Isolation and Identification of *Aspergillus* spp. from Brown Kiwi (*Apteryx mantelli*) Nocturnal Houses in New Zealand

Source: Avian Diseases, 58(1):16-24.
Published By: American Association of Avian Pathologists
DOI: http://dx.doi.org/10.1637/10589-061013-Reg.1
URL: http://www.bioone.org/doi/full/10.1637/10589-061013-Reg.1

AVIAN DISEASES 58:16–24, 2014

Isolation and Identification of *Aspergillus* spp. from Brown Kiwi (*Apteryx mantelli*) Nocturnal Houses in New Zealand

Travis R. Glare,[AD] Brett D. Gartrell,[B] Jenny J. Brookes,[A] and John K. Perrott[C]

[A]Bio-Protection Research Centre, P.O. Box 85084, Lincoln University, Lincoln 7647, New Zealand
[B]Wildbase, Institute of Veterinary Animal and Biomedical Sciences, Massey University, Palmerston North 4442, New Zealand
[C]Department of Natural Sciences, Faculty of Social and Health Sciences, Unitec Institute of Technology, Private Bag 92025, Mt. Albert, Auckland, New Zealand

Received 19 June 2013; Accepted 12 September 2013; Published ahead of print 12 September 2013

SUMMARY. Aspergillosis, a disease caused by infection with *Aspergillus* spp., is a common cause of death in birds globally and is an irregular cause of mortality of captive kiwi (*Apteryx* spp.). *Aspergillus* spp. are often present in rotting plant material, including the litter and nesting material used for kiwi in captivity. The aim of this study was to survey nocturnal kiwi houses in New Zealand to assess the levels of *Aspergillus* currently present in leaf litter. Samples were received from 11 nocturnal kiwi houses from throughout New Zealand, with one site supplying multiple samples over time. *Aspergillus* was isolated and quantified by colony counts from litter samples using selective media and incubation temperatures. Isolates were identified to the species level by amplification and sequencing of ITS regions of the ribosomal. *Aspergillus* spp. were recovered from almost every sample; however, the levels in most kiwi houses were below 1000 colony-forming units (CFU)/g of wet material. The predominant species was *Aspergillus fumigatus*, with rare occurrences of *Aspergillus niger, Aspergillus nidulans,* and *Aspergillus parasiticus.* Only one site had no detectable *Aspergillus*. The limit of detection was around 50 CFU/g wet material. One site was repeatedly sampled as it had a high loading of *A. fumigatus* at the start of the survey and had two recent clinical cases of aspergillosis diagnosed in resident kiwi. Environmental loading at this site with *Aspergillus* spp. reduced but was not eliminated despite changes of the litter. The key finding of our study is that the background levels of *Aspergillus* spores in kiwi nocturnal houses in New Zealand are low, but occasional exceptions occur and are associated with the onset of aspergillosis in otherwise healthy birds. The predominant *Aspergillus* species present in the leaf litter was *A. fumigatus,* but other species were also present. Further research is needed to confirm the optimal management of leaf litter to minimize *Aspergillus* spore counts. However, in the interim, our recommendations are that leaf litter should be freshly collected from areas of undisturbed forest areas and spread immediately after collection, without interim storage.

RESUMEN. Aislamiento e identificación de *Aspergillus* spp. de alojamientos nocturnos de kiwi marrón (*Apteryx mantelli*) en Nueva Zelanda.

La aspergilosis, enfermedad causada por la infección con *Aspergillus* spp., es una causa común de muerte en las aves a nivel mundial y es una causa no regular de mortalidad de kiwis en cautiverio (*Apterix* spp.). El Aspergillus spp. está a menudo presente en la descomposición de la material vegetal , incluyendo la cama y el material utilizado para la anidación del kiwi en cautiverio. El objetivo de este estudio fue examinar los alojamientos nocturnos de kiwis en Nueva Zelanda para evaluar los niveles de *Aspergillus* actualmente presentes en la cama de hojas. Se recibieron muestras de 11 alojamientos nocturnos de kiwi de toda Nueva Zelanda, con un sitio que suministró múltiples muestras con el tiempo. Se aisló y se cuantificó *Aspergillus* por recuento de colonias de las muestras de cama usando medios selectivos y temperaturas de incubación. Los aislamientos fueron identificados a nivel de especie mediante amplificación y secuenciación de regiones ribosomales ITS. Se recuperó *Aspergillus* spp. de casi todas las muestras, sin embargo, los niveles en la mayoría de los alojamientos de kiwi estaban por debajo de las 1000 unidades formadoras de colonias (UFC)/g de materia húmeda. La especie predominante fue *Aspergillus fumigatus*, con presentaciones poco frecuentes de *Aspergillus niger*, *Aspergillus nidulans* y *Aspergillus parasiticus*. Sólo un sitio no mostró *Aspergillus* detectable. El límite de detección fue de alrededor de 50 UFC/g de materia húmeda. Un sitio fue muestreado varias veces ya que tenía una alta carga de *A. fumigatus* al inicio del muestreo y estaba relacionado con dos casos clínicos recientes de aspergilosis diagnosticados en los kiwis residentes. La carga ambiental en este sitio con *Aspergillus* spp. se redujo pero no fue eliminada totalmente a pesar de los cambios de la cama. El hallazgo clave de este estudio clave es que los niveles basales de esporas de *Aspergillus* en los alojamientos nocturnos de kiwis en Nueva Zelanda son bajos, pero se presentan excepciones ocasionales que están asociadas con la aparición de aspergilosis en aves sanas. La especie predominante de *Aspergillus* presente en la cama de hojas fue *A. fumigatus*, pero otras especies también estuvieron presentes. Se necesita investigación adicional para confirmar el tratamiento óptimo de la cama de hojas para minimizar los conteos de esporas de *Aspergillus*. Sin embargo, mientras tanto, la recomendación es que la cama de hojas debe recolectarse fresca de áreas forestales no contaminadas y debe extenderse inmediatamente después de la recolección, sin almacenamiento temporal.

