

FORMULATING FUNGAL SPORES TO PREVENT INFECTION BY TRICHOSTRONGYLIDS IN A ZOOLOGICAL PARK: PRACTICAL APPROACHES TO A PERSISTING PROBLEM

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Abstract

Two different formulations containing a blend of spores of the filamentous fungi *Mucor circinelloides* and *Duddingtonia flagrans* have been assayed against trichostrongylids in wapitis captive in a grassy parcel from a zoological park and dewormed with fenbendazole. One approach (sprayed-on pellets) consisted of providing them, during 10 months, nutritional pellets sprayed the spores just before to be ingested, and the other (formulated pellets) involved the administration of pellets industrially manufactured with the spores for an identical period. Data collected on wapitis treated with fenbendazole without receiving spores during a 10-months interval were considered as controls. The effect of these strategies was evaluated through the analysis of feces collected directly from the soil, by estimating the reduction of the counts of eggs of trichostrongylids per gram of feces (EPG). The possibility of side effects in the digestive tract, respiratory apparatus or on the skin was assessed. The coprological analyses showed that, despite the successfulness of fenbendazole, the values of trichostrongylids egg-output increased four months later above 300 EPG. Through the administration of pellets sprayed spores, a significant EPG reduction of 69%

(30-100) was recorded, and 71% (36-100) by giving the wapitis pellets industrially enriched with the spores. No unfavorable effects were observed among the wapitis taking the spores. It is concluded that ingestion of a blend of spores of *M. circinelloides* and *D. flagrans* every two days affords a beneficial long-term effect on controlling the infection by trichostrongylids in wild captive animals.

Keywords: captive ruminants, gastrointestinal nematodes, biological control, predatory fungi, premixed feed, zoo

Introduction

Since several decades ago, the role of zoological parks comprises not only exhibition of animals for the enjoyment of visitors, but the possibility to educate in promoting conservation of endangered species as a contribution to preserving biological diversity (Schaaf, 1994). With the aim to participate in these strategies, many zoos are involved in keeping certain wild animal species under habitats focused on natural behavior, with the possibility to interact with the environment, forage and socialize (Okello et al., 2002). Accordingly, captive herbivores are maintained in grassy parcels where pasture is available almost throughout the year, depending on climatic conditions.

An important point to take into account in zoological parks relies on ensuring that captive animals have a safe and healthy environment. Certain helminth species complete an external phase of their life-cycle in the ground of areas with vegetation. Trichostrongylids are nematodes frequently infecting herbivores under grazing regimes, through the ingestion of third-stage larvae developing from eggs passed in the feces of infected animals (Turner & Getz, 2010), which is currently associated with diarrhea, anemia and weight loss (Pugh & Baird, 2011).

As occurs with livestock, control of these nematodes among wild captive animals entails the administration of efficient anthelmintics, and the choice and application of parasiticides appear among some of the challenges to wildlife veterinarians, due to the lack of knowledge on appropriate dosages or side effects. Owing to their confinement, high levels of ground contamination by parasites are achieved despite successful deworming is regularly applied (Fagiolini et al., 2010; Panayotova-Mencheva, 2016) (Table 1). Certain methods advised for controlling parasites in livestock as rotation of pastures or alternating animal species can not be observed in zoos due to enough numbers of parcels are not often available, and the movement of animals inside the park is impossible without causing them high levels of stress (Terry, 2013). Hence, the most useful procedures look the frequent removal of fecal samples from the parcels, besides the coprological analysis for the assessment of parasitic burden to establish the appropriate deworming to be administered at regular intervals (Singh et al., 2006; Aviruppola et al., 2016).

The control of parasites on wild animals captive in zoological gardens requires of preventive actions to avoid their infection, and the usefulness of certain filamentous fungi isolated from the soil to reduce the numbers and viability of some parasitic stages present in the ground has been proved. Based on their parasiticide activity these fungi are classified as ovicides (*Pochonia chlamydosporia*, *Mucor circinelloides*, *Trichoderma atrobrunneum*, *Verticillium chlamydosporum*) and nematode-trapping or larvicide (*Duddingtonia flagrans*, *Monacrosporium thaumasium*, *Arthrobotrys oligospora*) (Tavela et al., 2013; Arias et al., 2013b; Cazapal-Monteiro et al., 2015; Saumell et al., 2016; Luns et al., 2018). Prior investigations demonstrated the antagonistic effect of filamentous fungi on the eggs of helminths inside the feces of wild captive animals (three lynxes and four dromedaries) (Hernández et al., 2018c), whereas a three-year study performed on wild equids (two zebras, five European donkeys and four African wild asses) in a zoological park showed the successfulness of an approach involving the administration of pellets industrially

manufactured with a blend of spores of two filamentous fungi with ovicide (*Mucor circinelloides*) and larvicide (*Duddingtonia flagrans*) activity (Palomero et al., 2018). Nevertheless, this treatment has not yet tested in wapitis.

