

INCIDENCE DENSITY, PROPORTIONATE MORTALITY, AND RISK FACTORS OF ASPERGILLOSIS IN MAGELLANIC PENGUINS IN A REHABILITATION CENTER FROM BRAZIL

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Source: *Journal of Zoo and Wildlife Medicine*, 46(4):667-674.

Published By: American Association of Zoo Veterinarians

DOI: <http://dx.doi.org/10.1638/2013-0092.1>

URL: <http://www.bioone.org/doi/full/10.1638/2013-0092.1>

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INCIDENCE DENSITY, PROPORTIONATE MORTALITY, AND RISK FACTORS OF ASPERGILLOSIS IN MAGELLANIC PENGUINS IN A REHABILITATION CENTER FROM BRAZIL

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Abstract: Aspergillosis, an opportunistic mycosis caused by the *Aspergillus* genus, affects mainly the respiratory system and is considered one of the most significant causes of mortality in captive penguins. This study aimed to examine a 6-yr period of cases of aspergillosis in penguins at the Centro de Recuperação de Animais Marinhos (CRAM-FURG), Rio Grande, Brazil. A retrospective cohort study was conducted using the institution's records of penguins received from January 2004 to December 2009. Animals were categorized according to the outcome "aspergillosis," and analyzed by age group, sex, oil fouling, origin, prophylactic administration of itraconazole, period in captivity, body mass, hematocrit, and total plasma proteins. A total of 327 Magellanic penguins (*Spheniscus magellanicus*) was studied, 66 of which died of aspergillosis. Proportionate mortality by aspergillosis was 48.5%, and incidence density was 7.3 lethal aspergillosis cases per 100 penguins/mo. Approximately 75% of the aspergillosis cases occurred in penguins that had been transferred from other rehabilitation centers, and this was considered a significant risk factor for the disease. Significant differences were also observed between the groups in regard to the period of time spent in captivity until death, hematocrit and total plasma proteins upon admission to the center, and body mass gain during the period in captivity. The findings demonstrate the negative impacts of aspergillosis on the rehabilitation of Magellanic penguins, with a high incidence density and substantial mortality.

Key words: *Aspergillus*, captivity, incidence, Magellanic penguins, rehabilitation, Sphenisciformes.

INTRODUCTION

Aspergillosis is a respiratory fungal disease acquired by inhalation of *Aspergillus* spp. airborne conidia, which are dispersed in the environment.^{24,27} The main pathogenic etiologic agent is *Aspergillus* section *Fumigati*, and disease development is related to strain virulence, quantity of inoculum inhaled, and most important, host immunity.^{1,32}

Although considered uncommon in wild penguins,¹⁹ aspergillosis is a frequent cause of death

of penguins in zoos and aquaria,^{2,14} as well as in rehabilitation centers, where the animals are considerably debilitated by malnutrition, dehydration, or oil fouling.^{29,31,37} Clinical signs of aspergillosis are nonspecific, such as lethargy, anorexia, and dyspnea, among others, and it is thus necessary to perform laboratory and imaging diagnostic tests for presumptive diagnosis. There is no gold standard test for the disease, and definitive diagnosis is often obtained only through postmortem examination.^{20,32,40}

There are numerous published reports and case studies focusing on clinical, microbiological, and histopathologic aspects of aspergillosis in penguins,^{1,8,9,32} as well as studies on its diagnosis,^{11,12,15,20} treatment,^{3,13,18} and prevention.^{6,10,39} However, epidemiologic studies in the peer-reviewed literature remain scarce. In this study, we conducted a 6-yr retrospective epidemiologic survey of aspergillosis in Magellanic penguins (*Spheniscus magellanicus*) at a rehabilitation center in southern Brazil, aiming to determine the *Aspergillus* species involved, the incidence density, and proportional mortality due to aspergillosis and the main risk factors for this disease.

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MATERIALS AND METHODS

Study design and localization

A retrospective cohort study was conducted of aspergillosis in Magellanic penguins at Centro de Recuperação de Animais Marinhos, Museu Oceanográfico Prof. Eliézer de Carvalho Rios, Universidade Federal do Rio Grande (CRAM-FURG), located in Rio Grande, Rio Grande do Sul, Brazil (32°01'34"S, 52°06'21"W).

