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Effect of the Fungus *Pochonia chlamydosporia* on *Contraecaecum pelagicum* Eggs

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ABSTRACT

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The genus *Contraecaecum* (Ascaridida: Anisakidae) is a nematode parasite belonging to fish-eating birds throughout the world. *C. pelagicum* causes gastrointestinal problems in the *Magellanic penguin*. The objective of this study was to evaluate the effect of an ovicidal fungus (*Pochonia chlamydosporia*) on *C. pelagicum* eggs under different concentrations (500, 1500, 2000, and 3000 per Petri dish). The ovicidal effect was evaluated in accordance with the visualized morphological changes to the eggshell: type 1, physiological effect without morphological damage to the egg shell; type 2, lytic effect with morphological alteration of the eggshell; and type 3, lytic effect with morphological alteration to the egg shell and embryo in addition to hyphal penetration and internal egg colonization. At the end of the experiment, there was significant difference in egg destruction (effect type 3) in the four tested concentrations when compared to the control group. No difference was observed ($P > 0.01$) for ovicidal activity between the four concentrations used. At the concentration of 3000 chlamydospores, a 46.2% of *C. pelagicum* egg destruction was observed, after 7 days. A concentration of at least 500 chlamydospores was effective in destroying *C. pelagicum* eggs, suggesting a potential use for chlamydospores (*P. chlamydosporia*) against this nematode.

Introduction

The genus *Contraecaecum* (Ascaridida: Anisakidae), is a nematode parasite belonging to fish-eating birds throughout the world (Girisgin *et al.* 2012). There is usually a varied helminth fauna in waterfowl and thus, normally literature reports that the

helminths most found in these animals are nematodes of the Order Ascaridea, and the Anisakidae family, having great zoonotic potential (Martins *et al.* 2003, Carvalho *et al.*, 2012). Anisakids are associated to aquatic organisms (fish and marine

mammals) as well as fish-eating birds, and therefore considered in this context. The *Magellanic penguin* (*Spheniscus magellanicus*) is present in South America, with populations distributed along the Patagonian coast (Argentina and Chile) and the Falkland Islands, with the highest abundance among the penguins of temperate areas. According to Mayorga *et al.* (2011), some factors have been implicated in the weakening and mortality of Magellanic penguins during migration, among them are: (1) contamination of the oceans with oil and its derivatives, (2) accidents with fishing nets, (3) ingestion of anthropogenic waste, and (4) gastrointestinal parasites (Martins *et al.*, 2003; Carvalho *et al.* 2012).

The genus *Contraecaecum* (Railliet & Henry, 1912) contains approximately 50 nematode species, of which the mature ones parasitize fish-eating birds and mammals. Their complex life cycle consists of four larval stages and involves copepods, aquatic vertebrates, and fish as intermediate and/or paratenic hosts. Nematodes belonging to the Anisakidae family, such as the *C. pelagicum*, may cause a disease in humans known as anisakidosis. When in the stomach of the definitive host, L3 moults to L4 and both the larvae and the adults may negatively affect the host's health (Anderson *et al.* 2002, Martins *et al.* 2005, Karnek & Bohdanowicz, Girisgin *et al.* 2012).

In Brazil, several studies were conducted in watersheds mentioning the occurrence of helminths of hygienic-sanitary interest occurring in piscivorous fish and in waterfowl, and among these the *Magellanic Penguin* (Santos *et al.*, 1984; Garbin *et al.*, 2007; Ederli *et al.*, 2009). In these birds the changes observed by *C. pelagicum* are related to inflammatory processes and obstruction. In this context, Rezende *et al.* (2009), reports that the parasite community

of a species can reveal important information about their biology and ecology. Parasitological studies on penguins, as well as other migratory animals, may indicate seasonal changes in diet and temporary trophic relationships during migration.

Egg parasitism by means of fungi is an important biological phenomenon that occurs in species such as: *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium* Goddard), *Paecilomyces lilacinus* and *Dactyella ovoparasitica*; its main representatives with significant ovicidal activity (Lysek and Sterba, 1991). After an extended observational study, Lysek (1976) established a qualitative method used to sort ovicidal activity. This method, first proposed on *Ascaris lumbricoides* eggs, determined that the action mechanism of an opportunistic fungus is based on three basic types of ovicidal activity, with seven subtypes: (1) physiological, with biochemical effects and no morphological damage to the egg shell; (2) lithic biochemical effect, with progressive morphological alterations to the eggshell and damage to the embryo; and (3) lithic and morphological effect, with egg penetration, attack and death to the embryo.