Two commercial presentations containing chlamydospores of *D. flagrans* intended for livestock species have been recently registered (Healey et al., 2018; Braga et al., 2020), but until now, there is no possible to get commercial pelleted feed containing spores of parasiticide fungi (formulated pellets) for the nutrition of wild captive animals, except for research purposes. Hence, two formulations have been assayed in the current field trial comprising the administration of spores of *M. circinelloides* and *D. flagrans* to captive wapitis (*Cervus canadensis*). Firstly they have been provided pellets previously sprayed a blend of the fungal spores (sprayed-on pellets) to check their beneficial effect, and then pellets industrially manufactured with the spores (formulated pellets) with the aim to test which could occur if the sprayed-on pellets are replaced by this formulation.

Material and methods

Wapitis

The present investigation was conducted among five (one male and four females) adult wapitis (*Cervus canadensis*) in a parcel of approximately 1 Ha with grass and trees, belonging to a zoological park in NW Spain (Marcelle Natureza, Outeiro de Rei, Lugo) (43°4'14.71" N, 7°37'53.50" W). Supplementation based on nutritional pellets is administered every two days (ca. 1 kg pellets / wapiti / day).

Control of parasites consists of the quarterly administration of fenbendazole (5 mg / Kg bodyweight) (Panacur® 10, MSD Animal Health, Spain) in alternation with flubendazole (5 mg / Kg b.w.) (Laboratorios Syva, León, Spain). Removal of feces is practically done every day, early in the morning before the visitors arrive.

Climatic model

Information regarding the variations on climatic parameters (temperature, rainfall, relative humidity, frost days and water balance) was collected monthly between 2014 and 2018 from an automated meteorological station near (15 Km) to the Park and located at the same altitude.

Spores of parasiticide fungi

Spores of two parasiticide fungi, *M. circinelloides* and *D. flagrans* were co-cultured in the COPFr submerged medium (Arias et al., 2013b), until a concentration near to $2 \cdot 10^8$ / L was reached. Then two different formulations were prepared; firstly (sprayed-on pellets), the spores were sprayed onto commercial pelleted feed ($2 \cdot 10^6$ spores each fungus/kg pellets) (Arias et al., 2013a) just before feeding the wapitis. The other formulation (formulated pellets) consisted of adding the spores during the industrial manufacturing of the nutritional pellets ($2 \cdot 10^6$ spores each fungus per kg meal in the mixing phase) (Palomero et al., 2018).

Experimental design

Three assays were carried out during three years (2015 to 2018), from September to June:

Year 1 (controls): wapitis received anthelmintic treatment in September 2015 based on a single dose of 5 mg fenbendazole / Kg bodyweight (Panacur[®] 10, MSD Animal Health), and pelleted feed without spores every two days.

Year 2 (sprayed-on pellets): wapitis were dewormed in September 2016 with a single dose of fenbendazole (5 mg / Kg b.w.) (Panacur[®] 10, MSD Animal Health) and given (every two days) commercial pellets sprayed a blend of spores just prior to be ingested.

Year 3 (formulated pellets): wapitis were dewormed in September 2017 with a single dose of fenbendazole (5 mg / Kg b.w.) (Panacur[®] 10, MSD Animal Health) and provided (every two days) pellets added spores during the industrial manufacturing in a local feed mill.

Presence of parasites in feces

For the purpose to assess the presence of parasites, feces were collected monthly and directly from the ground, paying attention to take the upper portion for avoiding that free-living larvae could interfere the coprological analyses (migration). In this case, it was not possible to associate a fecal sample to a specific individual, then every sampling comprised the collection of ten fecal pats (Palomero et al., 2018). Each fecal sample was divided into three portions and analyzed through the sedimentation (Se= 30 eggs per gram of feces, EPG), flotation (Se= 30 EPG or 30 oocysts per gram of feces, OPG) and migration tests (Se= 30 larvae per gram of feces, LPG). The quantity of feces examined was five grams for the sedimentation, three grams by flotation and 10 grams through the migration test. All probes were conducted in duplicate, and microscopical examinations performed in triplicate for each analysis.

