Evidence of Amphotericin B Resistance in *Macrorhabdus ornithogaster* in Australian Cage-Birds

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Abstract

Amphotericin B is widely used for the treatment of *Macrorhabdus ornithogaster* infections. To date, however, there have been no randomised controlled trials confirming its efficacy where cure was confirmed by post mortem examination. To determine the efficacy of amphotericin B against *M. ornithogaster*, a three-part study was undertaken. Initially, treatment outcomes of *M. ornithogaster* infected birds that presented to the Sydney School of Veterinary Science over a 9-year period and were treated amphotericin B were reviewed. This was followed by a pilot treatment trial with two naturally infected birds (*Melopsittacus undulatus* and *Agapornis roseicollis*) administering amphotericin B at 100mg/kg twice daily for 30 days. Finally, a randomized controlled trial was performed using experimentally infected chickens that were treated with amphotericin B at 25mg/kg and 100mg/kg twice daily for 10 days. Nine years of clinical records indicated treatment failure in 80.4% of 36 cases that met the inclusion criteria. The pilot study in naturally infected birds showed that amphotericin B given twice daily for 30 days at 100mg/kg did not clear, but significantly decreased, *Macrorhabdus ornithogaster* burden and a profound rebound effect of the number of organisms shed in the faeces following treatment cessation. Finally, the randomised controlled trial found that amphotericin B given at 100mg/kg did not clear, but significantly decreased the burden of *M. ornithogaster* compared with both the 25mg/kg group ($p = 0.037$)

and the no treatment control group (p = 0.001), whilst amphotericin B at 25mg/kg twice daily showed no statistical difference to the no-treatment control group. A strong curvilinear correlation between body weight and M. ornithogaster infection burden was present in the infected chickens. These findings represent treatment failure in three scenarios and indicate that treatment with amphotericin B has poor efficacy against *Macrorhabdus ornithogaster*.

Keywords: Amphotericin B, *Macrorhabdus ornithogaster*, resistance, treatment

Introduction

Macrorhabdus ornithogaster is an anamorphic (asexual reproductive stage) ascomycete yeast that grows at the isthmus of the ventriculus and proventriculus in birds.[1] Infection may remain subclinical, but is commonly implicated in disease; causing proventriculitis and ventriculitis manifesting as vomiting, diarrhea, chronic wasting disease and death.[2,3] Disease occurs in individual birds and can also cause significant mortality in avicultural collections. Susceptible species include wild birds, pet and aviary birds, chickens, and other birds raised commercially and has a global distribution.[3-12]

Filippich first described the use of amphotericin B for the treatment of *Macrorhabdus ornithogaster* infection (macrorhabdosis) in 1993,[13] and it has became the mainstay of treatment in individual cage birds.[14] Recommended dosage rates range from 5 mg/kg by mouth twice daily to 100mg/kg by mouth twice daily, recommended durations of treatment range from 10 to 30 days.[13-15] A water-based formula with a dosage rate of 0.9-1.0mg/ml for 10 days is also commercially available and is routinely used to treat individual birds and avicultural collections.[14,15,13,16]

Filippich was also the first to report resistance to amphotericin B treatment when administered at a dosage rate of 5mg/kg by gavage for 10 days. Of 30 birds treated for *M. ornithogaster*, two were treated twice with 5mg/kg PO for 10 days, but remained persistently infected. Of the birds initially reported as being treated successfully, at least 5/24 birds were faecal positive for *M. ornithogaster*

by 10 weeks following treatment.[13] Most recently, a report assessing clinical outcomes of budgerigars treated with amphotericin B for macrorhabdosis, found that only 53% of birds treated with 100mg/kg PO BID for 30 days were treated successfully and of these, 17% re-presented with *M. ornithogaster* infection within 2.5 years, suggesting that relapse and apparent treatment failure also occurs in Germany.[17]

In this study, we investigate the efficacy of Amphotericin B for the treatment of macrorhabdosis (the clinical disease caused by *M. ornithogaster*). Efficacy is assessed through a retrospective case study, treatment trials in a naturally infected budgerigar (*Melopsittacus undulatus*) and peach-faced lovebird (*Agapornis roseicollis*) and in a controlled trial using day-old infected chickens.

