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Prevalence of and environmental factors associated with aerosolised *Aspergillus* spores at a zoological park

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ABSTRACT

Aspergillus is a significant pathogen in zoological species, although information on environmental variables influencing fungal prevalence in zoological settings are lacking. The objective of the study was to estimate the prevalence of and to identify environmental factors associated with aerosolised *Aspergillus* spores at a zoological park to advance the understanding of fungal exposure as a first step towards improved mitigation strategies for susceptible animals. Twenty-one locations were sampled for presence of *Aspergillus* species using the SAS Super 180 Microbial Air Sampler, while twenty-two environmental factors were evaluated every two weeks at SeaWorld of California during two 12-month periods. In each period, the frequency of investigated environmental factors was compared between samples classified as positive or negative for *Aspergillus* species using logistic regression. Prevalence of *Aspergillus* was higher (P<0.05) during the second 12-month period (110/525 or 21 per cent), compared with the first period (62/483 or 13 per cent). In both periods, positive *Aspergillus* samples were associated with indoor sites without high-efficiency particulate air (HEPA) filtration systems and other infection control measures (adjusted OR=4.33 and 5.19, P<0.01) or outdoor sites (adjusted OR=2.50 and3.79, P≤0.05), compared to indoor sites with HEPA filtration systems and other infection control measures, after controlling for season. Burden of airborne *Aspergillus* can be higher in indoor sites without HEPA filtration systems than in outdoor sites. The use of HEPA filtration systems and other infection control measures can mitigate the burden of *Aspergillus*. Risk-based surveillance systems that target indoor areas without HEPA filtration systems can be an efficient approach for early detection of high burden of *Aspergillus* at zoological parks.

INTRODUCTION

Aspergillosis is a fungal disease that can cause high morbidity and mortality in avian species at zoological institutions.[1–3] *Aspergillus fumigatus* is the most commonly implicated fungus in avian aspergillosis infections; other frequently isolated species include *A niger*, *A flavus*, *A glaucus* and *A nidulans*.[4] Inhalation of spores is the primary route of infection for *A fumigatus* infections in both avians and humans.[5] In the outside environment, the fungus is a saprophyte which produces conidiophores with chains of infectious spores.[6 7] The small particulate size of the spores allows the aerosolised pathogen to reach the lungs and air sacs of avian species.[8 9] The development of clinical disease is considered multifactorial, with inoculum burden and host immunity considered principle factors.[4] Aspergillosis is a well-described disease among wild birds in rehabilitation centres and managed birds in zoological institutions.[1 2 8 10] As such, reports detailing the pathogenesis, anatomic and histopathological features, diagnosis and treatment of aspergillosis are numerous.[3–5 9–11] However, epidemiological reports of *Aspergillus* in zoological institutions are limited at present.

Environmental air sampling has been used on occasion in zoological and avian rehabilitation settings to investigate disease outbreaks and quantify fungal burden.[1 2 8] However, reports of *Aspergillus* prevalence and data on environmental factors associated with the fungal pathogen in zoological settings are scarce. Two prospective studies of aerosolised *Aspergillus* have yielded useful data on fungal burden.[2 8] Although, in those studies environmental data were not acquired at the same time fungal air sampling was performed. Conclusions regarding environmental factors contributing to the fungal load were also postulated based on the typical climate of the facility (average air temperature, water temperature and humidity), temporal associations (the introduction of plant material in an avian exhibit), or general location characteristics (landlocked areas having higher organic debris compared with seaside sites).[2 8] Another study of aerosolised *Aspergillus* in an aviary recorded temperature and humidity at the time of sampling facilitating strong conclusions regarding those variables, however no other environmental data was evaluated.[12] A different study compared

fungal burden between indoor and outdoor sampling sites, however the dates at the various sampling locations were not the same and included different seasons in the comparison, influencing the validity of the conclusions.[8] Those authors concluded that further studies were needed for sampling over one or more years to assess the validity of their observations.[8] Therefore, objective data of environmental factors contributing to aerosolised fungus in zoological institutions and wildlife rehabilitation settings are limited and often highly speculative at present. Previous fungal air sampling studies have also lacked information regarding prevalence of the fungus in zoological parks.[2]

In this study, a two-year prospective cross-sectional evaluation of aerosolised *Aspergillus* species in indoor and outdoor sites at SeaWorld of California was conducted using standardised methodology. The objective of the study was to estimate the prevalence of and identify the most significant environmental factors associated with the presence of aerosolised *Aspergillus* by sampling 21 sites and documenting 22 environmental factors at the time of air sampling.