Case definition

The study included all Magellanic penguins admitted for rehabilitation at CRAM-FURG between January 2004 and December 2009. Animals that died with no available data on cause of death were excluded from analysis because of an inability to define a final outcome.

The case definition of interest was "aspergillosis." The diagnosis was established postmortem, through the observation of gross lesions at necropsy and histopathologic demonstration of hyaline, and septate and 45° branched hyphae in the affected tissues (lungs and air sacs). Furthermore, proven cases of the disease were subject to mycologic culture of respiratory tissue fragments on Sabouraud dextrose agar for the isolation and identification of the fungal species involved.^{23,27} All other penguins that died for whom aspergillosis was not proven in the postmortem and mycologic examination had their outcome categorized as dead from nonaspergillosis causes. Penguins that were rehabilitated and reintroduced to their natural habitat were classified as released.

Categorical variables analyzed

To determine the risk factors for mortality from aspergillosis, categorical variables, such as age group, sex, origin, oil fouling, and prophylactic administration of itraconazole, were analyzed.

The age group was classified as either juvenile or adult on the basis of their plumage.³⁶ Sex was determined through postmortem gonad dissection in deceased birds, whereas discriminant analysis of body measurements was used for live birds.³⁵ Origin was categorized as "local beach" for penguins found stranded on the beaches of the southern coast of Rio Grande do Sul state and sent directly to CRAM-FURG, or "other centers" for those rescued and rehabilitated by other rehabilitation centers in Brazil, then air-transported to CRAM-FURG for the final stages of the rehabilitation process and release. Oil fouling upon admission to CRAM-FURG and the use

of itraconazole prophylaxis (15 mg/kg per day for 15 days) were each categorized as either "yes" or "no."³⁰

Quantitative variables analyzed

Quantitative variables evaluated were body mass (g), body mass gain (g), hematocrit (%), total plasma proteins (g/L), and period in captivity at CRAM-FURG (days).

Body mass was determined upon admission to CRAM-FURG, and body mass gain was calculated through the difference between the body mass at final disposition (death or release) and admission. Hematocrit (Hct) and total plasma proteins (TP) were obtained 10–15 days after admission to the center, to avoid bias resulting from hemoconcentration associated with dehydration or stress-related adrenergic release resulting from air transportation. Period in captivity was calculated as the total number of days at CRAM-FURG, from admission to final disposition (death or release).

Statistical analysis

Mortality rate (incidence density) and the proportionate mortality due to aspergillosis (the proportionate mortality due to aspergillosis expressed as a proportion of all of the causes of death in the group) were calculated annually and for the entire study period. To investigate the association between factors and outcome, the Student's *t*-test was used for quantitative variables. The independent effect of factors was determined by logistic regression, calculating the odds ratios (ORs) and 95% confidence intervals (CI). The model quality was evaluated using the Hosmer–Lemeshow test. Data were analyzed using SPSS® 20.0. All tests were two-tailed and *P*-values lower than 0.05 were considered statistically significant.

RESULTS

Descriptive analysis of Magellanic penguin population

During the study period CRAM-FURG admitted 366 Magellanic penguins, of which 39 were excluded for not having enough information available about their cause of death, resulting in a total of 327 animals evaluated. The population density varied among the 6 yr, with the lowest value in 2007 ($n = 14$) and the highest in 2008 ($n = 163$) (Fig. 1).

