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Correspondence between urban bird roosts and the presence of aerosolised fungal pathogens.

Abstract

Habitat fragmentation in urban environments concentrates bird populations that have managed to adapt to these newly developed areas. Consequently, the roosts of these birds are potentially creating environments conducive to fungal growth and dissemination. Airborne fungi derived from these environments are relatively unstudied, as is the potential health risk arising from these fungi. This study documented the diversity of culturable airborne fungal propagules associated with forty urban bird roosts. Environmental variables from each site were recorded to allow us to analyse the correspondence between different bird species, the substrate they occupy and airborne fungal propagules. Associations were established between *Rhodotorula* and Pacific black ducks, wood ducks, myna birds and miner birds when in the presence of bare soil as a substrate. Further associations were established between *Penicillium*, *Scopulariopsis* and *Cunninghamella* and pigeons, sparrows and swallows living in areas with hard surfaces such as bitumen and rocks.

Keywords

Airborne fungi – *Rhodotorula* – Avian droppings – Guano – Environmental sources – Zoonosis

Introduction

Urban development eliminates or fragments native habitats, leading to a subset of resilient species becoming adapted to urban environments [1]. Some of the most adaptive animals to urban environments are birds. The communities of urban birds are usually dominated by few species, whose numbers are often in high abundance [2-4], and concentrated into relatively small pockets of suitable habitat.

Birds are well known to be vectors of human fungal diseases as well as creating a suitable environment for fungal pathogens via their nutrient-rich droppings (guano), which provides an ideal medium for the growth of some fungi, including a range of pathogenic species [5, 6].This has the potential to result in a public health threat, particularly if these areas are in close proximity to humans [7, 8].

Most studies that assess the occurrence and association between fungal pathogens with birds focus their attention to directly sampling the bird [9, 10], the guano or substrate present at bird roosts [11, 12] If air samples

are taken, they are limited to occupational safety risk assessment analysis scenarios [13, 14], as opposed to routine survey sampling. Further, these assessments tend to focus on a single pathogen (e.g. *Aspergillus fumigatus* [11] or *Cryptococcus neoformans* [12]) and its presence or absence in a given situation.

With an expanding urban landscape, it is paramount that a detailed assessment and evaluation of fungal pathogens and their potential sources are made. Similarly, bird species that carry and transmit fungal pathogens in addition to the environmental conditions that facilitate biosafety risks need to be identified.

The present study was undertaken to compare the diversity of fungal propagules present in air samples taken from sites with high urban bird frequency, to sites with relatively lower bird frequency. In doing so, an assessment of each site was undertaken, with a focus on establishing the correspondence between potential contributing environmental variables to the presence of fungal diversity that could pose a human health risk.

Methods

Sample Sites

Forty bird-frequented sites were selected from across Sydney, Australia. Sites were identified through visual inspection of the presence of urban birds at the site, where birds were either in obviously large abundances or were frequently seen, along with the presence of large volumes of faecal matter; roosting indicators such as feathers and broken egg shells and a degree of public use such that the risk of potential fungal pathogen exposure to the greater public was in evidence. A further eleven reference sampling sites were chosen, based on their low bird frequencies, including no evidence of bird colonies or any other predominant animal presence. Reference sites were otherwise similar to the sample sites. Locations of the samples sites are displayed in Fig. 1 and Fig. 2.

Whilst we deliberately sought sites with active bird presence proximal to human activity, we made an attempt to randomise the environmental conditions amongst the sites within an urban context. Given that the various bird species have specific habitat associations, a detailed assessment of the habitat characteristics of the sites was made and included in the data analyses.

Collection and Analysis of Samples

Collection of samples was carried out between 11th of September to the 3rd of November, 2014 as this time period coincides with seasonal conditions that have the greatest density of culturable fungal propagules in the

atmosphere for the study region [15]. Samples were conducted between 10.00 am and 3.00 pm, as this time corresponds with the heaviest human activity in the sampled areas, as the assessment of potential human exposure was a primary aim of this work.

At each sample site, a 20 m x 20 m target area at the centre of the 'site' was established. Air samples were taken walking down a straight line transect through the site, whilst the air sampler was operational. Air samples were taken 50 cm from the ground as the operator walked down a line running diagonally through the established area. The sampler was held in front of the operator so their movement minimised the aerosolisation of fungal material. Each air sample was 2 minutes in duration, which corresponds to 80 L of air – the approximate equivalent to 160 breaths or ten minutes total respiration, assuming the average human makes 16 breaths of 0.5 L per minute.

At each site, the species of birds seen in the vicinity at the time of sampling were recorded (Table 1) as were a number of environmental characteristics, including ground substrate (e.g. bitumen, soil, leaf litter etc.; Table 2 and 3). For analysis, these variables were qualitatively assessed and assigned a value on an ordinal scale. Meteorological factors; humidity and temperature, were included into the analysis and were supplied by the Australian Bureau of Meteorology [16]. No samples were taken if it was raining, and a minimum wait of two days was given after any rain event.