Materials and methods

Retrospective Case Series

Case selection and classification

One hundred and forty cases with a diagnosis of macrorhabdosis were retrieved from the medical records of the Avian, Reptile and Exotic Pet Hospital, The University of Sydney, Camden, NSW from January 2008 to June 2017 by searching for them using the keywords *Macrorhabdus*, *ornithogaster*, megabacteria, amphotericin, and AGY. Birds that had a confirmed ante-mortem diagnosis of *M. ornithogaster* infection based on a positive wet mount or Gram stain, received treatment with amphotericin B and had at least one follow-up wet mount, faecal examination or ventricular scraping from a deceased bird where included in the study. Thirty-six birds met the inclusion criteria and had complete clinical records available for assessment.

Treatment success was defined as consistent improvement or resolution of the patient's pre-treatment signs for more than 14 days after treatment and the absence of *M*. *ornithogaster* in the faeces 14 days or more after treatment.

Treatment failures where defined as birds that died during the treatment period, birds whose signs did not resolve and birds that were still shedding *M. ornithogaster* after treatment ceased.

Data collection and statistical analysis

Data was extracted from the medical records into a secure data portal, for analysis with statistical management software R.[18]

Initial treatment trial

A budgerigar and a peach-faced lovebird were surrendered from private breeding facilities after treatment was initially declined on cost-basis. Both birds came from large collections experiencing signs consistent with macrorhabdosis. The lovebird aviary had been treated with amphotericin B at 100mg/kg PO every 12 hours by gavage for thirty days. The lovebird was three years old according to the closed ID band on its leg and was of unknown sex. It was last treated with amphotericin B 90 days earlier. It had a pectoral muscle score of 3/5 with no overt signs of disease. The budgerigar was 3 years old and was a female based on her closed leg band and the colour of her cere respectively. She had a pectoral muscle score of 2/5 and was lethargic with a ravenous appetite and dark faeces, consistent with the signs of macrorhabdosis. Both birds were shedding *M. ornithogaster* in their feces.

The budgerigar and lovebird were housed separately in standard wire flight cages (AviOne, Kongs Australia Pty, Ingleburn, NSW, Australia). The birds were fed a high quality seed mix appropriate for the species and allowed access to clean water ad-libitum. All animal care practices and procedures were in accordance with the University of Sydney Ethics Committee approval 2016/1069.

Amphotericin B preparation

Amphotericin B 1000mg/g powder (PCCA, Houston, Texas, USA) was mixed with lactulose (Dulose, Aspen Australia Ltd, St Leonards NSW, Australia) to make a suspension that had a concentration of 50mg/ml. The suspension was made fresh every third day and stored in the refrigerator at 4°C in a lightproof container between treatments.

Detection and quantification of *M. ornithogaster* in faeces

Faecal samples and weights were collected twice weekly for two months prior to treatment, during the one month treatment trial and for one month after treatment. To collect faeces, the birds were weighed and then isolated in an all wire holding cage. The first faecal pellet produced was collected using a cotton tip applicator off a clean plastic tray, with care taken to avoid the urate portion of the faeces, and placed into a conical 1.5 microcentrifuge tube.

The faecal samples were processed within 20 minutes of collection. Samples were thoroughly mixed by agitation for 30 seconds in 0.5ml of sterile 0.9% NaCl (Provet Pty Ltd, Eastern Creek, NSW, Australia). The top 0.03ml of the meniscus was placed onto glass slide and covered with a cover slip. The slide was examined with direct light microscopy with the stage diaphragm down at 400X magnification. Ten randomly selected fields were examined, and the number of organisms was recorded from each field.

Treatment

The birds were treated with amphotericin B solution in lactulose at the dose of 100mg/kg PO BID for 30 days.[14]

Blinded randomised controlled treatment trial

Forty, day-old, white leghorn chickens (*Gallus gallus domesticus*) were obtained from a local producer (Red Lea Chickens Pty Ltd, Blacktown, NSW, Australia). Chicks were randomly assigned to groups using a random numbers table. The chicks were housed in raised, galvanised wire cages (AviOne, Kongs Australia Pty, Ingleburn, NSW, Australia) measuring 77cm x 46cm x 46cm. Chicks were fed a granular chicken starter crumble (Barastoc, Ridley Corporation, Melbourne, VIC, Australia) that did not contain antibiotics. Food and water were provided *ad*

libitum. The chicks were maintained at an ambient temperature of 26-28°C and supplementary heat was provided using a 250W ceramic heat globe that remained on 24h a day. Chicks were humanely euthanised with carbon dioxide at the conclusion of the trial. All animal care practices and procedures were in accordance with the University of Sydney Ethics Committee approval 2016/1069.

Infection trial

The chicks were divided into four groups of ten individuals using a random numbers table. Group 1 was treated with amphotericin B at 25 mg/kg PO BID, group 2 was treated with amphotericin B at 100 mg/kg PO BID, group 3 was administered sterile water at an equivalent volume to amphotericin B given to groups one and two PO BID and group 4 (uninfected) was not given amphotericin B nor sterile water.