Results were expressed as the numbers of eggs/oocysts/larvae per gram of feces (EPG, OPG or LPG, respectively).

Feces (10 g / month) were cultured at 25°C for 20 days to enhance the development of third-stage larvae of trichostrongylids. Identification at genus level was done according to morphological keys (van Wyk & Mayhew, 2013).

Evaluation of the parasiticide strategy

The effect of anthelmintic treatment was evaluated by analyzing the fecal samples obtained fourteen days after the deworming, according to the formula:

$$\text{FECR (Fecal Egg Count Reduction) (\%)} = [1 - (\text{EPG}_{\text{day14}} / \text{EPG}_{\text{day0}})] \times 100$$

Deworming was considered efficient when $\text{FECR} \geq 95\%$.

Comparison among the kinetics of parasites shed in feces was done by calculating monthly the percentages of reduction of the numbers of eggs in the feces collected during the second and third years:

$$\text{Reduction Sprayed-on Pellets (RSP) (\%)} = [1 - (\text{EPG}_{\text{year2}} / \text{EPG}_{\text{year1}})] \times 100$$

$$\text{Reduction Formulated Pellets (RFP) (\%)} = [1 - (\text{EPG}_{\text{year3}} / \text{EPG}_{\text{year2}})] \times 100$$

Statistical analyses

Because of data obtained through the coprological probes did not fit a normal distribution (Kolmogorov-Smirnov $Z= 2.414$, $P= 0.001$) and variances were not equal (Levene's statistics: 52.948 , $P= 0.001$), the non-parametric Friedman probe was performed by means of SPSS for Windows (v. 20.0; SPSS Inc., Chicago, IL, USA). Differences were established if $P < 0.05$.

Results

Climatic pattern

The variations in the climatic parameters between 2014 and 2018 are summarized in Figure 1. Significant differences concerning the year were not observed, then the climatic pattern was established by drawing the average values for each parameter. Maximum temperatures were recorded in summer while values dropped below zero during winter (January – March),

when frost days were also observed. The percentages of relative humidity ranged from 81 to 97% throughout the year. Rainfall peaked on winter and the lowest values were recorded in summer (July to September), together with negative values of soil water balance.

Parasites in feces

Eggs of trichostrongylids were observed in the fecal samples, and specimens belonging to the genera *Cooperia*, *Nematodirus* and *Trichostrongylus* were identified in the coprocultures during the first year of study. Elimination of oocysts of *Eimeria* spp. and eggs of *Trichuris* sp. were recorded erratically and as a consequence not considered in the current investigation.

Control of trichostrongylids

As shown in Figure 2, trichostrongylids FECs higher than 300 EPG were observed at the beginning of the trial (September 2015), then deworming based on the administration of fenbendazole was performed. A 99% FECR value indicated the anthelmintic treatment was successful. The counts of egg-output increased until May, when numbers higher than 350 EPG were recorded.

During the second year, an effective anthelmintic treatment was also administered in September (Fig. 2), as demonstrated by the 100% FECR. Eggs of nematodes were detected in the feces of the wapitis two months later, and the numbers maintained between 50 and 100 EPG until the trial ended (10 months).

In the third year, deworming administered in September was successful (FECR: 100%). Four months later, the coprological analyses revealed the presence of eggs of trichostrongylids, and counts lower than 50 EPG until the end of the assay (June) were observed.

The Friedman test showed statistical differences between the numbers of trichostrongylids in the first year and in the two other years ($\chi^2 = 53.063$, $P = 0.001$).

Comparison of the strategies applied for the control of trichostrongylids

Table 1 summarizes that the counts of trichostrongylids egg-output in the second year of trial reduced between 30 and 100% in respect to the first year, with an average value of 69% (Reduction Sprayed-on Pellets). In the third year, reduction percentages oscillating between 36 and 100% regarding the previous year were recorded, and the average was 71% (Reduction Formulated Pellets).

Unfavorable effects

No adverse consequences affecting the digestive tract, respiratory apparatus or the skin were detected from the time that spores of the parasiticide fungi were provided to the wapitis. Spraying spores on the pelleted feed did not cause their rejection, and the same occurred when pellets added spores during the industrial manufacturing were given to the wapitis.

