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INTEGRATED CONTROL OF DIGESTIVE HELMINTHS IN CAPTIVE HERVIBORES

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INTEGRATED CONTROL OF DIGESTIVE HELMINTHS IN CAPTIVE HERVIBORES

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The Doctoral candidate declares no conflicts of interest related to his Thesis.

FIGURES AND TABLES

All figures and tables included in this PhD dissertation are originals and belong to the author and the COPAR Research Group (GI-2120).

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Out of the night that covers me,
Black as the pit from pole to pole,
I thank whatever gods may be
For my unconquerable soul.
In the fell clutch of circumstance
I have not winced nor cried aloud.
Under the bludgeonings of chance
My head is bloody, but unbowed.
Beyond this place of wrath and tears
Looms but the Horror of the shade,
And yet the menace of the years
Finds and shall find me unafraid.
It matters not how strait the gate,
How charged with punishments the scroll,
I am the master of my fate,
I am the captain of my soul.

«Invictus» WILLIAM HENTLEY (1849–1903)

Más allá de la noche que me cubre,
negra como el abismo insondable,
doy gracias a los dioses que puedan existir
por mi alma inconquistable.

En las azarosas garras de las circunstancias
no he gemido ni llorado.
Sometido a los golpes del azar
mi cabeza sangra, pero está erguida.

Más allá de este lugar de ira y llantos
yace sino el horror de la sombra,
Y aún la amenaza de los años
me halla y me hallará sin temor.

No importa cuán estrecha sea la puerta,
cuán cargada de castigos la sentencia,
soy el amo de mi destino,
soy el capitán de mi alma.

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Abbreviations

AAEP: American Association of Equine Practitioners

AAZV: American Association of Zoo Veterinarians

B.C.: before Christ

BW: bodyweight

CECT: Spanish Type Culture Collection
EPG: eggs per gram of feces
ERP: egg reappearance period

FEC: Fecal Egg Counts
FECR: Fecal Egg Count Reduction
Fig.: Figure

GIN: gastrointestinal nematodes

Ha: hectare

IPCR: individuals positive to coprological tests

Kg: kilogram

L1, L2, L3: first, second or thrid stage larvae
mL: mililiter
mm: milimeter
OPG: oocystss per gram of feces
SSF: Soil Saprophytic Fungi

STHs: Soil-Transmitted Helminths

Susp: suspension

TPD: time lapsed from the previous deworming

WAAVP: World Association for the Advancement of the Veterinary Parasitology
WAZA: World Association of Zoos and Aquariums

1.- INTRODUCTION

1.1. THE HUMAN-ANIMAL BOND

Human evolution has occurred in parallel with their increasing ability, not ever positive, of dominating their environment, which involves land and animals. Accordingly, domestication of certain animal species served to ensure protein and energy needs could be easily attended, without hunting frequently.

The process of adapting wild animals for human use is called *domestication*. It is known that wolves were the first to be domesticated (between 33,000 and 11,000 years ago). People in Mesopotamia began to domesticate animals for meat, milk, whereas their skins were used for clothing, storage, and to build tent shelters. The first herbivores to be domesticated were small ruminants (goats and then sheep). Domestication of chickens in Southeast Asia dates back to 10,000 years ago, and larger animals as oxen or horses were domesticated later for plowing, transportation and to pull heavy loads.

Domesticating animals is strongly related to some traits as their nutrition, calm temperament, ability to breed in captivity and easy adaptation to changing conditions (Hoage & Deiss, 1996). Herbivores that graze on vegetation, as ruminants or horses, appear the easiest ones because the easiness of feeding them. By opposite, herbivores taking seeds and grain as chickens are more difficult due to the requirements of growing special crops, which are highly valuable also. Among the main consequences of domesticating animals, it is important to underline that these species are not feral, they were adapted to live alongside humans over centuries, and in many cases look different from their wild ancestors. Other point facilitating their domestication consists of animals living in herds are easier for humans to control.

The objective of domesticating was not sustained over time. In example, some dogs were domesticated to assist people in hunting, and hundreds of domestic dog species are known today. Despite many of them are still good hunters, most are pets. In general, three main groups can be considered regarding animal domesticating (Fig. 1).

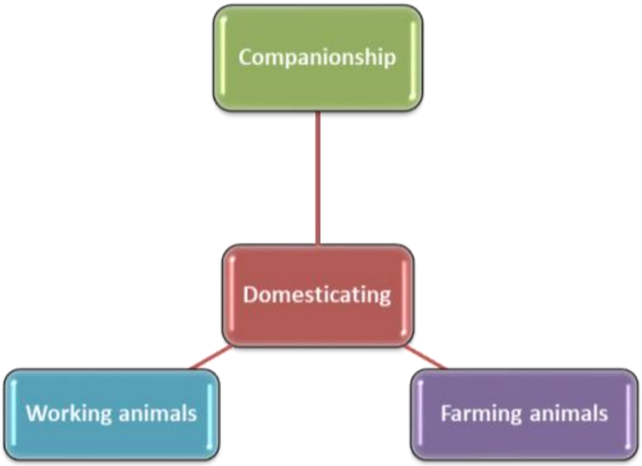


Fig. 1.- Domesticating animals are generally sorted into three groups.

It is important to note that *domesticating* and *taming* are not synonymous. Domestic animals have their origins in the selection and breeding in captivity by humans after multiple generations. As a result, they have developed morphological, behavioral and even reproductive changes with respect to their wild ancestors. It is assumed that these species are genetically determined to be tolerant of humans. Tamed animals are those that are habituated to humans, because their behavior can be conditioned so that they get used to coexist with them, but they are still wild. Some clear examples are tigers or elephants, which explains why a wild-caught animal should not be kept as a pet, even if it is quite docile. In the opposite side, other animal species experienced certain changes during their domesticating process, lacking some characteristics as their ability to survive in the wild because their predator instincts disappeared.

1.2. HISTORICAL BACKGROUND OF ANIMAL COLLECTIONS

Despite there are many animal species coming close to domesticating, very few meet the requirements mentioned above (easy nutrition, docility, ability for breeding under captivity) (Diamond, 1997). Accordingly, other species have been captured and maintained under captive conditions as collections of animals, without the purpose of domesticating. In some cases, the objectives were to underline power, wealth, status or military might.

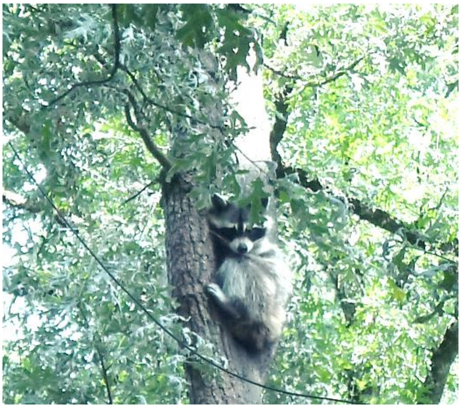


Fig. 2.- Animals in modern zoos are not kept in cages, but in an environment as close as possible to their natural habitat.

The earliest records of animals in captivity are attributed to Egyptians, Chinese and Greeks between 2000 and 500 B.C., describing caged animals as lions, birds and giraffes for the entertainment of the population, though predatory animals were preferred in most cases, due to they looked more impressive (Benbow, 2004). Accordingly, the main objective of Hittite kings displaying lions, tigers, wolves, leopards or bears, Egyptian Queen Hatsheput doing it with rhinoceroses, giraffes, or greyhounds, or Emperor Wu Di showing elephants, yak, giant pandas, cormorants, and herons, was to remark to others their ability to conquer their enemies, geography and the environment (Peart, 1993). Later, menageries or collections of wild animals emerged with Roman cities for the purpose of exhibition, symbolizing power and wealth.

In the Middle Ages, passion of highest social classes for captive animals spurred a global trade concerning especially wild species. As an example, it resulted very strange and infrequent that kings do not have an elephant or a zebra. In consequence, merchants captured, shipped, and sold legions of animals intended for royal and/or collections belonging to rich people. Besides this, some public spectacles as traveling fairs carted exotic animals (elephants, rhinoceroses, giraffes and hippopotamuses) from town to town.

The discovery of America by the Spaniards was an important contribution to the knowledge of new species of exotic animals. Between 1493 and 1496, Christopher Columbus gave sixty parrots and a macaw to the Catholic Monarchs, Queen Isabella and King Ferdinand, thus setting a precedent that meant a boost in the scope and symbolic importance of wild animals, which became the playthings of European empires. In this way, Hernán Cortés and his troops could observe the existence of a great variety of animal species (lions, tigers, bears, hawks, jaguars, snakes or eagles) in huge cages under the property of Moctezuma in Tenochtitlan, the floating city that gave rise to what is now known as Mexico City (Croke, 1997). In addition, the participation of specialists taking care for sick or injured individuals has been reported. During the fifteenth and sixteenth centuries, iguanas, bison, guinea pigs, llamas, turkeys, Muscovy ducks, squirrels, and many species of birds became highly frequent in Europe. One rhinoceros was gifted friendly from Sultan Muzaffar Shah of Malacca in 1515 to the governor of Portuguese India, and he sent it to Dom Manuel I, king of Portugal.

In the late 18th and early 19th centuries, the flourishing collections of exotic animals changed from private to public due to the transformation of kingdoms into nation-states, which meant a change in funding from private to public (Kohl, 2004). After the French Revolution, in 1793, the Jardin Royal des Plantes (formerly the menagerie of King Louis XIII) was transferred from royal to public ownership. Later, in a few decades, zoos in Madrid, London, Dublin, Antwerp, Melbourne, and Philadelphia opened their doors, and spread the world over by the end of the nineteenth century. This process was accelerated thanks to the appearance of a *middle class* in Europe and America, driven by that moment of significant population growth, industrial revolution and urbanization. New names were given to collections of animals, as zoological gardens, zoological parks, and colloquially *zoos*.

A case which undoubtedly illustrate this situation could be find in the history of Walter Rothschild (1868 - 1937). Although he was born into an important banking family, his main interest from his childhood was nature. During decades, he was collecting specimens on a family estate (Tring Park, Hertfordshire) built him a museum. The Walter Zoo Museum was opened to the public in 1892, and is now the Natural History Museum. During his lifetime he employed over 400 collectors, who sent specimens from over 48 different countries to the Museum. This collection became the largest private one ever accumulated by one person, and upon his death, the remainder of his research collections, the public museum, its contents, and the surrounding land were donated to the Natural History Museum in London.

Fig. 3.- The maintenance of animals in zoos is actually intended to provide them with adequate refuge and visitors with the chance to experience their natural habitat.

1.3. ANIMAL COLLECTIONS AT PRESENT

Currently, captive wild animals are mainly kept in facilities such as zoos, aquariums, marine and theme parks. Rehabilitation centers, sanctuaries, and private homes, where they are kept as companion animals, are less common, and the importance and number of circuses and traveling shows, as well as scientific research laboratories, has declined in many countries, as a result of public awareness (Hoage & Deiss, 1996). Recent estimations point about 10,000 zoos exist around the world, receiving yearly millions of visitors (WAZA, 2005; Holtorf, 2008).

As mentioned above, the transformation or evolution of menageries into modern zoos has been hard and tortuous, and consequently these places grew in size, housed a greater number of species, and international markets were established (Kisling, 2001). Despite the incorporation of more staff, mainly administrative, professional, laborers, veterinarians and zookeepers, the final results have not always been as expected, with changes still to come (Fa *et al*., 2011). Nowadays it is common to design and organize events and educational courses, important efforts for people to enjoy with their families. Restaurants, souvenir stores and recreational areas focused on children are also frequent in today's zoos, where technological updating measures are applied (Packer & Ballantyne, 2010). Modern zoos must satisfy different areas involving scientific research, wildlife conservation, public recreation, and education. Likewise, it should be noted that zoos have evolved in recent decades to become much more than just places to spend a fun afternoon. In this way, zoological gardens may act as spaces for the purpose of enriching people, areas for enjoy and entertainment, giving them scientific education, free of noise and pollution (Roe *et al*., 2014).

1.3.1. Captive animal species in zoos

Collections of animals were initially dedicated to exotic species, or those proceeding from other continents. In parallel to the historical evolution of the zoos, the aims changed from initially places of entertainment to institutions focused on conservation and education mainly (Patrick *et al*., 2007; Carr & Cohen, 2011), which seem more socially and morally acceptable for public opinion. Since 1970s and 1980s, zoos turned their activities to the conservation of endangered species and wildlife, and many of them became into refuges where both threatened and non-risky species are maintained. It is importat to stress that the rich diversity of the animal kingdom is not reflected by collections denominated ***charismatic megafauna*** (lions, tigers, giraffes, elephants, zebras, bears, hippopotamuses and rhinoceroses), then breeding animals in situ was significantly increased along the 20th century, which did facilitate that some species are interchanged among zoological parks, enriching their own collections (Conway, 2010). Although the vast majority of these animals would not (and could not) be introduced or reintroduced into natural habitats, this approach resulted in zoo populations of individuals being bred in captivity instead of being captured. This protective activity was supported in the slogan *Captivity for Conservation* as a clear definition for modern zoos (Keulartz, 2015).

1.3.2. Facilities for captive animals

All through the history of animal exhibits, most individuals were displayed in cramped cages, where drear inactivity, and visitors mistreatment and behavior (incessant staring, teasing, prodding, feeding) were responsible of their lives were often short. Despite cages still fill some zoos today, from the 19th century on, zoological parks acquired a new environmental role, which resulted in the introduction of significant changes in the enclosures to be less forceful and more naturalistic, in order to improve the housing conditions of captive animals, adapt them as much as possible to their wild life, and thus promote their welfare. In some cases, the effort was limited to replacing cages with larger areas such as fields or even forests.

In this regard, it must be remembered the contribution of Carl Hagenbeck in the 1890s, a German animal entrepreneur who set a new stage in the establishment of zoos by introducing animals of different species in the same enclosure, which had previously been conditioned with plants, soil and artificial rocks to mimic their natural habitat.

(Rothfels, 2002). This so-called *revolution* became the first attempt to build areas closer to the wild environment so that visitors could enjoy imagining the animals in their natural world. Accordingly, the size of zoo enclosures augmented notably, but they remained artificial.

Notwithstanding these good intentions, it is not always possible to reproduce environments for different animal species, because the requirements can be very diverse. Thus, the habitat of an ostrich bears no relation to that of a mountain goat or a giraffe.

1.3.3. Environmental enrichment

Throughout the last quarte of the 20th century, the concept of landscape immersion enclosures spread among zoological parks. This idea has been defined as the provision of stimuli which promote the expression of species-appropriate behavioral and mental activities in an understimulating environment (Reinhardt & Reinhardt, 1998), which connects with the guidelines proposed by Chamove and Moodie (1990) about the main goals for enrichment, (1) Increase behavioral diversity; (2) Reduce the frequencies of abnormal behavior; (3) Increase the range of normal (i.e., wild) behavior patterns; (4) Increase positive utilization of the environment; (5) Increase the ability to cope with challenges in a more normal way. Eight types of enrichment have been described by Hoy *et al.* (2010) (Fig. 4).

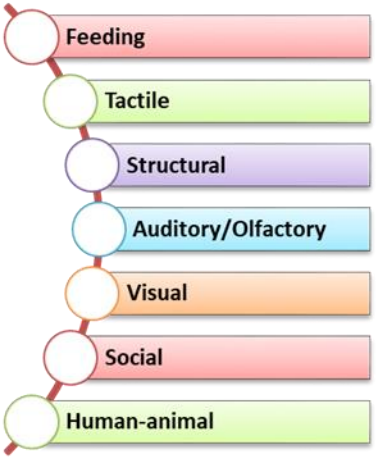


Fig. 4.- Enrichement provided to captive animals in modern zoos.

1.4. WILD HERBIVORES CAPTIVE IN ZOOLOGICAL PARKS

Animals adapted to a plant-based diet are known as *herbivores* (Cheeke & Dierenfeld, 2010). They represent around 80% of the mammals and are sorted into two main groups in order to the digestive processes, foregut fermenters (ruminants) and hindgut fermenters (monogastrics) (Ley *et al*., 2008). Currently, zoos host a large number of species belonging to both groups, as shown in Table 1.

Table 1.- Some of the animal species captive in zoos.

Foregut fermenters			**Hindgut fermenters**	
Okapi	***Okapia johnstoni***		**Elephant**	***Loxodonta africana***
Giraffe	***Giraffa camelopardalis***		**Rhinoceros**	
Mountain goat	***Oreamnos americanus***		**Equids**	***Equus ferus caballus***
Reindeer	***Rangifer tarandus***			***Equus asinus***
Wapiti	***Cervus elaphus***			***African wild ass***
Cuvier's gazelle	***Gazella cuvieri***			
Dama gazelle	***Nanger dama***			
Blackbuck	***Antelope cervicapra***			
Moufflon	***Ovis orientalis musimon***			
Bison	***Bison bison***			
Sitatunga	***Tragelaphus spekii gratus***			
Cobo	***Kobus kob***			
Axis	***Axis axis***			

In relation to their *feeding strategy*, herbivores are classified into three groups (Cheeke & Dierenfeld, 2010). **Grazers** have a large rumen, or ability to consume feed at a high rate, enabling ingestion of plants with high fiber and low energy content, and cattle, zebra or antelope belong to this group. The gastrointestinal tract in **browsers** is developed for taking advantage of low fiber forage, therefore species as giraffe, moose or roe deer can select low fiber portions as fruit, leaves and soft vegetable parts. Finally, **mixed feeders** (goats, sheep, elephant) can adapt to different environments, thus they can choice from browsing to grazing, regarding the availability of nutrients (Cheeke & Dierenfeld, 2010).

Fig. 5.- Herbivores are classified into grazers (left), browsers (center) and mixed feeders (right).

Non-territorial grazers with the ability to live in large herds and on limited areas could be said to be pre-adapted to a life in captivity and was most common candidates for domestication. The effort to domesticate species with less desirable traits, such as the moose and fallow deer was abandoned due to the difficulties maintaining them in captivity. Some species from the herbivore family was ignored completely because of complex mating systems, large home areas and territorial behavior (Tennessen & Hudson, 1981). Domesticated animals were and are used in the agriculture society, while some wild animals started to be captured and kept in captivity for other purposes.

1.4.1. Green areas

As explained previously, certain wild animal species were housed in zoos for entertainment and enjoyment of people. Currently, zoological parks are focused mainly on education and conservation, by considering that some wild species are in risk of extinction (Rabb, 2004). In the last decades, different programs aiming for the reintroduction of herbivores are being developed in some zoos (Gilbert *et al*., 2017; Gonzálvez *et al*., 2021). Based on the renewed role of these institutions, some of the most important changes have been focused on improving the environment for wild animals kept in zoos, by focusing on natural behavior, veterinary care and appropriate nutrition.

Fig. 6.- In many zoos, fenced parcels with grassy areas are created with the aim to simulate the original conditions of wild herbivores (Marcelle Natureza, Outeiro de Rei, Lugo, Spain).

Among the changes and attempts to improve the environment for herbivores, the presence of fenced paddocks with grass, where these animal species can forage and even socialize. This seems to be a significant contribution to their welfare, although the presence of grass depends mainly on the location or better climate. It is known that herbivores dedicate most of their time to grazing, and differences can be detected on the basis of social structure, nutritional strategy and gastrointestinal activity (Okello *et al*., 2002), ranging from 45% in Przewalski's horses (large grazers with hindgut fermentation), to 61% in goats and sheep (small mixed feeders with foregut fermentation)

(King, 2002; Pokorná & Hejcmanová, 2013). In any case, it must be taken into account that herbivores graze for half of the day or more.

Despite the undoubted benefits that herbivores receive by enjoying grassy and even wooded parcels, there are some disadvantages caused by the extended period they spend grazing throughout the day, which can increase the risk of exposure to different pathogens. Endoparasites, especially helminths, are known to develop part of their life cycle in the soil, where infective stages are reached (Fig. 7).

Fig. 7.- Third-stage larvae (left) are the infective stage of strongyles, and develop in the soil from eggs (right) passed in the feces of infected equids.

1.5. PARASITES AFFECTING WILD HERBIVORES CAPTIVE IN ZOOS

Animals reared in restricted environments appear highly susceptible to infection by gastrointestinal parasites transmitted by fecal-oral routes, therefore zoos are characterized as parasite-rich environments (Mir *et al*., 2016; Capasso *et al*., 2019).

Helminths represent the most frequently reported parasites in the animals inhabiting Europe's zoos (Gonzálvez *et al*., 2021). According to the frequency of the reports the most widely-spread among the helminths are the nematodes, followed by the cestodes and trematodes (Panayotova-Pencheva *et al*., 2013). Nevertheless, because of trematodes and cestodes need one or more intermediate hosts in their life cycle, the chance of animals captive in zoos become infected by these helminths is rather seldom, due to individuals are maintained in fenced parcels with little access to intermediate hosts (Atanaskova, 2011; Mirzapour *et al*., 2018; Dashe & Berhanu, 2020).

Diverse ways of explaining the occurrence of parasites in zoos have been put forward. In addition to ingestion of contaminated food (meat, fish, fruit, vegetables...), in the case of herbivores it seems more realistic and easier the participation of intermediate and paratenic hosts (snails, ants, cockroaches, other insects, worms, rodents, etc.), or even by recently acquired parasitized animals. The possibility of captive wild animals being placed in plots previously grazed by domestic or wild animal species must also be taken into account, because it could easily enhance the presence of certain parasite species and, therefore, the risk of infection.

1.5.1. Parasites identified in zoos

There is poor information about the parasites infecting animals captive in zoological parks. Eggs of certain species of gastrointestinal nematodes such as *Nematodirus*, *Capillaria* and *Trichuris* have been detected in the feces of captive ruminants (antelopes, gazelles and giraffids) from two zoos in Belgium (Goossens *et al*., 2005).