Key words: *Aspergillus fumigatus,* kiwi, *Apteryx* spp., aspergillosis, breeding, leaf litter

Abbreviations: BST = semiselective medium; CFU = colony-forming units; DRBC = *Aspergillus* selective agar; ITS = internal transcribed spacer; PDA = potato dextrose agar; rDNA = ribosomal DNA

The New Zealand native kiwi (*Apteryx* spp.) is in decline, with several species listed as critically endangered (17). Habitat destruction and predators have contributed to a decline in population levels to a point that intervention is now required to ensure survival of this national iconic bird. The captive breeding program and Operation Nest Egg are important tools in efforts to save the endangered kiwi of New Zealand. Mortality is high in chicks born in the wild, with estimates of 90% mortality in unmanaged areas, largely due to mustelid predation (22). Adults may only have two to three chicks over the course of lifetime because

[D]Corresponding author. E-mail: travis.glare@lincoln.ac.nz

of predation from possums, stoats, ferrets, cats, dogs, and pigs; therefore, captive rearing and breeding are one of the main methods used to stabilize and replenish wild kiwi populations. Consequently, there are a number of kiwi nocturnal houses throughout New Zealand that are used for advocacy and, to a lesser extent, for captive breeding.

Kiwi nocturnal houses are fully enclosed buildings that provide public viewing of kiwi, who are separated from people by either glass or low wall barriers. The kiwi enclosures have a soil base that is planted with native vegetation, and leaf litter is brought in at varying intervals to provide ground cover and to stimulate kiwi into displaying natural probing and foraging behavior for invertebrates. The enclosures are watered regularly to encourage plant growth and to prevent the soil from becoming compacted. The nocturnal houses are artificially lit and on a reverse daylight schedule to allow kiwi to display their normal nocturnal activity (7).

In captivity, there are other potential causes of mortality. Aspergillosis is most commonly a respiratory ailment caused by the fungi *Aspergillus* spp. (Trichocomacae: Ascomycota), primarily *Aspergillus fumigatus*. *Aspergillus* spp. are a naturally occurring ubiquitous soil-borne fungi, of which there are 180 known species, filling many ecologic niches from soil and leaf litter saprophytes to pathogens of a wide range of species, including mammals, birds, and humans (3). *Aspergillus fumigatus* accounts for more than 90% of all invasive aspergillosis cases (5). A review of the postmortem database held at the Institute of Veterinary, Animal and Biomedical Sciences, Massey University, showed that between 1997 and 2011, 17 captive kiwi had died of pneumonia and airsacculitis due to *A. fumigatus*.

Aspergillus spp. result in a common disease of wild and domestic birds around the world (23). Most infections occur through inhalation when birds are exposed to high levels of spores and damp conditions and/or if the birds are immunosuppressed. Infection can cause three main diseases in avian birds: infiltrative bronchopneumonia, granulomatous pneumonia and airsacculitis, and open fungal plaques in the air sacs (3). All typically form in the respiratory tract, but infection does depend on the spore size and where the fungus settles in the body. The fungus frequently invades blood vessels and may disseminate widely throughout the body (3).

Aspergillosis has been recorded regularly in other New Zealand bird species. The hihi or stitchbirds (*Notiomystis cincta*), a New Zealand honey eater, has been extirpated from mainland New Zealand and has been introduced into a number of islands to reestablish populations. Mortality rates have been high, and one study found high levels of aspergillosis, with 14% confirmed cases and a further 44% suspected on one island (1,21). The fungus, *A. fumigatus,* was confirmed for one case, and high levels of *Aspergillus* were found in hihi nesting material on Mokoia (20).

Aspergillus spp. are often present in rotting plant material, including the litter and nesting material used for kiwi in captivity. Generally, the presence of *Aspergillus* in these materials is not enough to cause disease. However, when spores are present in large numbers, the fungus can infect birds and cause disease. There have been sporadic deaths due to aspergillosis in captive kiwi over the last 20 yr; it has been suggested that the infections are due to high fungal spore counts in the leaf litter or immune compromise in the birds. However, there are no guidelines for safe levels of *Aspergillus* spores in the leaf litter used in kiwi houses and no studies reporting levels of *Aspergillus* in kiwi breeding areas.

Two adult brown kiwi in a nocturnal house in New Zealand concurrently developed severe pneumonia and airsacculitis, and the causative organism isolated from both clinical and postmortem samples was *A. fumigatus*. The sudden onset of this disease in two otherwise-healthy kiwi was strongly suspicious for a high environmental burden of fungal spores. The aim of this study was to survey nocturnal kiwi houses in New Zealand to assess the levels of *Aspergillus* currently present in leaf litter, especially *A. fumigatus,* which has been implicated in kiwi deaths in New Zealand.

Table 1. *Aspergillus* spp. CFU/g wet material from site 1 using either BST or DRBC selective media. Samples were received March 17, 2011. There is a highly significant linear relationship ($R^2 = 0.985$, $F = 467.28$, $P < 0.001$) between the results of the two quantitative methods used.

Sample	BST	DRBC
1 soil	20,000	50,000
1 leaf litter	700,000	350,000
2 soil	6000	9000
2 leaf	2000	3000
KP11 soil	0	0
KP 11 leaf	0	0
KP 12 soil	2000	0
KP 12 leaf	1000	2500

MATERIALS AND METHODS

Samples of breeding materials. A request for samples was sent to 16 nocturnal kiwi houses in New Zealand, along with collection instructions. A questionnaire was also included that asked for details of the number of nocturnal houses and kiwi, the regime of leaf litter management used in the houses, the source of leaf litter, vegetation type, and temperature and humidity of houses. A map of the collection sites was also obtained.