Discussion

Under temperate weather and humidity, areas provided of vegetation can represent a serious risk of infection for grazing animals due to the development of certain helminths species as nematode trichostrongylids can occur all year round (O'Connor et al., 2006; Nielsen et al., 2007). Certain pasture work as harrowing or ploughing has been shown beneficial to reduce the presence of parasitic stages among rearing livestock (Younie et al., 2004), but do not seem practical for zoological parks because of the difficulties to have sufficient areas to maintain the animals while performing them, and consequently deworming becomes into the only approach. In the current trial, one group of wapitis captive in a zoological park and infected by trichostrongylids received anthelmintic treatment consisting of fenbendazole, and regardless of the successful, counts of nematode EPG higher than 300 four months later denoted that deworming was needed again. These results are in concordance with previous

studies reporting a similar efficacy on wild species of equids (Arias et al., 2013a; Palomero et al., 2018).

Confinement of animals always in the same parcel represents a high risk of infection by nematodes, because of the development in the ground of their infective stages ensures the presence of infective stages throughout the year, or at least when mild temperatures, notable relative humidity and positive soil water balance concur (Fagiolini et al., 2010; Rees, 2011; Mir et al., 2016). In spite of the availability of efficacious dewormers, the presence of infective larvae in the soil is responsible for animals infect again quickly. The lack of useful and practical measures to limit the numbers of infective stages leads to increase the frequency of deworming, but several studies reported the frequent administration of parasiticides could result in lowering their effect, and selection of anthelmintic resistant parasitic strains (De Graef et al., 2012; Shalaby, 2013). Rotation of pastures has been indicated for the purpose to lessen the risk of infection through decreasing the exposure to infective stages, but periods about 3-9 months have been pointed, enlarging thus the numbers of paddocks required (Kumar et al., 2013), which can not be assumed for the majority of zoological parks.

On the basis of certain saprophytic soil filamentous fungi are able to destroy and feed on parasitic stages developing in the feces and ground, different investigations demonstrated a significant reduction on the viability of eggs of trematodes, ascarids and strongyles in the presence of *Pochonia chlamydosporia* (Dias et al., 2013; Thapa et al., 2018; Vieira et al., 2019) or *Verticillium chlamydosporium* (Cazapal-Monteiro et al., 2015; Zarrin et al., 2017); nevertheless, these fungi have not yet assayed on wild captive herbivores. The nematode-trapping fungus *D. flagrans* has been effectively given to captive giraffes during a 12-weeks trial infected by strongyles (Terry, 2013) and to wild equids for two years (Arias et al.,

2013a); the successfulness of a blend containing spores of *M. circinelloides* and *D. flagrans* against strongyles in wild equids has been also demonstrated (Palomero et al., 2018).

Appropriate feeding and care must be provided to captive animals (West & Dickie, 2007). Despite the undeniable advantages of keeping herbivores in parcels with grass, some action appears essential to avoid or minimize the risk of infection by certain helminths such as the trichostrongylids (Fagiolini et al., 2010). One valuable solution relies on limiting the viability of parasites and their possibilities to reach the corresponding infective stages. In view of the ability of certain filamentous fungi to grow and propagate in submerged cultures (Arias et al., 2013b), spraying of spores directly on the soil could represent an easy choice depending on the surface of the parcel, though captive animals might experience a notable stress. Other possibility consists of the oral administration of spores or mycelium in aqueous solutions to the animals (Paraud et al., 2005; da Silva et al., 2015), supported by the capability of some fungal species to survive the passage through the digestive tract without losing their activity (Araujo et al., 2012; Tavela et al., 2013; Hernández et al., 2018c). Consequently, fungal species would evolve in the feces in close contact with the parasitic stages (oocysts, eggs, larvae) passed in the feces, increasing the antagonistic activity which hinders the development of infective phases (Palomero et al., 2018). By considering the high complexity to provide liquids orally to wild captive animals, other interesting approach might involve the spores are added to the drinking water, but the possibility that each individual took the indicated dosage looks too low.