Macrorhabdus ornithogaster was isolated from a peach-faced lovebird (*Agapornis roseicollis*) showing signs suggestive of disease caused by a *M. ornithogaster* infection and grown *in vitro* as previously described.[19] The *M. ornithogaster* organisms were centrifuged to create a concentrated pellet. The growth media was decanted and the remaining pellet was diluted in sterile phosphate buffered saline (PBS) to make a concentration of 10^5 *M. ornithogaster*/ml.[10] The chicks from the three infection groups (1, 2 and 3) were inoculated with 1ml of the suspension by gavage tube into their crop. The negative control group (group 4) was inoculated with 1ml of phosphate buffered saline (PBS) using the same technique.

Seven days after inoculation, five infected chicks were randomly selected and euthanised, scrapings were collected from the proventricular isthmus and examined by direct wet mount microscopy to confirm infection. Once positive infection was confirmed in all of the euthanised individuals, treatment of the remaining infected chicks commenced.

The chicks were weighed twice daily prior to treatment and dose calculations were performed to adjust dose volume to match growth rate. All treatments were administered directly into the crop by the same investigator.

Faecal preparation

Faecal samples were collected from three random birds in each group, twice weekly prior to and during the treatment trial and processed as described.

Postmortem assessment of treatment efficacy

After ten days all chicks were euthanized. The proventriculus and ventriculus were removed and opened. The isthmus was divided in half longitudinally; the two sections were placed in 1ml of PBS and formalin (Fronine, Thermo Fisher Scientific Australia Pty Ltd, Riverstone, NSW, Australia) respectively. Within five hours of collection, the PBS suspension was agitated vigorously for 30 seconds and a wet mount of 0.3ml of the PBS solution was drawn from the meniscus and the concentration of the organisms was determined as described for the faecal samples and expressed as the number of *M. ornithogaster* organisms per high-powered microscope field (MO/HPF).

Data collection and statistical analysis

A generalised linear mixed model was used to estimate the effects of treatment group and bird weight on the number of *M. ornithogaster* organisms per slide, which was modelled as a Poisson random variable. The treatment and weight variables were included as fixed effects. The negative control group was excluded from the analysis as these chickens remained uninfected. Random effects due to individual birds and slides were included in order to acknowledge both between-bird variations in *M. ornithogaster* infection levels (e.g., due to differences in immune response) and inhomogeneous density of *M. ornithogaster* organisms across slides, respectively. The model was fitted in R using the package TMB.[18, 20]

Results

Retrospective case series

A total of 36 birds met the inclusion criteria. Of these, 23 (63.8%) died or were euthanised within three months of diagnosis. From the time of diagnosis, the mean survival time was 22 days with a median survival time of 14 days. Of the birds that died, eight had specific morbidities occurring in conjunction with their macrorhabdosis mentioned in their history, the majority being attributable to primary gastrointestinal disease (e.g. melena, enteritis, trichomoniasis) but others were not (e.g. *Cnemidocoptes* sp. and ataxia); one of the birds had moderate anaemia (14%) on presentation, was treated, but subsequently died and was found to have a myxosarcoma of the ventricular wall with no evidence of *M. ornithogaster* following treatment. Of the birds that survived, 7 (19.4%) remained positive following treatment with a median follow-up of 21 days and a mean of 53.9 days. There were 6 (19.6%) treatment successes, with birds recording negative faecal cytology results following treatment with a median follow-up of 20 days and a mean of 20.5 days.

The birds that met the inclusion criteria are represented in Table 1.

All birds were treated with amphotericin B at doses ranging from 5mg/kg to 100mg/kg PO BID. The following doses were used; 5mg/kg (1 bird), 25mg/kg (26 birds), 50mg/kg (2 birds) and 100mg/kg (5 birds). Three birds were treated in the water at 1mg/ml due to the owners' inability to medicate the bird. The treatment period varied from 1 day (those that died shortly after treatment) to 30 days.

The variety of doses presented in table two highlight the change in dose rate over time, in 2008 a dose of 5mg/kg PO BID was used (similar to the initial drug trials by Filippich in 1992),[13] during the ensuing 5 years 25mg/kg PO BID was the mainstay of treatment and in the last two years, the dosage regime was consistently 100mg/kg PO BID.