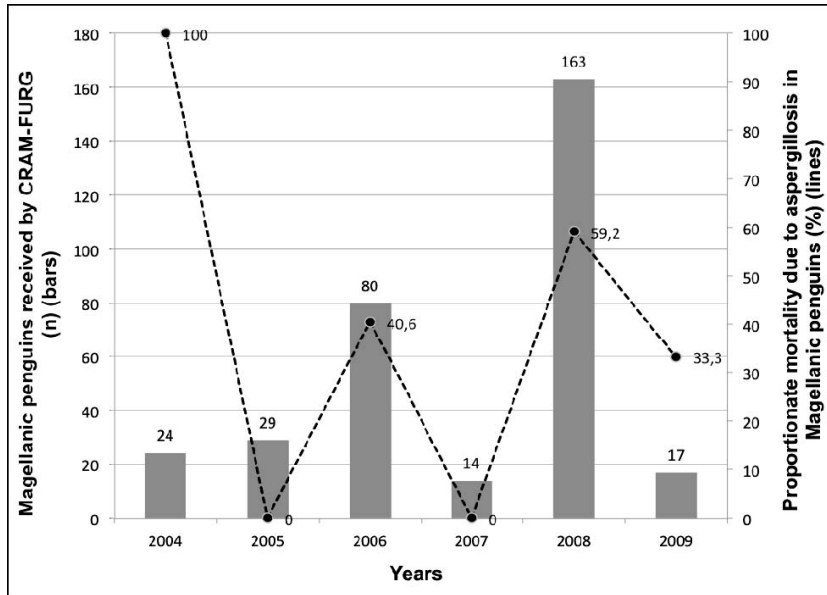


Figure 1. Distribution of Magellanic penguins received by CRAM-FURG and proportionate mortality due to aspergillosis in Magellanic penguins at CRAM-FURG between 2004 and 2009.

About 99.4% (154/155) of animals sent from other rehabilitation centers were juveniles and showed no signs of oiling. The 131 oiled animals in this study were all from the local beach, and represented 76.6% of the local beach group (131/171). Prophylactic itraconazole treatment was administered to Magellanic penguins that weighed less than 2.5 kg, according to the protocol used at CRAM (Fig. 2).^{26,30}

A physical examination of these penguins, upon admission to CRAM-FURG, revealed a median weight of 2,759 g, ranging from 1,574 g to 4,866 g (SD = 547.7). TP and Hct medians were 70.0 g/L (SD = 13.7 g/L) and 41% (SD = 6.8%), respectively, with values ranging from 20 to 100 g/L and from 11 to 56%.

Descriptive analysis of aspergillosis mortality in Magellanic penguins at CRAM-FURG

Among the 327 Magellanic penguins included in the study, 66 developed and died from aspergillosis at CRAM-FURG during rehabilitation. *Aspergillus* section *Fumigati* was the predominant etiologic agent identified in all cases, except one caused by *Aspergillus* section *Flavi*.

About 75.8% of aspergillosis cases (50/66) occurred in animals that had been air transported from other centers, 77.3% (51/66) occurred in animals that were not oiled, and 84.8% of cases (56/66) were animals that had not been treated

with prophylactic antifungal therapy. Most cases were observed in juvenile penguins, corresponding to 63 of 66 cases (95.5%), and their distribution by sex was similar, with 56.2% (36/66) in males and 43.8% (28/66) in females.

In 2005 and 2007, no cases of aspergillosis were diagnosed. The largest number of cases (45/66) occurred in 2008, representing 68.2% of all mortalities. Annually, proportionate mortality due to aspergillosis ranged from 0 to 100% (Fig. 1), with an overall proportionate mortality of 48.5% for the total study period.

Over 6 yr of monitoring, incidence density rate of aspergillosis was found to be 7.3 cases/100 penguins-month. Annual incidence density ranged from zero in 2005 and 2007 to 32 aspergillosis cases/100 penguins-year in 2008 (Fig. 3).

Risk factors for aspergillosis in Magellanic penguins at CRAM-FURG

Regarding age, 22.6% (63/249) of juvenile animals developed aspergillosis, whereas in adults this value was only 6.2% (3/48). Similarly, the disease occurred in 32.3% (50/155) of animals from other centers and in only 9.3% (16/172) of the penguins recovered from the local beach. In contrast, 11.5% of the penguins covered in oil developed the disease, and 26.2% of nonoiled died of aspergillosis. The group of penguins that

