

# Aspergillosis in a colony of Humboldt penguins (*Spheniscus humboldti*) under managed care: a clinical and environmental investigation in a French zoological park

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## Abstract

Aspergillosis is pervasive in bird populations, especially those under human care. Its management can be critically impacted by exposure to high levels of conidia and by resistance to azole drugs. The fungal contamination in the environment of a Humboldt penguin (*Spheniscus humboldti*) group, housed in a French zoological park next to numerous large crop fields, was assessed through three serial sessions of surface sampling in nests, in 2018–20: all isolates were counted and characterized by sequencing. When identified as *Aspergillus fumigatus*, they were systematically screened for resistance mutations in the *cyp51A* gene and tested for minimal inhibitory concentrations (MICs) determination. At the same time, the clinical incidence of aspergillosis was evaluated in the penguin population by the means of systematic necropsy and mycological investigations. A microsatellite-based analysis tracked the circulation of *A. fumigatus* strains. Environmental investigations highlighted the substantial increase of the fungal load during the summer season (> 12-fold vs. the other timepoints) and a large overrepresentation of species belonging to the *Aspergillus* section *Fumigati*, ranging from 22.7 to 94.6% relative prevalence. Only one cryptic species was detected (*A. nishimurae*), and one isolate exhibited G138S resistance mutation with elevated MICs. The overall incidence of aspergillosis was measured at ~3.4% case-years, and mostly in juveniles. The analysis of microsatellite polymorphism revealed a high level of genetic diversity among *A. fumigatus* clinical isolates. In contrast, one environmental strain appeared largely overrepresented during the summer sampling session. In all, the rural location of the zoo did not influence the emergence of resistant strains.

## Lay summary

- The incidence of aspergillosis in the penguin population of a French zoo was found elevated, mostly in juveniles.
- The fungal contamination was high in the surrounding environment, especially at the end of summer.
- Globally, a great level of genetic diversity was observed, except for one clonal strain.

**Keywords:** *Aspergillus fumigatus*, birds, Humboldt penguins, azole-resistance, *cyp51A*, microsatellite genotyping, *Aspergillus nishimurae*, MICs

## Introduction

*Aspergillus* spp. are environmental saprophytic molds that are responsible for opportunistic infection of the respiratory apparatus, or other tissues, which is referred to as aspergillosis. The latter can occur in a wide range of invertebrate or vertebrate animal species, ranging from corals to

humans, and including a large panel of avian species, especially burrowing birds and penguins.<sup>1</sup> Mostly found in humid and dusty soils, *Aspergillus* conidia are small and volatile. They may be vehicled by water or spread by air so that aspergillosis is usually acquired by inhalational route in birds.<sup>2</sup>

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Although it has been only rarely described in wild penguins,<sup>3–5</sup> aspergillosis is very frequently observed in Sphenisciformes birds living in zoological parks, aquaria, or rehabilitation centers.<sup>1,6–8</sup> In such facilities, its relative prevalence can reach 20–27% in some colonies,<sup>9,10</sup> and its prognosis is generally poor with mortality rates ranging up to 50%.<sup>10</sup> Local exposure to high concentrations of *Aspergillus* conidia and/or presence of certain transient or chronic immune deficiencies of the host are assumed to enhance the susceptibility to aspergillosis.<sup>11</sup> While it is well-known that environmental contamination can be seasonal and influenced by climatic parameters,<sup>12</sup> some authors suggested that the onset of clinical aspergillosis is probably age- and stress-dependent. Indeed in birds, the disease seems more commonly diagnosed in juveniles than adults,<sup>7,13</sup> and is more frequent when exposed to stressors (e.g., disturbance while on exhibition, transfers between institutions, ...) or to poor environmental conditions (e.g., bad ventilation, elevated levels of ammonia, inadequate nutritional input...)<sup>6,7</sup>

As is the case in humans, the clinical signs of aspergillosis in birds are not specific (e.g., in penguins: lethargy, anorexia and weight loss, open mouth-breathing and dyspnea, coughing and altered vocalization, self-isolation),<sup>6</sup> and diagnostic tools are not highly reliable.<sup>8,14</sup> Therefore, a definitive diagnosis is often obtained upon necropsy examination.<sup>15,16</sup> In previous works focusing on avian aspergillosis, the species *A. fumigatus* was involved in 95% cases.<sup>16</sup> In infected birds, empirical or curative treatment of aspergillosis usually relies on azole drug-based therapy, e.g., voriconazole or itraconazole.<sup>11</sup> In contrast to the intensive poultry farming practices where azole drugs may be preventively-distributed, the use of systematic antifungal mass prophylaxis is uncommon in penguin colonies. Indeed, it is usually restricted to susceptible individuals only, e.g., following transfers, during hospitalization, and/or antibiotic treatments.<sup>6,13</sup>

Since the end of the 20th century, azole drugs resistance in *A. fumigatus* strains has been considered as an emerging health problem to be taken into account for the management of both human and animal antifungal treatment. In a recent multicenter large international study, its prevalence was found at 3.2% in clinical strains retrieved from human samples.<sup>17</sup> Acquired resistance is generally—but not exclusively—associated with nucleotide mutation(s) inside the *cyp51A* gene encoding for lanosterol-C14- $\alpha$ -demethylase, the target protein of azole drugs. In some countries, massive use of azoles for the purposes of agricultural/floricultural practices or for the sawmill industry was demonstrated to be responsible for the occurrence of resistance mutation(s) in *A. fumigatus cyp51A* gene.<sup>18–20</sup>

The objective of the present study was to assess the impact of the environmental contamination on the clinical incidence of aspergillosis in penguins managed under human care. The ZooParc de Beauval was chosen for the investigations because this zoological facility hosts a large colony of Humboldt penguins (*Spheniscus humboldti*) and it is geographically located in the rural countryside of the Loir-et-Cher district, France.<sup>20</sup> We evaluated the level of fungal colonization inside the penguin habitat and the total prevalence of resistant strains among all *A. fumigatus* isolates collected. Meantime, we assessed the clinical incidence of aspergillosis within the penguin population and tracked the circulation of *A. fumigatus* strains within the zoological park.

## Materials and methods

### Description of the study population

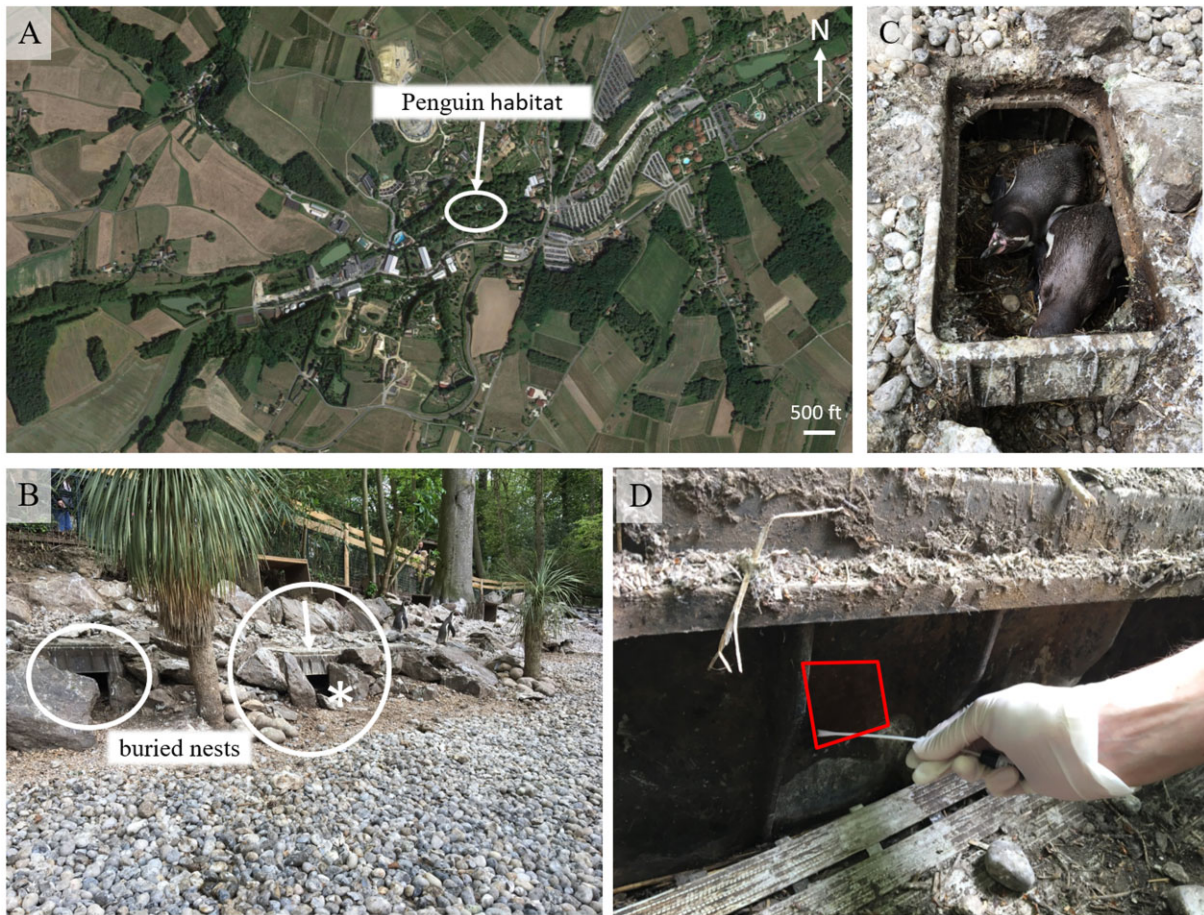
The study was conducted during the 2018–20 period at the ZooParc de Beauval (47°14'49.2"N – 1°20'56.1"E). This facility of 40 hectares hosts about 35 000 animals and 800 distinct species. The maximal population size of the Humboldt penguin group in ZooParc de Beauval was counted at 192 individuals in 2018, 185 in 2019, and 148 in 2020, including adults and chicks.