MATERIALS AND METHODS

Study site

This study was conducted at the SeaWorld of California zoological park in San Diego, California. The zoological park is located adjacent to the San Diego Bay, and houses a diverse collection of terrestrial and aquatic species in indoor and outdoor habitats across 172 acres. The park manages an extensive avian collection and their unique habitats include: indoor climate-controlled areas with chilled saltwater pools for five Antarctic penguin species and alcids, outdoor habitats with vegetation and chilled pools for the Magellanic penguins, and ambient pools for the Humboldt penguins. The aquatic bird rehabilitation centre consists of an indoor space with net-bottom pens, dry holding areas and a saltwater pool, while the outdoor space has multiple saltwater pools and vegetation. The Avian Center holds a large collection of non- aquatic birds in a variety of indoor and outdoor aviaries.

During March 12 to June 11, 2013, one major construction event occurred at the zoological park, the development of a public restroom. The distance between the construction sites and the two closest air sampling sites (No 15 and No 16) was 725 m (or approximately 0.45 miles). On sampling site No 15, the frequency of air samples that tested positive for *Aspergillosis* species was higher (5 of 8 air samples) during the four-month construction period, compared with the previous four months (1 of 7) or the later four months (1 of 8). On sampling site No 16, the frequency of air samples that tested positive for *Aspergillosis* species was lower (0 of 8) during the four-month construction period, compared with the previous four months (1 of 8) or the later four months (2 of 8).

Study design

Twenty-one sites at the zoological park were sampled every two weeks during two 12-month periods of (1) May 1, 2012 to April 23, 2013, and (2) May 16, 2013 to May 8, 2014. During each day of air sampling, 22 environmental factors were recorded. The frequency of investigated environmental variables was compared between samples classified as positive (≥10 colony-forming units (cfu)/m^3) or negative (0 cfu/m^3) for *Aspergillus* species in each 12-month period.

Collection of air samples and diagnosis of *Aspergillus* species

Designated park's indoor and outdoor sites (online supplementary appendix 1) were sampled using the portable Surface Air Systems (SAS) Super 180 Microbial Air Sampler using the following protocol (Bioscience International, Rockville, MD). First, the unit was prepared by removing the head of the device and the inner and outer surfaces were cleaned with 70 per cent isopropyl alcohol and a lint-free towelette. The air sampler was then positioned on the ground for 19 locations and placed 3 feet off of the ground at two locations, with the collection area facing upward. One hundred litres of air was aspirated at 180 l/minute through a sampling head with 401 0.75 mm holes. The laminar airflow was directed onto the Sabouraud dextrose agar plate, which was placed under the sampling head. After air sampling, the agar plate was removed and sealed immediately. One air sample was acquired at each testing location per sampling day, from which a single fungal plate was grown. The Sabouraud dextrose agar plates were incubated at 25°C–30°C under normal laboratory lighting conditions. Plates were checked every two days and counted under a dissecting microscope on the optimal day for fungal growth, which was typically the 10th day. Medical technologists used dichotomous keys based on microscopic and macroscopic features to identify the genera of fungi.[13] *Aspergillus* species fungal counts were recorded as cfu/m^3 of sampled air. To confirm the growth of *Aspergillus*, microscopic slide preparations of the collected fungi were made with tape impression procedures and the positive colonies were banked in a cryovial at −80°C. To confirm the air sampling unit itself was not contaminated, during each day of data collection a negative control sample was taken to verify that no fungal spores were grown.

Environmental variables

The following 22 environmental variables were recorded during each sampling: month and year of sampling, geographic location in the park (outdoor: yes/no); presence of a door connecting directly to the outside (outside access); animals present in the sampling location (animals present in area); breeding season for birds present at the sampling location (breeding season); presence of an active avian aspergillosis case (case of aspergillosis in area); direct or indirect sunlight present (sunlight exposure); presence of organic material

excluding wood (organic matter in area); wood material present (wood materials in area); substrate surface solid (asphalt, concrete, gunite), dirt, or snow (substrate type: concrete, dirt or snow); pool present; presence of moisture or standing water (substrate humid); ceiling or standing fans on (fans on: yes/no); construction within sampling area (construction in area); construction in the park (construction in park). Sampling locations were further separated into outdoor locations, indoor sites that possessed enhanced infection control (IC) measures such as HEPA filtration systems, daily cleaning procedures to remove organic material, and consistent cool ambient temperatures, and indoor sites without enhanced IC measures (infrastructure: outdoor, indoor with or without IC measures). During each day of data collection, the following weather parameters were recorded using the San Diego California regional weather report (The Weather Channel): precipitation (inches), wind speed (mph), wind direction (1–16 points on the wind compass), average temperature (°C) and humidity (per cent).