Each sample was taken from within the nocturnal houses and included material from the surface of the litter down to the soil layer. At least three samples from evenly spread locations within the houses were collected, and in most cases, more than 20 g of wet material was collected per sample. Samples were couriered to Lincoln University for processing. Samples were stored at 4 C until isolation (usually 1–4 days).

Isolation from soil, leaf, and litter. From each sample, a 20-g wet material subsample was placed in 180 ml of 0.01% Triton X-100 and shaken on a Stuart (Staffordshire, U.K.) flask shaker at 300–400 rpm for 30 min. Serial dilutions were then prepared in 0.01% Triton X-100. For each dilution, duplicate plates were prepared. One hundred microliters of each dilution was spread over the surface of an agar plate and incubated in the dark at 35 C. The method used selected for *Aspergillus* spp. that could grow at over 35 C, a temperature that precluded the growth of many other common environmental fungi. Therefore, *Aspergillus* spp. predominantly grew on the isolation plates. Two media were trialed: the *Aspergillus* selective agar DRBC (Difco, Franklin Lakes, NJ) and a semiselective potato dextrose agar (PDA)–based agar ''BST''. BST consisted of PDA (Difco) amended with 350 mg/L streptomycin sulfate (Sigma Chemical Co., St. Louis, MO) and 50 mg/L tetracycline hydrochloride (Sigma Chemical Co.). There was a highly significant linear relationship ($R^2 = 0.987$) between the quantitative levels of *Aspergillus* recovered using both media (Table 1), but BST was cheaper and easier to source, so it was used for the study. After 2–4 days, colonies were examined under a light microscope and the *Aspergillus* morphotypic colonies counted. Note was made of the number and frequency of each colony morphotype. Selected samples of each morphotype were subcultured on PDA (without antibiotics). Pure cultures were used for DNA extraction.

Molecular characterization. To identify the isolates to species level, a portion of the ribosomal DNA (rDNA) was amplified and sequenced from the 3′ end of the 16S rDNA gene to the 5′ end of the 26S rDNA, incorporating internal transcribed spacer (ITS)1–5.8S-ITS2 (4). DNA was isolated using the Viogene plant DNA extraction kit (Viogene, Taipei, Taiwan) following the manufacturer's instructions. DNA was extracted from mycelia grown in PDA for 2 days at 35 C via shaking.

Table 2. GenBank sequences used for comparison.

Species	Isolate code	GenBank accession No.
Used for comparison		
Aspergillus fumigatus	NRRL 6113	EF669999
Aspergillus fumigatus	SN-A4	HQ285578
Aspergillus fumigatus	T-HJ1	GU992275
Aspergillus fumigatus	ATCC 1022	HQ026746
Aspergillus niger	qy-Asp15	JF909353
Aspergillus niger	PTC08	JN384120
Aspergillus parasiticus	NRRL 3386	HQ340110
Emericella nidulans	OA/HCPF 9011	FJ878643
Neosartorya fischeri	A4	EU551199
Beauveria bassiana	IMI 361056	AJ560686
GenBank submissions (this study)	As in Fig. 2	KF020278–KF020313

Mycelium was harvested by filtering through Whatman no. 1 filters, and then less than 100 mg of wet hyphae was ground in liquid nitrogen before processing with the Viogene kit. DNA was resuspended in single distilled water.

The concentration of DNA used in the PCR reactions was determined empirically and ranged from 1 to 10× dilution of the initial isolation. PCR amplification of the rDNA was performed in 25-µl volumes consisting of 0.4 µM of each primer (Invitrogen, Carlsbad, CA), 200 µM deoxynucleotide triphosphates, 2.5 µl reaction buffer, 1.5 mM $MgCl_2$, 2 µl DNA, and FastStart Taq (0.7 U/reaction; Roche, Basel, Switzerland). Amplifications were performed in a Bio-Rad thermal cycler using a hotstart (95 C for 5 min), followed by 40 cycles of 45 sec at 95 C, 45 sec at 55 C, and 2 min at 72 C. Primers used were TW81 and AB28 (4). Amplified DNA was visualized on a 1% agarose gel and then sequenced by the Bio-Protection Research Centre (Lincoln, New Zealand) sequencing unit. Both strands were sequenced and the results aligned in Chromas Pro, then identification was confirmed using the BLAST programs (2). Sequences were aligned with reference sequences from GenBank (Table 2) using DNAMan (Lynnon Biosoft, Vandreuil, Quebec, Canada) and MEGA 5 (15,27) using the Neighbor-Joining method (24). Bootstrap test was conducted using 500 replicates (10), and the results are shown next to the branches (Fig. 1). The evolutionary distances were computed using the Kimura two-parameter method (14), and there were 389 positions in the final data set. Sequences obtained in this study have GenBank accession numbers KF020278–KF020313.

RESULTS

Samples were received from 11 nocturnal kiwi houses from throughout New Zealand, with one site supplying multiple samples over time. Samples were mainly received between April and August 2011.

Aspergillus spp. were recovered from almost every sample; however, the levels in most kiwi houses were below 1000 CFU/g of wet material (Table 3). The predominant species was *A. fumigatus*, with rare occurrences of *Aspergillus niger, Aspergillus nidulans,* and *Aspergillus parasiticus* (Table 2; Fig. 1). Sites 1, 2, 7, and 8 had higher levels of *A. fumigatus* than did other sites. Only one site (site 5) had no detectable *Aspergillus*. The limit of detection was around 50 colony-forming units (CFU)/g wet material.

Site 1 was repeatedly sampled, as it had a high loading of *A. fumigatus* at the start of the survey (Fig. 2) and had experienced two recent clinical cases of aspergillosis diagnosed in resident kiwi. Both enclosures had all leaf litter and topsoil replaced, so the samples over time reflect the effects of aggressive management. Despite the significant lowering of the *Aspergillus* levels, it could not be eliminated from the houses. There was a small background of *A. niger* and *A. parasiticus* present in some samples taken from site 1 on June 22, 2011, and at September 6, 2011, but no more than 500 CFU/g was ever recorded of these other species.