Fungal air samples were collected using an Reuter Centrifugal air sampler (RCS; Biotest Diagnostics Corporation, Denville, New Jersey, USA), which impacts particles onto an agar medium coating a plastic backing strip by utilising centrifugal force. Sabouraud's dextrose agar (SDX; Biotest AG, Germany) was used as the growth medium, as its low pH is known to inhibit most bacterial growth. The SDX formulation used did not contain cycloheximide, as this is known to suppress the growth of some common fungi, including pathogens such as *A. fumigatus* [17]. The sampler was disinfected thoroughly with 70 % ethanol between samples. After collection, strips were aseptically removed from the RCS sampler, re-sealed in their plastic sleeves and incubated at 25°C for a period of five to seven days. If fungal colony development was inadequate for identification after this period, strips were re-incubated until sufficient colony growth occurred (up to 21 days). After incubation, the strips were stored at 4 °C to prevent further colony growth until identification was conducted (2–4 weeks).

Fungal colonies were transferred to microscope slide and stained with lactophenol cotton blue. Mould colonies were identified to genus level using gross microscopic morphology, using descriptions and keys of Alexopoulos

et al. [18], Ellis et al. [19], and Klich and Pitt [20]. Colonies of *Aspergillus* were further identified to species level due to the incidence and prevalence of *Aspergillus* associated pathogenesis across both avian species and humans.

Whilst yeasts were not identified further than their general morphological form, any cultures resembling *Cryptococcus* spp. yeasts tested by using an india-ink preparation and observing whether distinct capsules were present. Encapsulated yeasts would be identified to species level if detected.

Statistical Analysis

Data are displayed as means ± the SEM. Fungal bioparticle counts for the various sample sites were expressed as mean colony-forming units per m^3 of air (CFU/m^3). All environmental variables and bird densities measured were given a value on an ordinal score on a scale of 1–10 for comparisons across sites. A general linear model analysis (GLM ANOVA) was used to analyse differences in total CFU/m^3 between sites with birds and those without.

Multivariate analyses was used to asses interrelationships between the fungal dataset, and both bird and environmental datasets simultaneously. Nonmetric multidimensional scaling (nMDS) was used to visualize the general patters in the fungal community dataset to indicate any clustering patters that may have been present, such as the accumulation of specific fungal taxa in the bird associated or reference sites. An analysis of similarities (ANOSIM) was used to compare differences among the structure of fungal communities at different sites, and, where significant differences between groups were found, the fungal taxa that best differentiated among sites were determined using similarity percentages analysis (SIMPER).

Canonical correspondence analysis (CCA) was used to determine whether the differential abundance of fungal genera across samples were related to the abundance of different birds, relative humidity, temperature or environmental variables.

Multivariate analyses were made using PRIMER v6.1.6 (Primer- E Ltd, 2006), apart from CCA, which was performed using CANOCO v 4.51 (Biometris 2003). Statistical significance was tested at alpha = 0.05.

Results and Discussion

The mean total concentration of culturable airborne fungi among sites frequented by birds was 1,820 ± 275 CFU/m^3, while sites with no birds had significantly lower concentrations , with a mean of 940 ± 90 CFU/m^3. These concentrations are surprisingly low, indicating that even proximal to bird roosts, the aerosolized fungal concentrations are not alarming high. However this may be due to the spores and other propagules being effectively dispersed from the source as opposed to the accumulation effect that invariably occurs in confined environments with high bird density such as poultry farms and egg handling plants [21].

Of the fungal species present at sites with birds, known pathogens were identified, with *Mucor* identified in 40% of samples, *Microsporum* identified in 17.5 % of samples, *Rhodotorula* identified in 15% of samples and *Aspergillus fumigatus* identified in 7.5 % of samples. Despite rigorous exploration, no *Histoplasma capsulatum*, *Sporothrix schenckii*, *Candida albicans* or *Cryptococcus* spp. were identified in this investigation. Yeasts and yeast-like fungi such as *Candida* spp., *Aspergillus* spp., *Microsporum* spp., *Trichophyton* spp., and cryptococci have previously been isolated from the cloaca of wild and migratory birds [8]. A number of other pathogenic fungi have been detected on feathers of migratory birds, including *Aspergillus flavus*, *A. nidulans*, *Microsporum gypseum*, *M. ripariae*, *M. persicolor*, and *Trichophyton mentagrophytes* [22, 23]. Guano from seagulls has been found to contain fungal pathogens such as *Candida* spp., predominantly *C. albicans*, along with *Rhodotorula* spp., *Trichosporon* spp., *Penicillium* spp., *Aspergillus* spp., and *Fusarium* spp. [24]. Although a theoretical risk of transmission of these pathogens to humans exists, there is a scarcity of empirical evidence to support it [7]. This trend notwithstanding, clearly wild birds harbour fungi and have the capacity to spread them throughout the environment. Many of these fungi may pose potential health risks for humans and represent a significant zoonotic concern, especially due to the vast distances birds cover between different areas. The roosts of these birds that occur in urban areas are commonly frequented by humans who are potentially at increased risk of contracting opportunistic diseases [25].

