – this gave two discrete clusters according to nestbox orientation (Fig. 1b). Entering both bacterial and fungal species into a PCA (Fig. 1c) produced a graph with some overlap in clustering, while the least defined clustering was provided when only bacterial species were used (Fig. 1a). Interestingly, all models contained the same one case as a statistical outlier:
304 the microbial community in one north-northeast facing nestbox (Nagshead box 104) was atypical. This box was on the edge of the ungrazed area, with a very open aspect, and might be subject to woodland edge effects [53], which are unusual at the Nagshead site. If this outlier is removed from the scatter plots visually, the clustering of datapoints in Fig. 1 (b and c) becomes more obvious.

308

Discriminant Function Analysis proved to be an extremely useful method of establishing the effect of orientation on nestbox microbial community by assigning nestboxes to the correct orientation group (south-southwest = 180-269° or north-northeast = 0-89°) objectively according
312 to microbial community. A classification accuracy of 65% was attained using bacterial and fungal community. This improved slightly on the 50% classification accuracy that could be attained *a priori* with two possible groups of equal size, but the model was not significant ($P = 0.152$). Classification accuracy decreased when classification was done solely on the basis of bacterial
316 community (accuracy = 60%; $P = 0.194$). However, when just fungal community was used, accuracy increased substantially to 85% and the model was significant ($P = 0.026$). The three cases assigned to the incorrect group had the lowest differences in group assignment probabilities (i.e. they were the most marginal cases for classification and were misclassified because they were
320 slightly unusual, not completely atypical). The most notably misclassified case was the outlier already identified above. Examination of the component loading matrix revealed that high weightings were given to *Epicoccum purpurascens* and *Cladosporium cladosporioides* in PC1 (both species that differed univariately with orientation), while high loadings were given to *C.*
324 *herbarum* and *Aspergillus flavus* in PC2, again species that showed some differences in prevalence (though not mean abundance) between boxes of different orientations (Table 1).

Relationships between microbial loading and avian offspring quality

328 Six microbial species occurred in ≥40% of nestboxes and could be usefully entered into a regression analysis: *Pseudomonas fluorescens*, *P. putida*, *P. veronii*, *Epicoccum purpurascens*, *Cladosporium cladosporioides*, and *C. herbarum* (Table 1). All-subsets regression was undertaken

to regress all combinations of these variables against offspring quality as quantified using wing

332 length; this generated 30 models that were compared post-hoc using AIC. Only one model had substantive support (delta AIC <2), and this contained the single variable describing the nestbox abundance of *E. purpurascens*. *E. purpurascens* was significantly and inversely related to offspring quality as measured by wing length at 15 days post-hatching ($r = 0.044$, $n = 20$, $P = 0.049$) such that

336 nestboxes with a high loading of *E. purpurascens* contained smaller (and thus low quality) offspring. The nestbox abundance of *E. purpurascens* explained 20% of the variation in offspring wing length. All other reduced models had noticeably higher AIC values (delta AIC >2) and were non-significant (tests not shown).

340

Unfortunately, low sample sizes precluded statistical analysis to investigate any negative effect of the pathogens on offspring quality (Table 1). However, offspring from the nests where these microbial species were present did not have atypically small wing length measurements.

344 Discussion

Microbial species

The most prevalent culturable bacteria isolated from great tit (*Parus major*) nestboxes were *Pseudomonas* spp., occurring in high numbers in almost every nest, then *Bacillus* spp. and
348 *Enterobacter* spp. These genera have been previously associated with birds or their nesting environments [3, 40, 42], and were three of the four genera of bacteria most often associated with house wren (*Troglodytes aedon*) nests [60]. In our study, the main *Pseudomonas* species were *P. fluorescens, P. putida* and *P. veronii*, which have been recorded before in nesting material of great
352 tits (*Parus major*) [20]. *P. fluorescens* has also been isolated from pharyngeal swabs of alpine accentors (*Prunella collaris*) [30] and from the faeces of 9% of wild birds caught in mist nets in Wisconsin, USA, apparently without detrimental effect on survival [8]. *Bacillus*, including *B. subtilis* isolated here, is a known keratinolytic bacterium found on bird plumage [11], while *Enterobacteriaceae*
356 species were the most common isolates from barn swallow (*Hirundo rustica*) nests [49].

The fungi associated with wild birds and their nesting environments has not been well researched [12, 28]. Microbial analysis has usually focused on bacterial species [e.g. 3, 60], and even in studies
360 that have considered fungi, isolates have typically been regarded as ‘fungi’ and not identified to family or genus [35, 42, 62]. A baseline study has demonstrated that the fungal communities of passerine nests can be diverse and can contain pathogens known to cause infection in young birds [see 20]. Potential pathogens were also found in this study, including *Aspergillus flavus,* which
364 can cause avian aspergillosis and aflatoxicosis [69], *Microsporum gallinae,* which can cause favus (ringworm) [15] and *Candida albicans*, a causative agent of candidiasis [46].