In the herbivores of the zoological garden "Peña Escrita" (Almuñécar, Spain), with an estimated extension of 600 ha, Pérez Cordón *et al*. (2008) reported that the highest prevalences were attained in protozoa (*Entamoeba* spp., *Endolimax nana* and *Eimeria* spp., mainly), followed by *Nematodirus* spp. This zoo is divided into different areas by wire fences, and the animals live in nature, with open natural zones with feeders, watering troughs, and hiding areas or caves.

Parsani *et al*. (2001) conducted a study in a municipal zoo in Gujarat (India) and found that Bluebull and Spotted Deer were infected by *Trichostrongylus* and *Balantidium coli*.

The anatomopathological examination of the digestive tract of a Cape giraffe (*Giraffa camelopardalis giraffa*) in the Aitana Zoo (Alicante, Spain) showed a total of 2724 nematodes, identified as *Trichostrongylus axei, Ostertagia ostertagi, Teladorsagia circumcincta, Teladorsagia trifurcata, Marshallagia marshalli, Trichostrongylus vitrinus, Trichostrongylus colubriformis, Spiculopteragia asymmetrica*, species observed also in mouflons, fallow and red deer (Garijo *et al*., 2004). Besides this, *Camelostrongylus mentulatus* and *Trichuris giraffae* were also identified.

In two Italian zoos located in Apulia and Tuscany, helminthic infections were more common than protozoan infections in all the mammal orders examined (Fagiolini *et al*., 2010).

The most frequent parasites found in feces from captive wild herbivores from a zoological park in Punjab were strongyles, then *Trichuris*, *Eimeria* and amphistomes (Singh *et al*., 2006).

By means of fecal analyses using the $ZnSO_4$ flotation method, Atanaskova *et al*. (2011) performed a study of the endoparasites affecting wild animals in a zoological garden in Skopje (Macedonia), and identified eggs of strongyloides, *Nematodirus*, *Trichostrongylus* and *Trichuris* in muntjac, deer, llama, pony horse, elands, camels and yaks.

Arias (2013) performed a coprological survey among animals captive in the Marcelle Zoological park (Outeiro de Rei, Lugo, Spain), and found the presence of protozoan and helminths (Table 2).

In zoo herbivores from Warsaw, Maesano *et al*. (2014) applied the FLOTAC for the analysis of fecal samples from ruminants and monogastric herbivores. They observed that gastrointestinal strongyles (60.5%) were prevalent in ruminants which resulted positive also to Coccidia (*Eimeria* spp.), *Trichuris* spp. and *Nematodirus*. *Strongyles* were the most frequent parasites in monogastric herbivores, and then *Parascaris equorum*. None of the animals showed any symptom associated with gastrointestinal parasitic infections.

Table 2.- Parasites detected in captive wild animals in the Marcelle Natureza Zoological Park (Lugo, Spain).

Species	Protozoa	Cestoda	GIN
Canidae		***Taenia***	
Ursidae	***Eimeria***		***Trichostrongylus***
Felidae	***Eimeria***		***Toxocara cati*** ***Toxascaris leonina***
Equidae			***Cyathostomum*** ***Strongylus*** ***Gyalocephalus*** ***Trichostrongylus*** ***Poteriostomum***
Bovidae	***Eimeria***		***Trichostrongylus*** ***Nematodirus*** ***Haemonchus***
Camelidae	***Eimeria***		***Trichostrongylus*** ***Trichuris***
Cervidae	***Eimeria***		***Trichostrongylus*** ***Nematodirus***

The analysis of fecal samples from Nilgai (*Boselaphus tragocamelus*), Spotted deer (*Axis axis*), Black buck (*Antelope cervicapra*), Sambar deer (*Cervus unicolor*), Hog deer (*Axis* *porcinus*), and Barking deer (*Muntiacus muntjak*) by sedimentation and flotation techniques showed the presence of strongyles (67%), Strongyloides spp., coccidia, *Trichuris* spp., ascarids and *Capillaria* spp., whereas no cestode or trematodes were detected during the study performed in a mini zoo in Punjab (India) (Mir *et al*., 2016).

Nosal *et al*. (2016) reported the presence of eggs of nematodes from the Trichuridae family (*Trichuris* sp., *Aonchotheca* (*Capillaria*) sp.) and some unspecified eggs of Strongylida in giraffes from two zoos in Poland (The Silesian Zoological Garden in Chorzów, and Kraków Zoological Garden). Camels resulted infected by the protozoan *Eimeria bactriani* and *E. dromedarii*, and by the GIN *Trichostrongylus* and *Cooperia*.

Eggs of strongyles and *Trichuris* were identified in the feces of herbivores captive in a zoo placed in Dehiwali (Sri Lanka) (Aviruppola *et al*., 2016).

In a zoological park of Madhya Pradesh (India), examination of a total of 374 fecal samples from wild herbivores (Chital *Axis axis*, Sambar *Rusa unicolor*, Nilgai *Boselaphus tragocamelus* and Chinkara *Gazella bennettii*) showed that majority of them had mixed infection by *Eimeria* spp., *Trichuris* spp., *Moniezia* spp., *Amphistome*, *Strongyloides* spp., *Balantidium* spp., and *Fasciola* spp. (Sengar *et al*., 2017).

Sprenger *et al*. (2018) reported that wild herbivores (alpacas, dromedaries, camels and llamas) captive in a zoo from the state of Paraná (Brazil) passed eggs of Strongyloidea family in their feces, and *Trichuris*.

A survey performed in four zoological parks from southern and central Italy showed that 80% of the fecal samples analyzed by the FLOTAC were positive to the presence of parasites (Capasso *et al*., 2019). The parasites identified were protozoa *Blastocystis* spp., *Giardia* spp. and *Eimeria* spp. Strongyles were the most frequent among the gastrointestinal nematodes, then *Trichuris* spp., *Parascaris* spp. and *Capillaria* spp.

Analysis of endoparasites affecting wildlife captive in Bangladesh showed the presence of gastrointestinal strongyles eggs in 40% donkeys (Nath *et al*., 2021).

1.5.2. Life cycle of the main parasites affecting herbivores captive in zoos

As occurs with livestock species, risk of infection by parasites among captive animals is directly related to their housing (Lim *et al*., 2008). In those animals that are kept in habitats with little or no vegetation, sandy or concrete soil, protozoa and *Trichuris* are recorded mainly (Hernández *et al*., 2018c) (Fig. 8). However, Arias *et al*. (2013a) found important levels of eggs of strongyles in the feces of African wild assess maintained in a sandy parcel from Marcelle Natureza (Outeiro de Rei, Lugo, Spain).

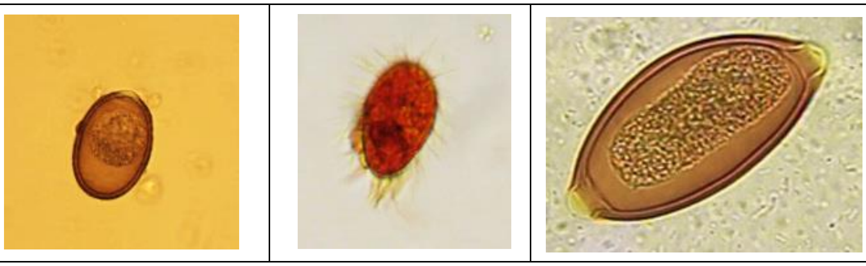


Fig. 8.- Wild animals kept into paddocks with concrete or sparsely planted soils eliminate oocysts of protozoa (*Eimeria, Isospora*; left; *Balantidium coli*, center), and eggs of *Trichuris* (right).

Non-sporulated oocysts of coccidia are shed in the feces of infected animals, mainly in those kept in limited spaces, where elevated humidity together with moderate to high temperatures, favor the development of sporocytes after a variable period (Molina & Ruiz, 2019).

The life cycle of ascarids involves that non-embryonated eggs are passed in the feces of infected animals (Fig. 9), and once in the ground, they transform into infective stages characterized by a second-stage larva developing inside the egg after celullar multiplication for one to four weeks (Cazapal-Monteiro *et al*., 2015) (Fig. 10).

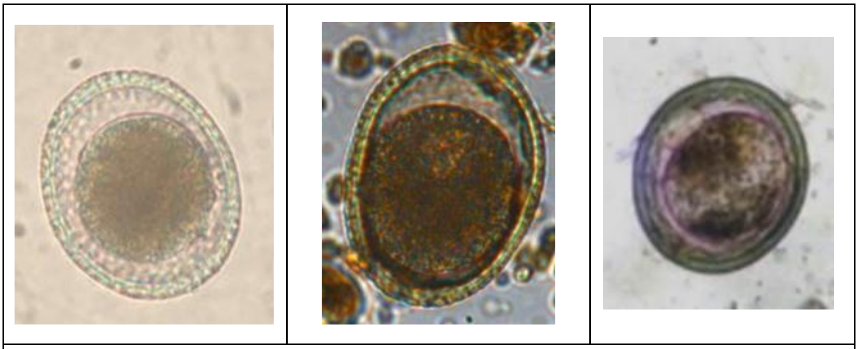


Fig. 9.- Eggs of ascarids as *Toxocara vitulorum* (left), *Toxocara canis* (center) or *Toxascaris leonina* (right) are commonly found in feces of captive wild animals.

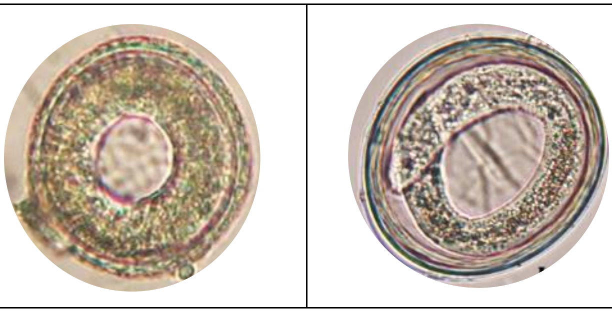


Fig. 10.- Infection by roundworms occurs by the ingestion of eggs with a second-stage larva inside.

Infective stages of *Trichuris* are eggs containing a first-stage larva, which originates from non-embryonated eggs (Lindquist & Cross, 2017).

When animals are captive in grassy or wooded areas, helminths, mainly nematodes, are most frequently detected, due to the possibilities of the external phase of their life cycle can successfully occur (Burke *et al*., 2009a) (Fig. 11). In case of strongyles, eggs passed in feces develop inside a first-stage larva (L1), which exits off and moult into L2 and finally L3, the infective stage which will be ingested together with grass by the herbivores while grazing (Voinot *et al*., 2021a) (Fig. 12).

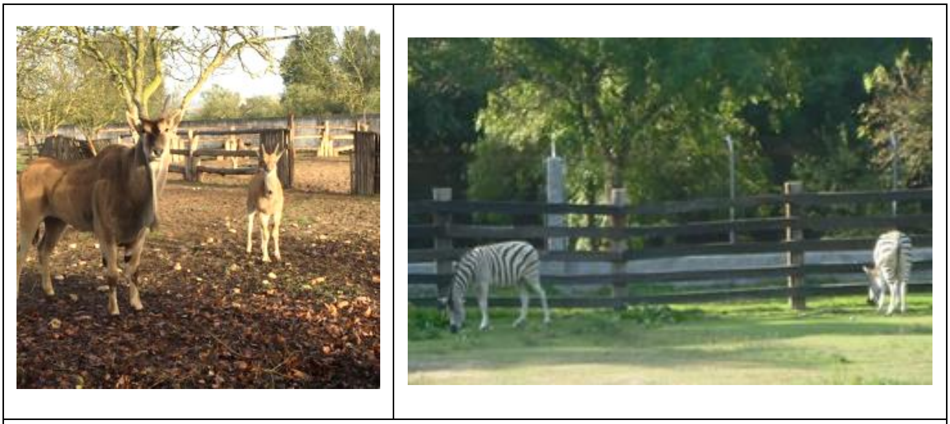


Fig. 11.- Captive wild animals kept in grassy areas are mainly exposed to certain helminths that develop the external phase of their life cycle in the soil. (Left: Quinta dos Plátanos, Alenquer, Portugal; right: Marcelle Natureza, Outeiro de Rei, Lugo, Spain).

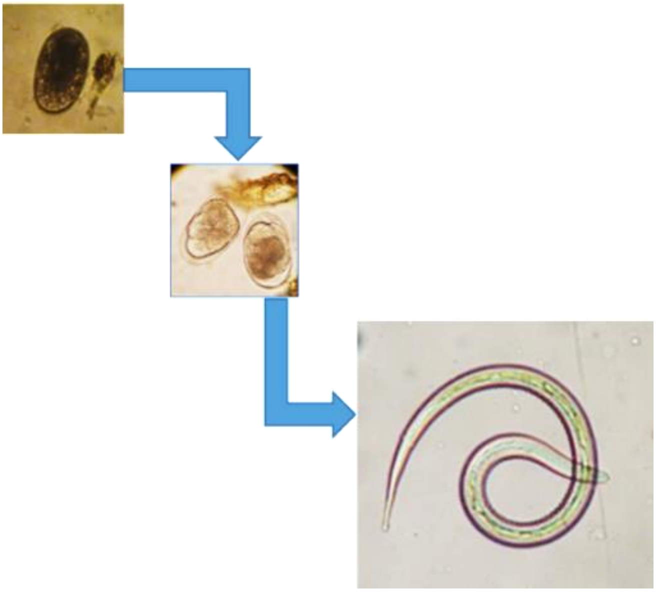


Fig. 12.- Unembryonated eggs of strongyles are shed in the feces of herbivores (up), then cellular multiplication occurs inside (center), and finally a first-stage larva (L1) exits off and moults into L2 and L3 (the infective stage) (down).

1.6. CONTROL OF PARASITES IN ZOOLOGICAL PARKS

In zoos, control of these parasites should primarily focus on lessening infection pressures by administering anthelmintics (Nath *et al*., 2021). It has been stated that captive individuals receiving regularly antiparasitic treatment do not exhibit clinical signs of infection (Parsani *et al*., 2001; Schieber & Štrkolcová, 2019), then this could be interpreted as deworming was successful and infection fully disappeared. Nevertheless, periodical administration of antiparasitic compounds does not prevent challenge infections among captive individuals, then it is suggested that lack of quarantine with new entered animals, and free-ranging stray animals, could be possible source for infection (Nath *et al*., 2021). Other issue needing attention relies on that the frequent use of broad-spectrum anthelmintics, in combination with inappropriate dose and administration methods may lead to the development of resistance to anthelmintics (Shalaby, 2013). In an experiment conducted by Young *et al*. (2000), several gastrointestinal strongyles in captive wild ruminants showed a higher level of resistance to common anthelmintics such as levamisole and avermectin. Based on the oral administration of mebendazole did not reduce nematode egg shedding in captive African gazelles, Ortiz *et al*. (2001) pointed differences in host metabolism, irregular administration of anthelmintics or anthelmintic resistance could be involved. In a giraffe acquired by the Lion Country Safari in Loxahatchee (Florida, USA), the existence of a strain of the nematode *Haemonchus contortus* resistant to benzimidazoles, imidazothiazoles, and macrocyclic lactones was demonstrated by means of the larval development assay (Garretson *et al*., 2009). To ensure anthelmintic efficacy, revision of parasite control strategies is required, including a greater emphasis on surveillance through periodic parasitological assessment.

For the purpose to limit the risk of infection by certain parasites, frequent cleaning of animal refuges and facilities is obliged, taking care of removing the feces to avoid that infective stages are attained. It is noteworthy to state that preventing environmental contamination by eggs and larvae of parasites represents one of the key steps to stopping the transmission of parasites to the wildlife. Regular monitoring for sanitation and cleaning is necessary to minimize the risk (Nath *et al.*, 2021).

1.6.1. Main strategies for the control of parasites in captive wild animals

Most common measures for parasite control in captive wild animals consist of periodical deworming, seldom supported in previous parasitological (coprological) analyses (Aviruppola *et al.*, 2016). As a matter of fact, the successful of treatment is infrequently estimated.

Captive wild herbivores are routinely administered the same anthelmintics used in the control of parasites affecting livestock (Terry, 2013) (Table 3). It should be underlined the absence of data relating not only to possible side effects, but also to appropriate dosage and expected efficacy. Because of the effect of deworming is seldom checked, the possibility of efficacy levels lower than expected increases, which could result in anhelmintic resistance (Shalaby, 2013).

Table 3.- Captive wild herbivores receive dewormers administered to livestock.

Species	Dewormer
Falabella	Equimax® oral paste
Zebra	
African donkey	
European donkey	
Dromedary	Panacur10® susp + Prolcen®
Eland	Panacur10® susp + Flubenol®
Antelope	
Reindeer	
Fallow deer	
Axis	
Wapity	
Mouflon	
Waterbuck	

Parasite control among captive herbivores in zoos also involves the manual collection of feces following aesthetic questions, every two or more days, before visitors arrive (Arias *et al.*, 2013a; Moreno *et al.*, 2019). Although this is an adequate measure to prevent parasites from being able to develop to infective stages on the ground, the results are inferior to those expected, as evidenced by frequent and early challenge infections are detected (Singh *et al*., 2006; Hernández *et al*., 2018c).

In order to reduce the use of anthelmintics among livestock, two approaches targeting the flock or the individual have been introduced (Calvete *et al*., 2020). Both are addressed on a proportion of the parasite population is kept in refugia (without deworming), with the aim to reduce the appearance of anthelmintic resistance (van Wyk, 2001). Targeted treatments (TT) characterize by treating all individuals in a group, based on knowledge of the risk or severity of infection. In targeted selective treatments (TST), deworming is performed at an individual level (Kenyon & Jackson, 2012). In both cases, mainly TST, it is necessary to define proper cut-off points for treatment, frequently supported on fecal egg counts (Charlier *et al*., 2014). Despite these procedures are mentioned in *The American Association of Zoo Veterinarians Infectious Disease Manual*, there is a lack of information concerning their observance in zoological parks (AAZV, 2020), and other parameters as the Body Condition Score could be helpful to support the neeed of deworming (Schiffman *et al*., 2017).

1.6.2. Alternatives to the deworming

Any parasite control program based solely on deworming is unlikely to achieve the expected effect. For that reason, different alternatives have been pointed. Despite most cases are combined with strategic deworming, the final objective consists of lessening the dependence of chemical dewormers (Williams *et al*., 2014).

a) Agricultural practices

In *The American Association of Zoo Veterinarians Infectious Disease Manual* (AAZV, 2020), it is stated that treatment and prevention of more than 35 species of nematodes belonging to the genus *Trichostrongylus* affecting different animal species captive in a zoo should involve control measures entailing deworming with benzimidazoles or macrocyclic lactones, and pasture rotation. In order to contribute to a more rational use of dewormers, different strategies have been advised for decades (Fig. 13). **Ploughing and plowing** are advised to make difficult both survival and development of certain parasites, which are faced to unfavorable conditions, mainly decreased humidity and direct exposure to sunlight (Voinot *et al*., 2021b).

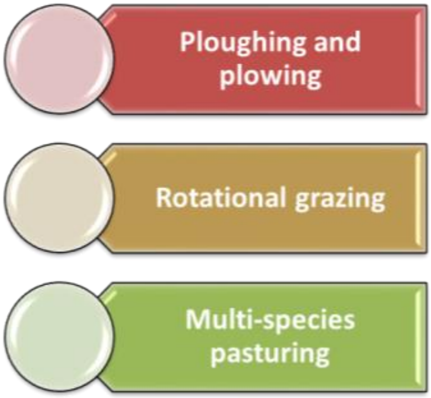


Fig. 13.- Some agricultural practices are suggested for reducing parasite survival in the environment.

Pasture rotation among grazing cattle is suggested, with the objective of limiting exposure to risk areas. However, in most cases, rotation is not adjusted to the life cycle of certain parasites, but depends on the nutritional needs of the animals (Hernández *et al*., 2018a).

The **alternation of grazing species**, also known as multi-species pasturing, implies that one species feeds on one pasture, and a period later other different species, in order to breaking the parasite life cycle. Concerning livestock species, cattle and horses could feed on the same plot as sheep and goats, because of the first did not share parasites with the last species. This idea aims to obtain safer pastures by breaking the parasite life cycle; the sequential passage of different species of herbivores results in reducing the presence of infective stages in the soil, and consequently the risk of infection (Cazapal-Monteiro *et al*., 2013; Mederos & Banchero, 2013).

Due to management of captive animals and the designing of the zoological gardens, ploughing and plowing appear the only measure really applicable. Pasture rotation looks inappropriate and almost impossible because of the requirements of land, together with the stress that herbivores could suffer by moving through the park, with probable visual contact with some predators. Although multi-species grazing in the same plot may be seen in some zoos, the ultimate goal is not parasite control, but rather to try to recreate some thematic zones (Fig. 14), as initially introduced by Hagenbeck at the end of the 19th century (Rothfels, 2002). For instance, an African area might contain waterbucks sharing the parcel with zebras, sitatunga and elands.

Fig. 14.- Modern zoos build thematic areas holding different species of captive wild animals grazing the same parcel (Safari Park, Manacor, Spain).

b) Plant-derived products

Certain natural plant extracts have long been administered as dewormers for livestock, even when scientific basis is not conclusive, and most of the active compounds remain unknown (Githiori *et al*., 2005). Condensed tannins (CT) are attributed the main anthelmintic activity, probably developed by alkaloids, terpenoids or polyphenols. These plant-derivatives can be obtained from forage legumes, trees, and shrubs collected in temperate and tropical zones, and some of them develop other activities as antioxidant or anti-inflammatory properties (Martínez-Micaelo *et al*., 2012). Among the most commonly species are bird's-foot trefoil (*Lotus corniculatus*), common sainfoin (*Onobrychis viciifolia*), willow-leaf red *quebracho* (*Schinopsis balansae*) or golden wattle (*Acacia pycnantha*). One interesting approach consists of seeding pastures with leguminous plants as jumbay (*Leucaena leucocephala*), black wattle (*Acacia* *mearnsii*) or neem (*Azadirachta indica*) (Chandrawathani *et al*., 2006).