Two models were fitted to compare the treatment groups: (1) a logistic regression model was fitted to test the null hypothesis that there was no

difference in treatment success probabilities, and (2) a parametric survival analysis regression model was fitted to test the null hypothesis that there was no differences in mean survival times. Neither null hypothesis was rejected, with p-values of 0.82 and 0.33, respectively. This is unsurprising; because the majority of birds were treated with 25mg/kg, the remaining treatment groups were left with small sample sizes, resulting in analyses that lacked statistical power. It was unlikely that statistical modelling would detect any effects that may exist.

Pilot case study

Treatment significantly ($p = 0.001$) decreased the number of *M. ornithogaster* shed in the faeces in both birds, and both birds had screening days during treatment when they were shedding where no *M. ornithogaster* was observed in their faeces (Figure 1). Immediately after the cessation of treatment both birds experienced a rebound in faecal shedding and began shedding higher numbers of *M. ornithogaster* than before treatment. In the month following treatment, the infected lovebird lost weight, displayed a ravenous appetite and became progressively lethargic and was humanely euthanised one month after the completion of treatment.

Blinded randomised controlled infection trial

Seven days after inoculation, the five birds that were euthanised had positive isthmus scrapes for *M. ornithogaster*. The remaining birds completed the treatment trial, of these, three birds from the 100mg/kg amphotericin B group were negative for *M. ornithogaster* on isthmus cytology. All the remaining birds in the three infected groups were positive at the completion of the treatment period, while the negative control group remained negative.

Likelihood-ratio tests provided strong evidence of differences between treatment groups ($p = 0.004$) and for a quadratic weight effect in the linear predictor ($p = 0.016$). These effects are shown in Figure 1.

The 100mg/kg group was significantly different from both the 25mg/kg group ($p = 0.037$) and the no treatment control group ($p = 0.001$). In comparison to the

100 mg/kg group, the average bird in the control and 25mg/kg groups were estimated to have 6.14 and 3.35 times the number of *M. ornithogaster* organisms per slide, respectively. These estimates had corresponding 95% confidence intervals of (2.22, 17.61) and (1.08, 11.63). The difference between the control and the 25mg/kg group was not statistically significant.

The final model included a quadratic effect, whereby birds of approximately 495g were estimated to have the largest number of *M. ornithogaster* organisms per slide, on average (Figure 1). The coefficient of the quadratic term was strongly significant ($p = 0.007$), providing support for this model over one with a linear relationship. The quadratic model was also preferred by AIC.

The faecal samples that were collected on day 0 (prior to inoculation) were negative for *M. ornithogaster.* From day 2, all samples collected from infected groups contained >10 organisms/HPF, confirming active infection and shedding of the organism. During the treatment trial, shedding results varied between groups with three birds in the 100mg/kg group having samples that returned <1 organism/HPF and two birds that returned negative faecal cytology results on day eight of treatment. Because samples were collected randomly from different chicks during the treatment trials, further statistical analysis was not carried out.

A t-test was used to compare the average weight of the birds in the negative control group to those that were infected with *M. ornithogaster*. The null hypothesis of no difference in average weight was rejected ($p = 0.022$); uninfected birds were estimated to be 53 g heavier than infected birds, with a 95% confidence interval of (8, 99)g.

Discussion

Macrorhabdus ornithogaster is a significant pathogen of avicultural and wild birds worldwide. Amphotericin B is the treatment that is most commonly recommended for *M. ornithogaster* infection. However, since Filippich first demonstrated amphotericin B's efficacy against *M. ornithogaster* there has been

only one additional study on its clinical efficacy.[13,17] In the present study, we assessed the current efficacy of amphotericin B for the treatment of *M. ornithogaster* by reviewing the outcomes of clinical cases of *M. ornithogaster*, doing controlled treatment trials in two naturally infected birds, and by treating experimentally infected chickens.

The retrospective study of client-owned birds diagnosed with *M*. *ornithogaster* infection treated with amphotericin B demonstrated that amphotericin B treatment was largely unsuccessful, curing only 6/36 (17%) of the treated birds. The cure rate may have been even lower, as repeated faecal examinations at the end of treatment were not always performed and some infections may have relapsed as seen in the budgerigar and lovebird in this study and a recent German study where a re-presentation rate of 17% of the treated budgerigars occurred. [17]

The success of amphotericin B treatment in the current study (17%) is considerably lower than the efficacy demonstrated by the study done in Germany where 36% of budgerigars infected with *M. ornithogaster* recovered from the infection with amphotericin B treatment at 100mg/kg PO BID for 30 days.[17] These results suggest that differences in the prevalence of resistance may occur in the two different study populations.