Built in 2007, the penguin habitat is entirely outdoors (Fig. 1A). It includes a 600 m<sup>3</sup> chlorine-treated swimming pool, an artificial beach, and above it, a 400 m<sup>2</sup> wooded area where several nests are set (Figs. 1B and C). Penguin nests are either composed of either buried 0.2 m<sup>3</sup> solid boxes (n = 24) or mobile overhead open constructions (n = 4–10). As the latter are sometimes changing in number and location over time (depending on the penguins' habits), they were not considered in the present study.

### Environmental investigations for *Aspergillus* contamination within the penguin habitat

Three periods of environmental sampling had been scheduled for 23 April 2018, 3 December 2018, and 16 September 2019. At each timepoint, the 24 buried nests were systematically sampled (except for the autumn campaign in 2019: only 22, because two nests had been deserted, totally fulfilled with soil and detritus, and thus were not exploitable), by applying wet swabs onto a 20 cm<sup>2</sup> surface of the floor and the walls, in duplicates (Fig. 1D). Then, 1 ml sterile saline was introduced into every swab container. Following a 1 min shaking step with Vortex® (LMS, Tokyo, Japan), each suspension sample was seeded onto a malt extract agar plate (Day-0, d0) that was subsequently incubated at 35°C. A first visual checkup of the fungal growth was programmed at 72 h (d3) and observed daily until d7. When fungal colonies were detected (between d3 and d7), they were individually numbered and identified. For the quantitative approach, an individual average of the number of colonies, expressed in mean colony-forming units (CFU), was calculated for each nest by compiling the four measures obtained from the four different swabbed surfaces. Thereafter, a global average of the fungal burden was calculated for every sampling session. For the qualitative approach, every distinct colony was taken into consideration and thoroughly identified. Characterization at the genus (or section) level was first addressed on the basis of macro- and microscopic criteria.<sup>21</sup> Thereafter, the species identification was confirmed by molecular biology based on the sequencing of the internal transcribed sequences of the 5.8S ribosomal rDNA (ITS1/2) and/or beta-tubulin ( $\beta$ -tubulin) regions.<sup>22</sup> For this purpose, conidia were harvested at the colony surface, after subculture on a Sabouraud-chloramphenicol agar slant tube incubated at 30°C, and placed into Precellys® tubes (Bertin Technologies, Montigny-le-Bretonneux, France). They were grinded by bead beating in the Cryolys/Precellys 24® device (Bertin Technologies, Montigny-le-Bretonneux, France). Nucleic acid of each ground was extracted using QiAmp DNA Mini Kit® extraction method (Qiagen, Hilden, Germany). Each DNA extract was then amplified in the thermocycler T3000® (Biometra, Göttingen, Germany) using the primers referenced in Supplementary Material 1,<sup>22</sup> and the quality of the amplicons was checked in 1% agarose gel. Nucleotide sequences were subsequently obtained with the BigDye





**Figure 1.** General presentation of the Humboldt penguin exhibit in ZooParc de Beauval. (A) Satellite photography of the zoological park with indication of the central location of the penguin habitat; (B) Picture of the wooded land of the penguin habitat where artificial buried nests are settled (the entrance for penguins is indicated with a white arrow); (C) Picture of a couple of Humboldt penguin hosted in its nest that had been opened by its maintenance lid; (D) Example of the swabbing procedure of the nest wall (the red parallelepiped indicates the outlines of the 20 cm<sup>2</sup> surface that was sampled). Each surface was systematically sampled in duplicate for every nest; the same procedure was applied to the nest floor.

Terminator® version 1.1 kit (Life Technologies SAS, Saint-Aubin, France) and the Applied 3130 Genetic Analyser® instrument (ThermoFisher Scientific, Montigny-le-Bretonneux, France), and analyzed using CodonCode Aligner 5.1.5® software (CodonCode Corporation, Centerville, MA, U.S.A.). Identification was eventually completed upon request of GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>). If BLAST-score < 1000 and/or query cover < 99% and/or percentage of identity < 98%, the Institut Pasteur Fungibank (<https://fungibank.pasteur.fr/>) was subsequently queried.

In order to screen for potential resistance mutation(s), all the strains, identified as *A. fumigatus sensu stricto*, were sequenced in the *cyp51A* gene using four sets of primers to detect the most common mutations (in amino acids G54, L98H, Y121F/T289A, G138, M220, and G448), and in a 234 pb region of its promoter, looking for a 34- or 46-bp tandem duplication sequence (TR34 or TR46, between positions –288 and –322 or –334) (Supplementary Material 1).<sup>23–25</sup> In the same time, minimal inhibitory concentrations (MICs) were determined, according to the broth microdilution method using the Micronaut-AM® kit (Biocentric, Bandol, France).<sup>26</sup> For this purpose, conidia were collected from a 48 h spore-forming subculture on Sabouraud-chloramphenicol agar tube and resuspended into 0.1% sterile saline Tween 20® polysorbate so-

lution to reach a 0.5 McFarland-turbidity suspension. Then, 600 µl were transferred into the Roswell Park Memorial Institute (RPMI) 1640 tube provided by the manufacturer. After homogenization, 100 µl of the mix were distributed per well in the Micronaut-AM® plate, and incubated at 37°C. MICs were optically evaluated for azoles (voriconazole, itraconazole, and posaconazole), but also for polyenes (amphotericin B) and echinocandins (caspofungin, micafungin, and anidulafungin), at 24 h and 48 h, according to the supplier's recommendations. Results were interpreted on the basis of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints.<sup>27</sup>

### Assessment of the clinical incidence of aspergillosis in penguins

Incidence of aspergillosis was assessed in the Humboldt penguin population of ZooParc de Beauval from 2018 to 2020. Laboratory investigations were carried out as soon as suggestive clinical signs and/or deaths were reported. During exploratory coelioscopy, the tissues of the trachea were sampled. During necropsy, the lungs or the surface of air sacs were systematically biopsied.

For the histology-based diagnostic approach, biopsied tissues were microscopically observed using hematoxylin and eosin stain and Grocott–Gomori’s methenamine silver staining, and examined for intra-tissular filaments compatible with *Aspergillus*-like morphological features.<sup>21</sup> For the mycology-based diagnostic approach, the microscopic direct examination was performed in biopsy affixing on a glass slide using calcofluor white impregnation.<sup>28</sup> Concomitantly, clinical specimens were sterilely cut and nucleic acids were extracted directly from them, being tested by quantitative polymerase chain reaction (qPCR), as reported previously<sup>29,30</sup> (Supplementary Material 1). Results of qPCR were considered negative when the *A. fumigatus* 28S ribosomal rDNA burden was < 1 fg.<sup>30</sup> An internal control for checking inhibition (Inhibition control plasmid®, Diagenode, Liège, Belgium), and positive/negative home-made controls were systematically used to assert validation of all the qPCR runs. Every biological sample was tested in duplicate to avoid false-positive or –negative, and re-rested, if necessary. Same procedures were applied to any other birds that were suspected to suffer from aspergillosis, during the study period.

At the same time, clinical specimens were seeded on two Sabouraud-chloramphenicol agar slants (BBL®, Becton, Franklin Lakes, NJ, U.S.A.), incubated at 30°C and 35°C for *in vitro* cultures for 7 days. In case of fungal growth, phenotypic identification of each colony was completed according to macro- and microscopic observation.<sup>21</sup> Confirmation was obtained by DNA sequencing as described previously.<sup>22</sup> Furthermore, *cyp51A* genes were sequenced and MICs were measured for every *Aspergillus* clinical isolate,<sup>31</sup> as described above.<sup>26</sup>

For the specific purpose of this study and since no consensus classification exists for animals,<sup>32</sup> the diagnosis of aspergillosis was classified as probable if mycological culture, and/or qPCR were positive, or as proven as soon as the direct examination (by either histology or calcofluor microscopic observation) was positive. All other situations were considered as cases of non-infection or only possible infection and thus were not retained for the calculation of the aspergillosis incidence.