Several harmful effects on feed intake, rumen microorganisms, nutrient utilization, and production performance of ruminant livestock, has been associated to the ingestion of elevated concentrations of tannins (Henke *et al*., 2017). Fidgett *et al*. (2003) pointed that inclusion of tannins is prohibitive due to their cost, and even to ecological considerations if *quebracho* is provided.

The Leguminosae family characterizes by a notable content of saponins, substances capable of provoking changes in cell permeability, which conducts to the formation of micelle-like aggregates. In consequence, the membrane is broken, resulting in the death of nematodes (Doligalska *et al*., 2017; Santos *et al*., 2018). The genus *Medicago* represents an important source of bioactive saponins, involving several forage crops such as alfalfa (*Medicago sativa*) and burr medic (*M. polymorpha*) (Piano *et al*., 2010). It has been demonstrated that saponins from *Medicago* species can exert different biological roles as hemolytic, antimicrobial, insecticidal, or anthelmintic against some plant nematodes (Avato *et al*., 2006, 2017; D'Addabbo *et al*., 2011; Vo *et al*., 2017).

The use of different herbs such as garlic (*Allium sativum*), pumpkin (*Cucurbita pepo*), thyme (*Thymus vulgaris*) or mugwort (*Artemisia vulgaris*) has been advised against parasites infecting livestock under organic regimes (Costa *et al*., 2008; Burke *et al*., 2009b; Castagna *et al*., 2021).

c) Copper oxide wire particles (COWP)

Two formulations have been assayed for the administration copper oxide wire particles, by means of gel capsules or included directly in the feed (Burke *et al*., 2010). Most of the trials performed focused on the action against gastrointestinal nematodes (Trichostrongylids) in sheep. Caution must be exercised if COWP is incorporated into a concentrate feed to goats on pasture without supplementation, which could cause grain overloading or acidosis if too much supplement is fed at once. Also, copper toxicity could occur if a small population takes most of the feed supplemented with these particles. Data concerning captive wild animals are scarce, and only one trial conducted on exotic artiodactylids has been reported (Fontenot *et al*., 2008).

d) Biological control strategies

Integrated management of pests needs of biological control procedures for reaching good results, especially concerning prevention of infection by different pathogens as parasites. Biological control has been defined as *the reduction of pest populations by natural enemies*, and normally involves an active human participation (Shelton, 2016). The difference with natural control relies on organisms are eliminated suppressed by naturally happening organisms and environmental factors, without human interference. Biological control encompasses action on insects, natural enemies of weeds and plant diseases, and pathogens affecting animal species.

For the purpose to reduce the risk of infection by different parasites, as well as the overuse of chemical anthelmintics with the objective of avoiding the decrease of the expected successful and the appearance of anthelmintic resistance, some strategies supported in the use of natural enemies have been tried.

The use of *Bacillus thurigiensis* for the parasitic control of gastrointestinal nematodes in grazing animals has been studied for some years, due to its capacity to eliminate the juvenile and adult stages, based on producing a series of crystals, which are solubilized when digested by the nematodes and give rise to protoxins, which alter their intestinal membrane (Hernández Linares *et al*., 2008; Salehi Jouzani *et al*., 2008; Ramezani *et al*., 2014).

Baculoviruses are the most widely used group of viruses for biological control, with very different characteristics from those that infect vertebrates, and they are also very safe to spread (Myers & Cory, 2015).

The knowledge of certain species of soil fungi that live as saprophytes, but can develop the ability to transform themselves into predatory parasites, has been taken into account in the design of sustainable strategies, initially aimed at controlling pathogens affecting plants and more recently animals (Hernández *et al*., 2017; Mendoza-de Gives, 2022).

Some carnivorous nematodes have been tested against *Rotylenchulus reniformis* and *Meloidogyne incognita*, parasites infecting plants crops (Wang *et al*., 2015).

1.7. CONTROL OF PESTS BY MEANS OF FUNGAL SPECIES

The kingdom Fungi contains five true phyla including *Chytridiomycota*, the *Zygomycota*, the *Ascomycota*, the *Basidiomycota*, and *Glomeromycota* (Aguilar-Marcelino *et al*., 2020).

Agricultural application of fungal species against plant pathogens has been widely developed in the second half of the 20th century, mainly focused on the control of unwanted vegetation by means of fungi with selective bioherbicide potential (Reichert Júnior *et al*., 2019). For the biological control of weeds, the use of phytopathogenic fungi called bioherbicides has been advised (Charudattan, 2001). The first record of this strategy was reported in 1971, consisting of the introduction of the species *Puccinia chondrillina* against *Chondrilla juncea* in Australia (Barton, 2004). Afterwards, eleven fungal-based herbicides were commercially available in 2009.

Other fungi have been shown helpful against pathogenic fungi (Punja & Utkhede, 2003). Consequently, different commercial formulations against some fungal disease targets can be found (Table 4).

Table 4.- Some fungal species are commercialized against certain plant diseases caused by other fungi.

Fungal species	Fungal disease target
Ampelomyces quisqualis M-10	Powdery mildews
Pythium oligandrum	Root rot
Fusarium oxysporum	Wilt
Coniothyrium minitans	Root rot
***Trichoderma* spp.**	Root rot
T. harzianum	Root rot
T. viride	Root rot, wilt

On the contrary, the knowledge on fungal species of interest under a veterinarian point of view has been significantly improved in the last 25 years. Different strains of soil filamentous fungi have been isolated and tested for gaining information concerning their activity, with *D. flagrans*, *Pochonia chlamydosporia*, *Mucor circinelloides* and *Monacrosporium thaumasium* being the most common employed species. Despite this, three commercial formulations based on the nematode-trapping fungus *D. flagrans* are available by now, Bioworma® and Livamol with Bioworma® in Australia (Healey *et al*., 2018), and Bioverm® in Brazil (Araújo *et al*., 2021).

1.7.1. Parasiticide mechanisms of action

a) Plants pathogens

Several mechanisms of biological control have been described concerning antagonistic activity against plant pathogens (Vujanovic & Goh, 2011) (Fig. 15). *Mycoparasitism* or *hyperparasitisim* can be considered the most direct type of antagonism (Jioty & Singh, 2017), and three possibilities are defined, (a) unilateral, (b) mutual and (c) no antagonism (Cook, 1993). Several species belonging to the filamentous saprophytic genera *Trichoderma* or *Clonostachys* are successfully utilized against important plant pathogens through biocontrol strategies (Motlagh & Samimi, 2013; Sun *et al*., 2019). In other line, predation is generally performed by different microorganisms, through a pathogen non-specific interaction.

It has been demonstrated that most fungi are able of releasing different compounds as well as secondary metabolites acting as *antibiotics*, associated to the phase of active growth (Keller, 2005). In concrete, some species of *Trichoderma* release a group of enzymes addressed against cell walls of pathogenic fungi.

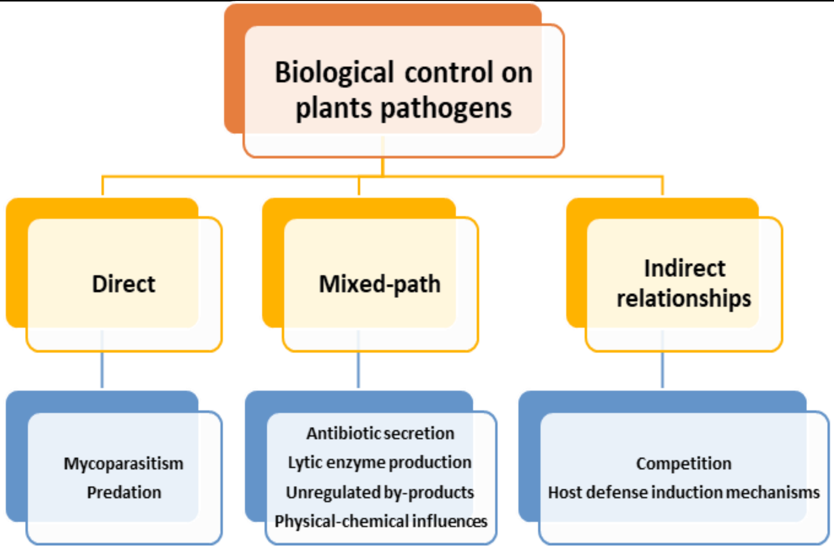


Fig. 15.- Fungi develop different mechanisms for the biological control of pathogens affecting vegetables.

The capability of *T. harzianum* for controlling the presence and survival of *Fusarium oxysporum* seemed attributable to the competence for rhizosphere colonization and nutrients (Tjamos *et al.*, 1992). More recently, Chen *et al.* (2019) reported that the resistance against *F. oxysporum* in cucumber roots is based on regulating reactive oxygen species (ROS), reactive nitrogen species (RNS), the redox balance, and energy flow.

b) Animal pathogens

Some saprophytic microorganisms often living in the soil can develop antagonistic activity against certain parasites, as occurs with several bacteria, viruses, acari, soil saprophytic filamentous fungi (SSF) or nematodes. The SSF are normally feeding on organic matter in decomposition, but a small percentage (less than 0.5%) of the SSF develop different structures focused on trapping larvae or penetrating cyst/oocysts/eggs of parasites, prior to digesting their inner contents (McInnes, 2003) (Figs. 16 & 17), especially addressed on taking nitrogen and carbon (Dackman & Nordbring-Hertz, 1992; Anan'ko & Teplyakova, 2011).

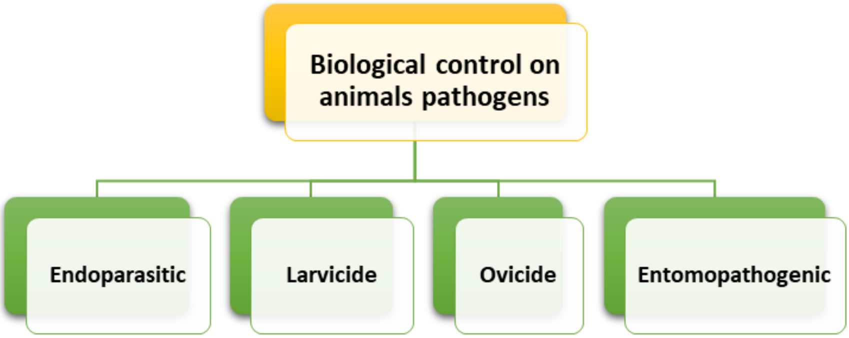


Fig. 16.- Fungi develop different mechanisms for the biological control of pathogens affecting animals.

Later, the nematicidal activity of *D. flagrans* on larvae of strongylids was demonstrated, as well as other trapping nematophagous fungi (*Arthrobotrys oligospora*, *Monacrosporium thaumassium*) (Mendoza de Gives, 1998; Braga *et al*., 2009b; Fitz-Aranda *et al*., 2015).

Some SSF have displayed activity against insect parasites of animals and plants, and *Beauveria bassiana* or *Metarhizium anisopliae* are the most known and tested enthomopatogenic species (Bittencourt, 2000).

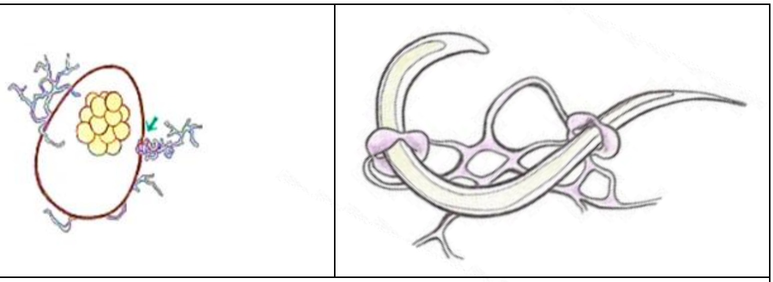


Fig. 17.- Some SSF can disrupt the eggshell of certain parasites, penetrating and taking the inner content (left); others elaborate specialized structures for trapping larvae of nematodes (Strongylids) (right).

1.7.2. Switching of saprofhytic to predatory behavior

The likely involvement of other soil microorganisms (bacteria) in the switching of saprophytic to predatory behaviour has been investigated previously, stating that possibly originated when living beings were extinguished in masse, leading to soils with high carbon content and low in nitrogen. Therefore, the ability to acquire nitrogen directly from living organisms afforded a significant competitive advantage (Yang *et al*., 2012).

The initial studies conducted on the trapping fungus *D. flagrans* noted the need of nematodes are alive for inducing trapping formation, identifying the movements and products excreted by nematode larvae as stimuli (Nordbring-Hertz, 1977; Liu *et al*., 2012). Despite these arguments, Arias *et al*. (2013a) pointed that *D. flagrans* developed fast and notably when cultured in the presence of dead specimens of *Fasciola hepatica*, *Calicophoron daubneyi*, *Parascaris univalens* and *Oxyuris equi*. Besides this, addition of their excretory/secretory antigens to the agar medium resulted a strong stimulus for hyphae development, and formation of traps and chlamydospores (Fig. 18).

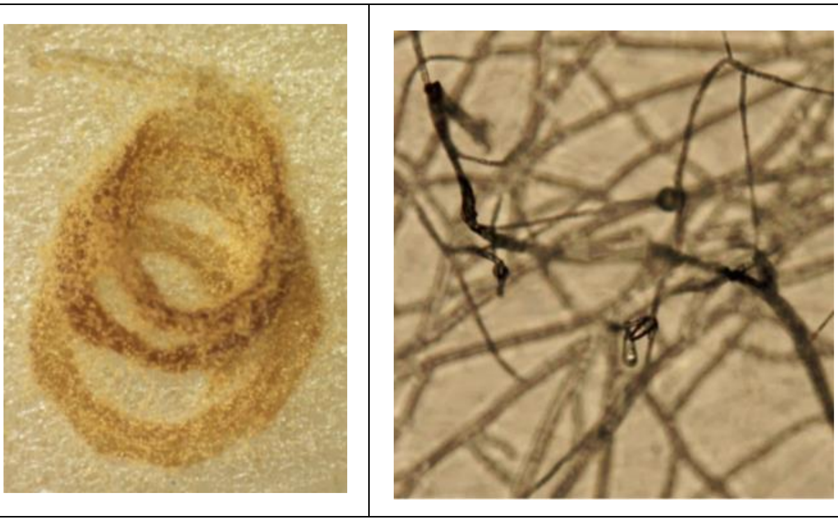


Fig. 18.- Improved production of chlamydospores of *D. flagrans* over an *Anisakis* L3 (left), and growth of mycelium in the presence of *C. daubneyi* excretory/secretory antigens (right).

The role of some aminoacids on trap formation by nematophagous fungi has been demonstrated later (Hsu *et al*., 2015), as well as the participation of *ascarosides*, molecules synthesized by nematodes (Hsueh *et al*., 2013).

Recently, the first small-secreted protein involved in the predatory relationship between fungi and nematodes, a cysteine-rich protein (CyrA), has been characterized during the exposure of *Caenorhabditis elegans* to *D. flagrans* (Wernet *et al*., 2021). Hsueh *et al*. (2017) reported that *Arthrobotrys oligospora*, a nematode trapping fungus, releases volatile compounds that attract the nematodes to the traps elaborated in their hyphae, and Yu *et al*. (2020) showed that a methyl-salicylic acid isomer (6-MSA) is produced by *D. flagrans* with the objective to lure *Caenorhabditis elegans* into fungal colonies.

1.8. SOIL SAPROPHYTIC FILAMENTOUS FUNGI (SSF) IN THE CONTROL OF PARASITES AFFECTING ANIMAL SPECIES

First observations related to the usefulness of SSF for the control of parasites were addressed on nematodes affecting plants (Barron, 1977). The species *Duddingtonia flagrans* resulted antagonist of larvae of the parasites *Meloydogine* spp., *Heterodera* spp. and *Globodera* spp., capable of injuring crops (Hussain *et al*., 2016).

1.8.1. SSF with parasiticide activity

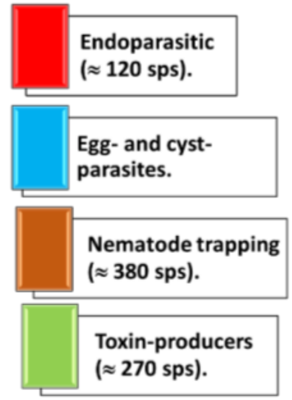


Fig. 19.- Nematophagous fungi are divided into four groups regarding their parasiticide activity.

Most of the knowledge acquired until now refers to the *mycelium* of fungi acting on nematodes (*nematophagous fungi*, NF). There have been described more than 700 species of NF, classified in four groups according to their infection mechanism (Zhang *et al*., 2011) (Fig. 19). However, there has been demonstrated antagonism against ectoparasites by the so called *entomopathogenic fungi*. As a consequence, the following classification appears more adequate (Li *et al*., 2015; Hernández, 2019; (Canhão-Dias *et al*., 2020):

- *Endoparasitic species* characterize by producing spores for the purpose to infect the parasite, by means of a passive way to penetrate it (ingestion is the most frequent).
- *Ovicidal species* are provided with structures specialized for breaking the external lawyer (eggshell) of oocysts, cysts or certain eggs of parasites, the denominated *appressorio* and *haustorio*.
- *Nematode-trapping fungi*, also known as *larvicidal*, have the capability of elaborating traps in their hyphae for catching mobile stages (larvae) of certain nematodes.

- *Toxin-producing fungi* release a toxin which immobilizes nematodes before penetration of hyphae through the cuticle.
- *Entomopathogenic fungi* can colonize and multiply inside ectoparasites.

a) Activity on immobile parasitic stages: eggs / cysts

As is widely known, infection by some parasites occurs by the ingestion of infective stages of cysts, oocysts or eggs present in the soil (feces). It has been demonstrated that certain species of fungi develop antagonistic activity on these immobile stages, and four phases have been defined for explaining the interaction between *Verticillium chlamydosporium* and eggs of the swine roundworm *Ascaris suum* (Fig. 20) (Lýsek & Stĕrba, 1991).

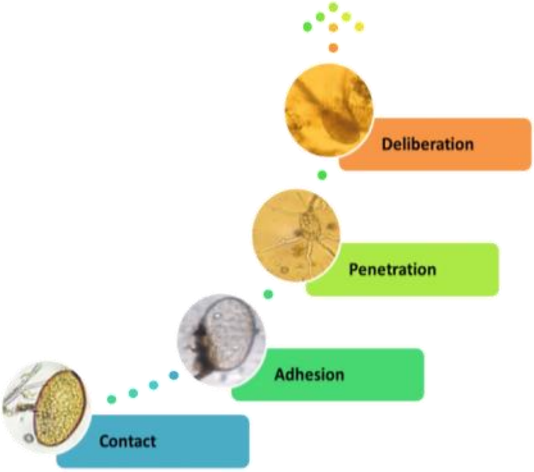


Fig. 20.- Ovicidal activity is exerted by certain fungi across four steps.

Those hyphae interacting perpendicularly with the parasitic surface can adhere to the eggshells (trematodes, nematodes) or capsule (protozoa) through a structural modification at their tips, the *appressorium*. With the assistance of other structure specialized, the *haustorium*, the nutrients are absorbed and carried out to the thallus (Fig. 21). This process needs of breaking the surface of the parasitic stage, which is improved by releasing different metabolites with different enzymatic activity (Maestrini *et al*., 2019).

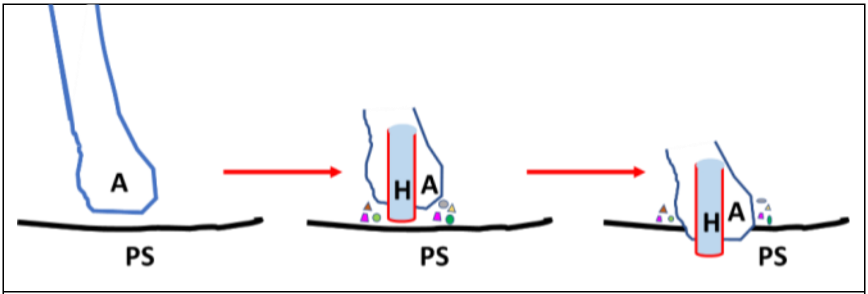


Fig. 21.- Hyphae from ovicidal fungi linking perpendicularly to the surface of the parasitic form (PS) (egg, cyst) through the *appressorium* (A), and the inner contents are absorbed by means the *haustorium* (H).

b) Activity on parasite mobile phases: larvae of nematodes

The spread of helminths belonging to Strongylidae or Ancylostomatidae involves a first stage larva (L1) developing within eggs that are passed in the feces to the soil, emerging and developing into L2 and then into the infective stage (L3). Because these forms are mobile, the antagonistic activity operated by ovicidal fungi may be too slow to prevent larval emergence. Several nematode-trapping fungi such as *Duddingtonia flagrans*, *Monacrosporium thaumasium* or *Arthrobotrys* spp. have been described, which are characterized by the ability to produce traps along their hyphae (mycelium) where nematode larvae are immobilized through *constrictor rings* (Canhão-Dias *et al*., 2020) (Fig. 22). Subsequently, *assimilative hyphae* originate and penetrate them to collect nutrients (Freiría, 2020).

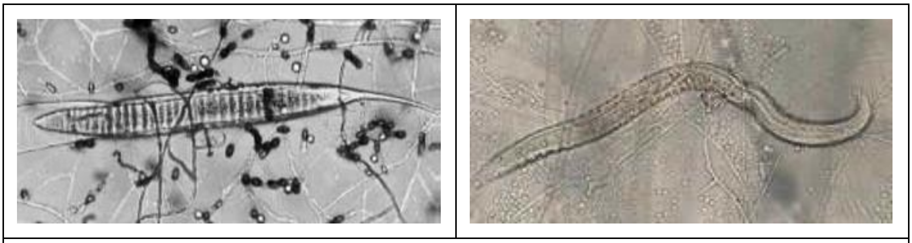


Fig. 22.- Cyathostomin larvae trapped by *D. flagrans* hyphae (zebra feces).