Data analysis

Environmental air samples were classified as positive or negative for *Aspergillus* species. Prevalence of air samples that tested positive for *Aspergillus* in each 12-month period was calculated by dividing the number of positive samples by the total number of samples tested. 95% CIs were calculated for each prevalence estimate using the software EpiTools (AusVet Animal Health Services, 2016). The null hypothesis that the annual proportion of air samples that tested positive for *Aspergillus* in each 12-month period was not different was tested by using a chi-squared test. In addition, among air samples classified as positive for *Aspergillus*, the distributions for the variable of *Aspergillus* fungal counts (cfu/m^3) were compared between the first and second study periods by using the Wilcoxon rank-sum test.

In each 12-month period, logistic regression analysis was used to model the odds of a positive finding of aerosolised *Aspergillus* spores as a function of investigated environmental factors.[14] Continuous variables (temperature, humidity, wind direction, wind speed, precipitation) were categorised into either two or three groups based on their frequency distributions (ie, median or 33rd percentiles). The variable for wind direction was grouped into onshore (directions 13–16) or along shore winds (directions 8–12), since the categories were deemed more relevant biologically. Adjacent categories of multinomial variables were collapsed whenever it was biologically justified and when those categories had similar stratum-specific odds for a positive finding of *Aspergillus* spores. Specifically, the 21 locations were collapsed into 'park geography', including west (locations 17–19), central (locations 12–16) and east (locations 1–11, 20, 21), and 'infrastructure', including outdoor (locations 6–8, 10, 11, 14, 17–19), indoor with IC measures (locations 1, 2, 4, 5, 12) and indoor without IC measures (locations 3, 9, 13, 15, 16, 20, 21). The variable for calendar months was collapsed from 12 into two categories (warm months of June to October: yes, no) because higher aerosolised fungus levels have previously been correlated with the warmer calendar months.[15] The new variable for warm months was defined as those months where the monthly average temperature is above the annual average temperature (17.7°C), based on National Oceanic Atmospheric Administration weather data.[16]

RESULTS

Prevalence of air samples that tested positive for *Aspergillus* species

Overall, the frequency of air samples that tested positive for *Aspergillus* was higher during the second 12-month period (110/525 or 21%; 95% CI 18% to 25%), compared with the first 12-month period (62/483 or 13%; 95% CI 10% to 16%) (p<0.01). In the first period, a high prevalence (ie, monthly prevalence ≥20 per cent) was observed in January 2013. In the second period, a high prevalence was observed in June, August and September 2013, as well as February, March and April 2014 (figure 1).

Among samples classified as positive, the distributions for the variable of *Aspergillus* fungal counts (cfu/m^3) were not different between the first study period (median=10; first quartile=10; third quartile=22) and the second study period (10; 10; 30) (p=0.86).

Environmental factors associated with air samples that tested positive for *Aspergillus* species

First 12-month period

In the univariable analysis, the variables for geographic location, infrastructure, pool present, substrate humid, fans on, organic matter in area, wood substrate in area, animals present in area, average temperature, average humidity, wind direction and precipitation had values of P≤0.20 (table 1). The variables for temperature, humidity, wind direction and precipitation were excluded because they were correlated (P<0.05) with the variable for warm months. The variable for animals present in the area was excluded because it was correlated with organic matter in the area. Finally, the variables for geographic location, pool present, substrate humid, fans on and organic matter in area were excluded because they were correlated with infrastructure (P<0.05).

In the multivariable analysis, the variables for infrastructure and warm months were retained in the final model (table 2). The odds of air samples testing positive for *Aspergillus* were 4.33 times higher in indoor sites without HEPA filtration systems (adjusted OR=4.33; 95% CI= 1.75 to 10.72) compared with indoor sites with HEPA filtration systems, after controlling for the variable of warm months. In addition, the odds of air samples testing positive for *Aspergillus* were 2.50 times higher (adjusted OR=2.50; 95% CI=1.00 to 6.25) in outdoor sites, compared with indoor sites with HEPA filtration systems, after controlling for the variable of