Molecular identification using sequencing of the rDNA demonstrated that the majority of colonies recovered were *A. fumigatus* (Fig. 1). The isolations were almost identical, except for a single base pair at around 70 bp from the 5′ end of the trimmed region. The change, between a T and C, did not correlate with any colony morphology or location. Morphology of colonies varied among the *A. fumigatus* that was not reflected in the sequencing analysis, although only one DNA region was examined. A single colony recovered from site 8 was identified as a close relative to the *A. fumigatus*, *Aspergillus fischerianus* (teleomorph = *Neosartorya fischeri*).

Effect of management strategies. Each manager who submitted samples for testing also completed a questionnaire regarding the kiwi house and management practices. The responses are summarized in Table 4. It is difficult to draw conclusions between the recovery of *Aspergillus* and management on this data, but there was some correlation between the age of the litter since last change and the level of *Aspergillus* (Fig. 3), although this was not supported by any statistical evaluation ($R^2 = 0.13$). The kiwi nocturnal house with high levels of *Aspergillus* present had stored leaf litter in sealed plastic bags for 48 hr between collection and distribution within the kiwi house.

DISCUSSION

Aspergillosis is a threat of holding kiwi in captivity. No research has previously looked at the background levels of *Aspergillus* in kiwi houses. In this study we found *A. fumigatus* present at almost all sites. *Aspergillus fumigatus* is a recognized cause of aspergillosis, so if present in high concentrations it would be dangerous to kiwi. Levels detected were generally low, under 100 CFU/g litter, but in several instances we found much higher levels. The level at which *Aspergillus* causes aspergillosis is not well documented and is dependent on a number of factors, including the health of the birds. The two main primary conditions under which inhalation of *Aspergillus* spores results in infection are either 1) overwhelming exposure to large numbers of spores, which can result in disease in otherwise-healthy birds, or 2) immune system compromise through concurrent disease, stress, or malnutrition (3,11). Kiwi are ground foraging birds that require the regular provision of leaf litter to indoor enclosures for foraging and enrichment; therefore, simply excluding the use of leaf litter is not a viable management option. Given the ubiquitous

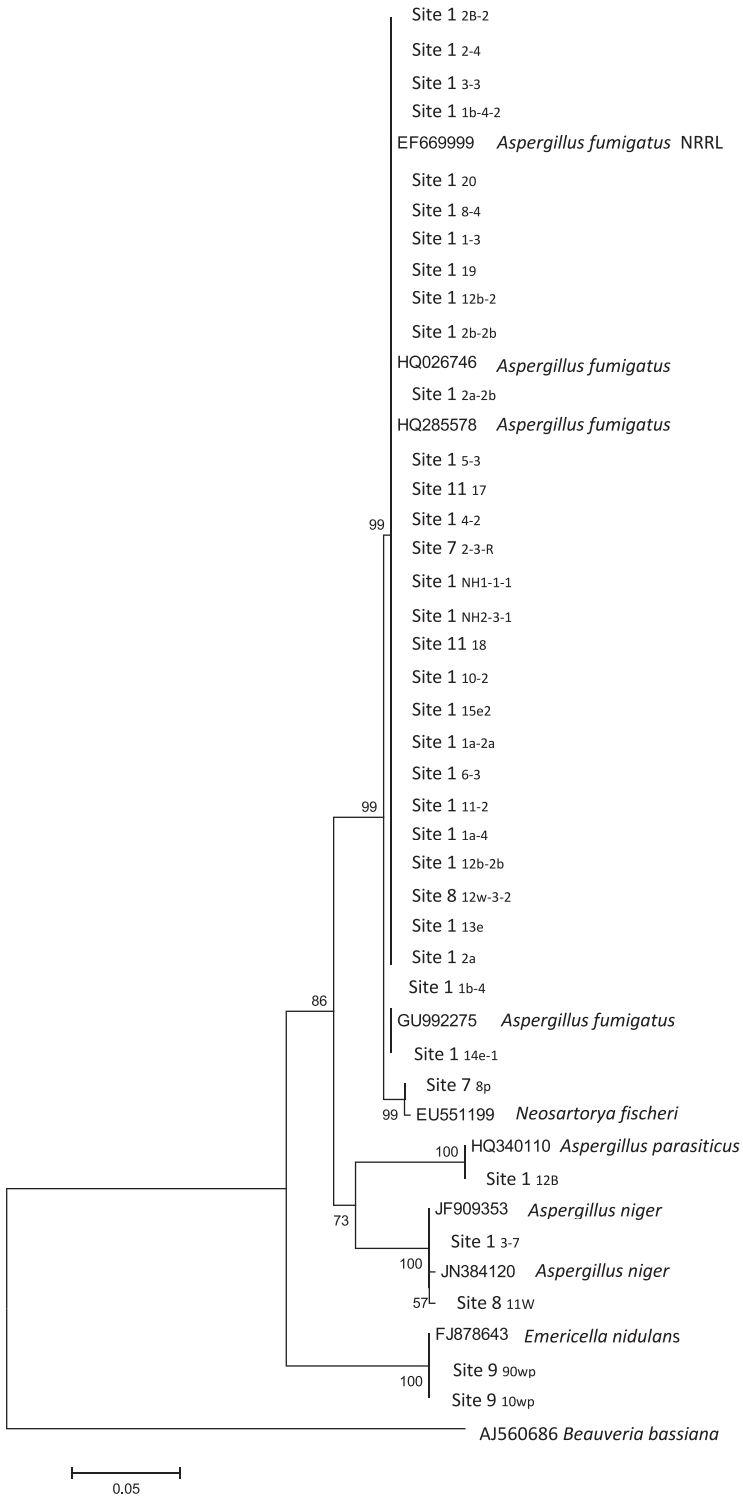

Fig. 1. Dendrogram of sequence comparison between *Aspergillus* isolates recovered (site and identification code shown corresponding to GenBank submission) and nearest GenBank relatives (inferred using the Neighbor-Joining method, MEGA5.2; Numbers above branches indicate bootstrapping at 500 replicates).

nature of *Aspergillus* spores in leaf litter and other decaying vegetable matter it is impractical to consider eradicating *Aspergillus* from the environment altogether. However, outbreaks of disease due to overwhelming spore exposure should be able to be prevented by managing the leaf litter used as substrate in kiwi nocturnal houses and by avoiding climatic conditions that favor fungal growth.