Three phases have been defined in the larvicidal activity of filamentous fungi (Fig. 23) (Hernández, 2019). Firstly, larvae are captured as they move, which requires that mycelium developed until a certain degree, and immobilized. Next, assimilative hyphae are created and penetrate the larvae; the final phase consists of the assimilation of nutrients.

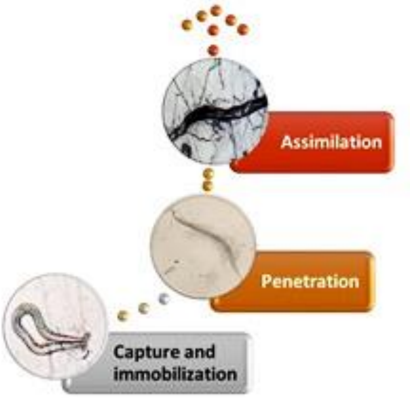


Fig. 23.- The larvicidal activity is carried out in three steps.

1.8.2. SSF of veterinary interest

a) Spores and mycelium of fungi

Most of the veterinary application of SSF has involved the use of their spores mainly, and to a lesser extent their mycelia. The main SSF species so far are listed in Table 5. During the second half of the 20th century, almost all trials were directed to estimate the effect of *D. flagrans* on Stronglylidae larvae, first in Petri dishes and then in field experiences (Nordbring-Hertz *et al.*, 1986; Grønvold *et al.*, 1993; Mendoza de Gives *et al.*, 1994; Ortiz *et al.*, 2017; Fernández *et al.*, 1999; Braga *et al.*, 2009a; Buzatti *et al.*, 2015; Zegbi *et al.*, 2021; Voinot *et al.*, 2021a).

The use of ovicidal fungi as parasiticidal agents is not a recent consideration, and data have been collected in experimental studies for several decades (Lýsek & Krajcí, 1987; Ciarmela *et al.*, 2005; Braga *et al.*, 2012). However, it should be noted that these fungi were not tested in field trials until practically the second decade of the 21st century (Dias *et al.*, 2012, 2013; Arias *et al.*, 2013a; Monteiro *et al.*, 2020; Viña *et al.*, 2020).

Despite the classification of SSF concerning their main parasiticide activity in larvicide or ovicide, it has been proposed the term of *helmintophagous* (Braga & Araújo, 2014) in the basis of it describes more precisely the effect of the aforementioned fungal species against different species of this group of parasites, the most profusely investigated both in animals and in humans (Araújo *et al*., 2021). In this line, it is striking to note that the usefulness of mixing fungal species with complementary ovicidal and larvicidal activity has not been considered until recently (Hernández *et al*., 2018a, b; Vieira *et al*., 2019; 2020; de Oliveira *et al*., 2021; Voinot *et al*., 2020, 2021a).

Table 5.- Main SSF utilized in veterinary.

Species	Activity	Target organism
Duddingtonia flagrans	Larvicide	*Strongyles* *Ancylostomatidae*
Arthrobotrys spp.	Larvicide	
Monacrosporium thaumasium	Larvicide	
Verticillium spp.	Ovicide	*Ascarids*
Pochonia chlamydosporia	Ovicide	*Trematodes* *Ascarids* *Trichurids*
Purpureocillium lilacinum	Ovicide	
Trichoderma spp.	Ovicide	
Mucor circinelloides	Helminthicide / Entomopathogen	*Trematodes* *Ascarids* *Trichurids* *Ixodidae*
Beauveria bassiana	Entomopathogen	*Ticks*
Metarhizium anisopliae	Entomopathogen	*Ticks*

b) Secondary metabolites

The hypothesis of the penetration of fungal hyphae inside eggs or larvae could be helped by the participation of certain substances led to consider the possibility of secondary metabolites secreted during the interaction between the two organisms could exert a notable effect on the pathogens, which could provide some explanations to the appearance of eggs or larvae of parasites with signs of damage but without evidence of attached hyphae or mycelium development. Lýsek *et al*. (1982) found that eggs of *A. suum* exposed to *Verticillium* *chlamydosporium* presented signs of damage though hyphae adhered to the eggshells were not observed. This was called Type-2 antagonistic effect (Carvalho *et al*., 2010; Silva *et al*., 2011).

It should be underlined the effect of metabolites secreted by some fungal specimens on the eggs of helminths can result in an *ovistatic* or an *ovicidal effect*. **Ovistasis** is the inhibition or delay in the development of the internal embryo, frequently reversible, and neither the morphology nor the integrity of the embryo is affected (Ko£odziejczyk *et al*., 2019). The **ovicidal activity** implies permanent damage of the eggs/cysts to non-viable forms, comprising mainly rupture of eggshell, vacuolization or destruction of the embryo (Cruz *et al*., 2012) (Figs. 24 & 25).

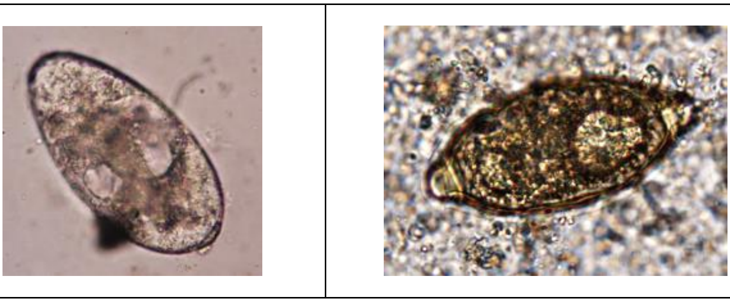


Fig. 24.- Vacuoles in *Calicophoron daubneyi* egg (left) and *Trichuris* spp. attributed to *M. circinelloides* metabolites due to the absence of hyphae (right).

Fig. 25.- Exposure of eggs of *T. canis* to secondary metabolites of *M. circinelloides* causes ovistasis (left) and ovicidal effects (center and right).

1.8.3. Administration of fungi to animal species

On considering that some of the known species of SSF are able to survive to the passage through the digestive tract and develop inside the feces, especially when donors are infected, livestock have been provided spores by oral route mainly (Table 6). Most of trials and experiments have been conducted on chlamydospores of *D. flagrans* (Mendoza de Gives *et al*., 2018; Ojeda-Robertos *et al*., 2009; Braga *et al*., 2009a; Ferreira *et al*., 2011; Araujo *et al*., 2012).

Fig. 26.- Petri plates with *D. flagrans*.

Initial studies comprised culturing of *D. flagrans* in solid media, principally Petri plates with agar enriched by adding different substrates, primarily cereals, or microbiologic media. After scraping the surface medium and rinsing with water (Fig. 26), chlamydospores were formulated in **liquid solutions** containing specific concentrations and given to the animals (Larsen *et al*., 1998). Sometimes, chlamydospores were cultured on cereals, washed and filtered prior to feeding different animal species (Santurio *et al*., 2009).

Another option consisted of distributing the spores in **small sachets of granulated content** (Chandrawathani *et al*., 2004). Voinot *et al*. (2021a) showed the efficacy of giving first-season pasturing ewes, three days a week, **milled cereal soaked** with a liquid medium containing chlamydospores of a mixture of *M. circinelloides* and *D. flagrans* (Fig. 27).

Fig. 27.- Milled cereal soaked in submerged medium facilitates the administration of spores to animal species.

Table 6.- Different formulations for the administration of fungal spores to domestic animal species have been assayed.

Fungal species	Formulation	Animal species	Reference
Duddingtonia flagrans	Water solution	Sheep	Larsen *et al*. (1998)
	Cereal sachets		Chandrawathani *et al*. (2004)
			Peña *et al*. (2002)
	Milled soaked cereal		Voinot *et al*. (2021a)
	Top-dressed pellets		Sanyal & Mukhopadhyaya (2003)
	Top-dressed cereals	Horses	Buzatti *et al*. (2015)
Arthrobotrys robusta	Handmade pellets	Cattle	Araújo *et al*. (2000)
Monacrosporium thaumasium			Alves *et al*. (2003)
Duddingtonia flagrans		Sheep	Rocha *et al*. (2007)
			Aguilar-Marcelino *et al*. (2016)
Pochonia chlamydosporia		Horses	Braga *et al*. (2010)
A. cladodes + P. chlamydosporia		Cattle	Vieira *et al*. (2020)
M. circinelloides + *D. flagrans*	Industrial pellets	Horses Cattle	Hernández *et al*. (2016) Voinot *et al*. (2020)
M. circinelloides + *D. flagrans*	Top-dressed pellets	Dogs	Hernández *et al*. (2018c)

Sanyal & Mukhopadhyaya (2003) tested concentrate feed that had been **top-dressed with desiccated chlamydospores** of *D. flagrans* on sheep infected by *Haemonchus contortus*, and the development of larvae in their feces was significantly reduced; the effect remained while the spores were being fed, but not for more than 4 days following discontinuous intake.

Based on the successful results in collecting high amounts of spores by supplementing the medium with excretory/secretory of several antigens (Arias *et al*., 2013a), and considering that liquid formulations could improve the delivery of parasiticidal fungal spores, a **submerged medium** (COPFr) containing water, mineral salts and a tegumental protein of *F. hepatica* was successfully developed (Arias *et al*., 2013b). This medium was fully appropriate for the mixed growth of two fungi with complementary activity on parasites, *M. circinelloides* (ovicidal) and *D. flagrans* (larvicidal) (Fig. 28), and by **top-dressing of nutritional pellets** with the medium prior to feeding equids (Arias *et al*., 2013a).

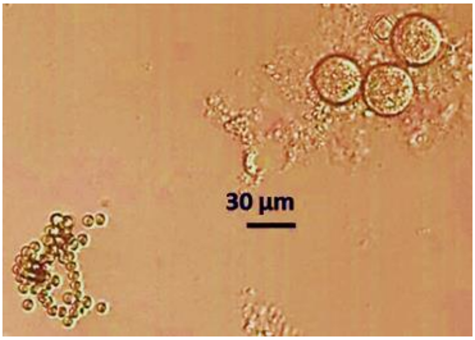


Fig. 28.- Chlamydospores of *M. circinelloides* (left) and *D. flagrans* (right) in the submerged medium COPFr.

For the purpose to improve the administration of chlamydospores of *D. flagrans*, **handmade pellets** were elaborated with chlamydospores or mycelium and given to ruminants infected by trichostrongyles, providing successful results (Alves *et al*., 2003; Rocha *et al*., 2007; Aguilar *et al*., 2009). Later on, by using the COPFr submerged medium, the industrial elaboration of **pelleted feed** enriched with chlamydospores of *M. circinelloides* and *D. flagrans* was possible for controlling strongyles affecting horses (Fig. 29) (Hernández *et al*., 2016, 2018a), trematodes and trichostrongylids in heifers (Voinot *et al*., 2020), and against soil-transmitted helminths in dogs (Hernández *et al*., 2018c; Viña *et al*., 2020). It is noteworthy that no signs of damage were found in any of the mentioned trials, confirmed later by an anatomopathological study (Voinot *et al*., 2021b).

Fig. 29.- Industrial fabrication of nutritional pellets enriched with *M. circinelloides* and *D. flagrans* improves the sustainable control of strongyles among grazing herbivores.

The evidence that chlamydospores of *D. flagrans* can survive freeze-drying and retain their biological activity made a significant contribution to the possibilities of developing strategies for biological control of helminths in animals, because in this way chlamydospores can be produced and stored for long periods of time (Santurio *et al*., 2009; LinJun *et al*., 2017).

Three formulations consisting of **powdered** chlamydospores of *D. flagrans* are commercially available for livestock (Healey *et al*., 2018; Braga *et al*., 2020; Rodrigues *et al*., 2021). Bioworma® and Livamol with Bioworma® are registered products of International Animal Health Products (Australia); the first consists of free-flowing meal containing chlamydospores of *D. flagrans* strain IAH 1297 ($\geq 10^5$ / g product), and the second is a palatable feed supplement aimed for mixing into feed and holding $\geq 3\text{x}10^4$ chlamydospores / g product.

Bioverm® is a fungal formulation containing *D. flagrans* strain AC001, manufactured by GhenVet (Brazil) as rice bran containing 10^5 chlamydospores / g product.

1.9. USE OF SSF IN ZOOLOGICAL GARDENS

The problem of controlling parasites affecting pasturing herbivores in zoological parks is the same to that occurring in grazing livestock, then useful strategies could be applied to prevent infection by certain helminths. Despite SSF have been utilized among domestic animal species, the number of applications of SSF for the control of parasites in zoos is scarce (Table 7).

Between 2010 and 2011, three studies were conducted at Disney's Animal Kingdom® (Bay Lake, Florida, USA) for checking the effect of giving chlamydospores of *D. flagrans* to giraffe, antelope and gerenuk (*Litocranius walleri*) (Terry, 2013). The first assay involved the administration of a suspension of $5x10^5$ chlamydospores of *D. flagrans* / kg BW for four consecutive days; in the second and third probes a powdered mixture with $3x10^4$ chlamydospores of *D. flagrans* per kg BW was added to the feed and administered through four consecutive days and eight weeks. Results indicated a notable reduction of L3 strongyles (57.6 to 96.5%) in the feces during the period of feeding.

In a posterior study conducted in the same zoo, Young (2018) assessed the effect of providing daily $3x10^4$ chlamydospores of *D. flagrans* / kg BW with standard feed to exotic ruminant ungulates (reticulated giraffe, *Giraffa camelopardalis reticulata*; scimitar-horned oryx, *Oryx dammah*; roan antelope, *Hippotragus equinus*). As results, the fecal egg counts of trichostrongyles did not decrease significantly, but a constant downward trend was recorded. Regarding the development and survival of larvae in coprocultures, a significant reduction was noted (<2% by 30% in the untreated-controls).

Assuming the fact that herbivores at Marcelle Natureza Zoological Park (Outeiro de Rei, Lugo, Spain) mainly passed eggs of strongyles (trichostrongyles) in their feces, while carnivores shed eggs of ascarids and trichurids, in 2011 it was considered useful to conduct a coprological survey to try to check for the possible presence of SSF in the different paddocks (Hernández *et al.*, 2017). Besides this, the choice of fecal material was done on considering that fungi could survive after passing through the digestive tract, and that damage had been not found. This strategy conducted to the isolation of several species of SSF identified as *Mucor circinelloides*, *Duddingtonia flagrans*, *Trichoderma atrobrunneum*, *Clonostachys rosea*, *Purpureocillium lilacinum* by the CECT (Spanish Type Culture Collection, Valencia), besides *Verticillium* spp., *Fusarium* spp., *Lecanicillium* spp. and *Penicillium* spp.

Table 7.- Administration of fungal spores to animals captive in zoos.

Fungal species	Formulation	Animal species	Reference
Duddingtonia flagrans	Top-dressed pellets	Giraffe Antelope Generuk	Terry (2013)
		European donkeys African asses Zebra	Arias *et al.* (2013)
	Powder	Reticulated giraffe Scimitar-horned oryx Roan antelope	Young (2018)
M. circinelloides + *D. flagrans*	Top-dressed pellets	Antelope	Miguélez *et al.* (2014)
		Mouflon	Cazapal-Monteiro *et al.* (2014)

Since 2011, tests have been carried out in this zoological park comprising the administration of spores of SSF. In 2013, *D. flagrans* chlamydospores were added every two days to nutritional pellets prior to be ingested by zebras, European and African assess (Arias *et al.*, 2013a) (Fig. 30). It was confirmed that anthelmintics are a temporary solution against strongyles; addition of chlamydospores of *D. flagrans* to the feedstuff was shown highly efficient to reduce the strongyles infective stages which might affect captive animals.

After the successful deworming of mouflons passing > 500 EPG trichostrongyles, Cazapal-Monteiro *et al.* (2014) provided them nutritional pellets soaked with a blend of *M. circinelloides* + *D. flagrans* (obtained simultaneously in the COPFr submerged medium), resulting in counts decreased and maintained by 50-150 EPG across a

1-yr period, then additional anthelmintic treatment was considered unnecessary.

Fig. 30.- Captive equids maintained in the Marcelle Natureza Zoo received *D. flagrans* chlamydospores.

The observation of counts of strongyles higher than 1500 EPG in the feces of antelopes led to their deworming together with the administration of pellets top-dressed with a solution of a blend of *M. circinelloides* and *D. flagrans* (Miguélez *et al*., 2014) (Fig. 31). Despite the counts of eggs of strongyles decreased for several months, high values were recorded again, so it was also decided to plow the field again. During an interval of 11 months, EPG numbers between 50 and 200 were obtained.

Fig. 31.- Antelopes in the Marcelle Natureza Zoo.

For the purpose to analyze the possibilities of limiting the viability of eggs of the roundworm *Baylisascaris procyonis*, Cazapal-Monteiro *et al*. (2015) tested the usefulness of *Mucor circinelloides*, *Verticillium* sp. and *Purpureocillium lilacinum* on feces of raccoons, and reported viability reduced significantly by 53–69% with *Mucor*, 52–67% with *Verticillium* and 45–62% with *Purpureocillium* (Fig. 32).

Fig. 32.- Raccoon in the Marcelle Natureza Zoo.

By means of an *in vitro* assay, Hernández-Malagón *et al*. (2018c) demonstrated that the SSF *M. circinelloides* and *Verticillium* sp. had an elevated antagonism against the eggs of *T. leonina* passed in the feces of captive lynxes (*Lynx lynx*), and the same occurred with *M. circinelloides* and *T. atrobrunneum* on eggs of *Trichuris* sp. shed by dromedaries (*Camelus dromedarius*). Development of eggs of *T. leonina* and *Trichuris* sp. in the feces was delayed in the presence of all fungi, and one third remained at the stage of zygote.

2.- UNITY AND THEMATIC AND METHODOLOGICAL COHERENCE

The idea to implement a study focused on the *integrated control of digestive helminths in captive herbivores* is supported by the knowledge of the difficulties for a successful control of parasites among those animal species captive in zoos, under a grazing regime in most of cases. For more than a decade, the close collaboration between the **Marcelle Natureza Zoo** and the **COPAR Research Group (GI-2120; USC)** has opened an interesting, necessary and fruitful channel to address a problem that affects essentially all zoos in the world where there has been an awareness to ensure that some species can enjoy an environment as close as possible to the original one.

Fig. 34. Graphical abstract summarizing the design of the current study.

Based on the results collected by the aforementioned research group on parasites affecting pasturing livestock and even pets, the design of the present study included the development of three trials conducted on herbivores kept in Marcelle, ruminants and equids, which were administered a mixture of soil saprophytic fungi (SSF) isolated from feces and soil samples from the zoo itself, *M. circinelloides* with ovicidal activity and *D. flagrans* with larvicidal action (Figs. 34, 35) (Hernández *et al.*, 2017). In this line, the aim in the **first assay** was to determine the most appropriate way to ensure the administration of a blend of two SSF to sylvatic bovids (wapiti, *Cervus canadensis*) captive in a plot where they can graze all day. These ruminants were infected by trichostrongylids, and for that reason fenbendazole was periodically given. However, the elevated counts of eggs per gram of feces (EPG) reached several months after treatment led to the veterinarian direction of the zoo to consider other strategies involving the reduction of the risk of parasitization to prevent further infections.

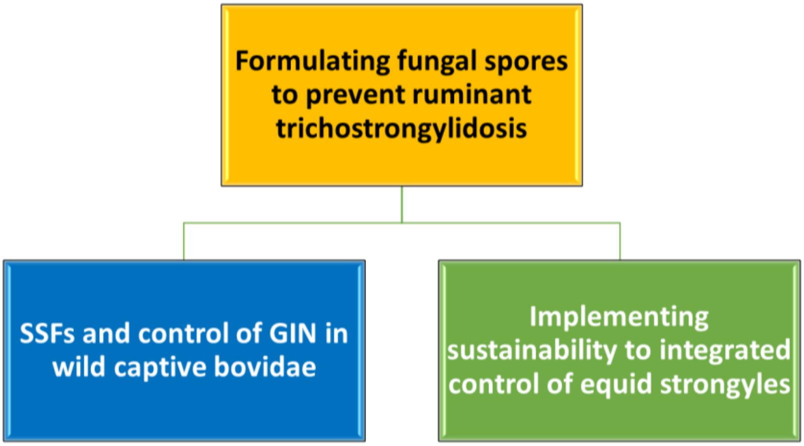


Fig. 35. Scheme of the trials performed in the present study.

Two formulations were prepared for the oral administration of the chlamydospores during a period of 10 months. First, they were sprayed-on nutritional pellets just before given to the wapitis, every two days. The other trial consisted of the industrial manufacturing of pellets enriched with the spores. Due to the impossibility to observe a control group, counts of EPG obtained on wapitis receiving fenbendazole only during 10 months (without chlamydospores) were taken as controls.

The effect of these strategies was evaluated on fecal samples taken directly from the ground, and analyzed by means of the coprological tests of flotation (McMaster) and sedimentation. Results were expressed as the counts of eggs of trichostrongylids per gram of feces (EPG). For gaining knowledge on the possibility of side effects could appear, special attention was paid to the digestive tract, respiratory apparatus and the skin.

Once demonstrated that the best formulation was the nutritional pellets enriched with chlamydospores of both SSF during the industrial manufacturing (10^4 –10^5 spores of each / kg meal), a **second assay** comprised the administration of that pellets, every two days for a period of 3.5 years, to wild bovidae belonging to the subfamilies Antilopinae (*Antilope cervicapra*, *Gazelle Cuvieri*), Caprinae (*Capra aegagrus hircus*, *Ovis orientalis musimon*), Bovinae (*Bison bison*, *Kobus kob*) and Reduncinae (*Tragelaphus spekii*). These wild ruminants were maintained in different parcels of a zoo, composed of red clover (*Trifolium pratense*), perennial ryegrass (*Lolium perenne*) and orchard grass (*Dactylis glomerata*); water is available ad libitum.