### Circulation of *A. fumigatus* isolates

*Aspergillus fumigatus* isolates obtained from environmental sampling and those retrieved from diseased penguins were analyzed by the means of microsatellite polymorphism genotyping in order to decipher their circulation within ZooParc de Beauval. Isolates from other bird species were included in this genotyping approach. Indeed during the study period, 23 birds (out of > 2000 birds housed at the zoological park), belonging to miscellaneous species, were suspected of aspergillosis, and 14 were actually diagnosed with: two vinaceous-breasted Amazon parrots (*Amazona vinacea*), one scarlet ibis (*Eudocimus ruber*), one great blue turaco (*Corythaeola cristata*), one lady Ross’s turaco (*Musophaga rossae*), one kea parrot (*Nestor notabilis*), one sulphur-crested cockatoo (*Cacatua galerita*), two great white pelicans (*Pelecanus onocrotalus*), one great grey owl (*Strix nebulosa*), one demoiselle crane (*Anthropoides virgo*), one griffon vulture (*Gyps fulvus*), one greater flamingo (*Phoenicopterus roseus*), and one Australia wood duck (*Chenonetta jubata*).

The DNA of each isolate was first quantified with Nanodrop One® (ThermoFisher Scientific, Montigny-le-Bretonneux, France). Three different multiple polymerase

chain reactions (PCRs) were then performed, each containing three pairs of primers that amplified the short tandem repeats (STR) of two-to-four nucleotides in nine microsatellite markers: 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B, and 4C.<sup>33</sup> We used similar sequences and 5'-fluorescent labels of primers than those previously described,<sup>33</sup> except for primers 2C, 3C and 4C that were tagged with a different label, i.e., NED instead of TET (NED and TET are yellow fluorescent dyes). The presence of PCR products was verified in a 2.5% agarose gel. Thereafter, 1 µl of each PCR product, diluted 1/10, was mixed with 0.25 µl of 400 HD ROX® size standard ladder and 8.75 µl of Hi-Di® formamide (ThermoFisher Scientific, Montigny-le-Bretonneux, France), and then run in the SeqStudio Genetic Analyzer® (ThermoFisher Scientific, Montigny-le-Bretonneux, France). The injection time was programmed on 7 sec. The DS-30 matrix Standard Kit® (Dye Set D, ThermoFisher Scientific, Montigny-le-Bretonneux, France) was used, which contained four oligonucleotides labeled with 6-FAM, HEX, NED, ROX, generating a multicomponent matrix required for the analysis of labeled fragments in SeqStudio® (ThermoFisher Scientific, Montigny-le-Bretonneux, France). The fluorescent PCR peaks (as ionic fragments), separated by the capillary electrophoresis of the SeqStudio®, were analyzed with the MSA® software (ThermoFisher Scientific, Montigny-le-Bretonneux, France). The relative fluorescent units (RFU) *vs.* base pairs curves were analyzed considering the range of 500–25 000 RFU as optimal. The size of each microsatellite fragment was measured to determine the number of repetitions according to de Valk et al.<sup>33</sup> Phylogenetic relationships between isolates were studied, generating minimum spanning trees based on goeBURST algorithm on PHYLOViZ® v2.0 (<https://www.phyloviz.net/>).<sup>34</sup>

### Ethics

No animal was anesthetized, euthanized, or sacrificed for the specific research purposes of this study. All the clinical specimens were collected after routine veterinary assessments. Research investigations were approved by Beauval Nature research program in April 2018.

### Statistics

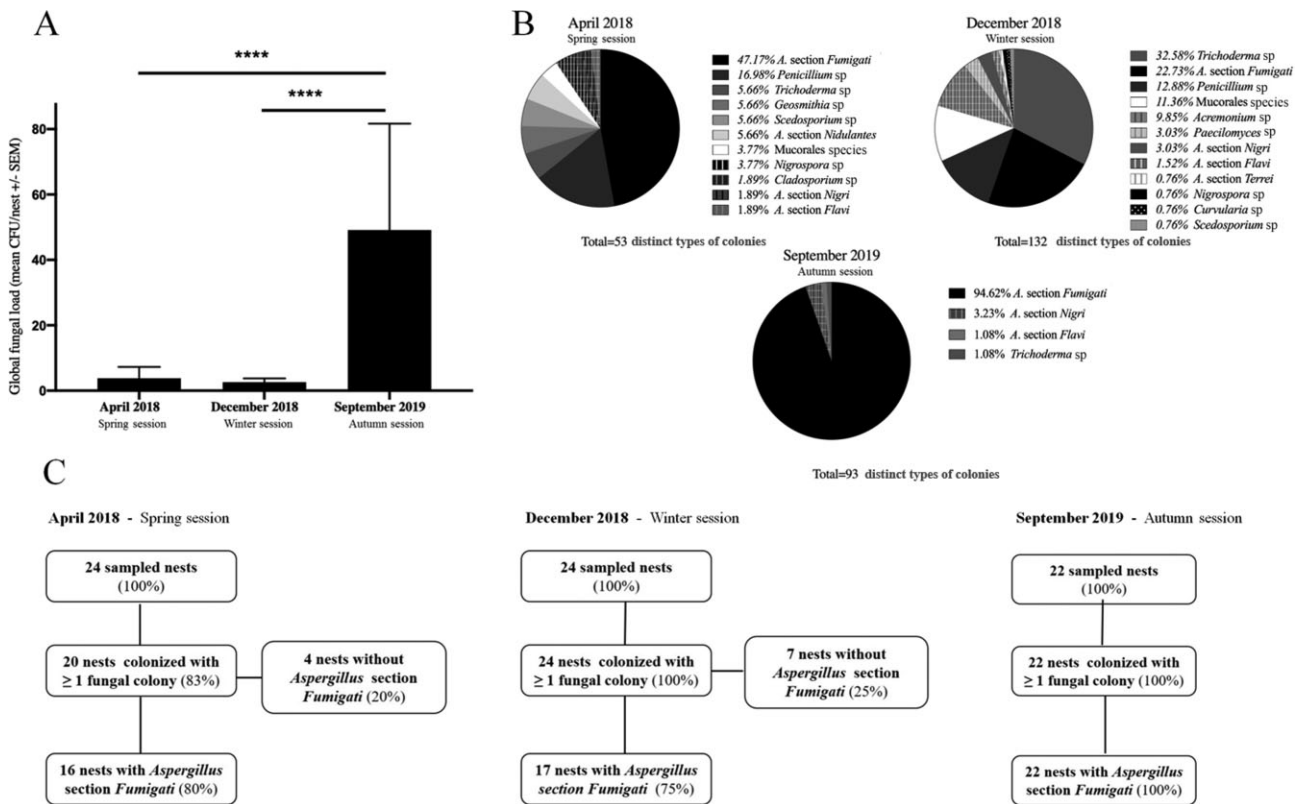
Quantitative results were expressed in mean and median values, and in standard error of the mean (SEM). Relative representations were expressed in percentages and in ratios. Statistical analysis was performed to achieve the global comparison and the two-by-two comparison between each set of environmental sampling about the fungal load, using parametric ANOVA and Tukey’s multiple comparison tests, respectively (the Bartlett’s test was beforehand used to test homoscedasticity). The significance level was set at  $P < .05$ .

## Results

### Environmental investigations for *Aspergillus* contamination within the penguin habitat

Cultures obtained from environmental sampling revealed a mean CFU/nest of  $3.8 \pm 3.3$  in April 2018,  $2.6 \pm 1.2$  in December 2018, and  $49.1 \pm 32.5$  in September 2019 (Fig. 2A). Fungal load appeared significantly 12.9- and 18.8-fold higher in September 2019 *vs.* the other dates ( $P < .0001$ ), respectively, but no difference was observed between the two timepoints of 2018 ( $P = .9716$ ).





