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Predatory effect of *Duddingtonia flagrans* on infective larvae of gastro-intestinal parasites under sunny and shaded conditions

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ABSTRACT

Duddingtonia flagrans is a natural strain of Nematophagous-Fungi isolated around the world. It has demonstrated efficacy and ease of use in laboratory as well as in field conditions. The fungus contributes to the prophylactic control of the worms by reducing the number of L_3 on pasture. The aims of this study were to test and analyze the predatory effect of *D. flagrans* under sunny and shaded conditions on the L_3 in the faeces, and to verify the reduction of translation to pasture during summer and winter seasons. Faecal Mass Units (FMUs) were assigned to two treated groups (groups treated with *D. flagrans* chlamydospores, TG) and two untreated groups (without *D. flagrans* chlamydospores, UG), in summer and winter, under sunny and shaded conditions. FMUs and herbage samples were taken for parasitological workup. Predatory activity of *D. flagrans* was evident under both conditions for the summer experiment but was not manifest for the winter experiment. In summer, an interaction between sunny and shaded conditions and predatory activity of *D. flagrans* was found. Environmental conditions on predatory activity should be considered when designing strategies for the implementation of *D. flagrans* in grazing systems to smooth the infectivity curve of L_3.

1. Introduction

Gastro-intestinal parasites affect the productive and reproductive performance of animals in grazing systems (Steffan et al., 2012). From an epidemiological viewpoint, only 5% of Gastro-intestinal Parasites (GP) are located within the animals, whilst the remaining 95% is on the pasture in the form of immature stages (Kenyon et al., 2009; Sagüés et al., 2011a,b). Knowledge of the population dynamics of immature stages of GP on pastures in relation to climatic variables (rainfall and temperature) is the basis for the development of prevention and control strategies (Stromberg and Averbeck, 1999).

The increasing demand for healthy food products obtained under animal welfare and safety standards (Clark et al., 2017; Wee et al., 2014) requires new alternatives for the control of GP (Ahmed et al., 2014; Sahoo and Khan, 2016; Thamsborg et al., 1999). The use of nematophagous fungi for the biological control of GP is one of the most promising strategies in the world (Braga and de Araújo, 2014; Grønvold et al., 1996; Larsen, 1999). *Duddingtonia flagrans* has demonstrated efficacy and ease of use in laboratory as well as in field conditions (Fontenot et al., 2003; Larsen et al., 1995). This agent can be formulated orally at different concentrations of chlamydospores (Grønvold et al., 2007; Ojeda-Robertos et al., 2008; Sagüés et al., 2011a,b). It passes through the gastrointestinal tract and is eliminated in the faecal–natural environment which allows the development of the fungus

Abbreviations: D. flagrans, *Duddingtonia flagrans*; FMU, Faecal Mass Units; SC, under Sunny Condition; SHC, under Shaded Condition; UG, Untreated Group without *D. flagrans* chlamydospores; TG, Group Treated with *D. flagrans* chlamydospores; GP, Gastro-intestinal Parasites; EPG, Eggs Per Gram; L_3, Infective Larvae; L_3.kg DM^{-1}, L_3 per Unit of Dry Matter

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D. flagrans in the presence of infective larvae (L_3) – along with GP eggs (Grønvold et al., 1999; Saumell et al., 1999; Silveira et al., 2017). In a theoretical and experimental framework, the fungus contributes to the prophylactic control of the worms by reducing the number of L_3 on pasture and minimizing the risk of reinfection (Knox et al., 2002; Mota et al., 2003).

The environment affects the seasonal dynamics of nematophagous fungi (Hasanzadeh et al., 2012; Saumell et al., 2015), both through abiotic (soil type, soil temperature, soil pH, water potential, redox potential, radiation) and biotic factors (vegetation, soil microbial activity, coprophilic fauna). The density (abundance per unit area) of different groups of nematophagous fungi studied in soil is higher in late summer and autumn, lower in winter and moderate in spring (Persmark et al., 1996). There is no clear association between this seasonal pattern and climatic factors such as temperature and humidity (Knox et al., 2002). Biotic and abiotic factors affect randomly distributed faeces under sun and shade (Grønvold et al., 1999; Wicklow et al., 1984) to varying degrees. The presence of shadow reduces climatic stress caused by the direct effect of sunlight on the faecal masses; it redistributes radiation and minimizes the impact on the microorganisms (Wicklow et al., 1984), thus creating a more favorable microclimate for their survival.