nMDS was used as a preliminary screen of the data. The analysis showed an apparent, although overlapping pattern between sites with no birds against sites with birds. To compare the fungal genus associations with bird species, we used ANOSIM with a presence / absence data transformation. There was a significant difference between sites with birds and reference sites (Global $R = 0.23$; $p = 0.009$). Several individual fungal genera varied in number between the two groups of sites, and which therefore appeared to show a clear bird association. SIMPER results ranked on mean differences, indicated that the largest influence on the differences observed between sites with bird colonies and reference sites were higher concentrations of yeasts, *Epicoccum*,

Penicillium and *Rhodotorula* at the bird sites. A CCA was conducted between airborne fungal community structure and both bird abundance scores and environmental variables (Fig. 3). Strong associations were found among several groups of bird species, in particular between mynas, ducks, seagulls and miners, indicating that these species were more abundant in sites with a common set of environmental conditions. There was also a marked association between this group of species and the presence of *Rhodotorula*, which was found only at sites with bird colonies. In general, *Rhodotorula* had a strong association with Pacific black ducks, wood ducks, mynas and miners in the presence of bare soil as a substrate. Furthermore, *Penicillium*, *Scopulariopsis* and to a lesser degree *Cunninghamella* may have an association with pigeons, sparrows and swallows when in the presence of hard impervious surfaces such as bitumen and rocks. Weak associations were found with the remaining substrates and bird species.

The diversity of fungal genera documented in the current study are not dissimilar to existing literature involving the fungal community associated with birds [26-29] and other animal excreta [30]. Forty one genera of fungi were identified in air samples from sites with birds, whilst 16 genera were identified in the air samples of reference sites (Table 3). Clearly, the presence of decaying avian material like faecal matter, feathers and broken egg shells facilitates a greater diversity of fungal genera relative to conditions when these factors are absent, even in an urban environment, which in many cases could be expected to provide depleted organic material. Rural areas are known to harbour greater concentrations of fungal spores and an increase in frequency of some genera [31], primarily due to the higher density of organic substrates present. Alternatively, as a consequence of urbanisation, the roosts surveyed in the current study occur in areas where substrates strongly associated with anthropogenic activity are present. This might be expected to reduce the frequency of natural saprophytes, thus reducing the rate in which guano or other bird derived substrates are broken down. As a consequence this could be predicted to potentially constrain the diversity of organisms present to those that either thrive in an urban environment or those that have an avian association, which could lead to the proliferation of human pathogens of zoonoeitc potential. Additionally, these roosts were present in urban areas within fragmented or isolated pockets of semi-natural habitat, such as reserves and forested land, where humans are attracted for recreational activities, including synanthropy with the birds themselves (e.g. feeding of birds), which may increase their exposure to the roost associated fungi identified in this study.. Health practitioners in urban environments should thus be mindful of the ecology of environmental contamination, as well as the zoonotic potential and burden of infectious diseases associated with urban determinant variables.

As meteorological factors are known to influence the production, release and dispersal of aeroallergens [32] further work that incorporates other seasonal times is required. Additionally, future research should investigate biochemical or molecular identification concurrently with culturable methods, to provide a more comprehensive assessment of bird-associated fungal threats.

Conclusion

The density and diversity of airborne fungal propagules present in air samples taken from urban bird roosts was significantly different to that of reference sites. Correspondence was established between *Rhodotorula*, and Pacific black ducks, wood ducks, myna birds and miner birds when in the presence of bare soil as a substrate. Furthermore, correspondence was established between higher densities of *Penicillium*, *Scopulariopsis* and *Cunninghamella* with pigeons, sparrows and swallows when in the presence of impervious surfaces. These results indicate possible health risks for sensitive individuals, and the roosts of urban bird colonies could present a risk of infection to those with a compromised immune system, and those with allergic rhinitis.

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Conflict of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Authors' contributions

PJI designed the study, BA and WLK collected samples, PJI and FRT analysed data, PJI, BA, WLK, MB and FRT drafted the manuscript. All authors read and approved the final manuscript.

Figure legends

Fig. 1 Map depicting the geographical locations of the sampling region and the sites sampled areas. Figure made with the R packages ggplot2 and ggmaps, utilising static maps from Google Maps.

Fig. 2 Map depicting the geographical locations of the central sampling region and the sites sampled areas. Figure made with the R packages ggplot2 and ggmaps, utilising static maps from Google Maps.

Fig. 3 Canonical Correspondence Analysis biplot showing multivariate correspondence between bird abundance scores, environmental variables and the airborne fungal community

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