A preliminary fecal analysis revealed infection by trichostrongylids, which were classified by stool cultures as belonging to the genera *Trichostrongylus*, *Nematodirus*, *Chabertia* and *Haemonchus*. Accordingly, deworming was administered to all ruminants at the onset of the trial. Anthelmintic treatment was also administered when a threshold of 300 EPG was surpassed. The effect of the controlling measures was assessed by fecal analyses to estimate the variations in the values of eggs of trichostrongyles per gram of feces (EPG).

Zoological parks contain a variable number of herbivores, mainly ruminants, althought the presence of feral equids as African wild assess, zebras or Faravella is not infrequent. With the aim to gain information about the effect of chlamydospores of *M. circinelloides* and *D. flagrans* on the infection by strongyles, in the **third assay** were utilized individuals of *Equus quagga*, *E. asinus*, and *E. africanus asinus*. While plains zebra and European donkeys feed on a grassland, African wild asses are maintained in a sandy area, where herbage is present only in the corners. On the basis of eggs of strongyles were detected at the beginning of the trial, all equids were provided ivermectin + praziquantel. The design of the study involved the administration of nutritional pellets added a blend of 10^4 - 10^5 chlamydospores of each fungi / kg meal during the industrial manufacturing, every two days for three years.

Fecal samples were collected periodically, directly from the ground in each plot, early in the morning. Feces were analyzed by means of the sedimentation and flotation (McMaster) tests. Data obtained was indicated as counts of eggs of strongyles per gram of feces (EPG). Calculation of the reductions in the fecal egg counts (FECR) and in the positive horses (PHR) was done fifteen days afer treatment. Other parameters as the egg reappearance period (ERP) and the time elapsed from the previous deworming (TPD) were also calculated.

3.- HYPOTHESIS AND OBJECTIVES

As happens with grazing livestock, captive wild herbivores in a zoo with access to grass are at risk of becoming infected with certain parasites, mainly gastrointestinal nematodes that develop the external phase of their life cycle in the grass. Despite successful dewormers addressed on the control of parasites in domestic herbivores are easily available for wild captive species, there is scarce information on the proper dosage, or side-effects. Besides this, a strategy based on antiparasitic treatment of grazing animals only has a temporary effect, and they become infected again soonly.

Under this situation, an interesting approach could rely on integrating the action on the animals and on the environment, for the purpose to contribute to decrease the levels of infective stages in the ground leads to minimize the risk of further infection and avoiding thus multiple or frequent deworming. In this way, the anthiparasitic shelf life is maintained, limiting the appearance of parasitic resistant strains.

The current study was supported in the hypothesis that a blend of soil saprophytic fungi (SSF), *Mucor circinelloides* and *Duddingtonia flagrans*, could interfere the survival and development of helminths passed in feces of herbivores, to the respective infective stages. Therefore, the study divided into three assays was designed, with the **specific goals**:

1. To assess the most appropriate formulation for the administration of SSF to herbivores captive in a zoological garden. (Paper 1).

2.- To analyze the usefulness of a blend of soil saprophytic fungi (SSF), *Mucor circinelloides* and *Duddingtonia flagrans*, to reduce the risk of infection by gastrointestinal nematodes among wild herbivores captive in a zoological park. (Paper 2).

3.- To determine the possibilites of improving integrated control of helminths infecting wild herbivores captive in a zoo. (Paper 3).

4.- To design a plan for the integrated control of parasites affecting wild herbivores kept in captivity as a way to contribute to the sustainable and integrated management of digestive parasitosis in centers and institutions dedicated to wildlife conservation (Papers 1, 2 & 3).

4.- METHODOLOGICAL TOOLS

4.1. PRODUCTION OF CHLAMYDOSPORES OF SSF.

4.1.1. Soil Saprophytic Fungi (SSF)

In the present research, two SSF were utilized. The strain CECT 20824 of *M. circinelloides*, with ovicidal activity, and CECT 20823 of *D. flagrans*, larvicidal, were isolated from feces of wild animals captive at the Marcelle Natureza Zoological Park (Outeiro de Rei, Lugo, NW Spain) (43º 4′ 14.71″ N, 7º 37′ 53.50″ W) (Hernández *et al*., 2017). Identification was performed by culturing them in specific media, and confirmed by the Spanish Type Culture Collection (Valencia, Spain).

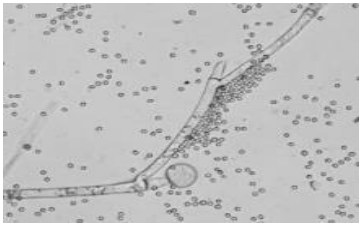
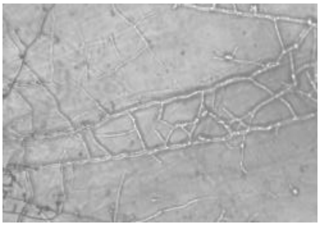
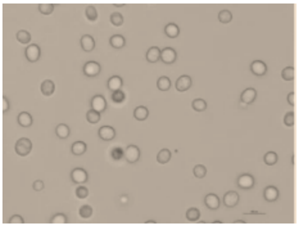
M. circinelloides	***D. flagrans***
	
	

Fig. 37. Identification of SSF is frequently based on the observation of mycelium characteristics (up) and chlamydospores (down).

4.1.2. Growing medium

According to Arias *et al*. (2013b), the submerged medium COPFr was prepared by mixing (per L H_2O) 7.1 g NaCl, 1.6 g $Na_2HPO_4{\cdot}12H_2O$, 0.423 mg FhrAPS recombinant protein of *Fasciola hepatica* tegument and 30.6 g *Triticum aestivum* (flour wheat) (patent PCT/ES2014/070110).

Once mixed vigorously, the medium was filtered for removing debrises, sterilized by autoclave (121ºC, 20 min). When temperature dropped to ≈50ºC, a total volume of 150 mL was transferred to 0.5 L recycled plastic bottles donored by a local catering establishment.

Bottles were addded an insert of each fungi, taken from corn meal agar Petri plates previously cultured, and maintained at RT until concentrations close to 10^6 spores / mL were reached (for two months at least). Counting was performed by taking 6 - 8 20 µL-aliquots of each bottle and examined under optical microscope at 10X.

4.1.3. Fungal formulations

For the purpose to administrate chlamydospores of the two SSF to captive herbivores, two oral formulations were prepared.

a) Sprayed-on nutritional pellets

Just prior to their administration, nutritional commercial pellets were sprayed-on a dosage of $2x10^6$ chlamydospores of *M. circinelloides* and $2x10^6$ chlamydospores of *D. flagrans*, at a ratio of 10 mL / kg meal (Arias et al., 2013a).

b) Formulated pellets

Based on previous investigations demonstrating that chlamydospores of both SSF survived at 75ºC for 5 min, $2x10^6$ spores of each fungus / kg meal were added during the mixing phase of the commercial fabrication of nutritional pellets (Hernández *et al*., 2016). This procedure was performed in *Piensos Flores* (Outeiro de Rei, Lugo, Spain).

4.2. ANALYSIS OF FECAL SAMPLES.

4.2.1. Collection of feces

The three trials composing the present research were performed at the Marcelle Natureza Zoological Park, and involved different species of herbivores, ruminants and equids kept in different plots where they can graze all the day (Table 8).

Table 8.- Species of captive animals at Marcelle Natureza Zoological Park involved in the present research.

Trial	Captive animal species	*N*	Plot surface (m^2)
1	Wapitis (*Cervus canadensis*)	5	10000
2	Antelope (*Antilope cervicapra*)	2	1925
	Gazelle (*Gazelle cuvieri*)	4	920
	Goat (*Capra aegagrus hircus*)	5	300
	Mouflon (*Ovis orientalis musimon*)	15	2250
	Bison (*Bison bison*)	5	10000
	Marshbuck (*Tragelaphus spekii gratus*)	4	9450
	Kob (*Kobus kob*)	5	2200
3	Plain zebra (*Equus quagga*)	2	4038
	European donkey (*E. asinus*)	5	2015
	African wild ass (*E. africanus asinus*)	4	2015

Despite it could be desirable for obtaining a more precise diagnostic and avoid the possibility of misdiagnosing parasites affecting plant species, the individual collection of feces from animals captive at a zoo is not possible, unless having personnel watching for the differen species. For that reason, sampling consisted of taking the superior part of fresh feces from the soil (Maesano *et al*., 2014). In consequence, this procedure could lead to most of samples belonged to a few individuals only. With the aim to solve this situation, it was considered more appropriate and accurate to collect two samples for every individual in each parcel.

4.2.2. Diagnosis of parasites in fecal samples

At the beginning of the trials, feces were analyzed by the coprological tests of flotation (McMaster), sedimentation and larval migration. Nevertheless, trematodes or lungworms were never observed, as expected (Mirzapour *et al*., 2018; Dashe & Berhanu, 2020).

a) Flotation (McMaster) test

Each fecal sample collected was examined by a quantitative flotation method, involving the homogeneisation of three grams feces in 42 mL water, then filtering all through a 150 µm mesh prior to fill two 12 mL tubes. After centrifuging at 1500 rpm / 10 min, the supernatant was discarded and sediment added 10 mL saturated sodium chloride solution (gravity = 1.2). A McMaster chamber was filled with this solution and observed under a light microscope (Leica DM2500) at 10X (MAFF, 1986; Voinot *et al*., 2020). Results were expressed as counts of eggs of parasites per gram of feces (EPG). A sensitivity value of 30 EPG was recorded (Hernández *et al*., 2018a).

b) Sedimentation test

Five grams of each fecal sample were thoroughly mixed with water and filtered through a 150 µm wire, transferred to a 1 L conic vessel and filled with water until 1 L. After 15 min, the supernatant was discarded, the sediment moved to a 0.5 L conic vessel and filled to 0.5 L. Fifteen minutes later, the same procedure was done but with a 100 mL vessel. Finally, the volume was dropped to 50 mL, and a McMaster chamber filled and examined under a light microscope at 10X. Results were indicated as numbers of eggs or oocysts per gram of feces (EPG, OPG), and sensitivity was estimated as 30 EPG / OPG (Hernández *et al*., 2018a).

c) Migration (Baerman) test

Ten grams of each fecal sample were placed on a filter paper, taken into a funnel with the lower end clamped, and water was added to cover the feces, After 10 - 15 h, the clamp was opened, the first volumes collected in 12 mL tubes, and observed in a Favatti chamber under an optical microscope at 10X. Identification of nematode genera was performed according to van Wyk and Mayhew (2013), and data expressed as counts of larvae per gram of feces (LPG).

4.2.3. Identification of nematode genera

Pools of 10 feces were prepared at the starting of each trial, then incubated at 25ºC during 18 days. Larvae were recovered by means of the migration test, and finally identified (van Wyk & Mayhew, 2013).

4.2.4. Assessment of anthelmintic efficacy

Fecal samples were taken at the beginning of each trial, and by fourteen days after deworming, according to the guidelines enunciated by the World Association for the Advancement of Veterinary Parasitology (WAAVP). With these data, the fecal egg count reduction (**FECR**) was obtainted as follows:

$$\text{FECR}\ (\%) = [1 - (\text{EPGday}_{14} / \text{EPGday}_{0})] \times 100$$

Efficacy was considered when FECR > 95% (Geary *et al*., 2012).

By taking into account the usefulness of the information obtained in previous investigations (Voinot *et al*., 2020), the reduction of the individuals positive to coprological tests (**IPCR**) was also estimated:

$$\text{IPCR}\ (\%) = [1 - (\text{Nr positive day}_{14} / \text{Nr positive day}_{0})] \times 100$$

To gain more information about the different genera/species of the gastrointestinal nematodes affecting the herbivores, fecal samples were cultured for 10–15 days at 25 – 27ºC to allow the development of eggs to the third-stage infective larvae. Then, these L3 were collected by means of the Baermann procedure and identified according to morphological keys (van Wyk *et al*., 2004).

The egg reappearance period (**ERP**) was calculated as the week afer treatment when the FECR decreased below a cut-of value of 90% (Larsen *et al*., 2011).

The time lapsed from the previous deworming (**TPD**) was established as the number of months elapsed from the previous administration of anthelmintics.

4.3. STATISTICAL ANALYSES.

Data collected along the study were checked for normality, by using the Levene test and the Kolmogorov – Smirnov test. In case of data were not normally distributed, non-parametric probes as the Friedman test were applied.

All analyses were performed using SPSS for Windows (v. 22.0; SPSS Inc., Chicago, IL, USA). Differences were established when $P < 0.05$.

5.- PUBLICATIONS

5.1. PUBLICATION NUMBER 1

https://www.sciencedirect.com/science/article/pii/S1049964420306939

Palomero, A.M., Cazapal-Monteiro, C.F., Viña, C., Hernández, J.Á., Voinot, M., Vilá, M., Silva, M.I., Paz-Silva, A., Sánchez-Andrade, R., Arias, M.S. Formulating fungal spores to prevent infection by trichostrongylids in a zoological park: Practical approaches to a persisting problem. *Biological Control, 152*, 104466. https://doi.org/10.1016/j.biocontrol.2020.104466.

Publisher: ACADEMIC PRESS INC ELSEVIER SCIENCE.
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Journal Impact Factor (2021): 3.857
5 Year Impact Factor: 4.141
Rank (2021): 22/165; Q1 (*Biotechnology & Applied Microbiology*).
Contribution of the PhD student

AMPS performed the experiments, data analysis, preparation of figures and writing of the essay.

Journal/publisher permission: Not needed:

Formulating fungal spores to prevent infection by trichostrongylids in a zoological park: Practical approaches to a persisting problem

Author: Antonio M. Palomero,Cristiana F. Cazapal-Monteiro,Cándido Viña,José Á. Hernández,Mathilde Voinot,María Vilá,María I. Silva,Adolfo Paz-Silva,Rita Sánchez-Andrade,María Sol Arias
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Antonio M. Palomero Salinero contributions to the paper have been on:

- Conceptualization
- Methodology
- Investigation
- Writing - Original Draft

5.2. PUBLICATION NUMBER 2

https://www.cambridge.org/core/services/aop-cambridge-core/content/view/907850303B4C2DE061299C7A90A29599/S0031182020000414a.pdf/soil-fungi-enable-the-control-of-gastrointestinal-nematodes-in-wild-bovidae-captive-in-a-zoological-park-a-4-year-trial.pdf

Palomero, A. M., Cazapal-Monteiro, C. F., Valderrábano, E., Paz-Silva, A., Sánchez-Andrade, R., & Arias, M. S. (2020). Soil fungi enable the control of gastrointestinal nematodes in wild bovidae captive in a zoological park: a 4-year trial. *Parasitology*, *147*, 791–798. https://doi.org/10.1017/S0031182020000414.

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ISSN: 0031-1820.
Journal quality indexes
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Contribution of the PhD student

AMPS performed the experiments, data analysis, preparation of figures and writing of the essay.

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Soil fungi enable the control of gastrointestinal nematodes in wild bovidae captive in a zoological park: a 4-year trial

Author: A. M. Palomero, C. F. Cazapal-Monteiro, E. Valderrábano, A. Paz-Silva, R. Sánchez-Andrade, M. S. Arias
Publication: Parasitology
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Antonio M. Palomero Salinero contributions to the paper have been:

- Conceptualization
- Methodology
- Investigation
- Writing - Original Draft

5.3. PUBLICATION NUMBER 3

https://www.hindawi.com/journals/bmri/2018/4267683/

Palomero, A.M., Hernández, J.A., Cazapal-Monteiro, C.F., Balán, F. A., Silva, M.I., Paz-Silva, A., Sánchez-Andrade, R., & Vázquez, M. (2018). Implementation of Biological Control to the Integrated Control of Strongyle Infection among Wild Captive Equids in a Zoological Park. *BioMed research international*, *2018*, 4267683. https://doi.org/10.1155/2018/4267683

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ISSN: 2314-6133.

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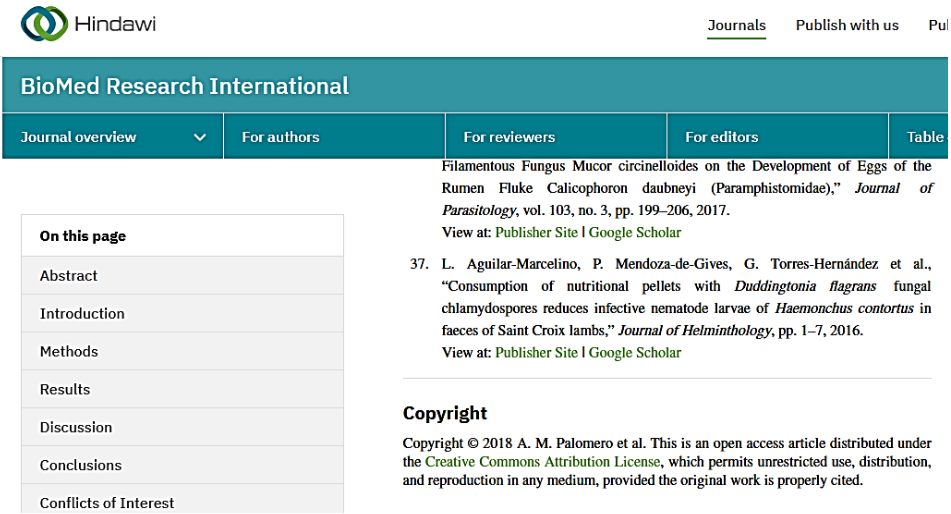
5 Year Impact Factor: 3.118

Rank (2021): 90/158; Q3.

Contribution of the PhD student

AMPS performed the experiments, data analysis, preparation of figures and writing of the essay.

Journal/publisherpermission: not needed.



Filamentous Fungus Mucor circinelloides on the Development of Eggs of the Rumen Fluke Calicophoron daubneyi (Paramphistomidae)," *Journal of Parasitology*, vol. 103, no. 3, pp. 199–206, 2017.
View at: Publisher Site | Google Scholar

37. L. Aguilar-Marcelino, P. Mendoza-de-Gives, G. Torres-Hernández et al., "Consumption of nutritional pellets with *Duddingtonia flagrans* fungal chlamydospores reduces infective nematode larvae of *Haemonchus contortus* in faeces of Saint Croix lambs," *Journal of Helminthology*, pp. 1–7, 2016.
View at: Publisher Site | Google Scholar

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Antonio M. Palomero Salinero contributed to the paper on:

- Conceptualization
- Methodology
- Investigation
- Validation

6.- GENERAL DISCUSSION

The changes introduced in recent decades in zoos to improve the conditions in which wild species are kept in captivity have resulted in measures aiming to reproduce, as far as possible, their original natural environment. This has been particularly noticeable in the preparation of landscaped areas where herbivores can graze throughout the day. To ensure the nutrition of livestock, different pasturing regimes are frequently implemented, and several types mainly classified as continuous or rotational, may be observed. Captive wild herbivores in zoos also have difficulties in being able to rotate from one plot to another, mainly centered on the high number of *resting paddocks* that would be necessary to achieve this particular purpose, as well as the need to provide and adequate number of feeders, waterers, and shelters. Another disadvantage is the movement of the animals around the zoo, since the possibility of establishing visual contact with some of their predators, could potentially lead to stressful situations with very unfavorable consequences. Therefore, the most realistic approach comprises herbivores are maintained always in the same plot, i.e. under ***continuous pasturing***.

Several investigations carried out in domestic pasturing species pointed out that those conditions very favorable for grass growing (moderate temperatures, humidity, rainfall) also promote some parasites, helminths in special, develop in the ground from stages shed in feces of parasitized animals to infective phases (O'Connor *et al*., 2006; Nielsen *et al*., 2007). This entails a serious risk of infection for herbivores while feeding on grass, which worsens if continuous grazing is observed. Among parasites affecting herbivores, gastrointestinal nematodes (GIN) belonging to Trichostrongylidae frequently infect ruminants, and Strongylidae often infect equids; but except for *Trichostrongylus axei*, these groups of herbivores do not share other GIN species.

Transmission of Trichostrongylidae occurs in a simple way through the ingestion of third-stage larvae, without the participation of intermediate hosts. These L3 infective stages originate from eggs passed in the feces of parasitized individuals, which once in the soil give rise to a first-stage larva (L1) develops inside the egg and subsequently hatches, feeding on organic matter in the feces and moulting to a second-stage larva which feeds also and finally reaches the third-stage larva (Smith & Sherman, 2009). Consequently, limiting the presence and/or survival of the infective stages in the soil looks very helpful to prevent the infection by these nematodes. It is important to underline that most of anthelmintics focuses on acting upon parasites inside the final hosts; a certain degree of action against eggs of horse strongyles has been reported with bencimidazoles only (Daniels & Proudman, 2016), and no information regarding others (macrocyclic lactones, imidazothiazoles, salicilanylides…) is available at the moment.