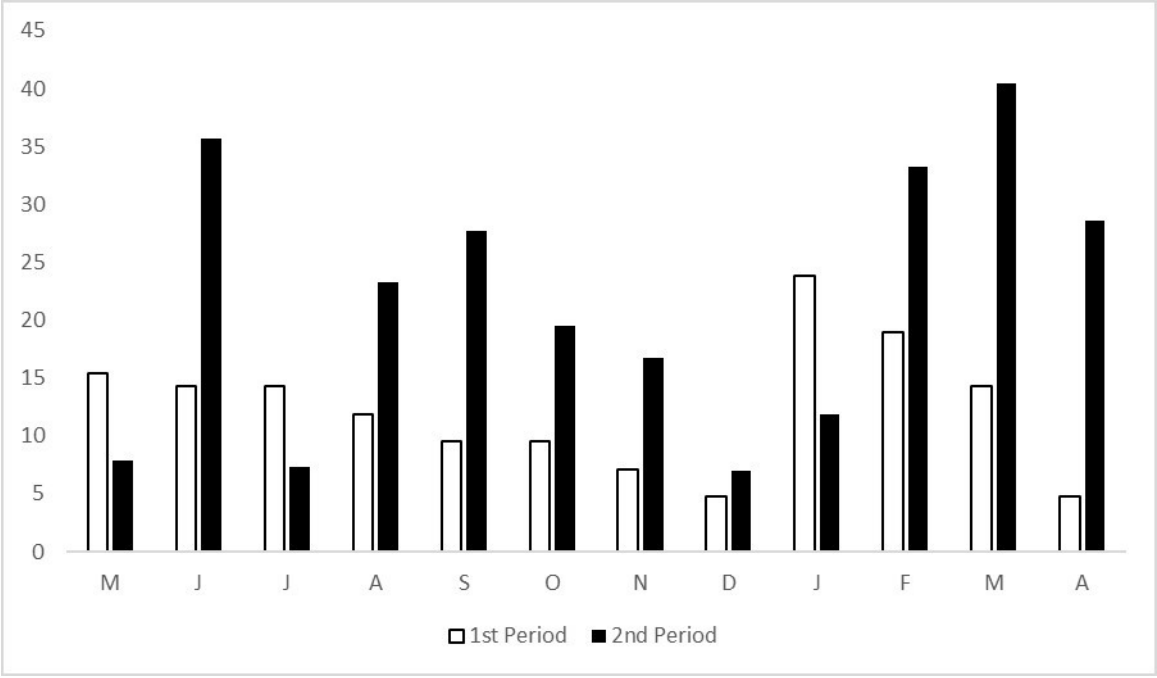

Figure 1 Monthly frequency of air samples that tested positive for *Aspergillus* species at the zoological park during May 2012 to April 2013 (first period) and May 2013 to April 2014 (second period).

warm months. The Hosmer-Lemeshow goodness-of-fit test (1.28; df=4; P=0.86) indicated there is no evidence of a poor fit for the data. In the final model, adding the variable for warmer months did not have an impact on the parameter estimate for infrastructure, indicating that its relationship with a positive finding of *Aspergillus* in air samples was not confounded by the variable of warmer months.

Second 12-month period

In the univariable analysis, the variables for infrastructure, outside access, fans on, organic matter in area, average temperature, average humidity, wind direction and precipitation had values of P≤0.20 (table 3). The variables for average temperatures, average humidity, wind direction and precipitation were excluded from the analysis because they were correlated (P<0.05) with the variable for warm months. In addition, the variables for outside access, fans on and organic matter in area were excluded from the analysis because they were correlated with the variable for infrastructure (P<0.05).

In the multivariable analysis, the variables for infrastructure and warm months were retained in the final model (table 4). The odds of air samples testing positive for *Aspergillus* were 5.19 times higher in indoor sites without HEPA filtration systems (adjusted OR=5.19; 95% CI=2.44 to 11.02) compared with indoor sites with HEPA filtration systems, after controlling for the variable of warm months. In addition, the odds of air samples testing positive for *Aspergillus* were 3.79 times higher (adjusted OR=3.79; 95% CI=1.80 to 8.01) in outdoor sites, compared with indoor sites with HEPA filtration systems, after controlling for the variable of warm months. The Hosmer-Lemeshow goodness-of-fit test (0.49; df=3; P=0.92) indicated there is no evidence of a poor fit for the data. The effect of air samples testing positive for *Aspergillus* was not confounded by warmer months.

DISCUSSION

This study is the first to objectively investigate the relationship between a broad range of environmental factors in a zoological setting as it relates to aerosolised *Aspergillus*. The main strengths of this study were that environmental data were collected at the same time air samples were collected allowing a cross-sectional examination of environmental factors and an unprecedented sample size was evaluated with rigorous standardised sampling methodology. Previous studies of aerosolised fungal burdens have lacked this degree of standardisation of air sampling methodology, sample size and objective documentation of environmental factors within each sampling site at the time of air sampling, minimising the validity of previous conclusions reported regarding environmental variables' relationship with aerosolised *Aspergillus*.[2 8]

This study provides epidemiological evidence demonstrating the use of HEPA filtration systems in combination with cool ambient temperatures to be the strongest determinant of aerosolised *Aspergillus* in the zoological environment among the 22 variables tested. Previous studies have speculated that humidity, ambient temperatures, organic debris and HEPA filtration systems, among others, to be significant factors influencing fungal burdens in zoological and avian rehabilitation settings.[2 8] The present study objectively evaluated such environmental factors in addition to 18 other variables and determined

