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Influence of size of sporocyst inoculum upon the size and number of sarcocysts of *Sarcocystis falcatula* which develop in the brown-headed cowbird

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Abstract

The influence of the number of sporocysts in the inoculum of *Sarcocystis falcatula* on the morphology of the sarcocysts has not been reported in the literature. To determine if there is a relationship, different number of sporocysts were inoculated orally into wild-caught cowbirds. After 14 weeks, the cowbirds were euthanised and muscle tissue was examined grossly and by histologic sections. Sarcocysts were compared based on the numbers which developed and their sizes. There was a linear increase in the number of sarcocysts as the size of the inoculum increased, however, the size of the sarcocysts became smaller with the increase in number of sporocysts inoculated.

Keywords: *Sarcocystis falcatula*; Cowbirds; Sarcocyst size; Sarcocyst development; Crowding effect

1. Introduction

Sarcocystis falcatula is a coccidian parasite which obligatorily alternates between definitive and intermediate hosts. The definitive host is the Virginia opossum (*Didelphis virginiana*) and the intermediate host can be several avian species such as grackles, cowbirds, canaries, budgerigars, and pigeons (Box and Smith, 1982). The opossum is host to sexual multiplication in its intestine. Infectious sporocysts are then passed in the feces. The sporocysts are ingested by the intermediate host where the sporozoites excyst, penetrate the

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gut wall, enter the bloodstream, and are carried to various organs. Schizogony occurs in endothelial cells throughout the body leading to the production of merozoites which lead to the eventual formation of sarcocysts filled with bradyzoites in striated muscle. The parasite can develop to different degrees in different intermediate host species (Box and Smith, 1982). The opossum then preys upon the intermediate host and becomes infected by the release of bradyzoites from the ingested sarcocysts and the cycle continues.

In a previous study, an arbitrary number of sporocysts were used to infect the cowbirds (Luznar et al., 2000). When the muscles from these cowbirds were examined, the sarcocysts had a different gross morphology in that they were present in higher numbers and were much smaller than sarcocysts found in naturally infected cowbirds. Because sarcocysts are the main stage used to differentiate species of *Sarcocystis*, we felt it important to determine if these different sized sarcocysts were the same species. Experimentally the sarcocysts were fusiform and were up to 5 mm long, but much narrower than those in naturally-infected birds where some cysts were as long as 9 mm (Box et al., 1984). It was hypothesized that because of the apparently large number of sporocysts used to infect the birds, that there was a crowding effect which caused the decreased size in the experimentally produced sarcocysts (Luznar et al., 2000).

Examining the potential role of *S. falcatula* with regards to equine protozoal myeloencephalitis, a neurological disease of horses is important (Dame et al., 1995). This parasite has been associated with the death of psittacines at the San Diego Zoo (Hillyer et al., 1991). The ongoing pursuit of knowledge regarding all aspects of this parasite is important in developing a plan for control, prevention, and treatment of the infected hosts. Thus determining the gross morphology of the sarcocysts under different conditions is important in the identification and classification of *Sarcocystis* spp.

2. Materials and methods

2.1. Inoculum

Feces from opossums experimentally infected with *S. falcatula* by feeding them brown-headed cowbird (*Molothrus ater*) muscles containing sarcocysts was used as a source of sporocysts. Sporocysts were harvested by the procedures of Luznar et al. (2000) from fresh opossum feces 5–7 weeks before inoculation.

To determine the concentration of the sporocysts in the solution a 0.1 ml sample was taken after thorough mixing and the sporocysts were counted using a light microscope (100× total magnification). Dilutions were made to create the following inocula: Group A — 1000 sporocysts; Group B — 500 sporocysts; Group C — 100 sporocysts; Group D — 10 sporocysts. For each group 1 ml was placed into 15 microfuge tubes.

2.2. Birds

Adult cowbirds were obtained from the USDA National Wildlife Research Center, Florida Field Station. These had been trapped from Alachua County the preceding autumn and had been in held in an opossum proof animal facility. A total of 60 adult cowbirds were

used, 12 for each inoculum size and 12 in the control group that was not inoculated. The 1 ml samples were centrifuged and 0.9 ml was aspirated and discarded. The remaining 0.1 ml and sediment was resuspended. The cowbirds were inoculated by using a 20 gauge needle and 1.0 ml syringe and allowing them to drink from the needle tip or the use of capillary action by pressing the needle against their closed beak. This was then followed with 0.1–0.2 ml of water to flush the needle and syringe. Each group of birds was inoculated this way except for the control group which received nothing. The cowbirds were returned to the USDA facilities 24 h post-inoculation where they remained housed by group until euthanasia.