Possible causes for treatment failure in these client-owned birds could include one or more of the following: Infection with amphotericin B resistant *M. ornithogaster* strains, problems with owner compliance, failure to obtain therapeutic concentrations of amphotericin B at the site of infection in birds treated with amphotericin B in the drinking water or failure to maintain therapeutic concentrations of amphotericin B at the site of infection because of rapid drug passage through the stomach. Failure to obtain therapeutic concentrations at the site of infection may be due to solubility in water. Amphotericin B is reported as being highly hydrophobic, making it insoluble in water at pH 6-7 and with a bioavailability of 0.1mg/ml at pH 2 and 11.[21] It is probable that poor water solubility when suspended in-water contributed to

these treatment failures. Various modifiers and suspensions have been trialed using lipid emulsification and solubilizing agents to increase bioavailability of amphotericin B in oral formulations, which may lead to better treatment efficacy, and these warrant future investigation in avian patients.[21,22,23]

To rule out the variables of owner compliance, route of administration and dosage rate, a preliminary treatment trial of a naturally infected budgerigar and lovebird was undertaken. While shedding stopped during treatment, it rebounded immediately after treatment was stopped, strongly suggesting that the strains infecting these two birds were resistant to amphotericin B, even when given at the highest recommended dosage rate (100 mg/kg, every 12 hours) for the longest recommended duration of treatment (30 days).

To provide additional evidence for the presence of amphotericin B resistant strains of *M*. *ornithogaster* a randomised controlled trial using experimentally infected chickens was undertaken using an isolate from a lovebird flock that had failed to respond to treatment. The results of this trial showed that treatment with amphotericin B at 25 mg/kg every 12 hours for 10 days, was ineffective based on its failure to cure any of the birds and failure to significantly reduce concentrations of the organism in the isthmus as compared to the infected control birds. Treatment with amphotericin B every 12 hours at 100mg/kg for 10 days had a negative impact on the *M. ornithogaster*, reducing its concentration at the isthmus, but only cured three of ten birds, also suggesting that this higher dosage rate would not be effective in most clinical cases. The rapid growth rate and heavy body weight made treatment for longer than 10 days cost-prohibitive (daily cost of the solution / day was approximately 100 Australian dollars for all chickens).

Combined, the three phases of this investigation show that treating birds with *M*. *ornithogaster* infections with oral amphotericin B, even at maximal dosage rates and strict adherence to dosage regimes, does not result in the definitive a cure of infected birds in the majority of cases. Based on the controlled treatment trials in chickens, the likely cause of these treatment failures is the presence of strains

of amphotericin B resistant *M. ornithogaster*. Whether this resistance is recently acquired or has always been intrinsic in *M. ornithogaster* is not known. A study by Filippich and Perry in 1993, where naturally infected *M. ornithogaster* budgerigars treated with the relatively low dosage rate of 5mg/kg amphotericin B orally every 12 hours for 10 days, suggests that some degree of resistance may have been present then prior to the widespread use of amphotericin. In this study, 30 naturally infected budgerigars were treated for *M. ornithogaster*, two were treated twice with 5mg/kg PO for 10 days, but despite this, remained persistently infected. The remainder of the budgerigars were reported as being negative on faecal cytology after 10 days of treatment. Of these birds reported as being treated successfully, at least 5/24 birds were faecal positive for *M. ornithogaster* by 10 weeks following treatment.[13]

There is limited information about the pharmacokinetics, dynamics or the efficacy of amphotericin B in different bird species. Amphotericin B is a fungicidal heptaene macrolide antimycotic that exerts a powerful and broad activity against a vast array of fungi and has a remarkably low rate of microbial resistance.[24] Resistance is reported most commonly in *Aspergillus terreus* and *Candida* spp. and despite extensive research, the mechanism for resistance remains poorly understood. [25,26] A number of mechanisms are thought to contribute. Amphotericin B - ergosterol binding was found to play a minor role in intrinsic amphotericin B resistance by forming aqueous pores in the lipid bi-layer and causing leakage of proteins and amino acids, disrupting membrane proton gradients.[25] Most recently however, researchers examining *A. terreus* strains resistant to amphotericin B found that the oxidative stress response plays a major role in modulating resistance. Resistant strains possessed an almost doubled basal superoxide dismutase activity when compared with susceptible strains, as well as exhibiting enhanced oxidative stress response when treated with amphotericin B. This work concluded that superoxide dismutase activity and oxidative stress response are crucial in the resistance of *A. terreus* to amphotericin B.[27]