It is important to note that this study identified microbial species through culture-based methods
368 and thus the microbes, particularly the bacteria, discussed here will only be a subset of those present. Given how few studies have been undertaken, the ratio of culturable to non-culturable microbes in avian nest material is unknown, however, the two approaches have previously revealed different microbial communities on bird feathers [58]. Accordingly, the current study
372 should not be taken as a full analysis of the influence of orientation on nestbox microbial community, but as a profile of culturable species. It should also be noted that of the culturable species present, only those that could initiate and sustain growth on the generalist media used here (see “Methods”) would be recorded, excluding, for example obligate anaerobes. Although these
376 limitations and potential biases are important to note, in this paper, we are comparing relative microbial loads, and any such bias should affect all nests equally.

Relationships between microbial loading and orientation

Although the presence of specific microbial species and their relative abundance in any one
380 nestbox is highly variable, nestbox orientation is an important determinant of the complete
microbial community, particularly the fungal community, of the nesting environment (Fig. 1a-c).
This demonstrates that nest-site characteristics (orientation) can influence the microbial
assemblage of the nesting environment, in a similar way to habitat influencing cloacal and plumage
384 bacterial assemblages in adult birds [11, 36], or feather number in a nest influencing the
microbiology of unhatched bird eggs [49]. The influence of other nest-site characteristics (for
example, height of nest cavity above the ground, proximity to water etc.) on the microbial
communities of nest sites would be useful avenues for further investigation, as would the amount
388 of sunlight received (a key influence on internal temperature) and nestbox internal humidity.

In addition to the general pattern of microbial community and orientation, orientation was significantly
associated with the abundance of three specific microbial species. *Epicoccum purpurascens* and
392 *Cladosporium cladosporioides* were more abundant in boxes facing south-southwest than north-
northeast, while the reverse was true for *Pseudomonas veronii*. The higher abundance of *E.
purpurascens* and *C. cladosporioides* in south-southwest oriented boxes is likely the result of a
warmer, and possibly moister, nestbox microclimate [13] as such boxes experience higher temperatures
396 during the hottest part of the day than boxes facing other directions and are oriented towards the
prevailing wind and rain (A. Goodenough, unpublished data). Different environmental conditions
might also be the reason why there is a difference in abundance of the bacterium *P. veronii* (lower
abundance in nestboxes facing south-southwest compared with those facing north-northeast) if this
400 species is better able to grow as slightly lower temperatures (and possibly in slightly drier
conditions). An alternative explanation is that lower abundance in south-southwest boxes could be a
direct result of the higher abundance of *C. cladosporioides* and *E. purpurascens* in south-southwest
oriented boxes, as both species, particularly *E. purpurascens*, have antibacterial properties [2, 37].
404 This hypothesis is given some support by a borderline-significant negative relationship between *P.
veronii* and *E. purpurascens* in this dataset (Spearman correlation $r_s = -0.435$, d.f. = 18, $P = 0.055$).

Relationships between microbial loading and avian offspring quality

408 Our findings agree with previous studies [e.g. 3] in that we found no relationship between total
culturable bacterial load and offspring quality. However, we did find a significant association
between abundance of the fungus *E. purpurascens* and offspring quality as measured by wing

length at day 15 post-hatching. Indeed the abundance *E. purpurascens* in the nesting environment
412 explains 20% of variance in offspring wing length. This is apparently the first time that the
abundance of a fungal species in the nesting environment has been empirically associated with
offspring quality. *Epicoccum purpurascens* is an important allergen, increasing specific IgE values
and causing histamine release [4, 5]. We hypothesise that this allergic attribute of *E. purpurascens*
416 might be the reason why high abundance is associated with low offspring quality. The other
possibility worthy of future research is that the higher abundance in south-southwest facing
nestboxes is reducing offspring quality indirectly, for example by inhibiting a bacterium
beneficial to chick growth [44].

420

As offspring quality can be influenced by many factors, not least genotype but also parental
foraging ability, availability of food, weather, and parasite burden, establishing that the abundance
of a single fungus explains a considerable amount of the variance in offspring quality is of
424 supreme importance. This is particularly true given that size and condition at fledging is a
fundamental influence on population dynamics, influencing immediate and first-winter survival
[43, 45], longevity [34] and reproductive success [26, 50].