Nematophagous fungi were first described more than a century ago, but very little is known about their ecophysiology (Persmark et al., 1996; Saumell et al., 2008). In this context, some aspects of the influence and interaction of environmental factors in relation to the effectiveness of *D. flagrans* against L_3 of GP cattle remain unknown. The aims of this study were to test and analyze the predatory effect of *D. flagrans* under sunny (SC) and shaded conditions (SHC) on the L_3 in the faeces, and to verify the reduction of pasture translation during summer and winter seasons.

2. Materials and methods

2.1. Experimental design

The study was conducted at the Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil. The experimental area consisted of a uniform parcel (25 m^2) covered with a pasture comprising ryegrass (*Lolium perenne*), barley grass (*Bromus catharticus*) and white clover (*Trifolium repens*), provided by the Instituto de Hidrología de Llanuras "Dr. Eduardo J. Usunoff", UNCPBA- CIC. Half of the experimental parcel was shaded with fine woven nylon. To avoid an effect of the nylon on the air temperature, it was placed at a height of 40–50 cm above the ground to enable ventilation. Faecal Mass Unit (FMU) dimensions and specifications were 15 cm × 3 cm (diameter x thickness), with a volume of 540 cm^3 and a wet weight of 500 g, and their appearance is shown in Fig. 1 and Fig. 2. For the purpose of this study, FMUs were assigned to two treated groups (with *D. flagrans* chlamydospores, TG) and two untreated groups (without *D. flagrans* chlamydospores, UG). FMUs were placed simulating the defecation pattern of a 5-month-old calf with a FMUs distance of 50 cm = 25 cm from the edge of each FMU. To compare the effects under SC and SHC, one TG and one UG for each situation were also set as shown in Fig. 2. The experiment was carried out twice, under summer and winter conditions.

2.2. Meteorological data

Mean, maximum and minimum temperatures (°C) and rainfalls data (mm) were obtained from the weather station belonging to the Institute of Hydrology of Plains "Dr. Eduardo J. Usunoff", UNCPBA- CIC, located where the study was carried out.

The historical average of mean, maximum and minimum monthly temperatures over a 21-year period (1991–2012) were 13.5 °C, 26.9 °C and −0.6 °C, respectively. The arithmetic mean for annual rainfalls for this period was 938.3 mm. Summer and winter had a historical average of accumulated rainfall of 137.8 mm and 275.2 mm, respectively.

Fig. 1. Faecal Mass Unit placed in the experimental area.

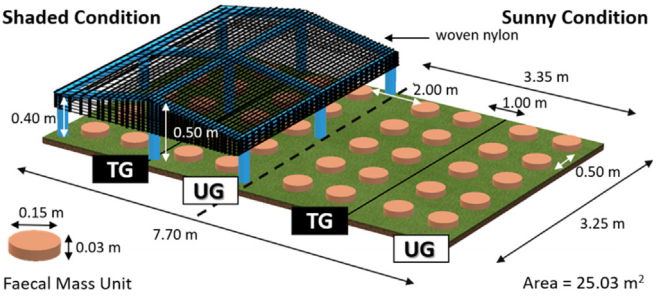

Fig. 2. Experimental design and Faecal Mass Unit dimensions. The dotted line indicates the spatial division between Faecal Mass Units under sunny and shaded condition. The solid lines indicate the spatial division between treated group (TG) and untreated group (UG). The arrows indicate distance, height and width of the components within the experimental design on the environment. Note: the drawings and graphics are not to scale and are intended for illustrative purposes only.

2.3. Source and processing of faeces

Fresh faeces were obtained from lactating Holando-Argentino calves between 4 and 6 months old, not subjected to any anthelmintic treatment. The calves were fed natural grasslands and pasture (*Lolium perenne*, *Bromus catharticus* and *Trifolium repens*). Faeces were pooled and thoroughly mixed for 5 min with an electric mixer.