**Figure 2.** Environmental investigations in the Humboldt penguin habitat of ZooParc de Beauval. (A) Bar graph showing the global average of the fungal burden in April 2018 (sampling session No. 1), December 2018 (sampling session No. 2), and September 2019 (sampling session No. 3); (B) Relative representation of the fungal species isolated during the three different sampling campaigns; (C) Flowcharts of the three different sampling sessions focusing on the *Aspergillus fumigatus* retrieving in the penguin nests. \*\*\*\*,  $P < .0001$ ; NS, Not significant

In April 2018, 80% of the nests (20/24) were colonized with at least one mold species, while 100% were colonized in December 2018 (24/24) and in September 2019 (22/22). *Aspergillus* section *Fumigati*, *Penicillium* spp., and *Trichoderma* spp. were the most frequently-isolated genera (Fig. 2B). *Aspergillus* section *Fumigati* was largely represented in each sampling session, given that it was isolated from 67% of nests in April 2018 (and from 80% of those colonized), 75% in December 2018, and 100% in September 2019 (Fig. 2C). Isolates belonging to section *Fumigati* were identified as *A. fumigatus sensu stricto* in 100% isolates in April 2018 (25/25), 97% in December 2018 (29/30), and 100% in September 2019 (88/88) (GenBank accession numbers: ON360074-360 141 and ON360447-360 340). Only one isolate collected in December 2018 from nest No. 18 was finally identified as *A. nishimurae* (GenBank accession number: MW822043). No mutations (G54, L98H, Y121F/T289A, G138, M220, G448, duplication TR34 or TR46) were found in the *cyp51A* gene (ON360142-360 270 and ON360448-360 672), except for one isolate recovered from nest No. 13 during the winter session 2018. In codon 138, a glycine amino acid was substituted with a serine (MW822042), because of a GGC→AGC mutation. For this isolate, MICs were  $> 4 \mu\text{g/ml}$  and  $0.25 \mu\text{g/ml}$  for itraconazole and voriconazole, respectively. The other environmental strains were found with low MICs (Table 1).

### Assessment of the clinical incidence of aspergillosis in penguins

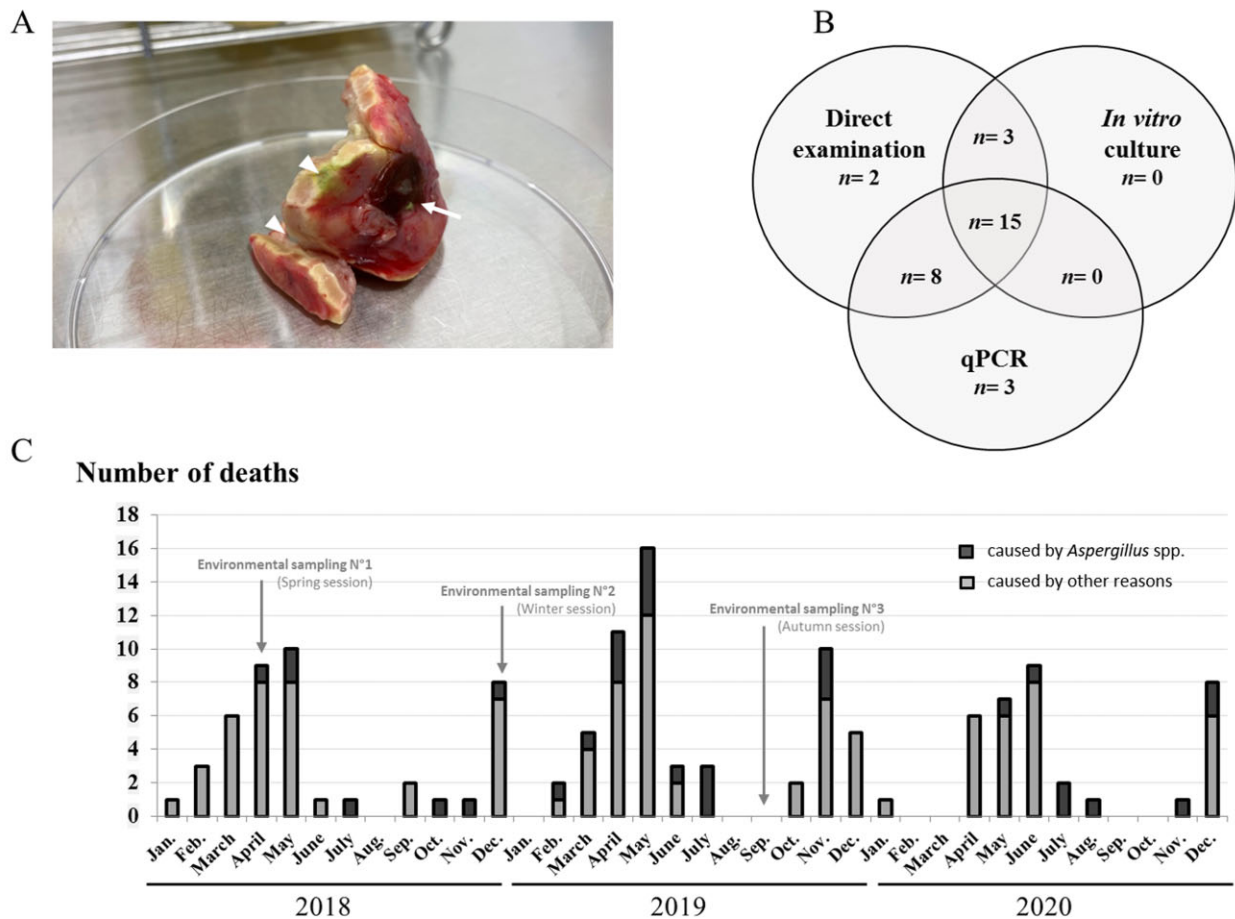
Within the colony of Humboldt penguins in ZooParc de Beauval, a total of 138 deaths, were reported during the study

**Table 1.** Antifungal susceptibility assessment of the *A. fumigatus* isolates retrieved from the environment in penguin nests. Results are expressed in median  $\mu\text{g/ml}$ . The estimation was conducted by the means of broth microdilution method and according to the EUCAST clinical breakpoints.

Antifungal drug	Median MIC ( $\mu\text{g/ml}$ ) [95%IQ]	Clinical breakpoints EUCAST ( $\mu\text{g/ml}$ )	
		S ≤	R >
Amphotericin B	0.125 [0.062–0.50]	1	1
Voriconazole	0.125 [0.031–0.25]	1	1
Itraconazole	0.25 [0.125–0.5]	1	1
Posaconazole	0.031 [0.015–0.125]	0.125	0.25
Micafungin	0.015 [0.002–0.031]	IE	IE
Anidulafungin	0.016 [0.002–0.031]	IE	IE
Caspofungin	0.031 [0.015–0.062]	IE	IE

IE, insufficient evidence; 95%IQ, interquartile range; MIC, minimal inhibitory concentration;  $\mu\text{g/ml}$ , micrograms per milliliter; R, resistant; S, susceptible.

period: 52 in 2018, 51 in 2019, and 35 in 2020. Overall, 78 carcasses were examined by histopathology and/or mycological investigations, including 14 penguins in 2018, 40 in 2019, and 24 in 2020 (the remainder 60 deaths were either not exploitable [e.g., cadavers totally autolyzed], or due to obvious alternative causes [e.g., drowning, trampling and crushing in the nest, lack of parental care and abandonment] and did not require specific diagnostic procedures). Overall, during the study, 22.5% of deceased penguins presented laboratory evidence of aspergillosis, i.e., 13.5% in