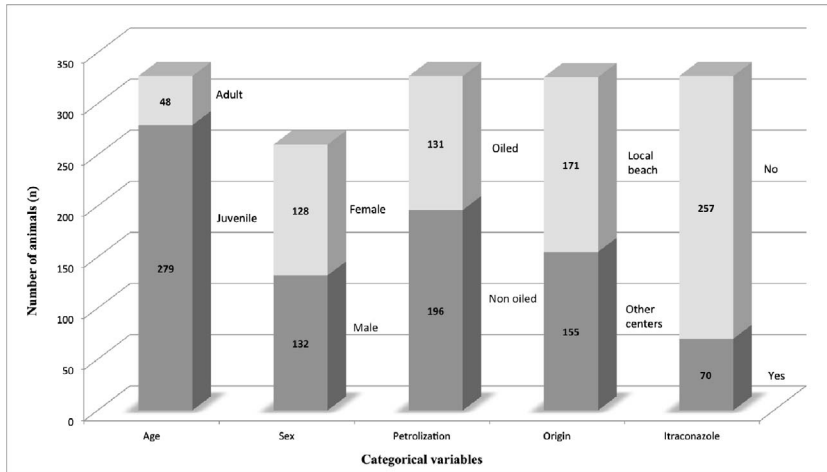


Figure 2. Descriptive analysis of categorical variables used in the study.

received itraconazole prophylactically had 1.8 times fewer animals with aspergillosis when compared with those without antifungal prophylaxis (12.2% versus 22.9%).

Multivariate analysis of these categorical variables showed that only the “origin” from other centers can be considered as a risk factor for the disease, with $P < 0.05$ (Table 1). The quality of model adjustment was proven by the Hosmer–Lemeshow test ($P = 0.246$). Significant difference ($P < 0.05$) in quantitative variables between the group of animals that developed aspergillosis and the nonaspergillosis group was observed in relation to the total period while in care, with mean values of 51.7 days ($SD = 76.8$) and 91.25 days ($SD = 97.05$) respectively; Hct, with mean values of 37.5% ($SD = 6.4\%$) and 40.3% ($SD = 6.8\%$), respectively; TP results, with values of 73 g/L ($SD = 13$ g/L) and 67 g/L ($SD = 14$ g/L), respectively; and weight gain during the captive period with average values of 127.9 g ($SD = 525.3$) and 864.4 g

($SD = 736.1$), respectively. In contrast, the average body weight of penguins when they were admitted to CRAM-FURG did not differ between the groups of animals with and without aspergillosis, with mean values of 2,856.6 g ($SD = 472.2$) and 2,775.1 g ($SD = 563.6$), respectively ($P = 0.25$).

DISCUSSION

This retrospective cohort study evaluated the epidemiologic data concerning aspergillosis in Magellanic penguins in rehabilitation at CRAM-FURG over a period of 6 yr, including 327 animals. Although many authors describe aspergillosis as one of the main problems found in captive penguins, no similar studies are available in the peer-reviewed literature.^{2,9,31,37}

Among the 66 cases of aspergillosis in Magellanic penguins included in this study, *Aspergillus* section *Fumigati* was the predominant etiologic agent in 98.5% of the cases, which is consistent with that described in the literature.^{2,14,16,22,28} This species of *Aspergillus* is the most pathogenic of the genus, and its virulence factors are well established, which include morphologic, physiologic, biochemical, nutritional, and reproductive characteristics of the fungus, as well as its adaptability to its hosts, even to the high body temperatures of penguins and other birds.^{4,24,25}

The nonhomogeneous distribution of aspergillosis cases among the 6 yr studied may be due to different factors. These can be related to the etiologic agent, such as variations between genotypes and predominant strains, and their availability in the environment of captive penguins,^{7,38} and also to those related to health conditions of the animals when admitted at CRAM-FURG,

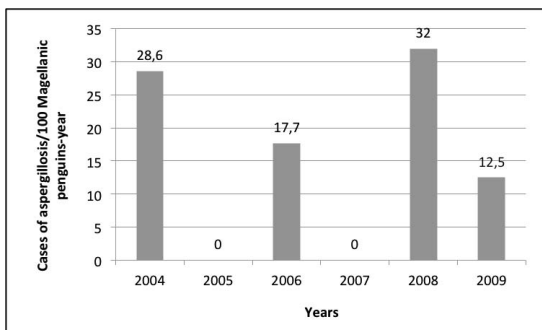


Figure 3. Incidence density of aspergillosis in penguins at CRAM-FURG between 2004 and 2009.

Table 1. Odds ratio and risk factors for mortality due to aspergillosis in Magellanic penguins at CRAM-FURG between January 2004 and December 2009 ($n = 327$).