The *Pochonia* genus is one of the most studied for the control of helminths potentially harmful to agriculture, and currently, combat against their eggs is also being emphasized. The fungus *P. chlamydosporia* was found in Alabama - USA, in 1981, parasiting *Meloydogine* sp eggs and females, being considered as one of the most promising agents for the management and control of problems caused by nematodes. It is also successfully employed in reducing the rate of hatching *Ascaris lumbricoides* eggs (Lysek, 1976; Hidalgo *et al.*, 2000). In this context, studies on the interaction of the ovicidal fungus,

Pochonia chlamydosporia, are justified by its importance in public health, due to the high resilience that geohelminths eggs have when present in the external environment, acting directly or indirectly as a source of contamination to humans, since the use of nematophagous fungi present in the environment as biological controllers and the viability of environmental dispersion of resistant fungal structures (chlamydospores) can act decontaminating the environment (Lysek *et al.*, 1982; Braga *et al.*, 2011b, Braga and Araujo, 2014).

Regarding the use of fungi with proven ovicidal activity, they may be useful as a complementary tool, even under experimental conditions, for control of helminth eggs, such as *C. pelagicum* eggs.

Thus, experimental studies that consider alternative measures for controlling these parasites are welcome, as the use of fungi helminth "eaters" (Braga and Araújo, 2012). In the present study we aimed to evaluate the effect of an ovicidal fungus on the eggs of the gastrointestinal nematode *C. pelagicum*.

Material and Methods

In the present study, an isolate of the nematophagous fungus *P. chlamydosporia* (VC4) was used. This isolate derived from Brazilian soil, and was maintained by means of continuous transfer of solid culture media in the Laboratory of Parasitology-Department of Veterinary Medicine from the Federal University of Viçosa.

C. pelagicum eggs were obtained by dissection of the uterus belonging to adult female specimens recovered during the necropsy of a *Magellanic penguin* (*Spheniscus magellanicus*) at the Institute for Research and Rehabilitation of Marine

Animals (IPRAM), located in the state of Espírito Santo. The identification of adult individuals and eggs followed the criteria established by Yamaguti *et al.* (1961), and Taylor *et al.* (2007). Later, the eggs were washed 10 times with distilled water by centrifugation at 1000 rpm for 5 min, discarding the supernatant after each centrifugation.

Then, from the edges of the fungal colonies grown free of contamination, agar disks with approximately 4 mm in diameter, were removed with the aid of a platinum loop and inoculated in Petri dishes containing 20 ml of YPSSA medium. Following, these plates were incubated in an environmental chamber at 25°C for 28 days and in the absence of light. After this period, the surfaces of the plates were washed with distilled water with the aid of a brush. The suspension contained in the plaques was passed through a sieve attached to a plastic container for removal of mycelium fragments. Chlamydospore identification was performed in accordance with Gams and Zare (2001). The spores recovered were quantified in ten counts in a Neubauer chamber and qualified (chlamydospores) according to three aliquots of 10µL. After quantification and qualification of the spores, the dilution for the desired concentrations (500, 1500, 2000, and 3000) was performed.

To compose the experimental assay, the *C. pelagicum* eggs were transferred over the surface of Petri dishes (9.0 cm in diameter) containing 2% WA medium in the following concentrations of chlamydospores (500, 1500, 2000, and 3000). Six repetitions were performed for each group. In the treatments, each plate contained one thousand *C. pelagicum* eggs with one of the concentrations; and the control group only one thousand eggs. In the intervals of 5 and

7 days, one hundred eggs were removed from each plate of both the treated and control groups, without chlamydospores according to the technique described by Araujo *et al.*(1995), and then evaluated in 40x objective according to the parameters established by Lysek *et al.*(1982).The data for each interval studied were subjected to the non-parametric Friedman test with 1% probability, Ayres *et al.* (2003).

Results and Discussion

The percentage results for the effect types 1, 2, and 3,during the 5 and 7 days of interaction in different chlamydospore concentrations (500, 1500, 2000, and 3000) of the fungus *P. chlamydosporia* (VC4) are shown in Figure 1, demonstrating the percentage of eggs showing only interaction (effect type 1); hyphae adhesion (effect type

2); and finally destruction of the eggs (effect type 3).

At the end of the experiment, there was a significant difference ($P < 0.01$) in the destruction of the eggs (effect type 3) in the four tested concentrations compared to the control group. No difference was observed ($P > 0.01$) for ovicidal activity between the four concentrations used. Through light microscopy, using the 40x and 10x objective, it was possible to observe *C. pelagicum* eggs destroyed by the fungus *P. chlamydosporia* (VC4), at the end of the experiment (Figs. 2A-I). The highest percentages for the effect type 3 were observed at concentrations of 2000 and 3000 chlamydospores, at the end of the experiment (7 days) with 45.3% and 46.2% destruction, respectively.