2.3. Necropsy

The birds were housed for 14 weeks to allow muscle stages to develop. They were euthanised, skinned, and examined grossly for muscle cysts. Muscle samples were taken from the leg and pectoral muscles and fixed in formalin for sectioning and H and E staining. Each slide contained tissues from one bird and consisted of cross-section and longitudinal section of the leg and pectoral muscles.

2.4. Measurements

The number of sarcocysts were counted within a 25 mm^2 area under a dissection microscope (25×). Three counts were made per slide by the same person. Diameters of the larger cysts on each slide were measured using an ocular microscope at 100× total magnification. All measurements are given in micrometers unless otherwise specified. Data were analyzed using analysis of variance (ANOVA) (SAS Institute, 1994; PROC GLM). Pairwise comparisons (using the Bonferroni method of correction for multiple comparisons) were made to determine if significant differences between treatment groups were present.

3. Results

3.1. Number of sarcocysts

The number of sarcocysts found in three random 5 mm × 5 mm areas on each slide were averaged for each group with the group averages shown in Fig. 1. Average number of sarcocysts differed significantly among treatments ($n = 112$, $F = 21.59$, $p = 0.0001$). Pairwise comparisons among all treatments (correcting for multiple comparisons) were significant (all $p < 0.004$), except for the Group A/B comparison ($p = 0.13$), the two highest inoculum treatments (Fig. 1). There was a linear relationship between inoculum size and resulting number of sarcocysts present in the muscle. However, once the inoculum concentration reached 500 sporocysts, the number of muscle cysts developing remained constant, possibly due to a threshold effect. Grossly, the muscles of birds with high numbers of sarcocysts were white. Individual sarcocysts could be visualized without magnification in infections receiving the smaller inocula. No sarcocysts were detected in the muscles of uninoculated muscles.

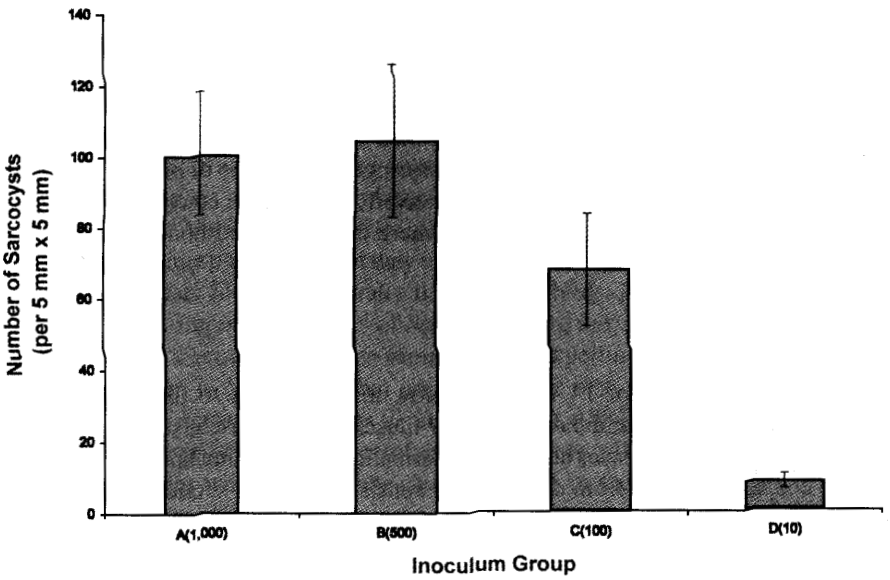

Fig. 1. Average number of sarcocysts detected in cowbirds ($n = 108$). Bars show standard error.