If the current protocols for treating *M. ornithogaster* with amphotericin B prove to be ineffective globally, then avian veterinarians maybe left with no proven effective and safe treatments for *M. ornithogaster* infection as all other potential therapeutic options have been shown to be ineffective, potentially toxic, or are of unknown efficacy. Fluconazole was shown to be effective at treating chickens experimentally infected with *M. ornithogaster* when administered at 100 mg/kg, but this dosage rate was toxic in budgerigars and lower dosage rates in budgerigars were not effective. An *in vitro* investigation, where growing *M. ornithogaster* was exposed to constant concentrations of sodium benzoate, potassium benzoate and potassium sorbate showed that all of these chemicals could inhibit their growth.[28] Unfortunately, *in vitro* susceptibility testing is complicated by the difficulty in obtaining pure culture of *M. ornithogaster* because of the specific and fastidious growth requirements of the organism outside the host.[19, 28] Flock treatment using sodium benzoate was reported to be effective in treating a budgerigar aviary with *M. ornithogaster*, however, the authors have seen both sodium toxicity in budgerigars and lovebirds treated with sodium benzoate and potassium toxicity in lovebirds treated with potassium benzoate (Baron and Phalen, unpublished 2015), indicating that if these chemicals are to be used they needed to be used cautiously. The potential efficacy of nystatin against *M. ornithogaster* is not known. *In vitro*, under conditions of constant contact, nystatin at a concentration of 10 units per ml, inhibited growth of *M*. *ornithogaster*[28] and there is a report in the literature where treatment using nystatin resulted in the cessation of *M. ornithogaster* shedding in budgerigars at a dose of 3,500,000iu/ml for two days, followed by 2,000,000iu/ml for 28 days.[29]. Filippich, however, was unable to stop *M. ornithogaster* shedding with nystatin in budgerigars.[13]

A potentially novel approach to treating *M. ornithogaster* infections would be concurrent oral treatment with pro-oxidants, such as L-ascorbic acid, and amphotericin B. *In vitro* studies using L-ascorbic acid resulted in an increased radical oxygen species generation and enhanced amphotericin B susceptibility in resistant strains of *A. terreus*, *Candida albicans* and *Aspergillus flavus* at classically therapeutic doses. All investigated strains displayed an *in vitro*

increase of amphotericin B susceptibility with increasing L-ascorbic acid concentrations.[24] These same results were mirrored when assessed using an insect model *in vivo* where L-ascorbic acid in combination with amphotericin B significantly improved the survival of larvae infected with resistant *A. terreus* compared with amphotericin B treatment alone.[24] The authors are currently investigating the efficacy of the addition of oral ascorbic acid to current amphotericin B treatment protocols of *M. ornithogaster*.

The treatment trials in the budgerigar and lovebird in this study showed that, although amphotericin B treatment was not able to induce a cure in these two birds and shedding recurred at the end of treatment, it did suppress shedding during treatment. This highlights the importance of repeat faecal testing at least a week after the cessation of treatment to determine if the treatment was effective and not assume that the cessation of shedding during treatment represents a cure. Why such a marked increase in shedding, and presumed regrowth in the isthmus, occurred in the budgerigar and lovebird treated in this study following the cessation of treatment is not known.

This is the second study to report the impact of *M. ornithogaster* infection on chickens infected at one-day of age. In the first study, *M. ornithogaster* infection had a significant impact on food conversion ratios and infected birds did not gain weight as fast as control birds, results supported in part by this study that found a significant difference in weight between the infected birds and the non-infected control group.[10] In our study, a non-linear relationship was observed between weight gain and the number of *M. ornithogaster* observed cytologically at the isthmus, post-mortem. This indicates that chicks with the heaviest burden were in the middle of the average weight range. This curvilinear weight relationship has not been previously described. Chicks with low and high body weights were likely to be infected with low numbers of MO/HPF, while the chicks with average body weights were likely to have higher numbers of MO/HPF. The highest shedding chicks were those nearest the weight 495g. The reason for this relationship is unclear, but may be related to the median weight range chicks having the most optimal growth environment for *M. ornithogaster* or the smaller

chicks, with lower burdens, being bullied away from food by those chicks with a higher *M. ornithogaster* burden and a more ravenous appetite. The heavier chicks may have begun to mount an immune response and were therefore harboring fewer organisms.

It is unclear from this study whether this model is applicable to experimentally infected birds in the early stage of disease or whether this is a consistent finding across all infected birds. In light of the fact that birds clinically affected by macrorhabdosis often present emaciated and with a ravenous appetite, the curvilinear nature of the weight to *M. ornithogaster* burden requires further investigation.