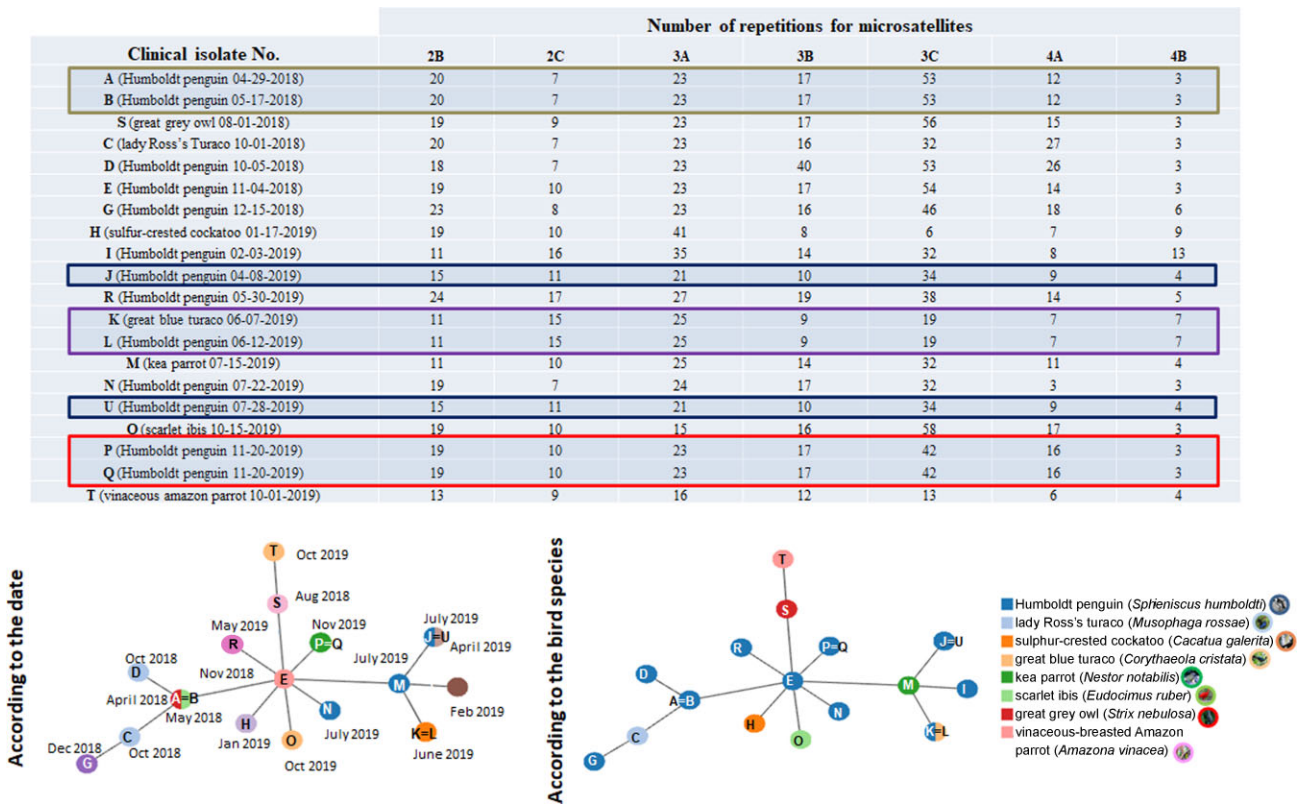


**Figure 3.** Detailed laboratory procedures for the diagnosis of aspergillosis in clinical specimens obtained from the Humboldt penguins of ZooParc de Beauval. (A) Gross observation of an infected lung lobe biopsied during necropsy. Hemorrhagic lesion is pointed with a white arrow. Invasive fungal foci are indicated with plain darts; (B) Contribution of the different laboratory approaches to the completion of the diagnosis of aspergillosis in diseased penguins, according to Venn diagram representation (the designation so called ‘microscopic examination’ encompasses findings based on both histology and direct fluorescence); (C) Incident number of aspergillosis cases in the Humboldt penguin colony of ZooParc de Beauval over the study period (January 1st, 2018–December 31st, 2020). The entire bars represent the crude overall mortality per month, expressed in absolute number, whereas the black top represents only the cases directly attributable to aspergillosis (dates of the environmental sampling campaigns are also indicated by dark arrows).

2018 (7/52 deaths), 31.4% in 2019 (16/51), and 22.9% in 2020 (8/35). For 28 cases, the diagnosis met the criteria that define proven aspergillosis (Fig. 3A and Supplementary Material 1), and for three others, the diagnosis of probable aspergillosis. For four penguins, another cause of death might be associated with aspergillosis: lack of maternal care, blood parasite disease, bacterial enteritis, and severe aortic atherosclerosis.

In detail, the direct microscopic examination, based either on histology staining or calcofluor white fluorescence (or both associated), exhibited an elevated sensitivity at 90.3%. The *in vitro* culture yielded a 58.1% sensitivity rate among the diseased cases (18/31). Fungal growth was observed within  $2 \pm 1.3$  days on average, and *A. fumigatus sensu stricto* was identified (GenBank accession numbers: ON360612-ON360336), except for one penguin that died in May 2019 with infection caused by a species belonging to section *Terrei* (ON360338). All clinical strains retrieved from cultures were found completely susceptible to all drugs (median MICs [ $\mu\text{g/ml}$ ]: amphotericin B = 0.375; itraconazole = 1; voriconazole = 0.5; posaconazole = 0.125; data not shown), except for one isolate collected from an adult penguin found dead

on 20 November 2019, and for which itraconazole MIC was evaluated at 4  $\mu\text{g/ml}$  (voriconazole and posaconazole at 0.5 and 0.125, respectively; no resistance mutation was detected in its *cyp51A* gene). A species of the mucorales order was also isolated once in culture (ON360336), in association with *Aspergillus* spp. and *Rasamsonia aspergillacea* (ON360339) (*Penicillium* sp. was isolated four times from diseased penguins, but systematically considered as a contaminant). Noteworthy, the *Aspergillus* qPCR allowed completion of the diagnosis in three diseased penguins, whereas all other aforementioned laboratory tests were negative (9.7% cases) (Fig. 3B). In contrast, in two positive cases, qPCR results were negative and so discrepant with those obtained by direct microscopy and/or culture, especially because of a very low fungal burden (and for three penguins, the qPCR run failed because of an insufficient amount of clinical material). In infected birds, the median quantitative cycle value (Cq) of qPCR was calculated at 30.5 cycles (mean = 29.6 cycles  $\pm$  2.3), and the median fungal burden at  $3.3 \times 10^1$  fg (mean =  $3.1 \times 10^4$  fg  $\pm$   $2.0 \times 10^4$ ). Thus, the sensitivity and specificity performance of *Aspergillus* qPCR were evaluated at 83.9% and 100%, respectively.



**Figure 4.** Genotyping approach according to the STR microsatellite polymorphism for studying the clinical *A. fumigatus* isolates. PHYLOViZ® v2.0 software and goeBURST algorithm were used to generate the tree.<sup>34</sup>

The mortality distribution was variable during the seasons, being more important in spring or at the end of winter (Fig. 3C). Upon these findings, the overall incidence rate of aspergillosis was then evaluated at 3.4% case-years in the colony over the study period (and even calculated at 8.6% when including 47 additional cases that had been classified as possible only). Aspergillosis affected mostly juveniles: 100% diseased penguins in 2018 (7/7 penguins), 87.5% in 2019 (14/16), and 75.0% (6/8) in 2020 were < 2 years old. The median age at diagnosis was 25 days. Regarding the four infected adults, the age ranged from 10 to 16 years; all were females, three were suffering from recent dyspnea ≤ 15 days, and one was very skinny and apathetic. Altogether during the study period, aspergillosis involved 9.7% of the chicks born in the institution in 2018 (9/93), 21.8% of those born in 2019 (12/55), and 8.9% (4/45) in 2020.

**Tracking the circulation of *Aspergillus* strains within the zoological center**

In all, genotyping was successfully performed in 94 *A. fumigatus* isolates. A systematic failure was noticed in the amplicon size of microsatellites 2A and 4C, so that only the polymorphism of the seven other loci was retained for comparative analysis.

In detail, a total number of 20 clinical *A. fumigatus* isolates were tested: 13 were collected from diseased penguins and 7 from other avian species. They represented 16 distinct genotypes (Fig. 4). Four identical genotypes were recovered from appaired isolates obtained from eight different birds. They involved seven penguins and one great blue turaco. For three

pairs, recovery of the two identical isolates occurred almost synchronously (between 0 and 18 days apart).

A total of 74 environmental *A. fumigatus* isolates were tested and eventually represented 25 distinct genotypes (Table 2). The same genotype was found for an environmental and two clinical isolates (referred to isolate K from a great blue turaco dead on 7 June 2019, and isolate L from a Humboldt penguin dead on 12 June 2019) (Fig. 4 and Fig. 5A). Another genotype was evidenced identical for the environmental isolate obtained in nest No. 2 during the spring session and the isolate recovered from a vinaceous-breasted Amazon parrot dead 8 months later on 1 October 2019. Altogether, a great genetic diversity was noticed for environmental samples obtained during the spring and winter sampling campaigns in 2018 (Table 2). In contrast, one genotype appeared largely overrepresented during the summer session in 2019, since it was found for 43/51 isolates, and in almost all the nests that were sampled during this campaign, except for nest No. 10 (Fig. 5B). Interestingly, this genotype had been actually already present during the first two sampling campaigns, since found once in each (Fig. 5A): e.g., nest No. 9 was evidenced contaminated with the same strain in winter 2018 and autumn 2019.

**Discussion**

The peculiar anatomic characteristics of the respiratory apparatus of birds make them particularly susceptible to aspergillosis: absence of epiglottis and diaphragm, limited distribution of pseudostratified ciliated columnar cells at the

**Table 2.** Genotyping approach according to the STR microsatellite polymorphism for studying the environmental *A. fumigatus* isolates.