With the aim to facilitate the presence of parasiticide fungi in the feces of wapitis passing eggs of trichostrongylids in a zoological park, two different formulations have been tested in the current investigation. The spraying of a mixture of spores of *M. circinelloides* and *D. flagrans* directly on nutritional pellets prior to be given to the wapitis resulted successful in lowering the levels of trichostrongylids egg-output, and a similar effect was recorded when

these wild ruminants fed on pellets enriched with spores during the industrial manufacturing. In both cases, average counts of trichostrongylids eggs below 100 were recorded, and the need for anthelmintic treatment during a period of ten months was considered unnecessary. These results agree with previous trials conducted on wild captive equids receiving pre-mixed food with spores of *D. flagrans* (Arias et al., 2013a) or commercial pellets containing spores of *M. circinelloides* and *D. flagrans* (Palomero et al., 2018), as well as on domestic horses under rotational grazing (Hernández et al., 2018a).

The impossibility to maintain a control group simultaneously in the zoological park makes difficult to establish a comparison among the effect of the measures developed for controlling the infection by trichostrongylid nematodes. No significant differences were observed in the climatic parameters throughout the current study, hence data collected during the first year (administration of anthelmintic treatment only) were considered as controls. By feeding successfully treated wapitis with pellets sprayed the fungal spores, a significant reduction of the counts of trichostrongylid egg-output to one third was recorded. When efficiently dewormed wapitis received pellets industrially enriched with the spores, the excretion of eggs decreased to a sixteenth. These results support the hypothesis that the administration of spores of *M. circinelloides* and *D. flagrans* interferes and reduces the numbers of infective stages (third stage larvae) of trichostrongylids in the ground, thus the risk of contamination in the parcel decreases; as a consequence, low levels of infection among the captive wapitis lead to consider that additional anthelmintic treatment during the mentioned periods was unnecessary. It should be taken into account that, opposite to that recorded in the first two years, at the beginning of the third year of investigation, counts of eggs of trichostrongylids lower than the cut-off value stated for deworming (300 EPG) were shed in feces, therefore the percentages of egg-output reduction could be biased by the administration of spores during the previous year. This is explained by the administration of pellets sprayed on spores to the wapitis (second year) seemed to reduce notably the

possibilities of third-stage larvae of trichostrongylids could develop and survival, decreasing thus the risk of infection among the captive herbivores in the following year.

One remarkable finding was the absence of adverse effects on the wapitis while receiving fungal spores through two formulations, as sprayed-on or formulated pellets, which points the innocuousness of this strategy, in agreement with prior trials conducted in horses (Hernández et al., 2018a) and dogs (Hernández et al., 2018b). Besides this, the inexistence of any effect of *D. flagrans* on non-parasitic soil nematodes has been previously demonstrated (Saumell et al., 2016), which confirms this constitutes an environmentally friendly approach.

The control of plant pests based on biological agents is widely spread, but their application against pathogens affecting animals has not developed to any great extent. There are several explanations to this, mainly centered on low levels of knowledge, few practical methods, the scarce number of field trials (especially long-term assays), and the broad association between disease and certain biological control agents as fungi. Data obtained in the present research point the usefulness of two feed formulations effortlessly applicable for developing sustainable measures to prevent the infection by trichostrongylids in captive wapitis. Two commercial formulations containing chlamydospores of *D. flagrans* intended for livestock species have been recently registered (Healey et al., 2018; Braga et al., 2020). In the present, study, very effective results have been possible by feeding the wapitis with pellets industrially manufactured with the fungal spores, which represents an easy method without additional task for animal keepers. However, due to this formulation is not yet commercially available and waiting to get it, other practical and fruitful solution could be based on spraying the spores onto the pellets before nourishing the animals.

Conclusions

The administration of a blend of spores of *M. circinelloides* and *D. flagrans* every two days to wild captive animals offers an innocuousness strategy to get a helpful long-lasting effect on preventing the infection by trichostrongylids. Highly successful results can be obtained by providing spores as sprayed-on pellets or as pellets industrially manufactured with the fungi. Aside from this last procedure appears easier for giving the spores to captive animals, in the absence of a commercial product available, possibly due to economical returns, a very practical and similarly successful effect can be achieved by spraying the spores on the pellets immediately before feeding the captive animals.

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Authors' contributions

Rita Sánchez-Andrade, Adolfo Paz-Silva, Cristiana Filipa Cazapal-Monteiro and María Sol Arias Vázquez: Conceptualization, Methodology, Data curation, Writing-Original draft preparation, Supervision, Funding acquisition. **José Ángel Hernández:**

Software. **Antonio Miguel Palomero, Cándido Viña**: Investigation, Validation. **Mathilde Voinot, María Vilá and María Isabel Torres**: Formal Analysis, Investigation.