Variable	Odds ratio (95% CI)	P-value	Odds ratio adjusted (95% CI)	P-value OR adjusted
Age (juvenile)	4.37 (1.31–14.55)	0.02	2.19 (0.56–8.53)	0.26
Sex (male)	1.34 (0.76–2.36)	0.31	1.27 (0.66–2.44)	0.46
Origin (other centers)	4.64 (2.51–8.59)	<0.0001	22.14 (2.91–168.63)	0.003
Petrolization (oiled)	0.36 (0.19–0.68)	0.002	6.13 (0.76–49.42)	0.09
Itraconazole prophylaxis (yes)	0.47 (0.23–0.97)	0.04	0.67 (0.29–1.57)	0.36

which fluctuate.³⁰ More than 65% of cases occurred in 2008, representing the highest incidence density of the disease at the center. This might be due to the high population density that occurred during this period, which corresponded to a number of animals 2 to 11.6 times higher than that observed in the other 5 yr of the study.

In fact, crowding results in organic matter accumulation, greater difficulty in maintaining adequate ventilation and environmental disinfection, and consequently offering greater nutritional support for rapid fungal development, generating high concentrations of infective conidia capable of being inhaled by birds.^{31,34} In addition, under these conditions, there is an increase in the number of people and in the intensity of professional activities within the center to provide all the necessary therapeutic interventions. This increased activity, although essential to fulfill the correct husbandry needs of the animals in rehabilitation, generates higher levels of stress, which can lead to increased immunosuppression due to the release of cortisol, and thus increasing their susceptibility to opportunistic infections such as aspergillosis.²⁵ The inversely proportional relationship between population density and immunity mediated by T cells has been described by Tella et al., analyzing the breeding colonies of Magellanic penguins and highlighting greater predisposition to diseases in crowded sites.³³

Considering the whole study period, the incidence density was found to be 7.3 aspergillosis cases per 100 penguins/mo of follow-up, and proportionate mortality due to aspergillosis was 48.5%. Other studies describing incidence density of the disease in penguins were not found in the literature; however, the proportionate mortality was similar to other studies as shown in Flach et al.,¹⁴ in which 41% of the gentoo penguins (*Pygoscelis papua*) died because of aspergillosis over a period of 24 yr, and in Russell et al.,³¹ in which proportional mortality due to aspergillosis reached 79% of the birds in rehabilitation during an oil-spill response in 1991.

Although these results cannot be extrapolated to other rehabilitation centers or other institutions keeping captive penguins because of numerous factors that differ in relation to the environment, as well as to the animal population, they corroborate the statement that this disease has a limiting character in the rehabilitation of penguins, as previously described.^{31,37} Preventive measures should be used.

Analyzing the annual proportionate mortality, we observed that the maximum value of 100% was reached in 2004, which can be explained by the fact that in this first year of the study an environmental disinfection routine was not yet established at CRAM-FURG. It began in 2005, resulting in improved air quality and consequently lower concentrations of infective inoculum of *Aspergillus* spp. to be inhaled by animals undergoing rehabilitation.³⁹ In this context, all animals susceptible to opportunistic infections in 2004 may have developed the mycosis by being exposed to high loads of *Aspergillus* conidia or spores.²⁶

The high risk of animals from other centers being affected by aspergillosis demonstrated in this study is most likely related to the stress of the transportation process. The penguins are transported to CRAM-FURG by air and later by truck, entailing several hours of transportation. Given that throughout the flight period the animals are subject to overcrowding, lack of ventilation, accumulation of organic matter, and consequently poor air quality, with likely proliferation of *Aspergillus* spp. conidia, the risk of infection is high.^{31,34,38} In addition to these factors, noise and disturbances also contribute to increased stress, which culminates with the release of corticosteroids into the bloodstream, causing immunosuppression and facilitating fungal proliferation.²⁵

Besides the process of air transport, the penguins from other centers spend time in other places for varying periods in unknown rehabilitation conditions. The first phase of the rehabilitation process (stabilization) is critical for success, and if procedures and interventions according to the protocols available are neglected it may

compromise the process and culminate in increased susceptibility to opportunistic infections.^{26,30,34} Maintaining a clean and low-stress environment and good air quality are critical for the process as well.^{31,34,39}