Environmental isolate No.	Number of repetitions for microsatellites						
	2B	2C	3A	3B	3C	4A	4B
A–2018 Spring	24	17	31	10	19	10	10
B–2018 Spring	13	9	16	12	13	6	4
C–2018 Spring	11	17	24	21	17	14	7
D–2018 Spring	17	12	40	18	6	11	21
E–2018 Spring	23	8	38	12	14	7	8
F–2018 Spring	18	7	36	8	8	3	9
G–2018 Spring	20	7	30	8	5	7	9
H–2018 Spring	19	8	33	8	90	9	7
I–2018 Spring	19	10	33	8	8	3	9
J–2018 Spring	11	15	25	9	19	7	7
K–2018 Spring	21	17	25	9	14	8	7
L–2018 Spring	11	7	27	8	19	8	8
45 distinct isolates (including one from 2018 Spring, one from 2018 Winter, and 43 from 2019 Autumn)	11	7	26	9	19	8	7
M–2018 Spring	19	7	34	8	6	7	9
N–2018 Winter	11	8	25	9	20	8	7
O–2018 Winter	20	7	29	8	5	7	9
P–2018 Winter	19	10	26	8	10	7	9
Q–2018 Winter	15	9	23	12	9	6	4
R–2018 Winter	22	14	33	10	18	12	8
S–2018 Winter	19	13	33	8	8	7	8
T–2018 Winter	11	8	25	9	20	8	7
U–2018 Winter	11	7	28	9	18	8	8
6 distinct isolates (all from 2019 Autumn)	11	7	26	12	19	8	7
V–2019 Autumn	13	9	16	12	31	6	5
W–2019 Autumn	18	11	26	9	35	19	10

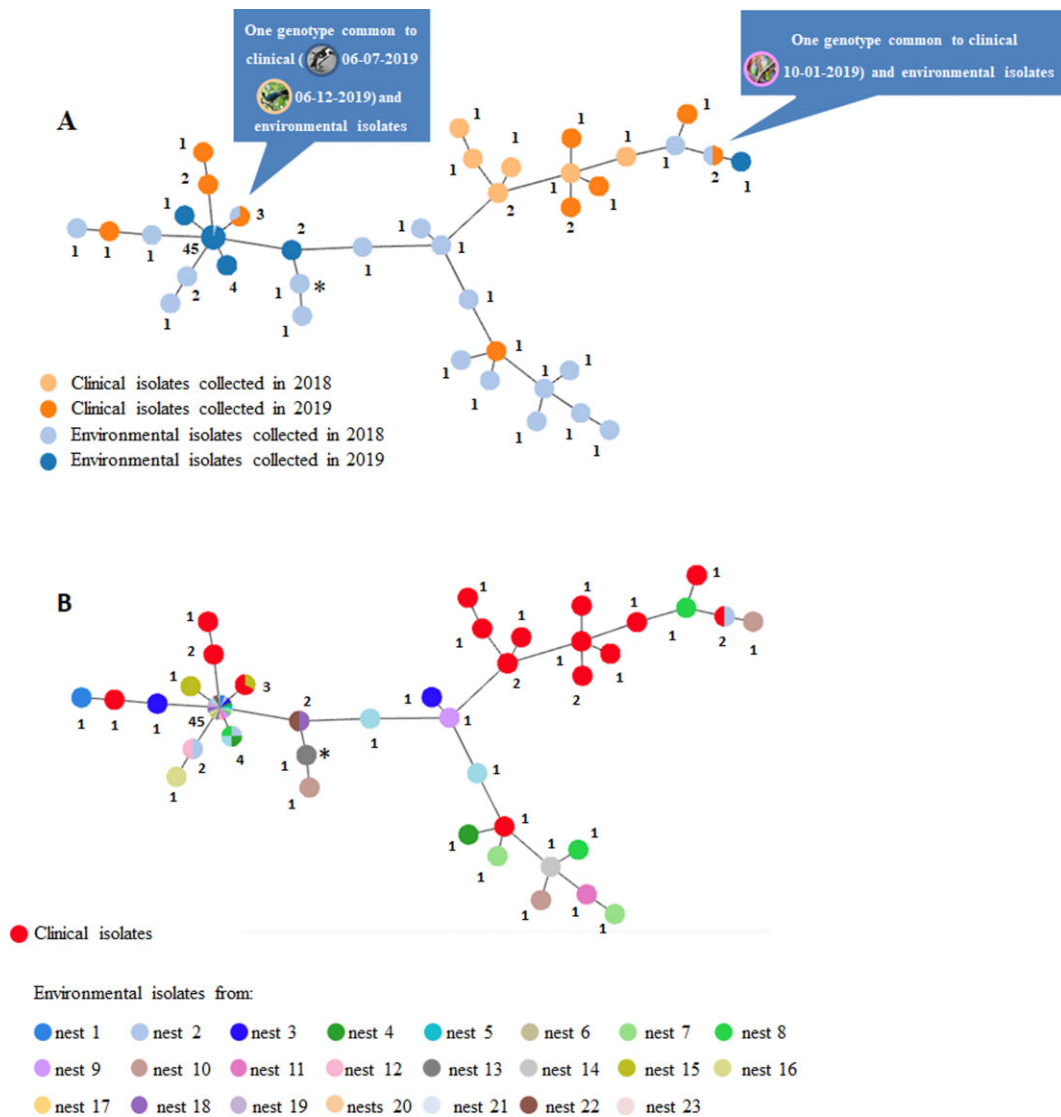
epithelium surface,<sup>16</sup> presence of air sacs where elevated moisture and local temperature (40–43.5°C in average) can favor fungal growth and facilitate proximal dissemination to lungs.<sup>35</sup> Aspergillosis is a devastating fungal disease for penguins that live in captivity under managed care,<sup>3–5,10,13,16,36</sup> whereas its overall incidence is assumed to remain very low into the wild.<sup>37</sup> This contrast suggests a critical role of the environment in which birds are supposed to thrive and evolve. Artificial habitats in parks may provide undesirable risk conditions that are particularly suitable for aspergillosis: dampness or excessive temperatures depending on the season,<sup>7</sup> accumulation of organic matter with high fungal loads and poor ventilation in the nests, exposure to aerosolized toxins resulting in mucosal irritation,<sup>13</sup> and increased stress. Size of the penguin population also seems to contribute to aspergillosis as shown in a study performed over 6 years in Magellanic penguins (*Spheniscus magellanicus*): 65% of the cases occurred during the year when the population density was the highest.<sup>10</sup> Excessive fungal loads in the environment, especially because of intensive agricultural practices conjugated to adequate climates, could also play a critical role by favoring high exposure and selecting mutant strains.<sup>20</sup>

In the present study carried out in ZooParc de Beauval, France, the mortality incidence directly attributable to aspergillosis was estimated close to 3.5% per year in Humboldt penguins and may be higher if all the penguin carcasses could have been totally investigated, while it was measured < 0.3% in all other birds of the zoological facility. This spectacular difference of occurrence may be explained by the metabolism and physiology specificities respective to each avian species: penguins' body temperature is slightly lower than those of other birds about 37.8–38.9°C vs. 40°C or more<sup>38</sup>—thus, more suit-

able for *Aspergillus* infection—and their burrower behavior more pronounced—thus, more exposed to *Aspergillus* spores clouds. Mostly penguin chicks, all born in the park, were affected rather than adults, and the highest mortality rate was observed following the nesting periods in April–May and in November–December. Juvenile birds, which stay confined in the nests for a long period of time, present an immature immune system possibly increasing their susceptibility to opportunistic infections like aspergillosis.<sup>13</sup> These results are consistent with those obtained by Flach et al. who reported that penguin chicks were the most susceptible population with global mortality of 14% in Edinburgh Zoo between 1964 and 1988<sup>39</sup> and those reported by Filho et al. with 95.5% of the cases of aspergillosis occurring in juveniles.<sup>10</sup>

In the wild, Humboldt penguin couples often nest in rocky cavities or aerated burrows. In contrast, the artificial nests of ZooParc de Beauval are partially buried in a totally outdoor habitat, i.e., continuously exposed to changing climates and air currents, and their open face is reduced which may stagnant the inside atmosphere and humidity, although all nests are oriented to the South or Southeast. During the present study, the global fungal load was systematically found elevated in penguin nests, throughout all timepoints. However, the fungal burden appeared significantly higher during the third sampling session, performed in September 2019, compared with those performed in April and December 2018 but with no obvious impact on the penguin mortality, and in spite of the advent of large building works in the park during the year 2018. Furthermore, species of the *Aspergillus* section *Fumigati* were largely overrepresented during these three periods. Similarly, in a study focusing on African penguins (*Spheniscus demersus*) in the Maryland Zoo in Baltimore,





**Figure 5.** Minimum spanning tree representing the genotypic relationship between clinical and environmental *Aspergillus fumigatus* isolates from the zoological park. PHYLOViZ® v2.0 software and goeBURST algorithm were used to generate the tree.<sup>34</sup> (A) Distribution of the genotypes according to the sampling period for clinical (in orange) and environmental (in blue) isolates; (B) Distribution of the genotypes according to the origin of the isolates. The numbers of isolates with the same genotype are indicated close to the corresponding nodes. \*the genotype of the resistant strain with the mutation in *cyp51A* gene, isolated from nest 13 in December 2018.