The number of GP eggs per gram (epg) in faeces was performed from five random samples using the modified McMaster technique described by Roberts and O'Sullivan (1950). In summer and winter, average epg quantified were 650 and 217, respectively. The analysis showed that more than 90% of the total eggs counts belonged to GP with spontaneous hatching and the remaining eggs count was represented by *Nematodirus* spp (thermal or mechanical stimulus is needed to hatch and develop).

The taxonomic keys by Niec (1968) were used to identify and classify the L_3 on coprocultures. In summer, faecal cultures showed the following relative distribution of GP genera per 100 L_3: *Ostertagia* spp. 60%; *Cooperia* spp. 29%; *Oesophagostomum* spp. 6%; and *Trichostrongylus* spp. 5%. Furthermore, in the winter experiment, faecal cultures showed a distribution of L_3 between *Ostertagia* spp. 52% and *Cooperia* spp. 48%.

2.4. Fungal material

A local isolate of *D. flagrans* 03/99 strain (Saumell et al., 2015), previously isolated from the same site where the trial was carried out, was used. The fungus was grown on pure agar cultures at 24 °C for 2 weeks, after which the chlamydospores on the agar plates were gently rinsed off with sterile water and counted using a Neubauer haematocytometer to estimate the number of chlamydospores per milliliter of water. According to the dose levels assessed by Grønvold et al. (2007) 25000 chlamydospores per gram were added to each FMU together with distilled water and mixed (46 chlamydospores.cm$^{3.-1}$ or 50 chlamydospores.g^{-1} of concentration per FMU treated with fungus).

2.5. Summer and winter experiments

The studies were conducted over a period of 15 days in summer (December 1st to December 16th, 2011) and 39 days in winter (July 20th to August 27th, 2012). In each study, 11 and 10 FMUs per group were placed on the soil, respectively. To monitor for the presence of L_3 through Baermann method (Fiel et al., 2011), 5–10 g faeces were daily sampled from the additional FMUs placed under SC and SHC conditions. After the study period was over, all FMUs were removed for parasitological workup.

2.6. Parasitological workup

To quantify the number of L_3 on the FMU, the Baermann method as described by Fiel et al. (2011) was performed. Each FMU was wrapped with gauze into a small package and immersed in warm distilled water inside a plastic container for 24 h. After that, the packages and the supernatant were removed while the remaining content was transferred and decanted into glass tubes. Once the supernatant of the glass tubes was discarded, the remaining 10% of each Baermann device was placed in a small glass receptacle. Four lugol drops (0.2 ml) were added, and the sample was observed under an optical microscope (40 × and 100 ×). In order to assess the predatory activity of *D. flagrans* and the percentage of reduction of L_3 in FMU, equation (1) was used.

$$Percentage\ reduction\ of\ L_3 = \frac{UG\ L_3 average - TG\ L_3 average}{UG\ L_3 average} \quad (1)$$

The methodology for cutting and washing the grass was taken from Fiel et al. (2011). After each trial (summer and winter), herbage samples were obtained at ground level around the faecal masses, starting from the edge and up to a distance of 25 cm. The sampling was always performed at the same time for all groups (9:00 to 11:00 h a.m.). With an interval of 24 h, sets of herbage samples from each group were washed twice in plastic buckets and the recovered liquid was put into Baermann devices. The remaining 10% of each Baermann device was placed in a small glass receptacle with the addition of some lugol drops and was observed under optical microscope (40 × and 100 ×). Herbage samples were oven dried under constant temperature (40 °C) with forced ventilation. In order to calculate the number of L_3 per unit of dry matter (L_3.kg DM^{-1}), equation (2) was used.

$$L_3\ per\ unit\ of\ dry\ matter = \frac{L_3 per\ group}{Forage\ dry\ matter\ weight\ (g)} * 1000 \quad (2)$$

Faecal cultures were performed using the modified Roberts and O'Sullivan technique described by Niec (1968). 60 g of calf faecal matter was mixed with 40 g of equine sterilized faeces. Each mixture was divided into 10 faecal cultures, each weighting 10 g, and left for 16 days in an oven under constant conditions (24 °C and 64–66% RH). After that, liquid collected from petri dishes was transferred and decanted into glass tubes. The remaining 10% of each Baermann device was placed in a small glass receptacle and some lugol drops were added to allow the microscopic observation (40 × and 100 ×) of the larvae.