Aspergillosis prophylaxis with oral itraconazole is indicated in several penguin rehabilitation treatment protocols.^{26,30,34} The procedure was followed by CRAM-FURG using compounded itraconazole and administered at a dose of 15 mg/kg per day for 15 days. A study on the pharmacokinetics and pharmacodynamics of itraconazole in Humboldt penguins (*Spheniscus humboldti*) showed that compounded itraconazole does not reach plasma concentrations required for antifungal activity, and that commercial itraconazole Sporanox (Janssen cilag, São Paulo, São Paulo, 05501-900, Brazil) must be used and administered at a dose of 20 mg/kg once a day, which may explain the suboptimal effectiveness of this drug for aspergillosis prevention in the present study.⁶

Although body weight at admission is described by Ruoppolo et al.³⁰ and used at CRAM-FURG as the parameter for an early prophylaxis of aspergillosis in Magellanic penguins, the average body weight upon arrival at the center of the penguins of the present study did not differ between the group that developed aspergillosis and the non-aspergillosis group. The data demonstrate the lack of efficacy in using this measure as defining risk groups for the disease and, thus, this parameter must be reviewed for future protocols.

On the other hand, a significant difference was found between groups in body weight gain during captivity, averaging about 850 g in the nonaspergillosis group and about 100 g in animals that died of aspergillosis. However, this variable was evaluated considering the body weight difference between admission and release or death of the animal, and not from a curve of weekly values. Therefore, it was not possible to determine whether the animals that developed aspergillosis maintained a relatively stable weight during the stabilization and recovery process, or if they gained weight with weight loss occurring once the disease began to affect them. This second hypothesis is more plausible, as one of the clinical signs of aspergillosis in penguins is anorexia and regurgitation, resulting in chronic weight loss.^{1,17,21,28}

The significant differences found in Hct and TP values between groups with and without aspergillosis in the present study suggest that the penguins received by CRAM-FURG might have been already infected. In this context, the signif-

icantly lower mean Hct in animals that developed aspergillosis may be explained by erythrocyte lysis caused by hemolysin produced and released by *Aspergillus* spp.²⁴ Also, the inflammatory processes in response to fungal tissue invasion associated with the increase in immunoglobulin production likely contributed to the average value of TP significantly increasing in the group with aspergillosis.^{1,5,17}

Some methodological limitations should be mentioned. First of all, we cannot say with certainty that released penguins did not have aspergillosis because the gold-standard diagnosis of the disease is still postmortem. It started from the principle that released animals were healthy, as all fulfilled release requirements in accordance with rehabilitation protocols, which in theory would prove their healthy condition.

We did not have all the baseline information for some of the animals sent from other centers. Some variables, such as weight variation during recovery in the other center, time in captivity before being sent to CRAM-FURG, oiled animals, and use of prophylactic itraconazole, were unknown. However, for those animals in which these variables were known, most were not oiled and most had not been treated with prophylactic itraconazole, which reinforces that the variable "origin" and its associated stress of transportation are important factors in increased risk of death. Also, it is possible that the reason for the observed result is a lack of power for the variables' petrolization and itraconazole prophylaxis. This hypothesis is feasible taking into account the large 95% CIs of the calculated OR. Finally, 39 of 366 penguins were excluded because of the absence of information for the cause of death. This proportion (8.9%) is in an acceptable range of losses, and analysis of age and origin in this group showed that, on the basis of these characteristics, they were not significantly different from those who were studied, minimizing any possible selection bias.

CONCLUSION

The proportional mortality of around 50% of the penguins at CRAM-FURG due to aspergillosis, mainly caused by *Aspergillus* section *Fumigati*, and the incidence density of aspergillosis in 7.3 cases per 100 Magellanic penguins/mo follow-up proves the importance of this disease for the species. In addition, this study highlights the necessity of adopting control and preventive measures to reduce mortality due to aspergillosis and to maximize release rates of rehabilitated penguins back to the

environment. The contribution of air transport in the development of aspergillosis in Magellanic penguins should be investigated further.

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Received for publication 8 July 2013