Table 1 Univariable analysis for identification of environmental factors associated with a positive diagnosis of *Aspergillus* species in air samples during the first 12-month period: May 1, 2012 to April 23, 2013

		Negative	Positive			
Variable	**Category**	**N = 421 (100%)**	**N = 62 (100%)**	**Crude OR**	**95% CI**	**P value**
Park geography	West	65 (15)	4 (6)	1	Reference	NA
	Central	93 (22)	22 (34)	3.84	1.27, 11.64	0.01
	East	263 (63)	36 (60)	2.22	0.77, 6.45	0.14
Infrastructure	Indoor with HEPA and other infection control measures	109 (26)	6 (10)	1	Reference	NA
	Indoor without HEPA and other infection control measures	130 (31)	31 (50)	4.33	1.75, 10.72	< 0.01
	Outdoor	182 (43)	25 (40)	2.5	1.00, 6.25	0.05
Infrastructure	Indoor with HEPA and other infection control measures	109 (26)	6 (10)	1	Reference	NA
	Indoor without HEPA and other infection control measures	130 (31)	31 (50)	4.33	1.75, 10.72	< 0.01
	Outdoor	182 (31)	25 (40)	2.5	1.00, 6.25	0.05
Outdoor	No	239 (57)	37 (60)	1	Reference	NA
	Yes	182 (43)	25 (40)	0.89	0.52, 1.53	0.66
Outside access	No	124 (29)	14 (23)	1	Reference	NA
	Yes	297 (71)	48 (77)	1.43	0.76, 2.68	0.26
Sunlight exposure	No	161 (38)	23 (37)	1	Reference	NA
	Yes	260 (62)	39 (63)	1.05	0.61, 1.82	0.86
Pool present	No	134 (32)	27 (44)	1	Reference	NA
	Yes	287 (68)	35 (56)	0.61	0.35, 1.04	0.06
Substrate humid	No	294 (70)	38 (61)	1	Reference	NA
	Yes	127 (30)	24 (39)	1.46	0.84, 2.54	0.17
Fans on	No	250 (59)	49 (79)	1	Reference	NA
	Yes	171 (41)	13 (21)	0.39	0.20, 0.74	< 0.01
Substrate type	Concrete	360 (85)	54 (87)	1	Reference	NA
	Dirt	39 (9)	7 (11)	1.2	0.51, 2.81	0.68
	Snow	22 (6)	1 (2)	0.3	0.04, 2.24	0.24
Organic matter in area	No	325 (77)	37 (60)	1	Reference	NA
	Yes	96 (23)	25 (40)	2.21	1.31, 3.99	< 0.01
Wood substrate in area	No	292 (69)	35 (56)	1	Reference	NA
	Yes	129 (31)	27 (44)	1.75	1.02, 3.00	0.04
Construction in area	No	413 (98)	60 (97)	1	Reference	NA
	Yes	8 (2)	2 (3)	1.72	0.36, 8.29	0.49
Construction in park	No	310 (74)	47 (76)	1	Reference	NA
	Yes	111 (26)	15 (34)	0.89	0.48, 1.65	0.71
Warm months of June to October	No	237 (56)	36 (58)	1	Reference	NA
	Yes	184 (44)	26 (42)	0.93	0.54, 1.59	0.79
Ave temperature (C)	11.7 to 16.7	163 (39)	26 (42)	1.31	0.67, 2.53	0.42
	17.2 to 18.9	131 (31)	16 (29)	1	Reference	NA
	19.4 to 25.6	127 (30)	20 (29)	1.29	0.64, 2.59	0.47
Ave humidity (%)	29 to 64	159 (38)	30 (48)	1.46	0.72, 2.99	0.29
	65 to 71	93 (22)	12 (19)	1	Reference	NA
	72 to 81	169 (40)	20 (33)	0.92	0.43, 1.95	0.82

Continued

Table 1 Continued

Variable	Category	Negative N = 421 (100%)	Positive N = 62 (100%)	Crude OR	95% CI	P value
Wind direction	On shore	351 (83)	48 (67)	1	Reference	NA
	Along shore	70 (17)	14 (23)	1.46	0.77, 2.80	0.25
Wind speed (mph)	2 to 5	157 (37)	32 (52)	1	Reference	NA
	6 to 12	264 (63)	30 (48)	0.56	0.33, 0.95	0.03
Precipitation (inches)	0	462 (100)	62	1	Reference	NA
	0.01 to 0.03	0 (0)	0	ND	ND	ND
Animal variables	No	210 (50)	24 (39)	1	Reference	NA
	Yes	211 (50)	38 (61)	1.58	0.92, 2.71	0.1
Aspergillosis case in the area	No	407 (97)	61 (98)	1	Reference	NA
	Yes	13 (3)	1 (2)	0.48	0.06, 3.68	0.47
Breeding season	No	383 (91)	57 (92)	1	Reference	NA
	Yes	38 (9)	5 (8)	0.88	0.34, 2.33	0.8

*During June to October, the monthly average temperature is above the annual average temperature (17.7°C) and the monthly average rainfall is less than 13 mm (0.5 inches) in San Diego, CA.
HEPA, high-efficiency particulate air filtration system; NA, not applicable; ND, not determined.