Amphotericin B is the recommended treatment for cage birds with macrorhabdosis and despite reports of treatment failure anecdotally, this report is the first to combine broad treatment failure in a retrospective case series analysis, case report and blinded, randomised controlled trial. We present amphotericin B treatment failure in three scenarios, despite being administered at the accepted dose and duration. This report highlights the need for further investigation into effective treatment modalities, strain specific resistance to amphotericin B and the importance of complete, consistent cessation of faecal shedding before withdrawal of treatment for *M. ornithogaster.* Further *in vitro* sensitivity testing assessing treatment success outside the host and larger-scale naturally infected controlled trials with treatment success confirmed by necropsy are warranted and a push for novel, successful and cost effective treatment modalities is required in order to better treat and eliminate *M. ornithogaster* from infected birds.

In conclusion, our work suggests that in at least one geographic area of Australia, that treatment of amphotericin B, even at the highest recommended dosage rate reported in the literature has limited efficacy. This creates an urgent need to determine if this is a pattern that is being seen in other countries and to develop alternate treatment protocols.

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Conflict of interest

The authors declare no conflicts of interest.

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Table 1. Species representation of birds that underwent treatment for *macrorhabdus orithogaster* with amphotericin B, highlighting the sex ratio and the clinical outcomes following treatment.

Species	Number	Male	Female	Unknown	MO Positive	MO Negative	Died
Mellopsittacus undulatus	22	10	7	4	5	3	14
Nymphicus hollandicus	3	1	0	2	0	1	2
Eolophus roseicapilla	2	0	0	2	0	0	2
Agapornis spp.	7	0	0	7	2	2	3
Alisterus scapularis	1	0	0	1	0	0	1
Polytelis alexandrae	1	0	0	1	1	0	0

Table 2. The variety of dose rates used in the retrospective case series and their corresponding treatment successes when using amphotericin B to treat *macrorhabdus ornithogaster*.

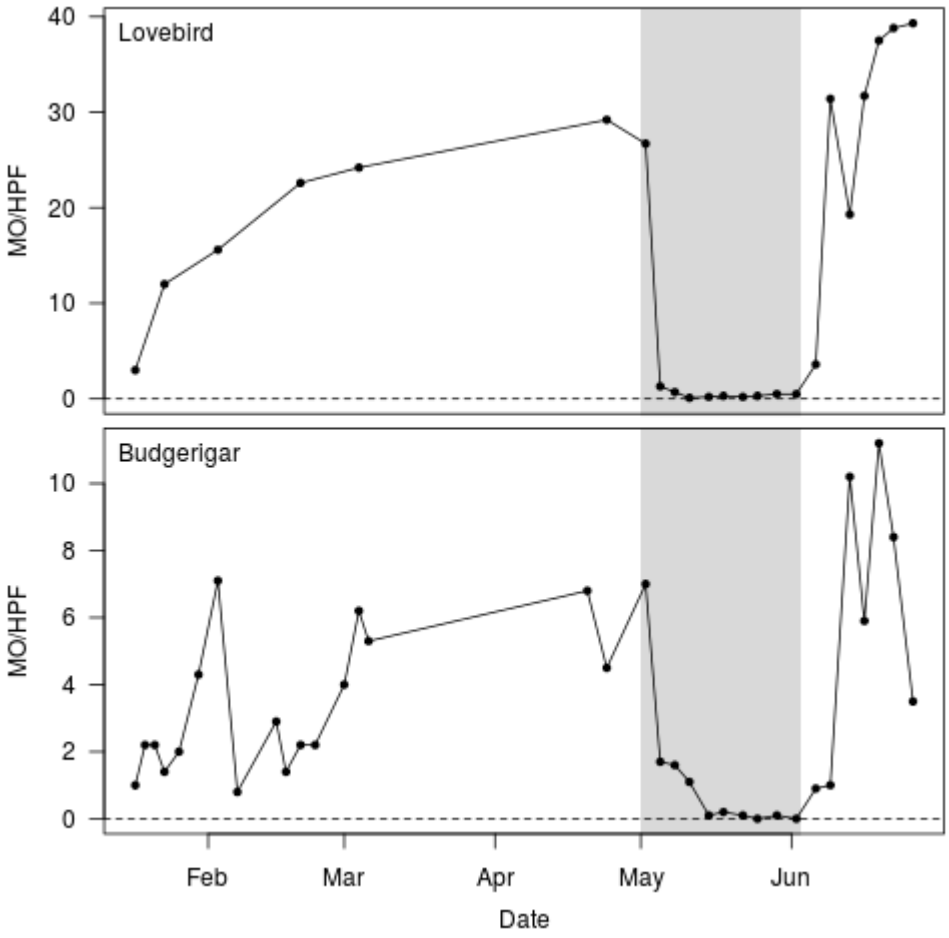
Dose Rate	Number of Birds	Treatment Success	Percentage of Treatment Success (%)
5 mg/kg	1	0	0/1 (0.00)
25 mg/kg	25	4	4/25 (16.00)
50 mg/kg	2	0	0/2 (0.00)
100 mg/kg	5	1	1/5 (20.00)
1 mg/ml in water	3	1	1/3 (33.33)
Total	36	6	6/36 (16.67)