USA, Rivas et al. found that *Aspergillus* environmental contamination was lower in January–April period and higher during warmer period that ranged from the end of spring to the beginning of autumn.<sup>40</sup> In the Loir-et-Cher district during the months preceding the sampling session of spring 2018, the temperatures were quite normal for the season, but rainfall was above normal, average + 20 mm. In contrast, summer 2019 was much warmer than usual: +2.0°C in June, +2.3°C in July, and + 0.9°C in August *vs.* mean anterior temperatures (<https://www.infoclimat.fr/>).<sup>41</sup> This particular climate might have played a role in the high level of environmental contamination specifically observed during the third period of sampling. This finding is supported by a previous study demonstrating that elevation of temperature was linearly associated with the increase of fungal population.<sup>42</sup> *Aspergillus* growth is almost twofold enhanced when passing from 20°C to 40°C. In the light of all these considerations, and in order to reduce aspergillosis incidence in zoological parks regardless of the cli-

mate features therein, one could question about the nests’ configuration to be redesigned or about the systematic decontamination/cleaning measures to apply. Besides, some conservation initiatives are creating artificial nest boxes to help birds breed in the wild, so it would be now worthwhile to investigate the putative fungal colonization in these artificial structures.

Through this study, it appears quite obvious that cryptic species of the *Fumigati* section (e.g., *A. lentulus*, *Neosartorya fischeri*, *N. udagawae*, *A. viridinutans*, ...) are rarely isolated, either from bird lesions or from the environment of the penguins as only one isolate was actually identified as *A. nishimurae*. This finding means that cryptic species probably have a very limited health impact in penguins.<sup>43</sup> These findings are in agreement with results of previous studies performed in Germany<sup>44</sup> and California,<sup>45</sup> where *A. fumigatus sensu stricto* was isolated from all clinical and environmental samples. Similar to human medicine, only a few case reports mentioned cryptic species as involved in actual diseases,<sup>46–49</sup> even though

their incidence has been probably neglected until the advent of molecular tools for their definitive identification.

Azole resistance in *A. fumigatus* currently represents an emerging problem in human and animal medicine.<sup>50</sup> Mutations in the *cyp51A* gene encoding the target protein of azole drugs, mostly L98H mutation associated with the TR34 in the promotor region, have been proven as generated by environmental exposition to the massive use of fungicides in intensive agriculture.<sup>18</sup> ZooParc de Beauval is located in a temperate region where 66% of the 3434 agricultural areas comply with the definition of farms with intensive practices (<https://centre-valde Loire.chambres-agriculture.fr>). However, in the present study, only one environmental *A. fumigatus* isolate exhibited a *cyp51A* mutation: the glycine amino acid was substituted with serine in the codon 138 which is believed responsible for multidrug-resistance. Indeed, the sensitivity profile of the detected mutated strain evidenced high MIC for itraconazole and, to a lesser extent, an increased MIC for voriconazole. Overall, our findings are in accordance with other articles focusing on avian aspergillosis in Belgium, Germany, China, or France.<sup>44,51,52</sup> In such circumstances, the use of itraconazole in prophylaxis may be recommended when re-designing the nest configuration is not easy to implement.<sup>53,54</sup> Nonetheless, it appears necessary to extend surveillance at a larger scale. Two studies performed in UK<sup>36</sup> and Poland<sup>55</sup> highlighted tremendous discrepancies with the aforementioned investigations,<sup>44,51,52</sup> as they reported a large representation of phenotypic resistance to itraconazole, without resistance to voriconazole, in *A. fumigatus* strains isolated from clinical and environmental samples. Although the rate of positive cultures in the UK study was quite similar to ours (68.0% vs. 58.1%), the itraconazole MICs for 100% isolates obtained from frozen clinical specimens of penguins with gross lesions (15/15) were elevated (median = 4 µg/ml). Notably, in the two latter studies, no molecular investigations were carried out to detect mutation(s) in the *cyp51A* gene. In the present work, only one clinical strain was evidenced with elevated MIC to itraconazole, but with no associated mutation in *cyp51A* gene. This phenotype may be caused by mutation(s) in other gene fragments that were not sequenced herein, or by other resistance mechanisms, independent of any mutations.

The genotyping approach of our study confirmed that clinical strains are usually different from those retrieved in the environment,<sup>56</sup> suggesting independent events for the contamination occurrences.<sup>43</sup> Some sporadic exceptions can be underscored: three pairs of isolates were found with identical genotypes in penguins that died at the same period. It must also be highlighted that a strain was first detected in the environment and in two birds that died 6 weeks later. Another single strain was isolated in the environment and a vinaceous amazon parrot died 8 months later. The large overrepresentation of a unique genotype during the environmental sampling campaign in September 2019 is a surprising result. This may be explained by our sampling procedure which was actually completed according to a one-shot swabbing within one unique day for each timepoint, and not according to a longitudinal continuous manner. Therefore, sporadic contamination by a unique transient source is possible.

The One Health concept considers health based on three pillars closely intertwined with each other: interactions between humans, animals, and the environment. This concept is actually a powerful weapon in the surveillance of infectious diseases, and the present study is a concrete example of such

a global approach to this problem: *Aspergillus* spp. are molds that fit relevantly in this context, in view of their ubiquity as well as their critical importance as pathogens for crops and for humans and avian species like penguins, and also because of the recent emergence of azole resistance. One should be aware of the complexity of the ecosystem and importantly address animal models of infectious diseases within the integrative framework of environment-human-animal interactions.

## Supplementary material

Supplementary material are available at *Medical Mycology* online.

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## Disclosure of conflicts of interest

None.

## References

1. Seyedmousavi S, Guillot J, Arné P et al. *Aspergillus* and aspergilloses in wild and domestic animals: a global health concern with parallels to human disease. *Med Mycol*. 2015; 53: 765–797.
2. Latge J-P. *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev*. 1999; 12: 310.
3. Graczyk TK, Cockrem JF. *Aspergillus* spp. seropositivity in New Zealand penguins. *Mycopathologia*. 1995; 131: 179–184.
4. Uhart M, Thijl Vanstreels RE, Gallo L, Cook RA, Karesh WB. Serological survey for select infectious agents in wild Magellanic penguins (*Spheniscus magellanicus*) in Argentina, 1994–2008. *J Wildl Dis*. 2019; 56: 66–81.
5. Ewbank AC, Duarte-Benvenuto A, Zamana-Ramblas R et al. Case report of respiratory aspergillosis and candidiasis in wild Magellanic penguins (*Spheniscus magellanicus*), Brazil. *Braz J Microbiol*. 2021; 52: 967–975.
6. Miller RE, Fowler ME. *Fowler's Zoo and Wild Animal Medicine, Volume 8 - Elsevieron Vital Source, 1er édition*, Saunders, 2014.
7. Terio KA, Mcaloose D, Leger JS, *Pathology of Wildlife and Zoo Animals, 1er édition*, Academic Press, 2018.
8. Desoubeaux G, Rodriguez M, Bronson E, Sirpenski G, Cray C. Application of 3-hydroxybutyrate measurement and plasma protein electrophoresis in the diagnosis of aspergillosis in African penguins (*Spheniscus demersus*). *J Zoo Wildl Med*. 2018; 49: 696–703.
9. Krol L, Moore RP, Mutlow AG, Brady SM, Dorsa D. A retrospective analysis of mortality in captive Magellanic penguins (*Spheniscus magellanicus*) in the United States, 2008–2018. *Zoo Biol*. 2020; 39: 405–410.
10. da S. Filho RP, Xavier MO, Martins AM et al. Incidence density, proportionate mortality, and risk factors of aspergillosis in