HEPA filtration systems to be the most impactful on fungal prevalence. Avian exhibits at SeaWorld of California associated with low fungal prevalence (Polar Alcid Exhibit, Penguin Encounter, Polar Nursery and Avian Cool Room) included areas with HEPA filtration, were climate controlled for cool temperatures (0°C–15°C for penguins and alcids) and had daily cleaning procedures to remove accumulated organic material (including dust, feathers, food and fecal matter), which validate previous studies' findings where less rigorous methodology was used.[2 12] Indoor locations that were associated with higher aerosolised *Aspergillus* prevalence lacked HEPA filtration systems and had warm environmental temperatures (water monitor lizard exhibit, Polar Exhibit penguin examination area, Avian Center holding enclosure, aquatic bird rehabilitation facility (Oiled Wildlife Care Center, OWCC), and an indoor theatre that periodically possessed birds for educational presentations). While the utility of HEPA filtration systems is well established in the human literature and regularly implemented in hospital settings for aspergillosis prevention,[17] their role in zoological institutions and wildlife rehabilitation centres as the strongest determinant of fungal spore levels above all other control measures had not been as well established. HEPA filters are therefore not always utilised in zoological institutions and wildlife rehabilitation centres. For example, in a report of wild Magellanic penguin rehabilitation aspergillosis was deemed a limiting factor for bird rehabilitation, therefore efforts were made to increase air quality and reduce aerosolised fungal candida by cleaning equipment with an antiseptic, while no mention of air filtration was described and would have likely had a greater impact on fungal burden based on our findings.[10] The present study underscores the importance of such IC measures when evaluated in context of the diverse array of environmental factors previously proposed to have significance and objectively evaluated here, thereby supporting institutions' efforts in directing resources towards implementing air filtration systems in all indoor avian spaces.

In this study, the odds of air samples testing positive for *Aspergillus* were higher in indoor spaces without HEPA filtration systems and warmer ambient temperatures than that in outdoor sites, compared with indoor sites with HEPA filtration systems and cool ambient temperatures during the two study periods. This differs from

Table 2 Final logistic regression model for identification of environmental factors associated with a positive diagnosis of *Aspergillus* species in air samples during the first 12-month period: May 1, 2012 to April 23, 2013

Variable	Category	Adjusted OR	95% CI	P value
Infrastructure	Indoor with HEPA and other infection control measures	–	–	–
	Indoor without HEPA and other infection control measures	4.33	1.75 to 10.72	<0.01
	Outdoor	2.50	1.00 to 6.25	0.05
Warm months	No	–	–	–
	Yes	0.93	0.54 to 1.60	0.79

Hosmer-Lemeshow statistic=1.28; df=4; P=0.86.
HEPA, high-efficiency particulate air filtration system.

Table 3 Univariable analysis for identification of environmental factors associated with a positive diagnosis of *Aspergillus* species in air samples during the second 12-month period: May 16, 2013 to May 8, 2014