Competing interests

All authors declare the absence of any financial or personal interests that could inappropriately influence the present investigation. The final article has been approved by all authors.

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CAPTION OF FIGURES

Figure 1.- Variations in climatic parameters in the area of study (Outeiro de Rei, Lugo, NW Spain). Points indicate the average values of data collected between 2014 and 2018.

Figure 2.- Kinetics of trichostrongyles egg-output in wapitis (*Cervus anadensis*) captive in a zoological park (Marcelle Natureza, Outeiro de Rei, Lugo, NW Spain). Points mean the average value and error bars 2SD.

Figure 1

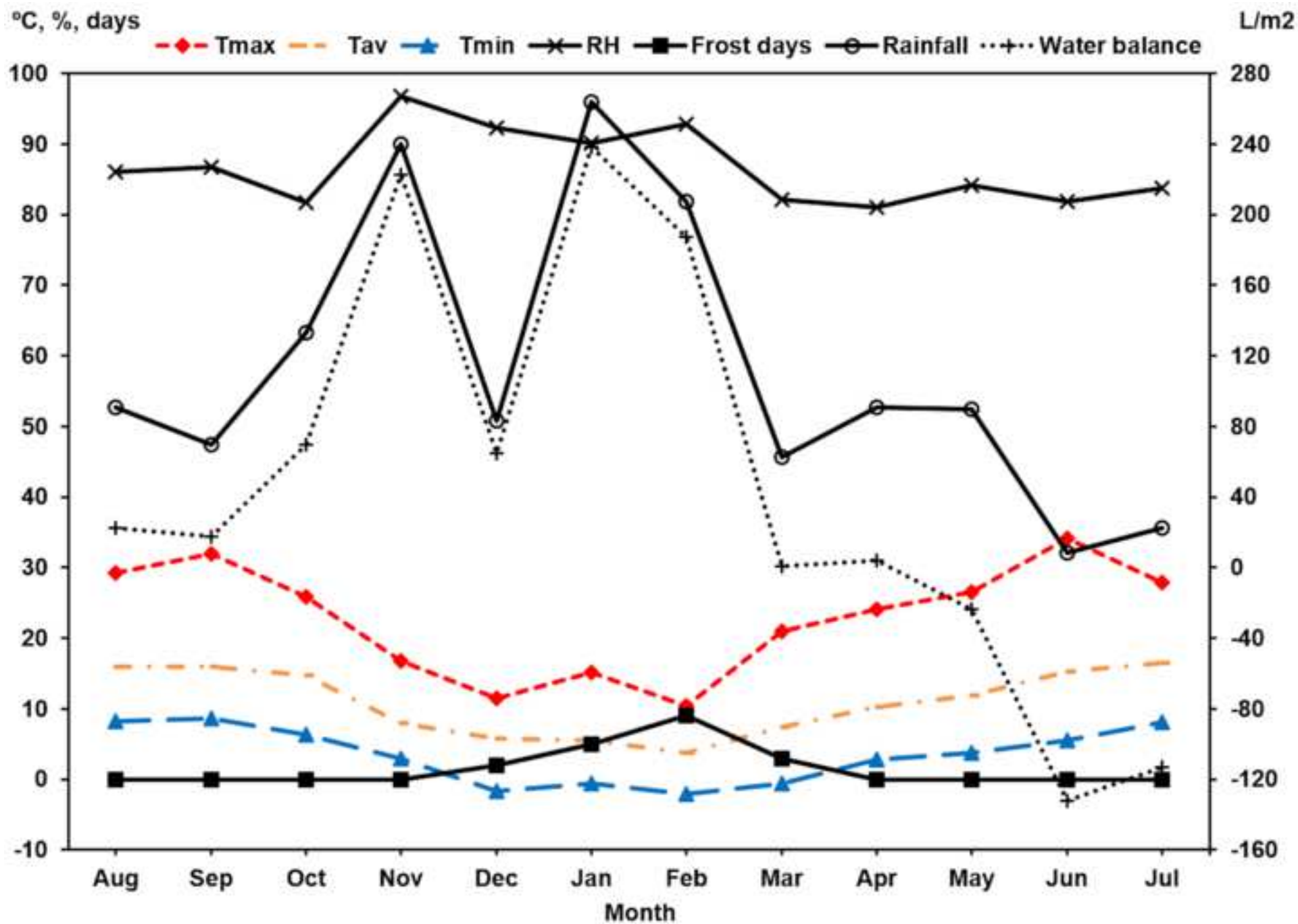
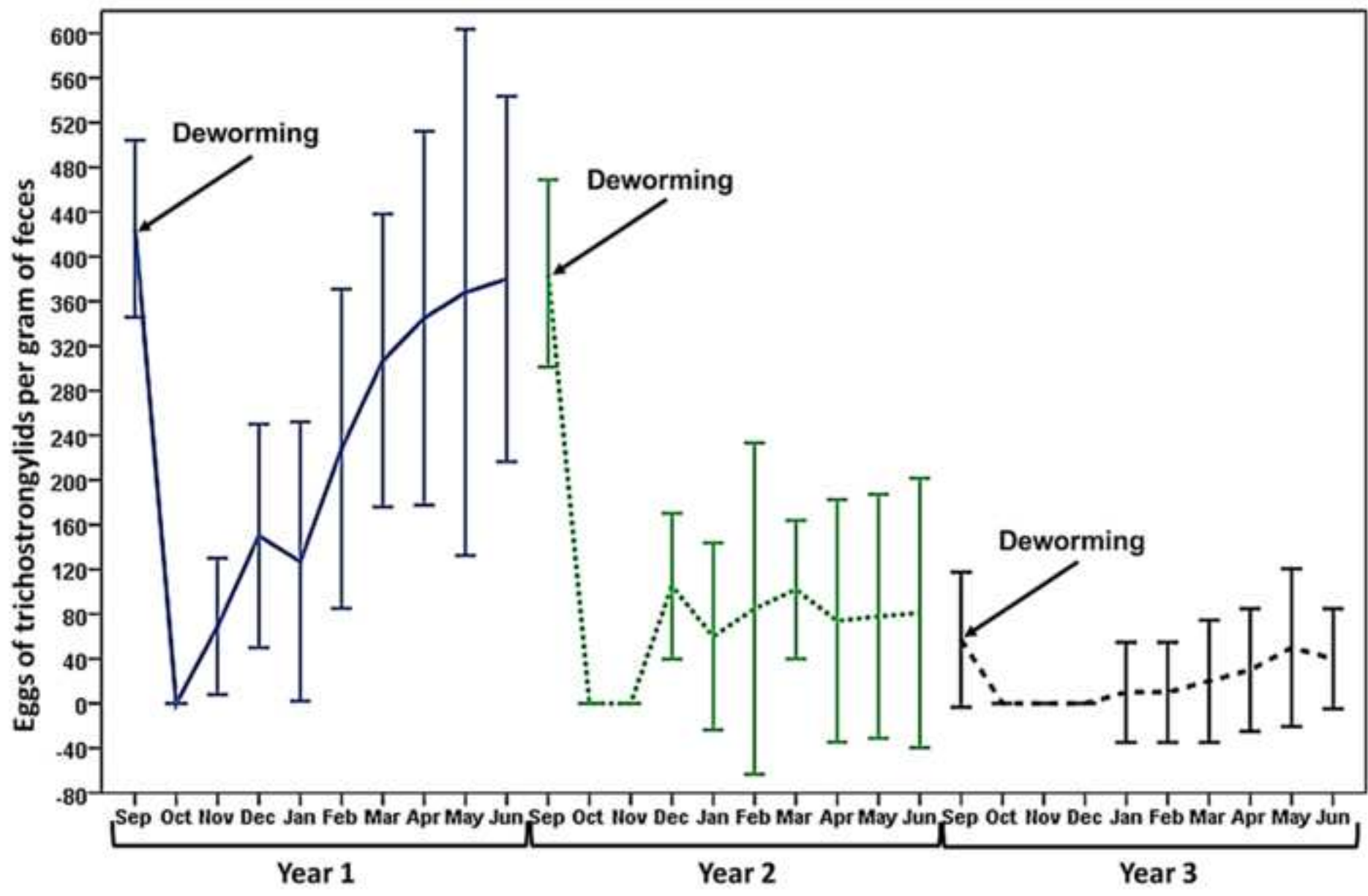
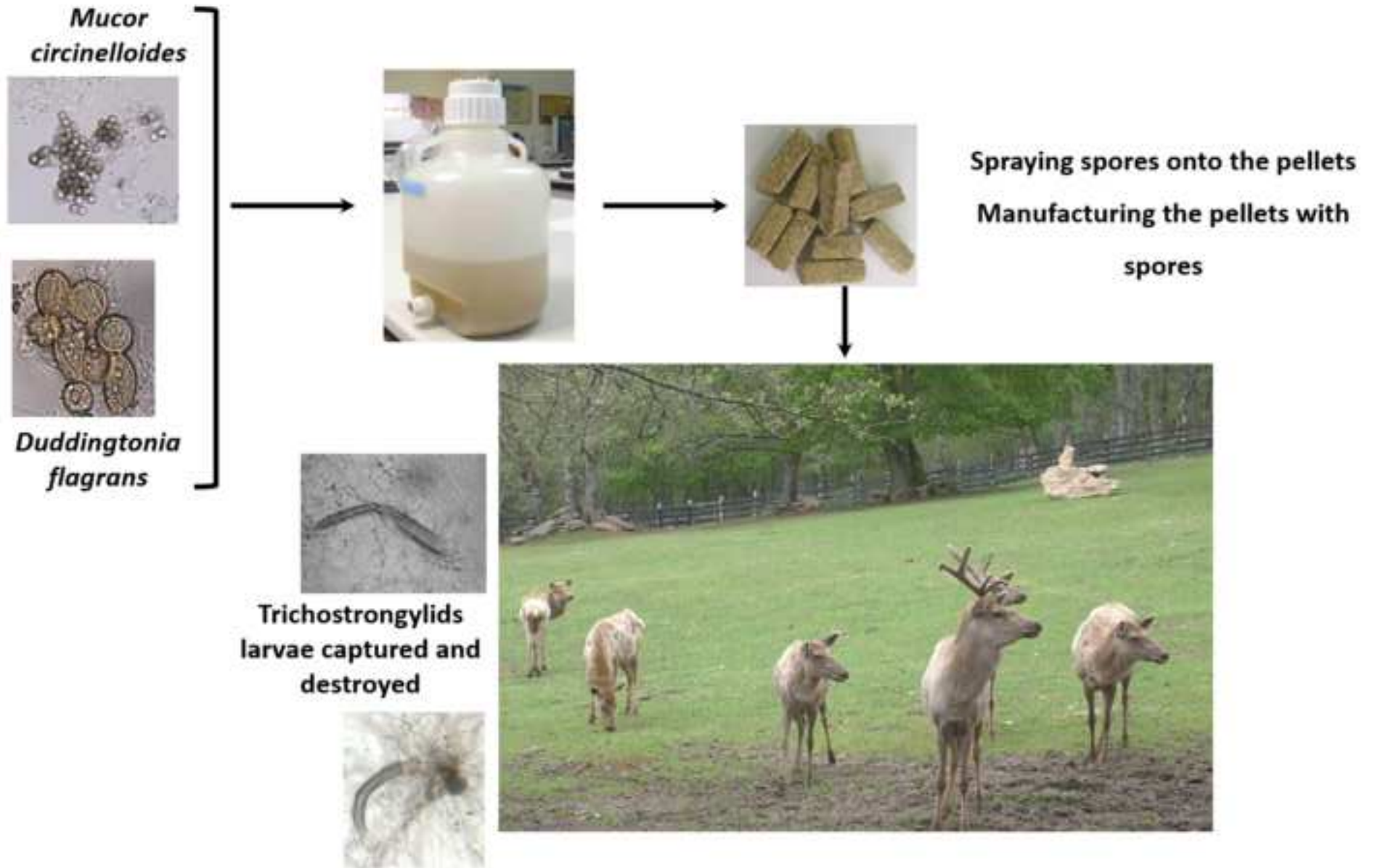
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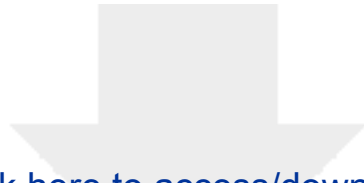


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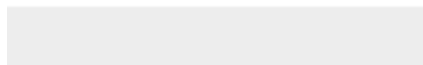
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Author Statement

Rita Sánchez-Andrade, Adolfo Paz-Silva, Cristiana Filipa Cazapal-Monteiro and María Sol Arias Vázquez: Conceptualization, Methodology, Data curation, Writing-Original draft preparation, Supervision, Funding acquisition. **José Ángel Hernández:** Software. **Antonio Miguel Palomero, Cándido Viña:** Investigation, Validation. **Mathilde Voinot, María Vilá and María Isabel Torres:** Formal Analysis, Investigation.

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