Figure.1 Percentages of the ovicidal activity for the effects of types 1, 2 and 3 to 5 and 7 days of interaction of chlamydospores of the fungus *Pochonia chlamydosporia* (VC4) at different concentrations (500, 1500, 2000 and 3000) on eggs of *Contracecum pelagicum* and the control group. The asterisk denotes a difference ($P < 0.01$)

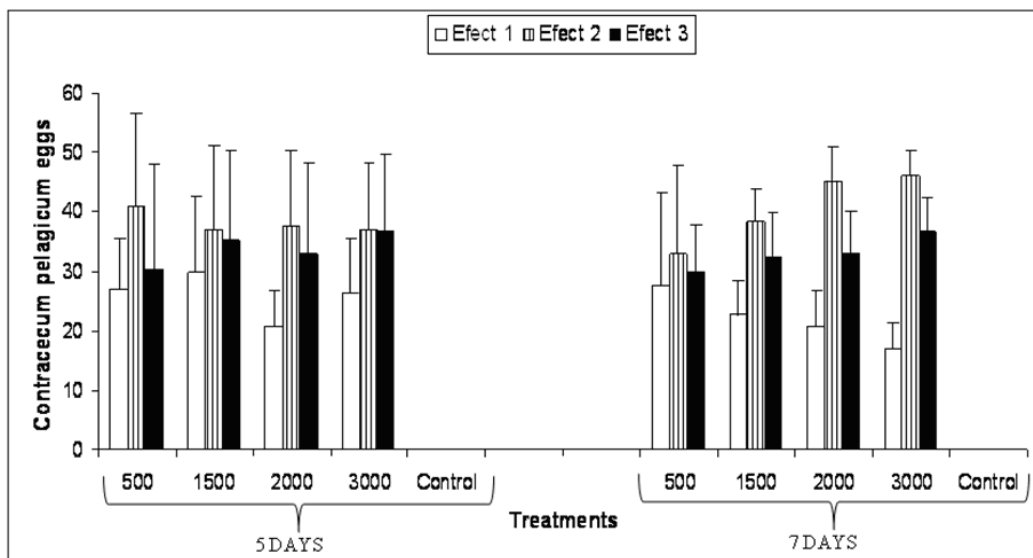
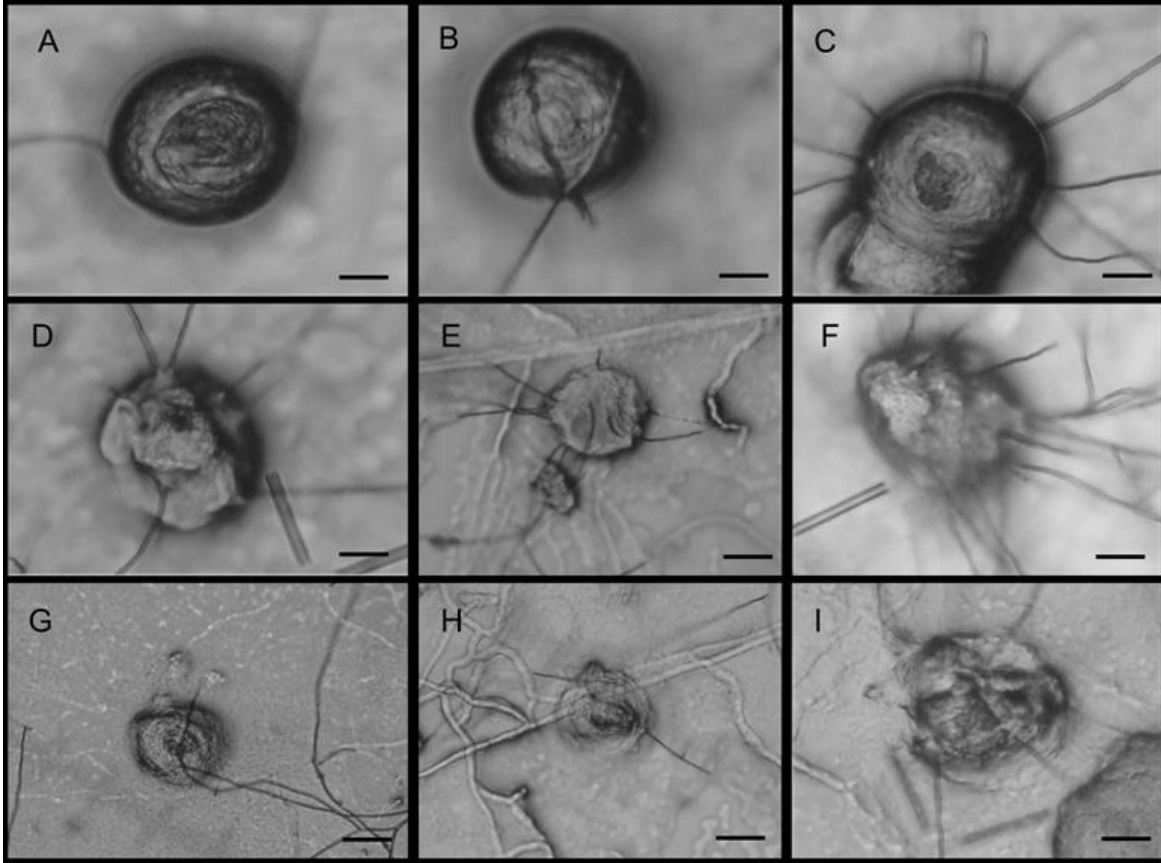