- Magellanic penguins in a rehabilitation center from Brazil. *J Zoo Wildl Med*. 2015; 46: 667–674.
11. Bechert U, Christensen J, Poppenga R, Le H, Wyatt J, Schmitt T. Pharmacokinetics of orally administered terbinafine in African penguins (*Spheniscus demersus*) for potential treatment of aspergillosis. *J Zoo Wildl Med Off Publ Am Assoc Zoo Vet*. 2010; 41: 263–274.
  12. Sautour M, Sixt N, Dalle F et al. Profiles and seasonal distribution of airborne fungi in indoor and outdoor environments at a French hospital. *Sci. Total Environ* 2009; 407: 3766–3771.
  13. Xavier MO, Soares MP, Meinerz ARM et al. Aspergillosis: a limiting factor during recovery of captive Magellanic penguins. *Braz J Microbiol*. 2007; 38: 480–484.
  14. Cray C, Watson T, Rodriguez M, Arheart KL. Application of galactomannan analysis and protein electrophoresis in the diagnosis of aspergillosis in avian species. *J Zoo Wildl Med*. 2009; 40: 64–70.
  15. Jones MP, Orosz SE. The diagnosis of aspergillosis in birds. *Semin Avian Exot Pet Med*. 2000; 9: 52–58.
  16. Tell LA. Aspergillosis in mammals and birds: impact on veterinary medicine. *Med Mycol*. 2005; 43: S71–S73.
  17. van der Linden JWM, Arendrup MC, Warris A et al. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. *Emerg Infect Dis J*. 2015; 21: 1041–1044.
  18. Snelders E, van der Lee HAL, Kuijpers J et al. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Med*. 2008; 5: e219.
  19. Jeanvoine A, Rocchi S, Reboux G, Crini N, Crini G, Millon L. Azole-resistant *Aspergillus fumigatus* in sawmills of Eastern France. *J Appl Microbiol*. 2017; 123: 172–184.
  20. Burks C, Darby A, Gómez Londoño L, Momany M, Brewer MT. Azole-resistant *Aspergillus fumigatus* in the environment: identifying key reservoirs and hotspots of antifungal resistance. *PLoS Pathog*. 2021; 17: e1009711.
  21. Desoubeaux G, Bailly É, Chandénier J. Diagnosis of invasive pulmonary aspergillosis: updates and recommendations. *Médecine Mal Infect*. 2014; 44: 89–101.
  22. Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol*. 1995; 61: 1323–1330.
  23. Morio F, Aubin GG, Danner-Boucher I et al. High prevalence of triazole resistance in *Aspergillus fumigatus*, especially mediated by TR/L98H, in a French cohort of patients with cystic fibrosis. *J Antimicrob Chemother*. 2012; 67: 1870–1873.
  24. Lavergne R-A, Morio F, Favennec L et al. First description of azole-resistant *Aspergillus fumigatus* due to TR46/Y121F/T289A mutation in France. *Antimicrob Agents Chemother*. 2015; 59: 4331–4335.
  25. Alanio A, Sitterle E, Liance M et al. Low prevalence of resistance to azoles in *Aspergillus fumigatus* in a French cohort of patients treated for haematological malignancies. *J Antimicrob Chemother*. 2011; 66: 371–374.
  26. Nuh A, Ramadan N, Schelenz S, Armstrong-James D. Comparative evaluation of MIRONAUT-AM and CLSI broth microdilution method for antifungal susceptibility testing of *Aspergillus* species against four commonly used antifungals. *Med Mycol*. 2020; 58: 1085–1090.
  27. EUCAST: Breakpoints for antifungals, (n.d.). <https://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals/> (accessed March 25, 2021).
  28. Desoubeaux G, Franck-Martel C, Caille A et al. Use of calcofluor-blue brightener for the diagnosis of *Pneumocystis jirovecii* pneumonia in bronchial-alveolar lavage fluids: a single-center prospective study. *Med Mycol*. 2017; 55: 295–301.
  29. Challier S, Boyer S, Abachin E, Berche P. Development of a serum-based Taqman real-time PCR assay for diagnosis of invasive aspergillosis. *J Clin Microbiol*. 2004; 42: 844–846.
  30. Chauvin D, Hust M, Schütte M et al. Targeting *Aspergillus fumigatus* crf transglycosylases with neutralizing antibody is relevant but not sufficient to erase fungal burden in a neutropenic rat model. *Front Microbiol*. 2019; 10: 600.
  31. Lass-Flörl C, Cuenca-Estrella M, Denning DW, Rodriguez-Tudela JL. Antifungal susceptibility testing in *Aspergillus* spp. according to EUCAST methodology. *Med Mycol*. 2006; 1: S319–S325.
  32. Donnelly JP, Chen SC, Kauffman CA et al. Revision and update of the consensus definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium. *Clin Infect Dis*. 2020; 71: 1367–1376.
  33. de Valk HA, Meis JFGM, Curfs IM, Muehlethaler K, Mouton JW, Klaassen CHW. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. *J Clin Microbiol*. 2005; 43: 4112–4120.
  34. Francisco AP, Vaz C, Monteiro PT, Melo-Cristino J, Ramirez M, Carriço JA. PHYLOViZ: phylogenetic inference and data visualization for sequence based typing methods. *BMC Bioinformatics*. 2012; 13: 87.
  35. Sverdlova NS, Lambert M, Witzel U, Perry SF. Boundary conditions for heat transfer and evaporative cooling in the trachea and air sac system of the domestic fowl: a two-dimensional CFD analysis. *PLoS ONE*. 2012; 7: e45315.
  36. Reed K, Macgregor SK, Stidworthy MF, Denk D, Guthrie A. The isolation and antimicrobial sensitivity of *Aspergillus fumigatus* from frozen respiratory tissues of penguins from zoological collections in the United Kingdom, 2007–2018. *J Zoo Wildl Med*. 2020; 51: 591–597.
  37. Smith KM, Karesh WB, Majluf P et al. Health evaluation of free-ranging Humboldt penguins (*Spheniscus humboldti*) in Peru. *Avian Dis*. 2008; 52: 130–135.
  38. Kazas S, Benelly M, Golan S. The Humboldt Penguin (*Spheniscus humboldti*) Rete Tibiotarsale - A supreme biological heat exchanger. *J Therm Biol*. 2017; 67: 67–78.
  39. Flach EJ, Stevenson MF, Henderson GM. Aspergillosis in gentoo penguins (*Pygoscelis papua*) at Edinburgh Zoo, 1964 to 1988. *Vet Rec*. 1990; 126: 81–85.
  40. Rivas A, Dykstra M, Kranz K, Bronson E. Environmental fungal loads in an indoor-outdoor African penguin (*Spheniscus demersus*) exhibit. *J Zoo Wildl Med*. 2018; 49: 542–555.
  41. Climatologie de l'année 2018 à Blois - Infoclimat, (n.d.). <https://www.infoclimat.fr/climatologie/annee/2018/blois/valeurs/07245.html> (accessed March 15, 2021).
  42. Manna M, Kim KD. Effect of temperature and relative humidity on growth of *Aspergillus* and *Penicillium* spp. and biocontrol activity of *Pseudomonas protegens* AS15 against aflatoxigenic *Aspergillus flavus* in stored rice grains. *Mycobiology*. 2018; 46: 287–295.
  43. Melo AM, Poester VR, Canabarro PL et al. Molecular epidemiology of aspergillosis in Magellanic penguins and susceptibility patterns of clinical isolates. *Med Mycol*. 2021; 59: 1076–1084.
  44. Barber AE, Scheufen S, Walther G, Kurzai O, Schmidt V. Low rate of azole resistance in cases of avian aspergillosis in Germany. *Med Mycol*. 2020; 58: 1187–1190.
  45. Sabino R, Burco J, Valente J et al. Molecular identification of clinical and environmental avian *Aspergillus* isolates. *Arch Microbiol*. 2019; 201: 253–257.
  46. Barrs VR, van Doorn TM, Houbraken J et al. *Aspergillus felis* sp. nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. *PLoS ONE*. 2013; 8: e64871.
  47. Verweij PE, Varga J, Houbraken J et al. *Emericella quadrilimeata* as cause of invasive aspergillosis. *Emerg Infect Dis J*. 2008; 14: 566–572.
  48. Gautier M, Normand A-C, L'Ollivier C et al. *Aspergillus tubingensis*: a major filamentous fungus found in the airways of patients with lung disease. *Med Mycol*. 2016; 54: 459–470.



49. Howard SJ. Multi-Resistant aspergillosis due to cryptic species. *Mycopathologia*. 2014; 178: 435–439.
50. Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science*. 2018; 360: 739–742.
51. Beernaert LA, Pasmans F, Van Waeyenberghe L et al. Avian *Aspergillus fumigatus* strains resistant to both itraconazole and voriconazole. *Antimicrob Agents Chemother*. 2009; 53: 2199–2201.
52. Wang DY, Gricourt M, Arné P et al. Mutations in the Cyp51A gene and susceptibility to itraconazole in *Aspergillus fumigatus* isolated from avian farms in France and China. *Poult Sci*. 2014; 93: 12–15.
53. Bunting EM, Madi NA, Cox S, Martin-Jimenez T, Fox H, Kollias GV. Evaluation of oral itraconazole administration in captive Humboldt Penguins (*Spheniscus humboldti*). *J Zoo Wildl Med*. 2009; 40: 508–518.
54. Zalesak SM, Sanchez CR, Pich AA, Papich MG. Preliminary pilot study of itraconazole after a single oral dose of a veterinary formulation solution in African penguins (*Spheniscus demersus*). *J Avian Med Surg*. 2020; 34: 52–56.
55. Ziółkowska G, Tokarzewski S, Nowakiewicz A. Drug resistance of *Aspergillus fumigatus* strains isolated from flocks of domestic geese in Poland. *Poult Sci*. 2014; 93: 1106–1112.
56. Valdes ID, van den Berg J, Haagsman A, Escobar N et al. Comparative genotyping and phenotyping of *Aspergillus fumigatus* isolates from humans, dogs and the environment. *BMC Microbiol*. 2018; 18: 118.