Control of GIN comprises periodical deworming with various anthelmintics, but despite the fact that successful compounds are commercially available, the presence of infective stages in the ground facilitates that herbivores become infected soon, thus deworming is required again. This occurred with wapitis in Marcelle Natureza, and though fenbendazole was successfully given at different times, high levels of eggs of trichostrongyles were observed in feces as early as three to four months after treatment. These findings underline the need to prevent infection among animals, and the most appropriate and practical approach appears to reduce the level of soil contamination by parasitic stages. With this idea in mind, several agricultural labors such as plowing, harrowing and disking that cause disturbance of the soil, which have been shown helpful to drop various the levels of stages of wireworms, the larval phase of click beetles (*Elateridae*) which affect potato (Parker & Howard, 2001; Vernon & van Herk, 2013). The beneficial action would be performed by directly destroying the oocysts, eggs or larvae of parasites in the soil, and indirectly by leading them to the surface where they would be exposed to adverse conditions such as sunlight or desiccation, as well as to natural antagonists such as arthropods and predatory birds (Younie *et al*., 2004). Despite the helpfulness of these measures has been shown in antelopes (Miguélez *et al*., 2014), it can be executed under particular circumstances only, because it implies that animals must be brought to other plots while this procedure is being carried out (the first plot is seeded again, the grass grows…). This does not seem practical for zoological parks because it entails to have sufficient areas to maintain the animals while performing them, and consequently deworming becomes into the only approach. In a **first assay** of the current trial, one group of wapitis (*Cervus canadensis*) captive in a zoological park and infected by trichostrongylids, received anthelmintic treatment consisting of a single dose of fenbendazole, with a 100% efficacy as shown by the FECR. Four months later, numbers higher than 300 EPG were recorded, in agreement with preceding investigations among wild captive equids (Arias *et al*., 2013a). The lack of useful and practical measures to limit the number of infective stages is currently being addressed by increasing the frequency of deworming, but several studies report that frequent administration of parasiticides could be responsible for efficacies lower than expected, and might even promote the selection of parasite strains resistant to specific anthelmintics (De Graef *et al*., 2013; Shalaby, 2013).

In view of the situation, it was considered the usefulness of soil saprophytic fungi with parasiticidal activity, *M. circinelloides* and *D. flagrans*, which have been successfully used in previous trials (Paz-Silva *et al*., 2011; Arias *et al*., 2013; Hernández *et al*., 2016). The strategy consisted of testing two oral formulations, sprayed-on nutritional pellets with a blend of chlamydospores of both fungi, and pellets enriched with the blend during the mixing phase of their industrial fabrication. By the spraying prior to giving pellets to the wapitis, the levels of fecal eggs of trichostrongylids were significantly lessened. The effect found by feeding the ruminants on pellets manufactured with the chlamydospores was also positive, and the need for anthelmintic treatment during a period of ten months was discarded when using both oral formulations, based on the observation of mean counts of trichostrongylids eggs lower than 100 EPG. These results are in coincidence with previous assays conducted on wild captive equids receiving pellets sprayed with spores of *D. flagrans* for two years (Arias *et al*., 2013a), as well as giving commercial pellets enriched with a blend of chlamydospores of *M. circinelloides* and *D. flagrans* to domestic horses for 1-yr under rotational grazing (Hernández *et al*., 2018a). The nematode-trapping fungus *D. flagrans* has been efficiently provided to captive giraffes infected by strongyles during a 12-weeks trial (Terry, 2013). Later, Young (2018) assessed the effect of chlamydospores of *D. flagrans* administered daily together with standard feed to ruminants, though a significant influence on the development and survival of larvae in coprocultures was observed only, and the numbers of eggs of trichostrongyles in the feces remained practically unaltered. At this point, it is necessary to highlight the complexity of properly examining the effect obtained through the periodic administration of chlamydospores of SSF, which appears highly linked to the surface plot where the animals are kept: the presence of parasiticidal fungi in the feces will decrease the chances that helminths can reach the infective stages in this medium, but a variable action will probably be acquired on the previously developed infective stages, able to survive for months under favorable conditions and maintaining an important risk of infection (Corning, 2009). By measuring the number of cyathostomin L3 larvae at a distance of 0 to 20 cm from the fecal pats of horses that received chlamydospores of *D. flagrans*, Braga *et al*. (2009a) recorded a 78.5% reduction compared to the observed in the feces of control horses (which were not given chlamydospores), and 82.5% at a distance of 20 to 40 cm. No data are available on wild captive herbivores by now, but some investigations pointed out significant reductions on the viability of eggs of trematodes, ascarids and strongyles when exposed to *Pochonia chlamydosporia* (Dias *et al*., 2013; Thapa *et al*., 2018; Vieira *et al*., 2019) or *Verticillium* sp. (Cazapal-Monteiro *et al*., 2015).

Preparation of grassy plots offers herbivores the possibility to nourish, and to socialize and interact with the environment, but the development of gastrointestinal nematodes requires some action to limit the risk of infection (West & Dickie, 2007; Fagiolini *et al*., 2010). As previously mentioned, the existence of natural soil microorganisms acting as antagonists serves to maintain a natural equilibrium among all the actors involved, that is hosts (mammals), pathogens (parasites) and antagonistic agents (bacteria, viruses, fungi, carnivorous nematodes…) (Nalubamba *et al*., 2012; Cazapal-Monteiro *et al*., 2013; Lahat *et al*., 2021). When the balance is disturbed (immunosuppressed hosts, reduction on antagonists), pathogens can exacerbate. According to certain SSF have the ability to grow and propagate in submerged cultures (Arias *et al*., 2013b), their distribution and spreading could be greatly enhanced. In example, spraying of spores directly on the soil could represent an easy choice depending on the surface of the paddock, though captive animals might experience a notable stress (Miguélez *et al*., 2014). On considering the capability of some fungal species to survive the passage through the digestive tract without losing their activity (Araujo *et al*., 2012; Tavela Ade *et al*., 2013; Hernández *et al*., 2018c), other likelihood would consist of the oral administration of spores or mycelium in aqueous solutions to the animals (Paraud *et al*., 2005; da Silva *et al*., 2015). Consequently, fungal species evolving in the feces in close contact with the parasitic stages might develop a notable antagonistic activity. Despite the helpfulness of water solutions to administrate spores of SSF to different livestock species, this formulation looks highly complex when refereeing to wild captive animals. A possible solution could rely on the spores are given with the drinking water, but the possibility that each individual taking the indicated dosage does not look great.

By feeding successfully treated wapitis with pellets sprayed with 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 plot 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 found 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) which seemed to reduce notably the possibilities of third-stage larvae of trichostrongylids could develop and survive, decreasing thus the risk of infection among the captive herbivores in the following year. For reasons that are considered so obvious that no further explanation is required, it is impossible to have a control group remaining untreated. Besides, no significant differences regarding the climatic parameters were found, so the interpretation of the results obtained was made in relation to the values obtained during the first year of trial.

One remarkable finding in the first assay consisted in adverse effects never being observed on the wapitis while fungal spores were given through two formulations, as sprayed-on or formulated pellets, which points out the innocuousness of this strategy, in agreement with prior trials conducted in horses (Hernández *et al*., 2018a) and dogs (Hernández *et al*., 2018b), and more recently on dairy heifers under rotational pasturing regimes (Voinot *et al*., 2021b). Besides, 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 at 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 registered, in Australia (Healey *et al*., 2018) and in Brazil (Braga *et al*., 2020). In the present, study, very effective results have been collected by providing pellets industrially manufactured with the fungal spores to wapitis captive at a zoological park under a continuous grazing regime, which represents an easy method without additional task for animal keepers. However, since 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 feeding the animals.

There is a scarce information regarding the monitoring of infections by parasites in animals maintained in zoological parks, and it appears that control measures comprising deworming mainly are not ever based on prior detection and identification of pathogenic agents (Arias, 2013; Aviruppola *et al*., 2016). Accordingly, the success of the antiparasitic strategy can result ineffective (Panayotova-Pencheva, 2016). For the purpose of adding knowledge on the helpfulness of implementing preventive measures based on SSF to reduce the development and presence of certain parasitic stages in the soil, a **second trial** was developed involving wild bovidae belonging to the subfamilies Antilopinae (*Antilope cervicapra*, *Gazelle Cuvieri*), Caprinae (*Capra aegagrus hircus*, *Ovis orientalis musimon*), Bovinae (*Bison bison*, *Kobus kob*) and Reduncinae (*Tragelaphus spekii*). These ruminants were maintained in different plots of a zoo, composed of red clover (*Trifolium pratense*), perennial ryegrass (*Lolium perenne*) and orchard grass (*Dactylis glomerata*); water was available ad libitum. Because of the finding of eggs of trichostrongylids by means of the flotation (McMaster) coprological test, anthelmintic treatment consisting of the administration of fenbendazole, ivermectin or ivermectin + praziquantel was done (Abaigar *et al*., 1995; Ortiz *et al*., 2001). Despite the deworming was successful, a total of four treatments through a 9-month period was needed for the blackbucks, and three for gazelles, goats, mouflons, bison, marshbucks and kobs during the same interval. These results reflect that the non-observance of preventive measures for the application of a parasite control program may be the main reason for the need to frequently administer anthelmintic treatments, since the development of the infective stages in the soil ensures an almost endless risk of infection throughout the year, especially in areas with mild climates. It has been proven that the strategies relying only on deworming result in a decrease in parasiticide efficacy, which can lead to the development of anthelmintic resistance (Kerry *et al*., 2000; Goossens *et al*., 2005; Shalaby, 2013). Likewise, deworming of wild species entails some problems, based on little information available regarding proper dewormers, dosages, frequency or side-effects. In this situation, the need to lessen the risk of infection among grazing individuals appears critical (Terry, 2013).

In view of the usefulness of certain soil filamentous saprophytic fungi to significantly reduce the presence and viability of the infective phases of some helminths in the feces and/or the ground (Campos *et al*., 2009; Hiura *et al*., 2015; Vieira *et al*., 2019), in the **second trial** successfully dewormed wild captive bovids were provided, every 2 days during 3.5 years, chlamydospores of a blend of parasiticide filamentous fungi with ovicide (*M. circinelloides*) and larvicide activity (*D. flagrans*). The numbers of eggs of trichostrongyles decreased to less than 120 EPG in the feces of captive Caprinae (goats and mouflon), Bovinae (bison and marshbucks) and Reduncinae (kobs); furthermore, anthelmintic treatment was not considered throughout this period on the basis of the values of egg-output did not exceed 300 EPG, the cut-off point established at the beginning of the trial. These data point out the beneficial effect which can be obtained by the complementary action of the two SSF administered to the ruminants, *M. circinelloides*, capable of penetrating the eggs of helminths as trematodes and ascarids and to destroy them (Hernández *et al*., 2018b), and *D. flagrans*, able to elaborate traps to capture larvae which proceed from eggs of strongyles (Mendoza-de Gives *et al*., 2018).

Data from the analyses of feces of Antelopinae species require a more detailed interpretation, because even though they were given pellets enriched with chlamydospores of the two fungi with parasiticide activity, two anthelmintic treatments were needed for blackbucks during a period of 16 months, and gazelles were administered one application. Among the possible explanations, it seems highly probable that contamination of soil reached an elevated level, as suspected in a previous experience (Miguélez *et al*., 2014). This could explain also that animals in the current study did attain high numbers of strongyle egg-output two or three months after the administration of efficient deworming, and underlines the requirement of safe environments to ensure animal health and welfare (Maesano *et al*., 2014). In a previous investigation, it was demonstrated that treatment with fenbendazole reduced egg shedding in feces, but infective larvae remained in the grass throughout the year (Mikolon *et al*., 1994). In this line, it has been reported the failure of an anthelmintic treatment in a zoological park could be attributable to residual or permanent contamination of the paddocks by nematode larvae surviving winter (Goossens *et al*., 2006).

Prevention of infection by parasites in wild captive herbivores present similar difficulties to those occurring among domestic species under continuous grazing. Maintenance of livestock under rotational pasturing consists of maintaining herbivores in plots with vegetation to ensure they receive appropriate nourishing; when grass is sparse, animals are taken to other grassland (Flack, 2016). With the intention of ensuring that herbage can grow again, a resting period needs to be observed which characterizes by the absence of animals feeding on it (Kerry *et al*., 2000). Although this strategy has been helpfully advised for preventing infection by helminths in grazing animals, a resting period longer than three months should be needed to break their life cycle (Undersander *et al*., 2002), which makes it too difficult for being applied in zoological parks. The efforts to introduce changes in the zoos, by displaying plots with vegetation, is a significant and valuable contribution to offer wild animals an environment close to the original, with more likelihoods to take forage, interact and enjoy nature. Rotation of plots cannot be considered, as opposed to livestock farms, because of the troubles raised to avoid stress when animals are moved to a different plot. It has been mentioned that this strategy would require a high number of proper plots, which do not make possible to comply with a resting period. Other proposals to limit pasture contamination in zoological parks comprise restrictions to pasturing by later turn-out, by overnight stabling, or by grazing on sandy to rocky enclosures (Goossens *et al*., 2006). Nevertheless, these procedures appear to be restrained to zoological parks with an elevated surface, enough to build different environments where the animals can be maintained.

The successful of an integrated program for the control of trichostrongyles, comprising the efficient deworming and prevention of infection through the administration of pellets with chlamydospores of *M. circinelloides* and *D. flagrans*, has been demonstrated in pasturing horses, both under continuous or rotational regimes (Hernández *et al*., 2016, 2018a). Besides this, no side-effects were observed among the bovids feeding the pellets enriched with spores, and none of them refused to take this kind of feed throughout the investigation, which is also in concordance also with previous information collected in horses (Hernández *et al*., 2018a, 2018c). Data presented in the current trial have been obtained among several species of wild bovids, and reflect the usefulness and innocuousness of giving chlamydospores of *M. circinelloides* and *D. flagrans* to wild captive bovids to ensure that low numbers of eggs of trichostrongyles are passed in their feces. Accordingly, these results can easily be extrapolated to most of herbivores under pasturing conditions. Finally, it is concluded the industrial manufacturing of pellets with spores of parasiticide fungi offers a viable and easy way to develop a preventive action against the infection by trichostrongyles among wild captive bovids, valuable to reduce the frequency of administration of anthelmintic treatment.

Numerous species of wild herbivores are exhibited in zoos, mostly ruminants. Among equids, the numbers of zebras are high also, and in a lesser importance, others as the exotic Falabella horse as different breeds of donkeys, that also benefit from the wise changes introduced in the last decades in zoological parks, addressed to improve the welfare and living conditions of captive animals, by enjoying an appropriate environment where herbage is present. As mentioned for ruminants, captive equids maintained in zoological parks are at risk of infection by parasites, and control strategies consist basically of deworming with drugs available for domestic horses also (Kerry *et al*., 2000). In the **third trial**, the effect of a strategy of integrated control of strongyles was evaluated in wild equids (plain zebras, European donkeys, and African wild asses) captive in a zoological park. At the beginning of the trial, anthelmintic treatment consisting in the administration of ivermectin + praziquantel was performed, and also if counts of 400 eggs per gram of feces (EPG) were surpassed (Arias *et al*., 2013). Additionally, equids were provided, every two days, commercial pellets industrially manufactured with chlamydospores of *M. circinelloides* (ovicidal activity) and *D. flagrans* (larvicidal activity). According to the World Association for the Advancement of Veterinary Parasitology guidelines (Coles *et al*., 1992), the effect of the deworming was measured fifteen days after the administration of anthelmintics, which provided FECR (Fecal Egg Count Reduction) values of 100%. The finding that none of the equids passed eggs at this time, together with a period of reappearance of eggs in feces (ERP) after eight weeks, confirmed the success of the deworming, in concordance with previous investigations (Arias *et al*., 2013; Stratford *et al*., 2014). Due to the fact that EPG numbers increased in the four months after treatment until reaching values higher than 400 (the initially established cut-off point), equids were dewormed again. The next treatment was administered fifteen months later, becoming fully effective. Finally, all the equids were successfully dewormed eighteen months after the last treatment. The administration of one dose of ivermectin to horses under continuous grazing, together with the daily intake of pellets containing chlamydospores of *M. circinelloides* and *D. flagrans*, resulted in no further anthelmintic treatment being required for 64 weeks (16 months) (Hernández *et al*., 2016). It has been noted that infection by strongyles occurs when animals take forage contaminated with third-stage larvae (L3s) (Von Samson-

Himmelstjerna, 2012), then as demonstrated for horses under a continuous grazing regime, wild equids captive in a zoo can be easily exposed to high levels of infective larvae. This problem is usually addressed by performing frequent treatments in equids to reduce the presence of eggs that could evolve to a high number of L3s in the soil (Matthews, 2014), but not all anthelmintics are ecofriendly for the environment, and toxicity on certain organisms (i.e., dung beetles) displaying a highly soil-enriching activity by decomposing manure has been denounced (González-Tokman *et al*., 2017; Verdú *et al*., 2018).

The issue of facilitating the prevention of horses strongyle infections has stimulated the development of different measures such as regular manual collection of feces (every 2-4 days) or rotation of pastures to reduce the risk of infection in grass plots (Corning, 2009; Corbett *et al*., 2014). At the zoo where the present research was conducted, feces are removed daily and early in the morning, before visitors enter the park, but captive equids are always housed in the same plot because the impossibility of rotating grasslands, a problem similar to that described for captive ruminants. In view of this situation, it was considered the usefulness of implementing a control program for strongyles as the one successfully applied in horses managed under a continuous grazing system (Hernández *et al*., 2016). Therefore, the effective deworming of the equids was supplemented with the administration of nutritional pellets made with chlamydospores of the two parasiticidal fungi every two days. As a consequence, a cumulative effect was registered, consisting in the extension of the period between the administration of the treatments, with consecutive intervals of four, fifteen and eighteen months. These results emphasize the helpfulness of the planned strategy, mainly regarding the possibility to decrease the frequency of deworming, with positive benefits in lessening the risk of appearance of anthelmintic resistance (Calvete *et al*., 2020). Besides, during a three-year period the wild equids were ingesting pellets with fungal chlamydospores, no undesirable effects concerning digestive and respiratory systems or the skin were reported, supporting thus the safeness of this strategy as prior pointed in domestic horses (Hernández *et al*., 2016).

Investigations conducted in lambs infected by *Haemonchus contortus* demonstrated a similar feed conversion when taking nutritional pellets with or without chlamydospores of *D. fagrans*, similar growth rates and values of Packed Cell Volume, Body Condition Score, and *H. contortus* EPG (Aguilar-Marcelino *et al*., 2016); by opposite, counts of L3s developed in the feces of lambs taking pellets with spores were significantly lower. One interesting finding was the observation of higher counts of eggs of strongyles in zebras and European donkeys than in African wild asses, possibly due to differences in the paddocks where they are maintained. Zebras and European donkeys are living in two grasslands with abundant forage, while African asses do it in a sandy parcel, where forage is rarely observed (at the corners). Hence, it could be supposed that these equids would hardly become infected. It has been reported that conditions favorable for hatching of strongyles eggs and larval development into infective larvae is enhanced when pasture and ground have enough moisture, and temperature is mild (Nalubamba *et al*., 2012).

Through the approach and performance of three trials on captive herbivores in a zoo, in the current study it is inferred that parasite control involves a problem identical to the one that has been reported for decades in the management of domestic species, and that basically could be summarized in an excessive dependence on the administration of antiparasitic drugs, and little or no effective and practical preventive measures that are feasible. Regarding the deworming, it should be desirable to support it on data collected from the analysis of different samples, although this presents several concerns. In a recent survey conducted for gaining information on husbandry practices of Bovidae in European zoos, most answers indicated that feces were collected from the ground (to avoid stress probably) and analyzed by means of a concentrating flotation technique (Moreno *et al*., 2019). Although the authors claim the application of sedimentation and Baermann probes for the detection of trematodes and lungworms, a more realistic solution could be based on examining the possibilities that these helminths can develop their life cycle in the respective zoological parks (Panayotova-Pencheva *et al*., 2013; Mirzapour *et al*., 2018; Dashe & Berhanu, 2020).

Other issue concerns that, though it has not yet been verified, the possibility of wild ruminants could metabolize and excrete certain benzimidazoles faster than domestic ones has been suggested (Mohammed *et al*., 2007). Assessment of the effect of deworming is done through the Fecal Egg Count Reduction (FECR), which allows also to suspect about the possibility of anthelmintic resistance (Young *et al*., 2000; Cabaret & Berrag, 2004; Goossens *et al*., 2005). Based on maintaining separate control groups is virtually impossible, the efficacy needs to be calculated in comparison with pre-treatment mean EPG (Goossens *et al*., 2006; Nalubamba *et al*., 2012). It appears that in very large zoological gardens a control group could be observed in the same ecosystem/area and isolated from the treated group; however, other requirements such as statistically comparable EPGs should be necessary, which makes it even more complicated. A pending question refers to the lack of comprehensive guidelines for captive wild herbivores (Nalubamba *et al*., 2012), as available for domestic animals (Wood *et al*., 1995; Coles *et al*., 2006).

In last years, it has been argued that low, but constant parasitic burden might favor immunity (Mathews *et al*., 2006); moreover, it has been considered that it is neither possible, nor desirable for organisms to be *parasite and disease free* (IUCN, 2013). In any case, there is no doubt that the immune system can be stimulated by a subclinical parasite burden, which should be of particular interest not only among those wild animals captive in a zoological garden for exhibiting purpose, but also for animals that are released into natural environments, due to the risk of exposure to parasites affecting wildlife species or livestock populations (Moreno *et al*., 2019).

Control of parasites among wild herbivores captive in zoological gardens needs to make possible strategies comprising programs of control involving the integration of successful deworming (when needed) and suitable and useful measures to prevent the infection. In the course of the present research it has been shown the oral administration of a blend of SSF with complementary activity on parasites (ovicidal and larvicidal) affords a very interesting and helpful tool to prevent that grass plots where wild herbivores are captive in a zoological park could attain an elevated level of contamination by infective stages, which would imply the animals are frequently dewormed to ensure an adequate health status. Proper distribution of fungal spores can turn also into a serious issue, due to the inconvenience to maintain a close contact with keepers. In a previous investigation, successful results in controlling strongyles affecting captive wild equids were obtained by giving them pelleted feed mixed previously with chlamydospores of *D. flagrans*, but this strategy needs keepers to elaborate the premix every two days (Arias *et al*., 2013). The demonstration of chlamydospores of *M. circinelloides* and *D. flagrans* did not resulted affected during the industrial manufacturing of pellets remarks the usefulness of this solution to the distribution of SSF without adding extra tasks to animal keepers (Arroyo *et al*., 2017, 2018).