428 Triangulated relationships

We have demonstrated that microbial loading of an avian nesting environment can be influenced
by the orientation that nestbox faces. Most notably the abundance of *E. purpurascens* is higher
(typically nearly treble) in south-southwest oriented boxes occupied by great tits compared with
432 those facing the diametrically opposite direction. Moreover, this same fungal species explains
20% of the variation in offspring quality in great tits. Given that previous research had already
revealed that offspring quality in great tits is related to orientation, being lowest in nestboxes facing
south-southwest [22], we have triangulated evidence for a nest-site selection pressure, with empirical
436 evidence for one correlation between nestbox orientation and avian offspring quality, another
correlation between orientation and the loading of *E. purpurascens* (and *P. veronii* and *C.
cladosporioides*) in the nesting environment, and a third correlation between *E. purpurascens* and
avian offspring quality. We thus conclude that the higher loading of *E. purpurascens* fungus in
440 boxes facing south-southwest could be, either directly or indirectly, responsible for reduced
offspring quality in these nestboxes, and that the observed parental patterns of nest-site selection
behaviour (lower occupation rates in boxes facing south-southwest) might have evolved to avoid
nest-sites simultaneously associated with high microbial loads and lower offspring quality.

444 Acknowledgements

We thank Hannah Stubbs for laboratory support and the Eric Hosking Charitable Trust for a grant to AEG to fund external microbe identification at specialist laboratories. We also thank two anonymous reviewers for their detailed comments on an earlier version of this paper.

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580 Table 1: Microbial communities of nestboxes used by great tits (*Parus major*) in relation to nestbox orientation, showing microbial species richness and percentage prevalence of individual isolates (species and genera) that occur relatively frequently, that differ substantially in prevalence with orientation category, or that are potential pathogens.

584

	Occupied nestboxes facing (S-SW) N = 10	Occupied nestboxes facing (N-NE) N = 10
Bacteria		
Total number of different species	20	18
Prevalence of different species (percentage):		
Pseudomonas: all isolates (n = 7)	100	95
P. fluorescens biotype B	80	50
P. putida biotype B	40	40
P. veronii	20	60
P. syringae	40	20
P. agarici	20	10
P. stutzeri	10	-
P. luteola	-	10
Bacillus: all isolates (n = 9)	40	70
B. circulans	10	10
B. subtilis	20	-
Staphylococcus: all isolates (n = 5)	70	40
S. hominis	10	-
*S. hyicus**	10	40
S. lentus	20	30
S. lugdunensis	20	-
Other important isolates		
*Enterobacter cloacae**	-	10
*Aeromonas hydrophila**	-	10
Paenibacillus macerans	-	20
Roseomonas gilardii	10	-
Mean number of species per nestbox	3.8	4.0
Fungi		
Total number of different species	9	9
Prevalence of different species (percentage):		
Cladosporium: all isolates (n = 2)	100	60
C. cladosporioides	80	60
C. herbarum	60	20
Arthrinium: all isolates (n = 2)	30	-
Mucor: all isolates (n = 2)	40	50
Other important isolates		
Epicoccum purpurascens	100	90
Chrysosporium tropicum	10	10
*Aspergillus flavus**	-	40
*Candida albicans**	-	10
*Microsporum gallinae**	-	10
Mean number of species per nestbox	2.9	3.4

* = potential pathogens. See text for more detailed discussions

Table 2: Differences in the microbial load of nestboxes occupied by great tits (*Parus major*) facing different orientations. Data were analysed using paired sample *t* tests after *ln*+1

588 transformation (d.f. = 9 in all cases; * = significant at 0.05).

	Mean nestbox load (x 10^9)		*t*	*P*
	S-SW (180-269°)	N-NE (0-89°)		
Bacteria	1,240	985	-0.561	0.588
Fungi	2,078	1,022	3.111	0.013*
Pseudomonas veronii	56	160	-2.561	0.031*
Epicoccum purpurascens	1,156	400	2.423	0.038*
Cladosporium cladosporioides	389	190	2.502	0.034*
Bacteria other than *P. veronii*	1,235	969	-0.308	0.765
Fungi other than *E. purpurascens* and *C. cladosporioides*	543	471	0.796	0.447

Figure 1: Scatter plots of PC1 and PC2 from Principal Components Analyses with Verimax
592 orthogonal rotation undertaken on the microbial community of nestboxes occupied by great tits (*Parus major*) using (a) bacterial species; (b) fungal species; and (3) bacterial and fungal species. The variance in the microbial community explained by PC1 and PC2 is 23.1%, 45.70% and 39.6%, respectively. All models contain the same one outlier, a north-northeast facing nestbox
596 (Nagshead box 104) which was atypical, possibly due to woodland edge effects. If this outlier is removed from the scatter plots visually, the clustering of datapoints in (a) and (c) becomes more obvious.

600

a)

Principal Component 2

Principal Component 1

b)

Principal Component 2

Principal Component 1

c)

Principal Component 2

Principal Component 1

●South-Southwest ○North-NorthEast