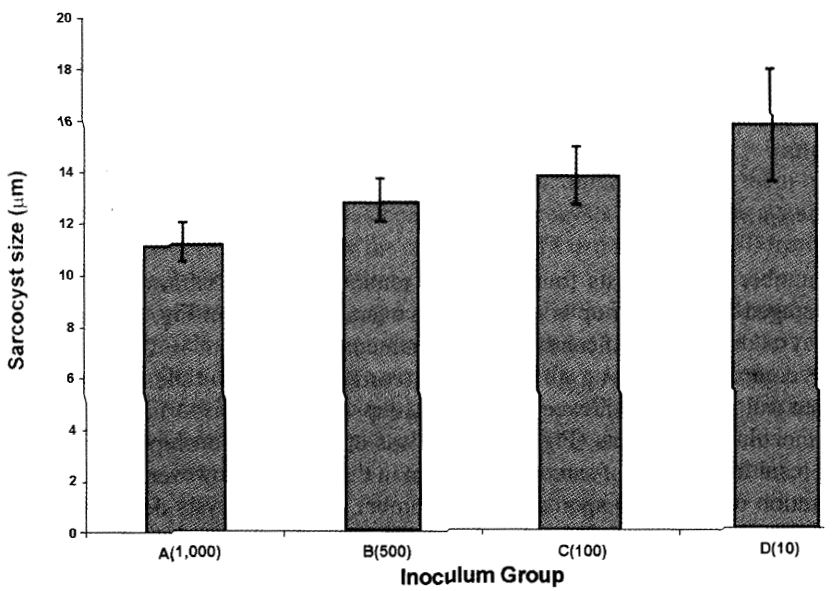

Fig. 2. Average sarcocyst size from each treatment of cowbirds ($n = 108$). Bars show standard error.

3.2. Size of sarcocysts

The average size of the sarcocysts for each group are presented in Fig. 2. Overall, there was a significant difference in mean sarcocyst size among treatments ($n = 108$, $F = 5.21$, $p = 0.0022$). Pairwise comparisons between all treatments (using the Bonferroni correction for multiple comparisons) indicate significant differences between most groups (all $p < 0.02$), the exception being between C and D, the two lowest inoculum treatments ($p = 0.10$) (Fig. 2).

Three cowbirds died before being euthanised. Two of the birds, one from Group B and the other from Group D died less than 5 weeks after inoculation. At necropsy they showed no evidence of infection, however, it takes at least 6 weeks for the sarcocysts to develop. The third bird was from Group A and died 11 weeks post-inoculation. The average number of sarcocysts found corresponded with the numbers found in the other birds in Group A. The average diameter of the cysts was 63.3 μm which was almost half of the average size in Group A most likely due to the reduced time for development.

4. Discussion

The number of sarcocysts and their distribution in the tissues can be affected by a number of factors such as the number of sporocysts ingested, the *Sarcocystis* species, the host species, and the host's immunological state (Dubey et al., 1989). There was lot of individual variation regarding the number of sarcocysts found in each bird. This may be attributable to the individual bird's ability to resist development of the infection. Statistically, using Bonferroni's method, there were significant differences between all of the groups ($p < 0.05$) except between groups receiving 500 sporocysts and 1000 sporocysts, which verifies that there may be a threshold effect.

The size of the sarcocysts can be affected by the length of time they are allowed to develop, the type of host cell in which it is located, and the techniques used for study (Dubey et al., 1989). Only the pectoral and leg muscles were examined for this study and the technique was consistent throughout to minimize variation. Groups A and B were inoculated 1 day before Groups C and D and all birds were killed and necropsied on the same day. In Group D one bird had an average sarcocyst diameter of 310 μm which was approximately twice the size of the sarcocysts found in the birds of the same group. This was probably due to the bird being naturally infected at time of capture which would have allowed a longer time for development and thus the larger sarcocyst size. This bird was removed from the analysis. The prevalence of sarcocysts in cowbirds from Alachua county was 4% (Luznar et al., 2000) and thus in a sample of 60 cowbirds, 2–3 might be expected to harbor sarcocysts of natural origin. Therefore, finding a naturally infected cowbird would not be surprising. The sizes between the different groups did show significant differences. There are many variables which can affect the size of the sarcocysts. Other factors which may affect the size of the sarcocysts are the fixation and possibly the type of fixative. Also, since the sarcocysts are located in striated muscle, the size will depend on whether the host cell was in a relaxed or contracted state at the time of fixation (Dubey et al., 1989). To minimize variation, the same tissues were

collected from each bird using the same technique and type of fixative (formalin) was used.

Overall, there was a slight increase in the average size of the cysts as the number of cysts and inoculum concentration decreased. Again using the Bonferroni method for multiple comparisons, there was a significant difference in size as Groups C and D were different from Groups A and B. There was a crowding effect on the size of the sarcocysts as the number of sporocysts in the inoculum increases above some level.

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