Several other *Aspergillus* were found in low numbers. *Aspergillus parasiticus* is a known producer of aflatoxins, belongs to the Flavi section of *Aspergillus,* and can be difficult to distinguish from closely related species (8). *Aspergillus niger* is a common clinical isolate and has also been implicated in aspergillosis (28). *Aspergillus nidulans* is used as a model organism for mycologic studies and is also implicated

Table 3. CFU/g of *Aspergillus* spp. recovered from litter material.

Site	Sample	Sample received	Total *Aspergillus* CFU/g wet material[A]	Non–*A. fumigatus* CFU/g wet material
2	1	May 2011	500	—
	2		1000	—
	3		2500	—
3	1	May 2011	500	—
	2 and 3		0	—
4	1	May 2011	500	—
	2 and 3		0	—
5	1, 2 and 3	May 2011	0	—
6	1-1	May 2011	500	—
	1-2		0	—
	1-3		50	—
	2-1 and 2-2		0	—
	2-3		150	—
7	1-1	June 2011	650	—
	1-2		3250	—
	1-3		1775	—
	2-1		675	—
	2-2		725	—
	2-3		950	50
	3-1		7750	—
	3-2		1925	—
	3-3		1100	50
8	1	June 2011	500	50
	2		2725	500
	3		8000	1675
9	1	June 2011	300	100
	2		1175	50
	3		500	50
10	1-1	June 2011	500	—
	1-2		350	—
	1-3		500	—
	2-1		0	—
	2-2		1000	—
	2-3		100	—
11	1 and 2	July 2011	0	0
	3		1050	0

[A]Samples were mainly identified as *A. fumigatus*.

in some diseases (12). *Neosartorya fischeri* (anamorph *A. fischerianus*) is a sexual *Aspergillus* species and the closest relative to *A. fumigatus* (25).

The diagnosis of aspergillosis in birds at postmortem examination is based on the gross and histologic findings and fungal cultures (11). The diagnosis of the disease in live birds is more challenging (3). Suspicion of aspergillosis is often based on clinical signs of lower respiratory tract disease, with supportive evidence from ancillary diagnostic techniques such as radiography and hematology (11). However, many birds may show no clinical signs of illness prior to death (3). The ubiquitous nature of the organism means that serologic diagnosis is unreliable in birds (11). Confirming the diagnosis of aspergillosis in live birds usually requires more invasive tissue biopsies and cultures using endoscopy (11). Treatment of aspergillosis in birds requires weeks of medication with systemic and nebulized antifungal agents and has a guarded prognosis (3). Given the difficulties with diagnosis and treatment of aspergillosis, prevention of the disease is worthwhile.

The effects of habitat disturbance on disease occurrence can be subdivided. Habitat disturbance may increase stress levels in the host organisms, reducing immunocompetence (9,16,30), or it may increase the prevalence of the disease-causing organism in the environment (6,19,21). This distinction is critical for disease management. If the effect is due to stress, management can be based solely on the autecology of the host species and may involve improving factors, such as nutrition or shelter. If the effect is due to increased prevalence of the pathogen, it is necessary to understand the ecology of the pathogen in relation to disturbance. For obligate parasites, an increase in prevalence could be related to an intermediate host. For facultative parasites such as *A. fumigatus*, an increase could be associated with any aspect of the organism's environment (21).

Factors that have been suggested to increase the load of spores within a captive facility include a warm environment, high humidity, poor ventilation, poor sanitation, and the long-term storage of feed (3); however, no firm data support these suggestions. Our results suggest that, in general, the current management of New Zealand kiwi nocturnal houses does not promote the development of high levels of spores in the captive environment. While not conclusively determined in this research, it is strongly suspected that the high environmental loads of *Aspergillus* spores in one kiwi nocturnal house resulted from the storage of leaf litter in sealed plastic bags for 48 hr before it was spread out through the nocturnal house. This temporary storage of the material would have resulted in ideal warm, humid conditions for the proliferation and sporulation of the fungus. The kiwi house with the high spore count had two birds clinically affected with aspergillosis, both of which died despite treatment.

One key recommendation based on our findings is that leaf litter collected for use in kiwi houses should be transported and placed as