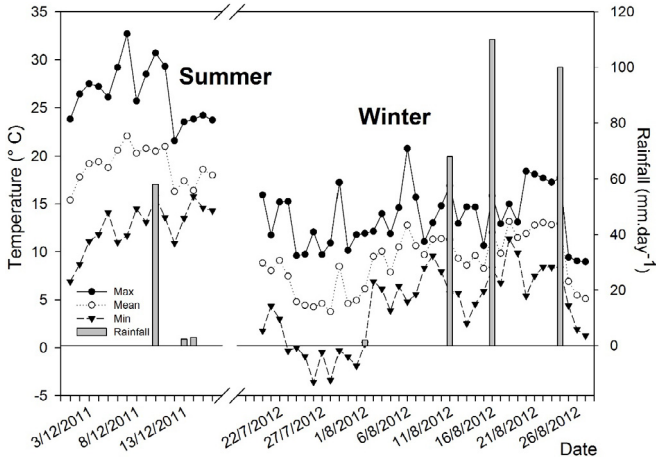

Fig. 3. Rainfall data and maximum, mean and minimum temperatures at the experimental site.

2.7. Statistical analysis

The percentages of reduction and the differences in L_3 counts were analyzed through ANOVA for parametric data, with log transformation of the L_3 variable in order to fit the ANOVA assumptions. L_3 average groups were compared using the LSD Fisher test and interaction effect between SC and SHC in predatory activity on TG with *D. flagrans* was searched. Statistical analysis was performed with the statistical software InfoStat 1.0 (Di Rienzo et al., 2011).

3. Results

3.1. Meteorological data recorded at the experimental site

The meteorological data recorded at the experimental site during the summer trial (Fig. 3), showed accumulated rainfalls surpassing the 63 mm (58 mm was fallen in a single day) and an average thermal range of 13.9 °C between the minimum and maximum temperatures, related to a wet and warm weather as expected in Tandil. In the winter experiment, thermal range fluctuated around 9.5 °C, more than twenty days had minimums under 5 °C, and a mean temperature of 8.9 °C for the period was observed with heavy rainfalls that accumulated 280 mm in three days (more than the historical average rainfall accumulated for the entire season).

3.2. Infective larvae (L_3) counts, genera and predatory activity assessment of Duddingtonia flagrans in faecal masses

Predatory activity of *D. flagrans* was evident under both conditions for the summer experiment (Fig. 4, a). The percentage of reduction of L_3 for SC was 58.7 ± 19.3% ($P < 0.01$), while for SHC the percentage was 92.3 ± 1.3% ($P < 0.0001$) in TG and UG, respectively. An interaction was found between SC and SHC conditions and predatory activity of *D. flagrans* ($P < 0.0001$).

Predatory activity of *D. flagrans* was unclear under both conditions for the winter experiment (Fig. 4, b). The averages of L_3 counts did not differ significantly between TG and UG in SC as SHC conditions without reductions ($P = 0.1478$). A lower L_3 count was found in UG under SHC compared with UG in SC ($P < 0.001$).

3.3. Infective larvae (L_3) counts and percentages of distribution of gastrointestinal parasites genera on herbage samples

The observation of data collection on Tables 1 and 2 showed a lower L_3 count on herbage samples of TG. There were remarkable differences between L_3 quantified under SHC compared with SC conditions in the