One of the most important issues linked to deworming consists in the appearance of strains of parasites resistant to some antiparasitic drugs which can transmit this ability (Shalaby *et al*., 2013). It has been widely explained that the degree of development of anthelmintic resistance could be reduced by maintaining a proportion of the parasite population in refugia (unexposed to anthelmintic) (Van Wyk, 2000). For this purpose, treatment approaches targeting the flock have been counseled to improve the sustainable use of anthelmintics (Calvete *et al*., 2020. Targeted treatments are characterized by the whole group is treated since knowledge of the risk or parameters that quantify the severity of infection (Kenyon & Jackson, 2012). This looks to be the case of wild herbivores captive in zoos, where the need of deworming is mainly based on fecal analyses, and clinical examination. Along the same line, the visual body condition scoring in zoo animals could add further information about the health status of the animals, and bear in mind when considering the need for deworming (Bray, 1999; Schiffman *et al*., 2017).

Based on the results obtained, it can be concluded that the administration of chlamydospores of *M. circinelloides* and *D. flagrans* every two days to captive wild herbivores provides a safety procedure to obtain a helpful and long-lasting effect on avoiding the infection by gastrointestinal nematodes. Data presented demonstrate that this strategy can be supported in two oral formulations, sprayed-on pellets or as pellets industrially manufactured with the fungi, the latter being easier for ensuring the appropriate dosage is administered. This formulation provides a novel and sustainable tool for developing programs of integrated control of strongyles involving anthelmintic deworming also, but if faced with the lack of a commercial product available very practical and similarly successful effects can be attained by spraying the spores on the pellets immediately before feeding the animals.

Finally, special attention must be paid to the surface of the grazing area where the herbivores feed, as this will determine the effect on parasitic free-living stages, as well as the time required to achieve the expected results. The trials conducted in this study were carried out in plots with vegetation of between 300 and 10000 m^2, and satisfactory results were collected as soon as one year. Data concerning the possibility of applying this strategy to zoos involving many species of wild animals which can share larger grasslands are not available by now, thus results obtained in the current research can not directly extrapolated. The extent of grazing area also affects the way for administering the SSF, because in the current experience, wild captive herbivores are supplemented with nutritional pellets every two days, which enhances the administration of the fungal chlamydospores. Further investigations are needed if trying to adapt to husbandry conditions different to those in the present study.

7.- CONCLUSIONS

The tests carried out in this study led to the conclusion that:

1. The administration of a blend of spores of *M. circinelloides* and *D. flagrans* every two days to wild captive ruminants offers an innocuousness strategy to get a helpful long-lasting effect on preventing the infection by trichostrongylids.

2. Although highly successful results can be obtained by providing spores as sprayed-on pellets or as pellets industrially manufactured with the fungi, this last procedure appears easier for giving the spores to captive herbivores. However, in the absence of a commercial product available, a very practical and similarly successful effect can be achieved by spraying the spores on the pellets immediately before feeding the captive animals.

3. Low strongyles egg counts are maintained in feces of wild captive bovids receiving spores of *M. circinelloides* and *D. flagrans*, resulting very valuable to prevent their infection, and to reduce the frequency of administration of anthelmintic treatment.

4. Commercial manufacturing of pelleted feed with spores of the flamentous fungi *M. circinelloides* and *D. fagrans* affords a very useful tool to their administration to wild equids captive

at a zoological park. This procedure provides a novel and sustainable strategy for developing programs of integrated control of strongyles involving anthelmintic deworming also.

5. A program for the control of parasites affecting wild herbivores which integrates deworming with anthelmintics (benzimidazoles or macrocyclic lactones) together with periodical administration of chlamydospores of the soil saprophytic fungi *Mucor circinelloides* and *Duddingtonia flagrans* provides a novel and helpful contribution to the sustainable management of digestive infections by parasites in zoological parks.

8.- SUMMARY / RESUMEN / RESUMO

8.1. SUMMARY

The possibilities of developing the *integrated control of digestive helminths in captive herbivores* was planned in a study divided into three field trials conducted in the Marcelle Natureza Zoological Park (Outeiro de Rei, Lugo, Spain), within the collaboration agreement signed a decade ago between this zoo and the COPAR Research Group (GI-2120; USC). The main intention was to propose a practical solution to a problem that had been dragging on for many years, the control of parasites affecting wild captive herbivores. Since many decades, that is, the deworming of these animals has been practically the only measure taken into account, and for this purpose efficient antiparasitic drugs widely commercially for the control of parasites among livestock have been administered to species kept at zoological gardens. In other words, the problem of parasite control is being handled in the same way as in animal farms, which leads to inheriting the same disadvantages that explain the failures that have been occurring for decades, mainly centered on the lack of preventive measures.

On the basis of control of parasites can not be supported in deworming only, due to different parasites, as trichostrongylids or strongyles develop in the soil from eggs passed in the feces of infected animals to first-stage larvae (L1) which exit off and moult into L2 and finally into L3, the infective phases, the utility of attempting to limit L3 development was considered as an effective tool to prevent infection among captive herbivores maintained in a zoo. Accordingly, the successful deworming of wild species of ruminants and equids was articulated with a strategy comprising the utilization of some strains of soil saprophytic fungi isolated previously from samples of ground and feces of different animals in the mentioned zoo. Concretely, a blend of two fungi was used, *Mucor circinelloides*, able to adhere to eggshell, penetrate and take the inner contents of eggs or cysts (ovicide), and *Duddingtonia flagrans*, which elaborates traps into the mycelium where larvae of nematodes are catched and then the contents of their interior are taken.

By considering previous data collected among domestic animals, the central idea in the present study revolved around the practical possibilities of contributing to the integrated control of parasites among herbivores captive in a zoological garden. The oral administration of spores of this mixture of spores was contemplated, taking into account that in previous research studies it was demonstrated that some SSF were able to survive passage through the gastrointestinal tract and reach the exterior with the feces without losing their biological activity, which in this case consisted of antagonisc effect against the stages (oocysts, eggs, larvae) of certain parasites. **In the first assay**, the aim was to determine the most appropriate formulation to ensure that sylvatic bovids (wapiti, *Cervus canadensis*) captive in a plot where they can graze all day, could be provided a blend of the two aforementioned SSF. On the basis of these ruminants were shedding eggs of trichostrongylids, the anthelmintic fenbendazole was periodically given, but in view of high counts of eggs per gram of feces (EPG) were reached several months after treatment the veterinarian direction of the zoo pondered other strategies involving the prevention od further infections by limiting the risk of parasitization. In this sense, a blend of chlamydospores of *M. circinelloides* and *D. flagrans* were formulated as two oral presentations, sprayed-on nutritional pellets just before provided to the wapitis every two days, or as enriched pelleted feed during the industrial manufacturing in a commercial factory. Both procedures were assessed on fecal samples taken directly from the ground, due to the impossibility of collecting them directly from the rectum. Other important point relies on the observation of a control-untreated group with identical characteristics is not possible. Fecal samples were analyzed by the coprological tests of flotation (McMaster) and sedimentation, and results expressed as the fecal egg counts (FEC) of trichostrongylids EPG. With these data, the FECR (fecal egg count reduction) was estimated, considering the EPG values obtained on wapitis receiving fenbendazole only during 10 months (without chlamydospores) as controls. Besides, special attention was placed on the possible side effects that could affect to the digestive tract, respiratory apparatus and the skin. The absence of significant differences regarding the climatic parameters along the study (2014 to 2018) led to the joint analysis of the data obtained as an unique climatic pattern.

The analysis of feces was negative by the sedimentation, and eggs of trichostrongylids were observed in the fecal samples. By performing coprocultures, larvae were classified and sorted into the genera Cooperia, Nematodirus and Trichostrongylus. Because of oocysts of protozoa (*Eimeria* spp.) and eggs of *Trichuris* sp. were found intermittently, these data were not included in the present investigation. The values of FEC were higher than 300 EPG at the beginning of the trial (September 2015), and deworming with fenbendazole yielded a 99% efficacy. The numbers of eggs increased in feces through May above 350 EPG. Administration of flubendazole in the second year resulted successful (FECR= 100%). Sprayed-on pellets were given throughout this year, and eggs of nematodes appeared again in the feces of the wapitis two months after treatment, maintaining counts between 50 and 100 EPG until the trial ended (10 months). In the third year, administration of fenbendazole in September was successful (FECR= 100%); during this year, when pellets enriched with chlamydospores were provided to the wapitis, eggs of trichostrongylids were not observed until the fourth month after treatment, and counts lower than 50 EPG until the end of the assay (June) were observed. Significant differences between the trichostrongylids egg-output in the first year and in the two other years were found by using the non-parametric Friedman test (χ^2= 53.063, P= 0.001).

The combined analysis of the results achieved demonstrated a 30–100% (average: 69%) reduction in the numbers of eggs of trichostrongylids in the second year in respect to the first year; a reduction of 36-100% (average 71%) was recorded in the third year. It needs to be underlined the absence of adverse consequences affecting the digestive tract, respiratory apparatus or the skin while spores of the parasiticide fungi were administered to the wapitis. Wapitis did not reject to take sprayed-on nutritional pellets or enriched pellets. The failure of useful and practical measures to limit the risk of infection by trichostrongylids has been tried to be solved by increasing the frequency of deworming, but this strategy could evolve in lower efficacies than expected, and even promote the selection of parasite strains resistant to specific anthelmintics and able to transmit it. Results collected in the current trial showed that the administration of a blend of chlamydospores of *M. circinelloides* and *D. flagrans* affords a high beneficial effect in decreasing the infection by trichostrongylids in wapitis maintained in a grass paddock. It appears interesting to underline that grass plots are considered so that the herbivores can nourish, socialize and interact with the environment, but these plots present suitable conditions for certain gastrointestinal nematodes to easily develop the external phase of their life cycle, which points out the need for proper measures to limit the risk of infection for grazing herbivores. There is a natural equilibrium in the enviroment which involves hosts (mammals, mainly herbivores), pathogens and soil microorganisms acting as pathogen antagonists. When the equilibrium is disturbed (immunosuppressed hosts, reduction of antagonists), pathogens can exacerbate. Results achieved in the current study reinforce the hypothesis that the spores of *M. circinelloides* and *D. flagrans* reduce the numbers of infective stages (third stage larvae) of trichostrongylids in the ground, thus the risk of contamination in the paddock lessens. A similar efficacy was recorded by means of the two formulations tested, though the administration of nutritional pellets enriched with the chlamydospores makes it easier for their distribution to be realised among the captive herbivores since additional tasks are not added to the animal keepers.

Once demonstrated that the best formulation was the nutritional pellets enriched with chlamydospores of both SSF during the industrial manufacturing (10^4–10^5 spores of each / kg meal), a **second assay** was performed on wild bovidae belonging to the subfamilies Antilopinae (*Antilope cervicapra*, *Gazelle cuvieri*), Caprinae (*Capra* *aegagrus hircus*, *Ovis orientalis musimon*), Bovinae (*Bison bison*, *Kobus kob*) and Reduncinae (*Tragelaphus spekii*), feeding on red clover (*Trifolium pratense*), perennial ryegrass (*Lolium perenne*) and orchard grass (*Dactylis glomerata*) in different plots of a zoo. The design involved the deworming of the herbivores, based on the presence of eggs of trichostrongylids in the feces. Anthelmintic treatment was administered again when a threshold of 300 EPG was surpassed. A preventive measure was also considered, consisting in the administration of pellets enriched with the chlamydospores, every two days for 3.5 years.

The evaluation of the integrated control measures was done by the coprological analyses of samples collected from the upper portion of feces directly from the ground in each plot, because as evident, individual collection is impossible. Feces were analysed by means of the sedimentation [sensitivity (S)= 30 EPG] and flotation tests (S= 30 EPG or OPG, oocysts per gram of feces), and results described as the numbers of eggs/oocysts per gram of feces. After performing coprocultures, nematode general were identified according to morphological keys, by preparing pools of 10 g feces taken at the beginning of the study and incubating at 25°C for 18 days. The effect of deworming was assessed by examining feces 14 days after treatment and calculating the percentage of fecal egg count reduction (FECR). This parameter was also estimated across the trial, as well as the period in which feces did not represent a risk for contamination of the soil (NRFP, non-risky feces period), defined as the interval (months) occuring from the last administration of a successful deworming to the reappearance of eggs in the feces. Furthermore, the possibility of damage to the digestive or respiratory system, and the skin, was examined.

By means of the McMaster (flotation) test, eggs of trichostrongylids were observed; oocysts of *Eimeria* spp. and eggs of trichurids (*Trichuris* spp.) were rarely detected throughout the trial, hence these data were not included in the analyses. Examination of coprocultures indicated the presence of third-stage larvae of the genera *Trichostrongylus*, *Nematodirus*, *Chabertia* and *Haemonchus*.

The efficacy of the deworming administered throughout the trial oscillated from 97 to 100% without observing significant differences. Between March and August, the values of NRFP were 0–1 months for the blackbucks (*Antilope cervicapra*), and 1 month for the gazelles (*G. cuvieri*), goats (*Capra aegagrus hircus*), mouflons (*Ovis orientalis musimon*), bison (*Bison bison*), marshbucks (*Tragelaphus spekii*) and kobs (*Kobus kob*). Since November, these values increased to 1–2 months for the blackbucks and goats, and to 3 months for the other ruminants. During this period, values of fecal counts of eggs of trichostrongylids in the Antelopinae (blackbucks and gazelles) higher than 300 EPG were detected by four times (blackbucks) and three times (gazelles). Between November and March, EPG numbers higher than 300 were found twice (blackbucks) and once (gazelles). Since that month, counts of trichostrongylids egg-output around 100–150 EPG were recorded. In the Caprinae, the deworming cut-off point (300 EPG) was surpassed once in goats and twice in mouflons until November. From December to the end of the trial, the values of egg-output maintained at 80–120 EPG in their feces. Finally, in the Bovinae an Reduncinae the cut-off was exceeded three times until November, then remained between 50 and 110 until the end of the study. Significant differences were observed in all the captive species, mainly during the first two years. No adverse effects on the digestive or respiratory system were detected in the wild bovids receiving pellets enriched with fungal spores. The absence of skin lesions was also confirmed, whereas refusal to take the pellets was never observed. These results reveal the beneficial effect which can be obtained by integrating two efficient measures, successful deworming along with the administration of a blend of two SSF rising a complementary activity. The fungus *M. circinelloides* is capable to penetrate the eggs of helminths as trematodes and ascarids and to destroy them, and *D. flagrans* elaborates traps along the mycelium for capturing larvae. It is also concluded that in the absence of preventive measures, infective stages (L3s) developing in the soil ensure an almost never-ending risk of infection throughout the year, especially in areas with mild climates.

Aiming to gain information about the usefulness of integrating two measures, successful deworming and prevention of infection in a program for the control of parasites in wild captive equids, a **third trial** was developed comprising individuals of *Equus quagga*, *E. asinus*, and *E. africanus asinus* passing eggs of strongyles in feces. While plains zebra and European donkeys feed on a grassland, African wild asses were maintained in a sandy area, where herbage is present only in the corners. The strategy consisted in equids being treated with ivermectin + praziquantel at the beginning of the trial, and nutritional pellets added a blend of 10^4-10^5 chlamydospores of each fungi / kg meal during the industrial manufacturing was given to the equids, every two days for three years. As mentioned previously, fecal samples were collected periodically, directly from the ground in each parcel, early in the morning, before the visitors arrival. Samples were analyzed by means of the sedimentation and flotation (McMaster) tests. Data obtained was expressed as strongyles EPG. The effect of the strategy for the control of parasites was assessed by calculating the FECR and in the positive horses (PHR). Besides, the egg reappearance period (ERP) and the time elapsed from the previous deworming (TPD) were also estimated.

Fecal analyses showed the presence of eggs of strongyles, and eggs of *Trichuris* were seldom found, without evidence of coccidia, trematodes, cestodes, or lung worms. Fifeen days afer the first administration of anthelmintics, the FECR values were 100% for European donkeys, plains zebra, and African wild asses, and the PHRs were 100% as well (none of the equids shed eggs in the feces). An ERP of 8 weeks was recorded for zebras and European donkeys and of 12 weeks for the African asses. The kinetics of fecal egg-output peaked at December, when values higher than 400 EPG were attained and thus anthelmintic treatment was administered again, with full efficaccy (FECR= 100%; PHR= 100%). The ERP was 12 weeks for zebras and European donkeys and 16 weeks for the African asses. Despite the counts decreased significantly, a new increment from March was recorded, when numbers higher than 400 EPG were observed in the feces of all equids, thus the necessity of a new treatment was considered (15 months after the last treatment), with successful results as demonstrated by only one individual from the European and the African donkeys remained passing eggs of strongyles by feces, and the ERP in all equids was 24 weeks. Finally, anthelmintics were administered 18 months afer the last deworming, becoming fully efective, since one of the European donkeys and one of the African asses were positive to the flotation test, with an ERP of 28 weeks in all captive equids. When considering the phases between deworming, the mean values of EPG were reduced by 13-30% in the second period (March 2016) and by 34-48% in the third period (September 2017). Ingestion of pellets was completely accepted, and equids took all the feed provided.

Through the approach and performance of three trials on captive herbivores in a zoo, in the current study it is inferred that parasite control involves a problem identical to the one that has been reported for decades in the management of domestic species, and that could basically be summarized in an excessive dependence on the administration of antiparasitic drugs, and little or no effective and practical preventive measures are feasible. Regarding the deworming, it should be desirable to support it on data collected from the analysis of different samples, although this presents several worries.

Other issue concerns that, though it has not yet been verified, wild ruminants could metabolize and excrete certain benzimidazoles faster than domestic ones has been suggested. Assessment of the effect of deworming is done through the Fecal Egg Count Reduction (FECR), which allows also to suspect about the possibility of anthelmintic resistance. Since maintaining separate control groups is a virtually impossible task, the efficacy needs to be calculated in comparison with pre-treatment mean EPG. It appears that in very large zoological gardens a control group could be observed in the same ecosystem/area and isolated from the treated group; however, other requirements such as statistically comparable numbers of EPG should be necessary, which makes it even more complex. A pending question refers to the lack of comprehensive guidelines for captive wild herbivores, as available for domestic animals.

Control of parasites among wild herbivores captive in zoological gardens needs to make possible for strategies comprising programs of control to involve the integration of successful deworming (when needed) and suitable and useful measures to prevent the infection. In the course of the present research it has been shown that oral administration of a blend of SSF with complementary activity on parasites (ovicidal and larvicidal) became a very interesting and helpful tool to prevent that grass plots where wild herbivores are captive in a zoological park could attain an elevated level of contamination by infective stages, which would imply the animals are frequently dewormed to ensure an adequate health status. The proper distribution of fungal spores can turn into a serious issue, due to the inconvenience to maintain a close contact with keepers.

Special attention must be paid to the surface of the grazing area where the herbivores feed, as this will determine the effect on parasitic free-living stages, as well as the time required to achieve the expected results. The trials conducted in this study have been carried out in parcels with vegetation of between 300 and 10000 m^2, and satisfactory results were collected as soon as one year later. Data concerning the possibility of applying this strategy to zoos involving many species of wild animals sharing larger grasslands are not available by now, thus results obtained in the current research can not be directly extrapolated. The extent of grazing area also affects the way for administering the SSF, because in the current experience, wild captive herbivores are supplemented with nutritional pellets every two days, which enhances the administration of the fungal chlamydospores. Further investigations are needed for trying to adapt to husbandry conditions different to those in the present study.

From the results obtained, it can be concluded that the administration of chlamydospores of *M. circinelloides* and *D. flagrans* every two days to captive wild herbivores provides a safe procedure to obtain a helpful and long-lasting effect on avoiding the infection by gastrointestinal nematodes. Data presented demonstrate that this strategy can be supported in two oral formulations, sprayed-on pellets or as pellets industrially manufactured with the fungi, the latter being easier for ensuring the appropriate dosage is administered. This formulation provides a novel and sustainable tool for developing programs of integrated control of strongyles involving anthelmintic deworming also, but if faced with the lack of a commercial product available very practical and similar successful effects might be attained by spraying the spores on the pellets immediately prior to feeding the animals. Finally, it is concluded that a program that integrates deworming with anthelmintics (benzimidazoles or macrocyclic lactones), together with the periodic administration of chlamydospores of the saprophytic fungi *M. circinelloides* and *D. flagrans*, offers a novel and useful contribution to the control of parasites affecting wild herbivores in zoos.

8.2. RESUMEN

Con objeto de aclarar las posibilidades del control integrado de helmintos digestivos en herbívoros cautivos, se planteó un estudio dividido en tres ensayos de campo realizados en el Parque Zoológico Marcelle Natureza (Outeiro de Rei, Lugo, España), dentro del convenio de colaboración firmado hace una década entre este zoológico y el Grupo de Investigación COPAR (GI-2120; USC). La intención principal consistió en proponer una solución práctica a un problema que viene de lejos, el control de los parásitos que afectan a herbívoros silvestres en cautividad. Desde hace muchas décadas, la desparasitación de estos animales ha sido prácticamente la única medida que se ha tenido en cuenta, administrándose a las especies mantenidas en los parques zoológicos fármacos antiparasitarios eficaces y ampliamente comercializados para el control de los parásitos del ganado. En otras palabras, el problema del control de parásitos se está manejando de la misma manera que en las granjas de animales, lo que lleva a heredar los mismos inconvenientes que explican los fracasos que se vienen produciendo en el curso de los años, centrados principalmente en la falta de medidas preventivas.