		Negative	Positive			
Variable	**Category**	**N = 415 (100%)**	**N = 110 (100%)**	**Crude OR**	**95% CI**	**p value**
Park geography	West	63 (15)	12 (11)	1	Reference	NA
	Central	97 (23)	28 (25)	1.52	0.72, 3.20	0.27
	East	255 (62)	70 (64)	1.44	0.74, 2.82	0.28
Infrastructure	Indoor with HEPA and other infection control measures	116 (28)	9 (8)	1	Reference	NA
	Indoor without HEPA and other infection control measures	125 (30)	50 (45)	5.16	2.43, 10.94	< 0.01
	Outdoor	174 (42)	51 (47)	3.78	1.79, 7.96	< 0.01
Outdoor	No	241 (58)	59 (54)	1	Reference	NA
	Yes	174 (42)	51 (46)	1.2	0.78, 1.83	0.4
Outside access	No	125 (30)	25 (23)	1	Reference	NA
	Yes	290 (70)	85 (77)	1.47	0.90, 2.15	0.12
Sunlight exposure	No	163 (39)	37 (34)	1	Reference	NA
	Yes	252 (61)	73 (66)	1.28	0.82, 1.98	0.27
Pool present	No	133 (32)	42 (38)	1	Reference	NA
	Yes	282 (68)	68 (62)	0.76	0.49, 1.18	0.22
Substrate humid	No	245 (59)	65 (59)	1	Reference	NA
	Yes	170 (61)	45 (41)	1	0.65, 1.53	0.99
Fans on	No	223 (54)	84 (76)	1	Reference	NA
	Yes	192 (46)	26 (34)	0.36	0.22, 0.58	< 0.01
Substrate type	Concrete	352 (85)	98 (89)	1	Reference	NA
	Dirt	38 (9)	12 (11)	1.13	0.57, 2.25	0.71
	Snow	25 (6)	0 (0)	ND	ND	ND
Organic matter in area	No	256 (62)	60 (55)	1	Reference	NA
	Yes	159 (38)	50 (45)	1.34	0.88, 2.05	0.17
Wood substrate in area	No	237 (57)	63 (57)	1	Reference	NA
	Yes	178 (43)	47 (43)	0.99	0.65, 1.52	0.97
Construction in area	No	410 (99)	109 (99)	1	Reference	NA
	Yes	5 (1)	1 (1)	0.75	0.09, 6.51	0.79
Construction in park	No	364 (88)	98 (89)	1	Reference	NA
	Yes	51 (12)	12 (11)	0.87	0.45, 1.70	0.69
Weather variables	No	242 (58)	73 (66)	1	Reference	NA
Warm months	Yes	173 (42)	37 (34)	0.71	0.46, 1.10	0.12
Ave temperature (C)	11.7 to 16.7	40 (10)	2 (1)	0.13	0.03, 0.54	< 0.01
	17.2 to 18.9	212 (51)	82 (75)	1	Reference	NA
	19.4 to 25.6	163 (39)	26 (24)	0.41	0.25, 0.67	< 0.01
Ave humidity (%)	29 to 64	124 (30)	44 (40)	1.99	1.21, 3.27	< 0.01
	65 to 71	196 (47)	35 (32)	1	Reference	NA
	72 to 81	95 (23)	31 (28)	1.83	1.06, 3.14	0.02
Wind direction	On shore	372 (90)	90 (82)	1	Reference	NA
	Along shore	43 (10)	20 (18)	1.92	1.08, 3.43	0.02
Wind speed (mph)	2 to 5	138 (33)	30 (17)	1	Reference	NA
	6 to 12	277 (67)	80 (73)	1.33	0.83, 2.12	0.23

Continued

Table 3 Continued

Variable	Category	Negative N = 415 (100%)	Positive N = 110 (100%)	Crude OR	95% CI	p value
Precipitation (inches)	0	318 (77)	81 (75)	1	Reference	NA
	0.01 to 0.03	77 (23)	28 (25)	1.43	0.87, 2.34	0.15
Animals present in area	No	206 (50)	49 (45)	1	Reference	NA
	Yes	209 (50)	61 (55)	1.23	0.80, 1.87	0.34
Aspergillosis cases in area	No	396 (95)	106 (96)	1	Reference	NA
	Yes	19 (5)	4 (4)	0.79	0.26, 2.36	0.66
Breeding season	No	347 (84)	95 (86)	1	Reference	NA
	Yes	68 (16)	15 (14)	0.81	0.44, 1.47	0.48

HEPA, high-efficiency particulate air filtration system; NA, not applicable; ND, not determined.

a previous study of a seabird rehabilitation centre in Northern California in which higher counts of *Aspergillus* were detected in air samples collected from outdoor sites when compared with indoors.[8] However, in that study the lack of samples evaluated was noted as a weakness in validity, with longer studies recommended such as the time frame used in the present study to evaluate such observations.[8] The finding in the present study is particularly significant for institutions that manage avians indoors in warm environmental conditions, and in potentially immunocompromised rehabilitating wildlife such as seabirds. Therefore, based on the results of this study, housing avians indoors without HEPA filtration systems may expose birds to higher fungal prevalence than if managed outdoors.

In this study, the variable for warm months (June to October) was not associated with *Aspergillus*, demonstrating a lack of seasonality with fungal prevalence. This is a unique finding since fungal burden seasonality has been widely reported in the literature at other geographical locations.[12 18] The finding in the current study can be explained by mild climate conditions in Southern California (San Diego) which can favour proliferation of the fungus throughout the year, compared with other regions in the USA which possess winters with colder temperatures and longer duration that are inhospitable for fungal growth and sporulation.[2 17 19] Furthermore, in previous studies in Alaska, Nevada, North Carolina and Washington, the burden of *Aspergillus* in air samples was higher during summer months compared with winter months.[2 12 17 18] In those studies of human buildings across the USA as well as a North Carolina aviary, indoor fungal spore loads followed seasonal outdoor variation and air temperature trends, differing from the present study.[12 18] The lack of fungal seasonality identified at this institution underscores the importance of year-round fungal surveillance at zoological institutions with mild climates.