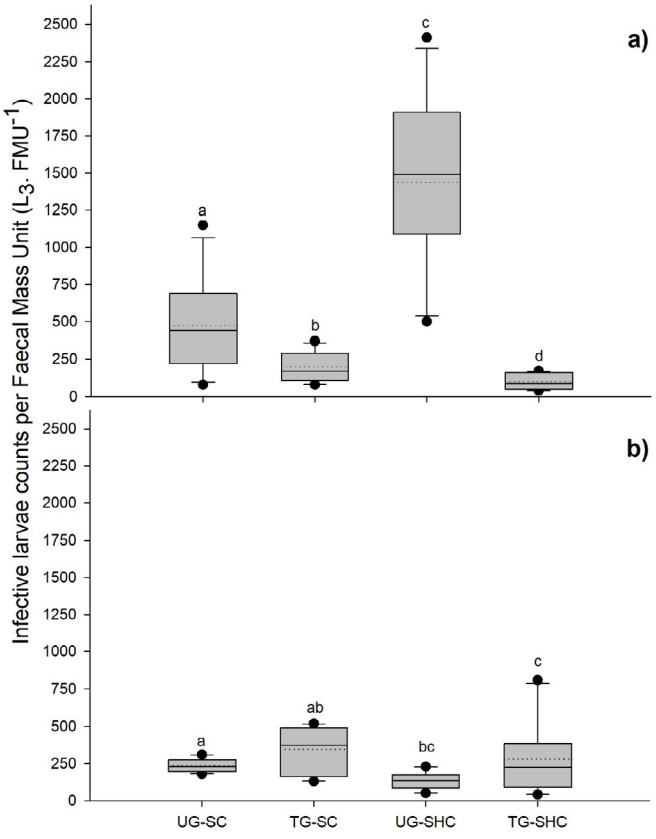

Fig. 4. Infective larvae (L_3) counts per Faecal Mass Unit for a) summer and b) winter experiment. Box plots show 10th, 25th, 50th, 75th and 90th percentiles. The dotted line is the mean and the dots are the 5th and 95th percentile values.UG-SC: untreated group under sunny condition; TG-SC: treated group under sunny condition; UG-SHC: untreated group under shaded condition; TG-SHC: treated group under shaded condition. Different letters indicate statistically significant differences between groups ($p < 0.05$).

Table 1
Infective larvae count and relative distribution of gastro-intestinal parasites genera for herbage samples from Untreated Groups (L_3.kg DM^{-1}) in both experiments.

	Summer		Winter	
Gastro-intestinal parasites genera	SC	SHC	SC	SHC
Ostertagia spp.	–	80%	53%	60%
Oesophagostomum spp.	25%	–	–	–
Cooperia spp.	75%	20%	47%	40%
L_3 counts	265	1344	4633	3566

Table 2
Infective larvae count and relative distribution of gastro-intestinal parasites genera for herbage samples from Treated Groups (L_3.kg DM^{-1}) in both experiments.

	Summer		Winter	
Gastro-intestinal parasites genera	SC	SHC	SC	SHC
Ostertagia spp.	–	–	49%	26%
Oesophagostomum spp.	100%	–	–	–
Cooperia spp.	–	100%	51%	74%
L_3 counts	96	156	4560	3439

summer experiment, while the winter experiment showed only slight differences among groups.

4. Discussion

Predatory activity of *D. flagrans* was influenced by the conditions and climatic factors considered in this study. According to Larsen et al. (1995) and Su et al. (2007), the activity, species diversity and density of nematophagous fungi are modified under extremely hot or cold conditions, with impact on dynamic and seasonal distribution (Fernández et al., 2007; Githigia et al., 1997; Persmark et al., 1996).

In the summer experiment, exposure to *D. flagrans* resulted in a reduction of L_3 of 60–90%, in SC as well as in SHC conditions, in accordance with the data presented by Healey et al. (2018) and Saumell et al. (2005). The differences observed between TG and UG under SC and SHC conditions could indicate the existence of associated factors that promoted L_3 development and predatory activity of *D. flagrans* in SHC compared with SC conditions. On the other hand, the number of L_3 quantified in TG under SHC was lower than L_3 counts of TG in SC. SHC generates a microenvironment that decreases the impact of solar radiation and physical damage by rainfalls on faecal masses (Blaustein et al., 2016; de Mendonca et al., 2014) and this could favor predatory activity of nematophagous fungi (Saumell et al., 2008). In SC, the lower amount of L_3 collected per FMU might be associated with a low water potential, increases in evaporation and drying on the faecal layer (de Mendonca et al., 2014; Jaffee, 1996). Accordingly, Dimander et al. (2003) found lower L_3 counts in a dry year on a 3-year plot study.