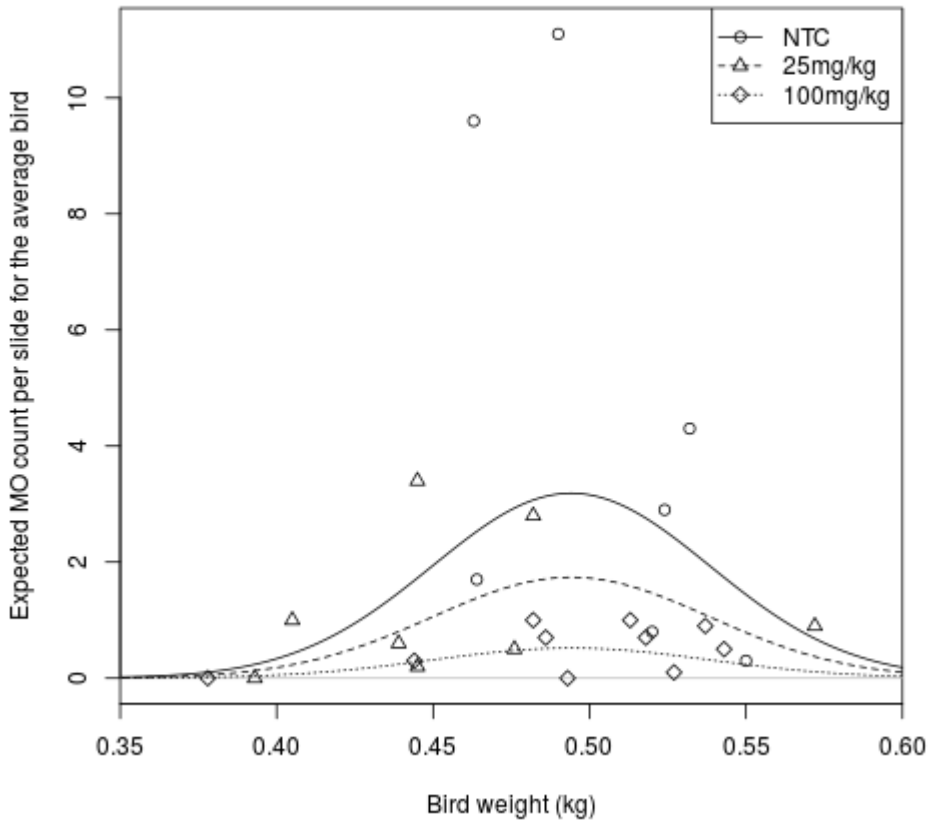
Table 3. Four treatment groups demonstrating the average *macrorhabdus ornithogaster* / high powered field (MO/HPF) and average bodyweight, with the body weight range in each group in grams.

Treatment Group	MO/HPF	Average weight (g)	Range (g)
1 -- Amphotericin B 25mg/kg PO BID	1.15	457.12	393 - 572
2 -- Amphotericin B 100mg/kg PO BID	0.78	492.10	378 - 543
3 – Sterile water (equivalent volume) PO BID	4.4	506.14	463 - 550
4 -- Negative Control	0	538.2	345 - 624

Figure 1. Budgerigar and lovebird *macrorhabdus ornithogaster* per high-powered field in faeces measured over a five-month period. The shaded area represents the treatment period when amphotericin B was administered twice daily at 100 mg/kg by gavage tube.

Figure 2. Quadratic weight effect in the linear predictor ($p = 0.016$). Quadratic effect highlighting the finding that regardless of group, individual birds, of approximately 495 g have the largest number of *macrorhabdus ornithogaster* organisms per slide.


Lovebird
MO/HPF
40
30
20
10
0
Budgerigar
MO/HPF
10
8
6
4
2
0
Feb
Mar
Apr
May
Jun
Date


NTC
25mg/kg
100mg/kg
Expected MO count per slide for the average bird
0
2
4
6
8
10
0.35
0.40
0.45
0.50
0.55
0.60
Bird weight (kg)