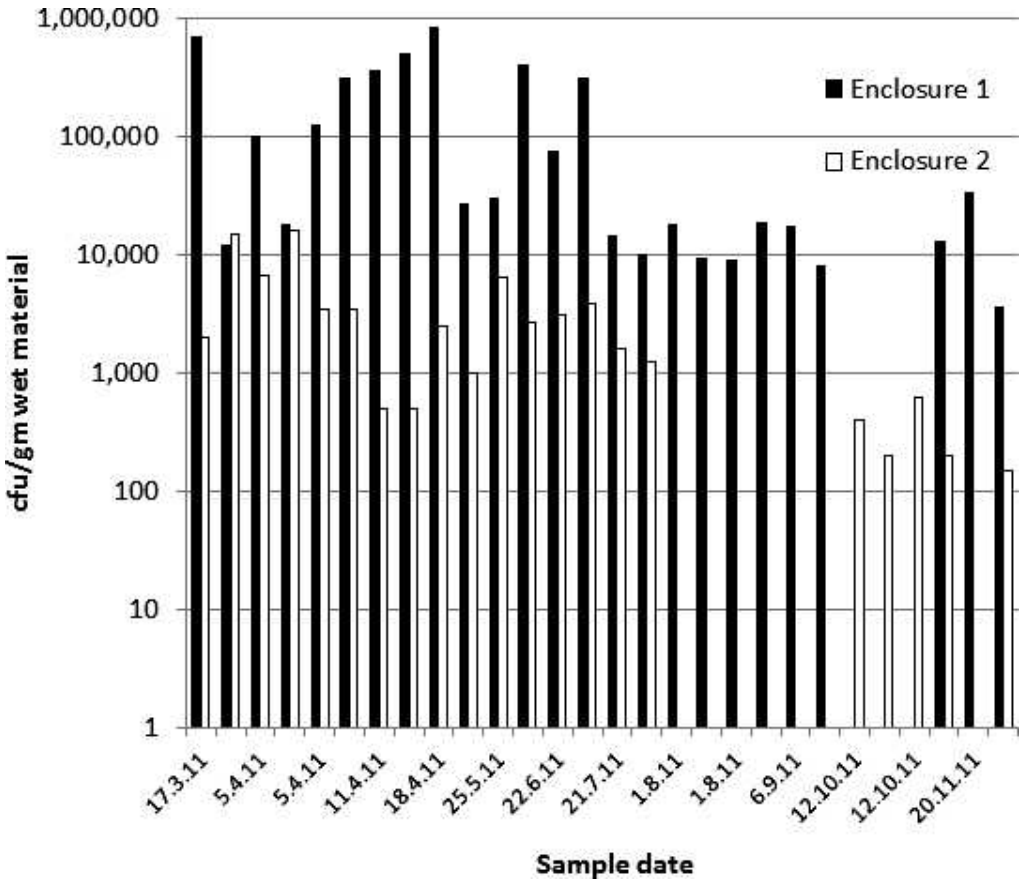

Fig. 2. CFU from repeat sampling of two enclosures from site 1 (log scale). The figures represent *Aspergillus* spp. growing on semiselective medium at 35 C; the majority of colonies were *A. fumigatus*. The minimum level of detection is 50 CFU/g.

substrate with minimal interim storage. However, we recognize that this is complicated by the need to screen leaf litter for metallic foreign bodies before use. Kiwi are known to ingest such metal objects, and nails, screws, and wire have caused the death of captive birds as a result of gastrointestinal perforation (18).

We suggest there is a need for further study to identify the optimal management of leaf litter to minimize *Aspergillus* spore burdens. Factors that may influence this include the frequency of leaf litter changes, the effect of light regimes, and the temperature and humidity of the enclosure. Wicklow (29) and Perrott (20) demonstrated that by removing leaf litter in forest sample plots, conditions favored the establishment of *A. fumigatus*. Leaf litter removal experiments were conducted to investigate whether leaf litter removal is a factor influencing the abundance of *A. fumigatus*. Results showed that leaf litter disturbance and drought conditions increase the abundance of *A. fumigatus* in the soil. Perrott (20) observed an average 24% increase in *A. fumigatus* densities 1 mo following leaf litter removal. Both treatment and control plots showed strong seasonal changes in *A. fumigatus* densities, and these were closely correlated. *Aspergillus fumigatus* densities showed a biannual cycle, with density peaks in spring and autumn. It should be noted that our study did not consider seasonal changes in fungal loads in leaf litter. While the indoor nature of the nocturnal houses will buffer seasonal changes within the nocturnal house, the collection of leaf litter at different times of year has significant effects on fungal load and species diversity.

In addition, results indicate a high persistence of *A. fumigatus* 2 yr following the disturbance event. Leaf litter removal favors the establishment of fungal species capable of growth at elevated soil temperatures and able to survive periods of excessive drought through the production of resistant propagules (e.g., sclerotia in the case of *A. fumigatus*). *Aspergillus* are among a small group of cellulose-degrading fungi capable of growth at high temperatures (above 50 C) and low soil water levels of around 0.75 relative humidity (26). Management considerations are difficult to develop because of the broad environmental tolerances and facultative nature of *A. fumigatus*. More information on the ecology of *A. fumigatus* is required; however, we can suggest some basic management considerations. The recommended site for collection of leaf litter is from mature forests away from forest edge areas, as these areas have been previously shown to have the lowest mean levels of *A. fumigatus* (20).

High temperatures (e.g., >25 C) and/or excessive drying out of leaf litter increase the abundance of *A. fumigatus* and decrease the diversity and abundance of less tolerant microflora. Hong *et al.* (13) found that the intraspecific genetic variation of *A. fumigatus* was low, but morphologic variation was high. Our results were similar, with colony morphology varying but with little genotype variation. However, only a small fragment of a highly conserved region was analyzed. According to recent studies, specific genotyping of *A. fumigatus* is achieved using small portions of the b-tubulin (*btub*) and rodlet A (*rodA)* genes (25). The benefit of using ITS in our study was that many representative *Aspergillus* species sequences were available in GenBank, ensuring a species match.

The key findings of our study are that the background levels of *Aspergillus* spores in kiwi nocturnal houses in New Zealand are low, but that occasional exceptions occur and are associated with the onset of aspergillosis in otherwise-healthy birds. The predominant

Table 4. Summarized responses to the questionnaire regarding the kiwi houses and management practices.