In the winter experiment, no reduction between tested groups was found. In agreement with Blaustein et al. (2016) and Santurio et al. (2011), part of the explanation may be the eviction and scatter effect on L_3 induced by heavy rains and wet weather (Fig. 3). In the same way, this phenomenon affects the chances of success of the nematophagous fungi since adhesive action of networks occurs within faecal masses. Recent studies have shown the negative effect of cool temperatures on chlamydospores germination, fungal structures development, mycelial growth, competitive saprophytic ability and metabolism of nematophagous fungi (Hasanzadeh et al., 2012; Kredics et al., 2003).

The GP genera identified by L_3 counts match the seasonality and population dynamics observed by Fiel et al. (2012) in the Flooding Pampas region. However, L_3 counts of herbage samples showed differences with regards to GP genera and percentages identified in faecal cultures. The absence of *Trichostrongylus* spp. in herbage samples in the summer experiment could be explained by a less likely L_3 translation from FMU to forage due to the low L_3 counts of faecal cultures. Despite the presence of *Nematodirus* spp. epg counts in both experiments, L_3 from this species were not found in faecal cultures or herbage samples. As reported by Oliver et al. (2014); Van Dijk and Morgan (2009); Yazwinski and Tucker (2006), this peculiarity could be explained by a lack of thermal or mechanical stimulus and short time for L_3 to hatch and develop, respectively. The differences between GP genera identified in the environment and faecal cultures are associated with multiple variables that interact with FMU (Fiel et al., 2012; Saumell et al., 2015; Saumell et al., 2008), in contrast to optimal and controlled laboratory conditions. Interestingly, herbage samples of TG showed a reduction and even a reversal on percentages of GP genera identified as predominant in faecal cultures. This would enable the development of research strategies to reduce prairie infectivity and moderate the manifestations of GP seasonality.

The implementation of *D. flagrans* as a key component within a strategic integrated parasite management plan might be useful to diminish the farmers dependence on anthelmintics with less frequency of application (Healey et al., 2018). In the light of our findings, and taking into consideration the epidemiology of gastrointestinal parasites of ruminants, the treatment of cattle in late spring or early summer may be important to reduce the number of inhibited L_3 in grazing heifers and steers and to avoid subsequent clinical events (Fiel et al., 2012).

Considering the principles of refugia (Muchiut et al., 2018; Fiel et al., 2017), low infection levels of the environment and a high risk for the selection of resistance in this period would indicate a "window of opportunity" to use *D. flagrans* and, in turn, prevent the transmission of L_3 to weaning calves through faeces in the early autumn of the following year. According to Healey et al., (2018), in order to decrease subclinical effects from gastrointestinal parasites, the application of *D. flagrans* should be recommended in autumn when weather conditions are conducive to L_3 development (above 5 °C) and guarantee the predatory activity of the fungus. Despite the negligible or low activity of *D. flagrans* in winter, chlamydospores or other fungal structures remain in the faeces or are reingested by animals and disseminated to the grass, giving continuity to the environment cycle (Santurio et al., 2009). Therefore, differences in prevalence and abundance of gastrointestinal parasites, the different geographical locations (considering both local ecological and climatic zones), and the recommended parasite management will determine the strategic use of *D. flagrans* in grazing systems.

5. Conclusions

Predatory activity of *D. flagrans* was evident under both conditions for the summer experiment with a significant interaction between SC and SHC conditions, with being SC negative to larvae and fungus development. Looking forward to new treatment plans, the absence of predatory effects between tested groups in winter with biological control was not a minor finding to GP management. With the purpose of designing strategies for the implementation of *D. flagrans* in grazing systems, larger studies under environmental conditions to long-term are needed. The complex influence of the environment on predatory activity of nematophagous fungi, as well as the assessment of biotic and abiotic factors, require more research in order to smooth the infectivity curve of L_3 through integration between laboratory and field tests. This, in turn, would allow researchers to enhance current knowledge aiming at the application of nematophagous fungi in livestock systems with an holistic approach.

Compliance of interest

To ensure objectivity and transparency in research and to ensure that accepted principles of ethical and professional conduct have been followed, the authors would like to point out that the animals mentioned in this study were not involved in the experiment and only provided faecal masses to perform the assays. Therefore, this study is in agreement with the proceedings issued by the Animal Welfare Committee located in the Faculty of Veterinary Medicine, Tandil UNCPBA. The data of animal management in the farm where faecal masses were collected were provided by the farmer. The authors have no conflicts of interest to declare.

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