An additional strength of the present study when compared with previous avian air sampling studies is that non-animal exhibit areas were sampled for *Aspergillus* to provide data on fungal prevalence around the zoological park.[1 2] These data are particularly useful considering *Aspergillus* susceptible animals are commonly taken to areas outside their regular exhibits for veterinary exams, temporary holding areas, guest interactions, educational presentations or behavioural enrichment. Through regular air sampling of a variety of geographical locations across the zoological park (21 sites total), specific areas were elucidated as high-fungal prevalence regions. For example, when locations in the public walkway (Environmental East and West) were sampled, significantly higher fungal prevalence in the East site was found year-round. These data can be utilised to facilitate evidence based management strategies to mitigate fungal risk. Furthermore, in some institutions African penguins or flamingos are guided on walks through public areas

Table 4 Final logistic regression model for identification of environmental factors associated with a positive diagnosis of *Aspergillus* species in air samples during the second 12-month period: May 16, 2013 to May 8, 2014

Variable	Category	Adjusted OR	95% CI	P value
Infrastructure	Indoor with HEPA and other infection control measures	–	–	–
	Indoor without HEPA and other infection control measures	5.19	2.44 to 11.02	<0.01
	Outdoor	3.79	1.80 to 8.01	<0.01
Warm months	No	–	–	–
	Yes	0.70	0.45 to 1.10	0.11

Hosmer-Lemeshow statistic=0.49; df=3; P=0.92.
HEPA, high-efficiency particulate air filtration system.

for behavioural enrichment and public education, which could be modified based on air sampling data to mitigate fungal exposure. As such, this manuscript offers a tool for other zoological institutions to employ similar fungal surveillance to identify potential areas of concern with the intention of improving the care of managed species.

In this study, the prevalence of aerosolised *Aspergillus* was higher during the second period (21 per cent) compared with the first period (13 per cent); however, among samples that tested positive for *Aspergillus* fungal counts were similar in both periods (10–30 cfu/m^3 v 10–22 cfu/m^3). Climate data for the San Diego area and the construction event in the first period did not explain the higher frequency of positive air samples observed in the second period. Rainfall was lower during the second period (135 mm or 5.32 inches), compared with the first period (159 mm or 6.27 inches) (Western Regional Climate Center, Reno, NV). The major construction at the zoological park (a public restroom) during the first period was associated with a higher frequency of aerosolised *Aspergillus* species during the four months of construction on one of the two sampling sites that were closest in location. This contrasts previous studies that have reported an association between construction and increased fungal counts.[20] The study results suggest that burden of aerosolised *Aspergillus* is not related to ambient temperature and rainfall, as well as construction events located at least 0.45 miles at the zoological park. Rather, these findings suggest that the environmental factors in direct proximity to the sampling site or microenvironment may have a greater effect on aerosolised fungal prevalence than weather or major construction events removed great distances. It is possible that volumetric air sampling may not detect *Aspergillus* spores that have settled to the ground following construction events, where fungal swabs of surfaces may positively detect the fungus.[21] However, in a previous study of aviary *Aspergillus* gravitometric and swab sampling did not yield fungi, while volumetric air sampling was reported to be the most sensitive methodology for detecting fungal spores.[1]

This study has several limitations. Recovery rates of aerosolised fungus can be higher when using the RCS Plus sampler (Biotest Diagnostics, Denville, NJ) compared with the SAS Super 180 air sampler, which was used in this study.[21] Thus, it is possible the burden of *Aspergillus* could have been higher than that observed at SeaWorld of California during the study periods. However, the frequency of sample collection (every two weeks for 24 consecutive months) is adequate to properly examine the monthly burden of *Aspergillus* in air samples. Fungal identification to the species level was not pursued in the current study since previous studies have evaluated such data in zoological settings.[2]

In conclusion, of the 22 environmental factors evaluated in this study, HEPA filtration systems in conjunction with cool ambient temperatures were found to be the most significant environmental factors associated with limiting aerosolised *Aspergillus* in the zoological setting. Burden of airborne *Aspergillus* can be higher in indoor sites without HEPA filtration systems and warm ambient temperatures when compared with outdoor sites. These data are particularly significant for the management of indoor avian exhibits which are common in zoological facilities and avian rehabilitation settings, for which HEPA filtration systems are not exclusively utilised. Year-round fungal surveillance employed at this zoological institution allowed for improved monitoring of fungal prevalence and elucidated a lack of seasonality in Southern California, facilitating optimisation of management strategies. This manuscript offers a tool for institutions to employ similar fungal surveillance practices to mitigate fungal exposure and improve the care of susceptible wildlife and zoological species.

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