NOC house sample No.	Frequency of leaf litter replacement	Sampled leaf litter time in house	Leaf litter source	Main vegetation type used as leaf litter	Temperature inside the kiwi house, C	Water practice
1	Approx. four times a year; not changed, just added to existing litter	Added, up to 3 mo (multiple samples)	Native bush reserve	Mixed native broadleaf; e.g., mahoe, tawa, coprosma, pittosporum, and fivefinger	17	Irrigated 2–3 times/wk depending on time of year
2	Usually added to as it decomposes	<1 wk	Native forest, supplemented with leaf litter of unsprayed lawn areas	tawa, kahikatea, mahoe, and ash	~10–15	Sprinkler system 2–3 times/wk
3	Leaf litter not changed yet	<1 mo	Regenerating bush	Ferns, coprosma, tree ferns, some rimu, and other young podocarps	10–22	Water plants about 15 min every second day
4	New leaf litter/logs introduced every 2 wk	<1 wk	Native forest	Kamahi, tree fern, and rata vine	n/a	Plants 2–3 times/wk
5	Average of monthly	<2 wk	Local forest	Mix of pine and native leaf litter	16–23	When required; approx. every second day, but seasonal variance
6	New leaf litter monthly (approx.); do not remove old leaf litter, it just mulches down	Added ~3 wk	Pristine native beech forest	Native beech	~15–20	Water plants daily, hose for cleaning daily
7	Leaf litter added approx. every 2 mo	~3 mo	Native forest	Rewarewa, tawa, mahoe, and other rotten logs also	18–20	Soil is lightly forked to aerate once a week and watered every morning
8	Leaf litter added monthly	None removed, added monthly	Native areas	Gum, pine, tree lucerne, pohutakawa, cabbage tree, punga, and pittosporum	~15	Plants watered daily
9	New leaf litter is added every 2–4 wk; contaminated leaf litter is removed daily	~3 wk	Native areas	Mixture of native leaf litter such as coprosma, broadleaf, lemonwood, akeake, wineberry, and lacebark, very occasionally maple leaf	n/a	Irrigated between 1 and 3 times/wk for ~15–30 min, depending on the time of year
10	Replaced monthly	~1 mo	Native bush	Mature rewarewa forest with very little understory and wineberry and ponga on edge	n/a	Daily misting
11	Leaf litter added two to three times per week; old leaf litter is not removed	Not removed, new added <1 wk	Bush	Unknown; a variety is used	~17	Sprinkler system 3 times/wk

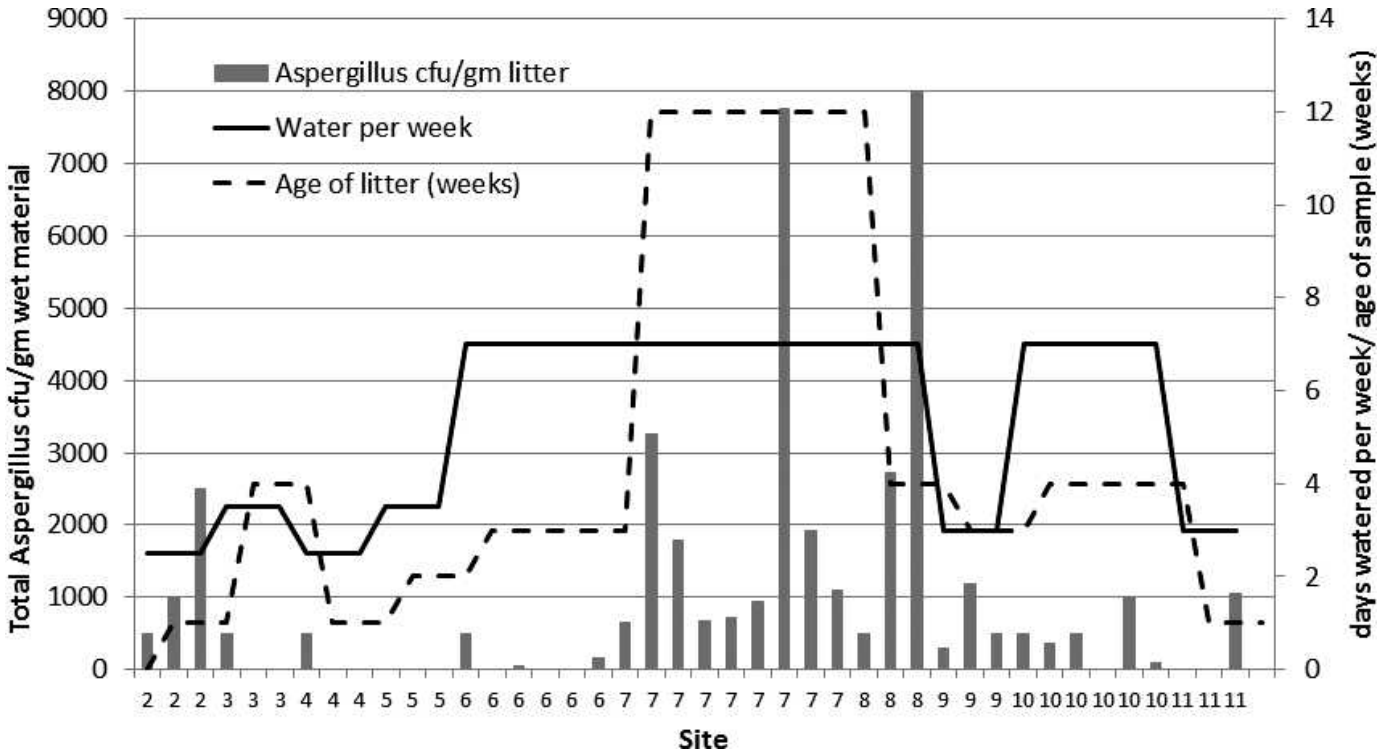

Fig. 3. Comparison of *Aspergillus* spp. CFU/g litter recovered, number of waterings per week, and age of litter.

Aspergillus species present in the leaf litter was *A. fumigatus,* but other species were also present. Further research is needed to confirm the optimal management of leaf litter to minimize *Aspergillus* spore counts. However, in the interim our recommendations are that leaf litter should be freshly collected from areas of undisturbed forest areas and spread immediately after collection, without interim storage.

REFERENCES

1. Alley, M. R., I. Castro, and J. E. B. Hunter. Aspergillosis in hihi (*Nofiomystis cincta*) on Mokoia Island. N. Z. Vet. J. 47:88–91. 1999.

2. Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. Basic local alignment search tool. J. Mol. Biol. 215:403–410. 1990.

3. Beernaert, L. A., F. Pasmans, L. Van Waeyenberghe, F. Haesebrouk, and A. Martel. *Aspergillus* infections in birds: a review. Avian Pathol. 39:325–331. 2010.

4. Curran, J., F. Driver, J. W. O. Ballard, and R. J. Milner. Phylogeny of *Metarhizium*: analysis of ribosomal DNA sequence data. Mycol. Res. 98:547–552. 1994.

5. Dagenais, T. R. T., and N. P. Keller. Pathogenesis of *Aspergillus fumigatus* in invasive aspergillosis. Clin. Microbiol. Rev. 22:447–465. 2009.

6. Deem, S. L., W. B. Karesh, and W. Weisman. Putting theory into practice: wildlife health in conservation. Conserv. Biol. 15:1224–1233. 2001.

7. Fraser, I., and T. Johnson. Brown kiwi (*Apteryx mantelli*) husbandry manual. Zoo & Aquarium Association, Auckland, New Zealand. 2009.

8. Godet, M., and F. Munaut. Molecular strategy for identification in *Aspergillus* section Flavi. FEMS Microbiol. Lett. 304:157–168. 2010.

9. Griffin, J. F. T. Stress and immunity: a unifying concept. Vet. Immunol. Immunopathol. 20:263–312. 1989.

10. Felsenstein, J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791. 1985.

11. Fudge, A., and B. Speer. Selected controversial topics in avian diagnostic testing. Semin. Avian Exotic Pet Med. 10:96–101. 2001.

12. Henriet, S. S. V., P. E. Verweij, and A. Warris. *Aspergillus nidulans* and chronic granulomatous disease: a unique host-pathogen interaction. J. Infect. Dis. 206:1128–1137. 2012.

13. Hong, S. B., S. J. Go, H. D. Shin, J. C. Frisvad, and R. A. Samson. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. Mycologia 97:1316–1329. 2005.

14. Kimura, M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16:111–120. 1980.

15. Kumar, S., J. Dudley, M. Nei, and K. Tamura. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief. Bioinformatics 9:299–306. 2008.

16. Lochmiller, R. L., and C. Deerenberg. Trade-offs in evolutionary immunology: just what is the cost of immunity. Oikos 88:87–98. 2000.

17. Miskelly, C. M., J. E. Dowding, G. P. Elliot, R. A. Hitchmough, R. G. Powlesland, H. A. Roberston, P. M. Sagar, R. P. Scofield, and G. A. Taylor. Conservation status of New Zealand birds, 2008. The Ornithological Society of New Zealand Inc. Notornis 55:117–135. 2008.

18. Morgan, K. J. Kiwi first aid and veterinary care [Internet]. Department of Conservation, Wellington, New Zealand, [cited 16 August 2011]. Available from: http://www.doc.govt.nz/upload/documents/science-and-technical/sap245entire.pdf. 2008.

19. Patz, J. A., T. K. Graczyk, N. Geller, and A. Y. Vittor. Effects of environmental change on emerging parasitic diseases. Int. J. Parasitol. 30:1395–1405. 2000.

20. Perrott, J. K. The ecology of *Aspergillus fumigatus* and implications for wildlife conservation in modified environments. Unpublished Ph.D. Thesis. Massey University, Palmerston North, New Zealand. 2001.

21. Perrott, J. K., and P. Armstrong. *Aspergillus fumigatus* densities in relation to forest succession and edge effects: implications for wildlife health in modified environments. Ecohealth 8:290–300. 2011.

22. Robertson, H. A., R. M. Colbourne, P. J. Graham, P. J. Miller, and R. J. Pierce. Experimental management of brown kiwi *Apteryx mantelli* in central Northland, NZ. Bird Conserv. Int. 21:207–220. 2011.

23. Rosen, M. N. Aspergillosis in wild and domestic fowl. Avian Dis. 8:1–6. 1963.

24. Saitou, N., and M. Nei. The Neighbor-Joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425. 1987.

25. Serrano, R., L. Gusmão, A. Amorim, and R. Araujo. Rapid identification of *Aspergillus fumigatus* within the section Fumigati. BMC Microbiol. 11:82. 2011.

26. Smith, S. L., and S. T. Hill. Influence of temperature and water activity on germination and growth of *Aspergillus restrictus* and *Aspergillus versicolor*. Trans. Br. Mycol. Soc. 79:558–560. 1982.

27. Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28:2731–2739. 2011.

28. Walsh, T. J., E. J. Anaissie, D. W. Denning, R. Herbrecht, D. P. Kontoyiannis, K. A. Marr, V. A. Morrison, B. H. Segal, W. J. Steinbach, D. A. Stevens, J.-A. van Burik, J. R. Wingard, and T. F. Patterson. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin. Infect. Dis. 46:327–360. 2008.

29. Wicklow, D. T. Microfungal populations in surface soils of manipulated prairie stands. Ecology 54:1302–1310. 1973.

30. Zuk, M. Immunology and the evolution of behaviour. In: Behavioral mechanisms in evolutionary ecology. L. A. Real, ed. University of Chicago Press, Chicago. pp. 354–368. 1994.

ACKNOWLEDGMENTS

We thank the staff from the following institutions who provided samples and completed the survey for this publication: The National Aquarium of New Zealand; West Coast Wildlife Centre; Whangarei Museum & Kiwi House; Nga Manu Nature Reserve; Kiwi Birdlife Park, Queenstown; Orana Wildlife Park; Wellington Zoo; Pukaha Mt. Bruce; Otorohanga Kiwi House; Kiwi Encounter, Rainbow Springs; and the Auckland Zoo. We wish to thank the Kiwi Recovery Group, which gave us permission to carry out this work.