Figure.2A-I Hyphae of the fungus *Pochonia chlamydosporia* (black arrow) and the eggs of *Contracaecum pelagicum* destroyed (white arrow) at the end of the experiment. Light microscope. Magnification: 10 x and 40 x objective lens. Bars: A – 156 µm; B – 182 µm; C – 182 µm; D - 182 µm; E – 124.8 µm; F – 182 µm; G -119.6 µm; H-104 µm and I-182µm



In Magellanic penguins the changes observed by *C. pelagicum* are related to inflammation and obstruction, leading to death in these animals. On the other hand, according to López-Serrano *et al.* (2000), these parasites have significant public health importance, with description of infections in humans resulting in gastrointestinal perforations, obstructive and allergic reactions. Furthermore, although there are no reports in literature regarding human infection due to *Contracaecum* sp., mammals have been experimentally infected, resulting in harmful effects to the organism and therefore indicating its importance (Barros *et al.*, 2004).

Regarding the use of nematophagous fungi, much has been studied about the percentage differences for their predatory activity (Braga *et al.*, 2011; Araujo *et al.*, 2012). Thus, in this study, the fungus *P. chlamydosporia* (VC4) used at different concentrations, was effective in destroying *C. pelagicum* eggs throughout the experiment. This information indicates that this fungus could be successfully used in the biological control of potentially zoonotic helminths (Araujo *et al.*, 2012). The production of chlamydospores is one of the main characteristics that possibly show if a fungus could be used in the environmental control of gastrointestinal parasitic helminth eggs.

These structures allow nematophagous fungi the capability of passing through the gastrointestinal tract belonging to domestic animals, as well as being readily dispersed in the external environment then acting as natural dispersers (Araújo *et al.*, 2004).

Another important aspect observed on the efficiency of helminth egg destruction by *P. chlamydosporia* is its growth from culture media known to be "poor". At this point, literature mentions that this fungus behaves in different ways according to the culture medium being used, maintaining their ovicidal ability through contact with the eggs. Eren and Pramer (1965), mention that the periodic supply of nematodes to nematophagous fungi, in a culture medium poor in nutrients, reduces its saprophytic growth increasing its activity as natural antagonist. Starting from this premise, in a recent study Braga *et al.* (2011) proposed that not only the culture medium, but also the amount of chlamydo spores being used in the *in vitro* control of helminth eggs, were two important characteristics from an abiological point of view. In the present study, we observed that the concentrations of 2000 and 3000 chlamydo spores presented the highest ovicidal percentages. Thus, the results once again suggest that the chlamydo spore concentration and the 2% WA medium, even though considered to be a "poor" culture medium, may influence egg predation.

Paperna (1964), reports that fish can serve as definitive, intermediate, or paratenic (transport) hosts in the life cycles of many parasite species and usually affect the marketability of commercially produced fish, thus raising a lot of public health concerns, especially in areas where raw or smoked fish are consumed. In this sense, a wide variety of helminth genera suggest the use of "bio controller agents" in several studies under laboratory conditions. In this

context, literature also reports some studies where nematophagous fungi species have demonstrated their activity on helminth eggs and larvae belonging to birds, which can represent, in the future, an alternative control (Braga *et al.*, 2012, 2013). Yáñez *et al.* (2012,) described gastric ulceration caused by *Contracaecum* in mammals and birds (such as penguins), but this is the first report of an ovicidal fungus infection on *C. pelagicum* eggs from a *M. penguin*.

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