En base a la idea de que el control parasitario no se puede apoyar solamente en la aplicación de tratamientos convencionales, debido a que parásitos como los tricostrongílidos o los estróngilos se desarrollan en el suelo a partir de los huevos eliminados en las heces de individuos infectados, a larvas de primer estadio (L1) que eclosionan y mudan a L2 y finalmente a L3, las fases infectivas, se consideró la utilidad de tratar de limitar el desarrollo de L3 como una herramienta eficaz para prevenir la infección entre herbívoros cautivos en un zoológico. En consecuencia, el éxito de la desparasitación de especies silvestres de rumiantes y équidos se articuló en una estrategia que comprendió la utilización de algunas cepas de hongos saprófitos del suelo, aisladas previamente a partir de muestras de suelo y heces de diferentes animales del citado zoológico. Concretamente, se utilizó una mezcla de dos hongos, *Mucor circinelloides*, capaz de adherirse a la cáscara del huevo, penetrar y tomar el contenido interior de los huevos o quistes (ovicida), y *Duddingtonia flagrans*, que elabora trampas en el micelio donde quedan atrapadas las larvas de los nematodos, ingiriendo a continuación su contenido interno.

Teniendo en cuenta los datos recogidos en animales domésticos, la idea central del presente estudio giró en torno a las posibilidades prácticas de contribuir al control integrado de los parásitos entre los herbívoros cautivos en un jardín zoológico. Para ello, se planteó la administración oral de esporas de esta mezcla de esporas, teniendo en cuenta que en investigaciones anteriores se demostró que algunos SSF eran capaces de sobrevivir al paso por el tracto gastrointestinal y llegar al exterior con las heces sin perder su actividad biológica, que en este caso consistía en el efecto antagónico contra los estadios (ooquistes, huevos, larvas) de ciertos parásitos. En el **primer ensayo**, el objetivo fue determinar la formulación más adecuada para suministrar una mezcla de los dos SSF mencionados a bóvidos silvestres (wapitíes, *Cervus canadensis*) cautivos en una parcela donde pueden pastar todo el día. Dado que estos rumiantes eliminaban huevos de tricostrongílidos en las heces, se administraba periódicamente fenbendazol, pero a la vista de los elevados recuentos de huevos por gramo de heces (EPG) que se alcanzaban varios meses después del tratamiento, la dirección veterinaria del zoo se planteó otras estrategias que implicaran la prevención de nuevas infecciones, limitando el riesgo de parasitación. En este sentido, se formuló una mezcla de clamidosporas de *M. circinelloides* y *D. flagrans* en dos presentaciones orales, en forma de pellets nutricionales pulverizados justo antes de suministrárselos a los wapitíes cada dos días, o en forma de pienso pelletizado enriquecido durante su elaboración industrial en una fábrica comercial. La valoración de ambos procedimientos consistió en el análisis de muestras fecales tomadas directamente del suelo, debido a la imposibilidad de recogerlas directamente del recto. Otro punto importante es que no es posible observar un grupo de control no tratado con idénticas características. Las heces se analizaron mediante las pruebas de flotación (McMaster) y sedimentación, y los resultados se expresaron como recuentos de huevos de tricostrongílidos por gramo de heces (HPG). Con estos datos se estimó el FECR (porcentaje de reducción de los recuentos de huevos en heces), considerando como testigos los valores de HPG obtenidos en wapitis que recibieron sólo fenbendazol durante 10 meses (sin clamidosporas), durante el primer año del ensayo. Además, se prestó especial atención a los posibles efectos secundarios que pudieran afectar al tracto digestivo, al aparato respiratorio y a la piel. La ausencia de diferencias significativas en cuanto a los parámetros climáticos a lo largo del estudio llevó al análisis conjunto de los datos obtenidos como un patrón climático único.

El análisis de las heces fue negativo a la sedimentación, y con la flotación se observaron huevos de tricostrongílidos. Mediante la realización de coprocultivos, las larvas se clasificaron y ordenaron en los géneros *Cooperia*, *Nematodirus* y *Trichostrongylus*. Debido a que se encontraron ooquistes de protozoos (*Eimeria* spp.) y huevos de *Trichuris* sp. de forma intermitente, y por ello estos datos no se incluyeron en la presente investigación. Los valores de HPG fueron superiores a 300 al inicio del ensayo, y la desparasitación con fenbendazol tuvo una eficacia del 99%. El número de huevos aumentó en las heces hasta mayo por encima de 350 HPG. La administración de flubendazol en el segundo año resultó exitosa (FECR= 100%). Durante este año, los wapities recibieron pellets previamente pulverizados con una mezca de los dos hongos parasiticidas mencionados, demostrándose que los huevos de nematodos volvían a aparecer en las heces dos meses después del tratamiento, manteniéndose entre 50 y 100 HPG hasta el final del ensayo (10 meses). En el tercer año, la administración de fenbendazol en septiembre fue completamente eficaz (FECR= 100%); durante este año, cuando se suministraron pellets enriquecidos con clamidosporas a los wapitis, no se observaron huevos de tricostrongílidos hasta el cuarto mes después del tratamiento, y los recuentos fueron inferiores a 50 HPG hasta el final del ensayo (junio). Se encontraron diferencias significativas entre la producción de huevos de tricostrongílidos en el primer año y en los dos restantes mediante la prueba no paramétrica de Friedman ($\chi^2 = 53{,}063$, $P= 0{,}001$).

El análisis combinado de los resultados obtenidos demostró una reducción del 30-100% (media: 69%) del número de huevos de tricostrongílidos en el segundo año respecto al primero, con un valor medio del 69%; en el tercer año se registró una reducción del 36-

100% (media: 71%). Hay que subrayar la ausencia de consecuencias adversas en el tracto digestivo, aparato respiratorio o a la piel mientras se administraban esporas de los hongos parasiticidas a los wapitis. Los wapitis no rechazaron tomar los pellets nutricionales rociados ni los pellets enriquecidos. El fracaso de medidas útiles y prácticas para limitar el riesgo de infección por tricostrongílidos ha intentado solucionarse aumentando la frecuencia de desparasitación, estrategia que podría evolucionar en eficacias inferiores a las esperadas, e incluso en la selección de cepas de parásitos capaces de resistir al tratamiento con antihelmínticos específicos, y de transmitirlo. Los resultados del presente ensayo mostraron que la administración de una mezcla de clamidosporas de *M. circinelloides* y *D. flagrans* proporciona un elevado efecto beneficioso en la disminución de la infección por tricostrongílidos en wapitis mantenidos en una parcela de hierba. Es interesante subrayar que el objeto de estas parcelas es que los herbívoros puedan alimentarse, socializar e interrelacionarse con el medio, pero al mismo tiempo concurren las condiciones idóneas para que ciertos nematodos gastrointestinales puedan desarrollar fácilmente la fase externa de su ciclo vital, lo que señala la necesidad de tomar medidas adecuadas para limitar el riesgo de infección de los herbívoros que pastan. En el medio, existe un equilibrio natural en el que intervienen los hospedadores (mamíferos, principalmente herbívoros), patógenos y ciertos microorganismos del suelo que actúan como antagonistas de los patógenos. Cuando el equilibrio se altera (huéspedes inmunodeprimidos, reducción de antagonistas), los patógenos pueden exacerbarse. Los resultados obtenidos en el presente estudio refuerzan la hipótesis de que los hongos *M. circinelloides* y *D. flagrans* reducen el número de estadios infectantes (larvas de tercer estadio) de los tricostrongílidos en el suelo, con lo que disminuye el riesgo de contaminación en la parcela. Se registró una eficacia similar con las dos formulaciones ensayadas, aunque la administración de pellets nutricionales enriquecidos con las clamidosporas facilita su distribución entre los herbívoros cautivos, ya que no se añaden tareas adicionales a los cuidadores de los animales.

Una vez demostrado que la mejor formulación era la de pellets nutricionales enriquecidos con clamidosporas de ambos SSF durante la fabricación industrial (10^4 -10^5 esporas de cada uno / kg de concentrado), se realizó un **segundo ensayo** en bóvidos silvestres de las subfamilias Antilopinae (*Antilope cervicapra*, *Gazelle Cuvieri*), Caprinae (*Capra aegagrus hircus*, *Ovis orientalis musimon*), Bovinae (*Bison bison*, *Kobus kob*) y Reduncinae (*Tragelaphus spekii*), que se alimentaban en diferentes parcelas de un zoológico con trébol rojo (*Trifolium pratense*), raigrás perenne (*Lolium perenne*) y dáctilo (*Dactylis glomerata*). El diseño incluyó la desparasitación inicial de los herbívoros, basada en la presencia de huevos de tricostrongílidos en las heces, aunque este tratamiento se repitió siempre que se superaba un umbral de 300 HPG. Además, se consideró una medida preventiva, consistente en la administración de pellets enriquecidos con clamidosporas, cada dos días durante 3,5 años.

La evaluación de las medidas de control integrado se realizó mediante los análisis coprológicos de las muestras recogidas de la parte superior de las heces directamente del suelo en cada parcela, ya que, como es evidente, la recogida individual no es posible. Las heces se analizaron mediante las pruebas de sedimentación [sensibilidad (Se)= 30 EPG] y de flotación (Se= 30 HPG u OPG, ooquistes por gramo de heces), y los resultados se describieron como el número de huevos/ooquistes por gramo de heces. Tras realizar los coprocultivos, se identificaron los nematodos siguiendo claves morfológicas, preparando *pools* de 10 g de heces recogidas al principio del estudio e incubándolos a 25°C durante 18 días. El efecto de la desparasitación se evaluó examinando las heces 14 días después del tratamiento y calculando el porcentaje de reducción del recuento de huevos en heces (FECR). También se estimó este parámetro a lo largo del ensayo, así como el periodo en que las heces no representaban un riesgo de contaminación del suelo (NRFP, *non-risky feces period*), definido como el intervalo (meses) que transcurre desde la última administración de un antihelmíntico con éxito hasta la aparición de huevos de nuevo en las heces. Además, se examinó la posibilidad de que se produjeran daños en el sistema digestivo o respiratorio y en la piel.

Mediante la prueba de McMaster (flotación) se observaron huevos de tricostrongílidos; rara vez se detectaron ooquistes de

Eimeria spp. y huevos de tricúridos (*Trichuris* spp.) a lo largo del ensayo, por lo que estos datos no se incluyeron en los análisis. El examen de los coprocultivos indicó la presencia de larvas de tercer estadio de los géneros *Trichostrongylus*, *Nematodirus*, *Chabertia* y *Haemonchus*.

La eficacia de la desparasitación administrada a lo largo del ensayo osciló entre el 97 y el 100%, sin observar diferencias significativas. Entre marzo y agosto del primer año, los valores de NRFP fueron de 0 a 1 mes para los antílopes (*Antilope cervicapra*), y de 1 mes para las gacelas (*G. cuvieri*), cabras (*Capra aegagrus hircus*), muflones (*Ovis orientalis musimon*), bisontes (*Bison bison*), sitatungas (*T. spekii*) y cobos (*Kobus kob*). A partir de noviembre, estos valores aumentaron a 1-2 meses para antílopes y cabras, y a 3 meses para los demás rumiantes. En este periodo se detectaron valores de HPG de tricostrongílidos superiores a 300 en los Antelopinae (antílopes y gacelas) en cuatro ocasiones (antílopes) y tres (gacelas). Entre noviembre y marzo del año siguiente, los recuentos fueron superiores a 300 HPG en dos ocasiones (antílopes) y en una (gacelas). A partir de ese mes, se registraron valores de huevos de tricostrongílidos en torno a 100-150 HPG. En los Caprinae, el punto de corte de desparasitación (300 HPG) se superó una vez en cabras y dos veces en muflones hasta noviembre del primer año. Desde diciembre hasta el final del ensayo, se mantuvieron valores entre 80 y 120 HPG. Por último, en los Bovinae y Reduncinae se superó el corte en tres ocasiones hasta noviembre del primer año, manteniéndose después entre 50 y 110 hasta el final del estudio. Se observaron diferencias significativas en todas las especies en cautividad, principalmente durante los dos primeros años. No se detectaron efectos adversos en el sistema digestivo o respiratorio en los bóvidos salvajes que recibieron pellets enriquecidos con esporas fúngicas. También se confirmó la ausencia de lesiones cutáneas, mientras que nunca se observó el rechazo a tomar los pellets. Estos resultados ponen de manifiesto el efecto beneficioso que puede obtenerse integrando dos medidas eficaces, la desparasitación eficaz junto con la administración de una mezcla de dos SSF que desarrollan actividad complementaria. El hongo *M. circinelloides* es capaz de penetrar los huevos de helmintos como trematodos y ascáridos y destruirlos, y *D. flagrans* elabora trampas a lo largo del micelio para capturar larvas. También se concluye que, en ausencia de medidas preventivas, los estadios infectantes (L3) que se desarrollan en el suelo entrañan un riesgo casi infinito durante todo el año, especialmente en zonas con climas suaves. Es conveniente recordar que las estrategias que se basan únicamente en la desparasitación dan lugar a la disminución de la eficacia de los parasiticidas, lo que puede conducir al desarrollo de resistencia a los antihelmínticos.

Con el objetivo de obtener información sobre la utilidad de integrar dos medidas, desparasitación eficaz y prevención de la infección, en un programa de control de parásitos en équidos salvajes cautivos, se desarrolló un **tercer ensayo** con individuos de *Equus quagga*, *E. asinus* y *E. africanus asinus* que eliminaban huevos de estróngilos en las heces. Mientras que la cebra y los asnos europeos se alimentan en una pradera, los asnos salvajes africanos se mantuvieron en una parcela arenosa, con hierba sólo en las esquinas. La estrategia consistió en que los équidos se trataron con ivermectina + praziquantel al principio del ensayo, y los équidos ingirieron pellets nutricionales con una mezcla de 10^4 - 10^5 clamidosporas de cada hongo / kg de concentrado, cada dos días durante tres años. Como en los ensayos anteriores, se recogieron heces periódicamente, directamente del suelo en cada parcela, a primera hora de la mañana, antes de la llegada de los visitantes. Las muestras se analizaron mediante las pruebas de sedimentación y flotación (McMaster). Los datos obtenidos se expresaron como recuentos de huevos de estróngilos por gramo de heces (HPG). El efecto de esta estrategia se evaluó mediante el cálculo de la reducción de los recuentos de HPG y de los caballos positivos (PHR). Además, también se estimó el periodo de reaparición de los huevos (ERP) y el tiempo transcurrido desde la desparasitación anterior (TPD).

Los análisis fecales mostraron la presencia de huevos de estróngilos, y rara vez se encontraron huevos de *Trichuris*, sin evidencia de coccidios, trematodos, cestodos o pulmonares. Diez días después de la primera administración de antihelmínticos, los valores de FECR fueron del 100% para los burros europeos, cebras y asnos africanos, con valory los PHR también eran del 100% (ninguno de los équidos eliminaba huevos en las heces). Se registró un ERP de ocho semanas para las cebras y los asnos europeos y de 12 semanas para los asnos africanos. La cinética inicial de huevos en heces llegó a su máximo en diciembre, con valores superiores a 400 EPG y, por tanto, se volvió a administrar antihelmíntico, con eficacia total (FECR= 100%; PHR= 100%). El ERP fue de 12 semanas para las cebras y los asnos europeos y de 16 semanas para los asnos africanos. A pesar de que los recuentos disminuyeron significativamente, aumentaron de nuevo a partir de marzo hasta números superiores a 400 HPG en las heces de todos los équidos, por lo que se consideró la necesidad de un nuevo tratamiento (15 meses después del último tratamiento), con resultados exitosos como lo demuestra que sólo un individuo de los burros europeos y un asno africano continuaban eliminado huevos de estróngilos en las heces, con un ERP de 24 semanas en todos los équidos. Finalmente, se administraron antihelmínticos de nuevo 18 meses después de la última desparasitación, que resultaron completamente eficaces, ya que uno de los burros europeos y uno de los asnos africanos fueron positivos a la prueba de flotación, con un ERP de 28 semanas en todos los équidos cautivos. Considerando las fases entre la aplicación de la desparasitación, los valores medios de HPG se redujeron en un 13-30% en el segundo periodo y en un 34-48% en el tercero. No se detectaron problemsa con la ingestión de pellets, y los équidos tomaron todo el alimento suministrado.

A través del planteamiento y realización de tres ensayos en herbívoros cautivos en un zoológico, en el presente estudio se infiere que el control parasitario conlleva una problemática idéntica a la que se viene denunciando desde hace décadas en el manejo de las especies domésticas, y que básicamente se podría resumir en una excesiva dependencia de la administración de antiparasitarios, y unas medidas preventivas poco efectivas y prácticas. En cuanto a la desparasitación, sería deseable apoyarla en datos recogidos del análisis de diferentes muestras, aunque esto presenta varios problemas. Otra cuestión es que, aunque todavía no se ha verificado, se ha sugerido la posibilidad de que los rumiantes salvajes puedan metabolizar y excretar ciertos benzimidazoles más rápidamente que los domésticos. La evaluación del efecto de la desparasitación se realiza a través de la Reducción del Recuento de Huevos en Heces (FECR), que permite también sospechar acerca de la posibilidad de resistencia a los antihelmínticos. Basándose en que el mantenimiento de grupos testigo separados es prácticamente imposible, la eficacia debe ser calculada en comparación con los valores medios antes del tratamiento. Es posible que en los parques zoológicos que cuentan con grandes extensiones se podría observar un grupo testigo en el mismo ecosistema/área y aislado del grupo tratado; sin embargo, deberían ser necesarios otros requisitos como HPG estadísticamente comparables, lo que lo hace aún más complicado. Una cuestión pendiente se refiere a la falta de directrices exhaustivas para los herbívoros silvestres en cautividad, como las disponibles para los animales domésticos.

El control de los parásitos entre los herbívoros silvestres cautivos en los parques zoológicos debe posibilitar estrategias que comprendan programas de control que incluyan la integración de una desparasitación eficaz (cuando sea necesaria) y medidas adecuadas y útiles para prevenir la infección. En el transcurso de la presente investigación se ha demostrado que la administración oral de una mezcla de SSF con actividad complementaria sobre los parásitos que se encuentran en el medio (ovicida y larvicida) constituye una herramienta muy interesante y útil para evitar que las parcelas con hierba donde se encuentran cautivos los herbívoros silvestres en un parque zoológico puedan alcanzar un elevado nivel de contaminación por estadios infectantes, lo que implicaría la desparasitación frecuente de los animales para asegurar un adecuado estado sanitario. De otro lado, la correcta distribución de las esporas fúngicas puede convertirse en una seria dificultad, debido a la inconveniencia de mantener un estrecho contacto con los cuidadores.

Hay que prestar especial atención a la superficie de la zona de pastoreo donde se alimentan los herbívoros, que determinará el efecto sobre los estadios parasitarios de vida libre, así como el tiempo necesario para conseguir los resultados esperados. Los ensayos realizados en este estudio se han llevado a cabo en parcelas con vegetación de entre 300 y 10000 m^2, y se han recogido resultados satisfactorios a partir de un año como mínimo. Por el momento no se dispone de datos sobre la posibilidad de aplicar esta estrategia a parques zoológicos en los que participen muchas especies de animales salvajes que compartan praderas de mayor tamaño, por lo que los resultados obtenidos en la presente investigación no pueden extrapolarse directamente. La extensión de la zona de pastoreo también afecta a la forma de administrar los SSF, ya que en la experiencia actual, los herbívoros silvestres en cautividad reciben un suplemento nutricional cada dos días, lo que favorece la administración de las clamidosporas fúngicas. Es necesario realizar más investigaciones para tratar de adaptarse a condiciones de cría diferentes a las del presente estudio.

A partir de los resultados obtenidos, se puede concluir que la administración de clamidosporas de *M. circinelloides* y *D. flagrans* cada dos días a herbívoros silvestres en cautividad proporciona un procedimiento seguro para obtener un efecto útil y duradero en la prevención de la infección por nematodos gastrointestinales. Los datos presentados demuestran que esta estrategia puede apoyarse en dos formulaciones orales, en forma de gránulos pulverizados o en forma de gránulos fabricados industrialmente con los hongos, y esta última parece más fácil para garantizar la administración de la dosis adecuada. Se trata de una formulación que proporciona una herramienta novedosa y sostenible para el desarrollo de programas de control integrado de estróngilos que incluyan también la desparasitación antihelmíntica; teniendo en cuenta la falta de un producto comercial disponible, se pueden conseguir efectos muy prácticos y de similar eficacia pulverizando las esporas sobre los pellets justo antes de proceder a la alimentación de los animales. Finalmente, se concluye que un programa que integra la desparasitación con antihelmínticos (bencimidazoles o lactonas macrocíclicas), junto con la administración periódica de clamidosporas de los hongos saprofitos *M. circinelloides* y *D. flagrans*, ofrece una contribución novedosa y útil para el control de parásitos que afectan a herbívoros silvestres en parques zoológicos.

8.3. RESUMO

O presente traballo afonda nas posibilidades de desenvolver o control integrado de helmintos dixestivos en herbívoros cautivos, realizando para iso unha investigación dividida en tres ensaios de campo realizados no Parque Zoológico Marcelle Natureza (Outeiro de Rei, Lugo, España), dentro do convenio de colaboración asinado hai unha década entre este zoolóxico e o Grupo de Investigación COPAR (GI-2120; USC). A intención principal foi propoñer unha solución práctica a un problema que arrástrase dende hai moitos anos, o control dos parasitos que afectan aos herbívoros silvestres en catividade. Dende hai décadas, a desparasitación destes animais ven sendo practicamente a única medida que se ten en conta, e para iso as especies mantidas nos parques zoolóxicos receben fármacos antiparasitarios eficaces e amplamente comercializados para o control dos parasitos do gando. Noutras palabras, o problema do control de parasitos está a manexarse da mesma maneira que nas granxas de animais, o que leva a herdar os mesmos inconvenientes que explican os fracasos que véñense albiscando dende décadas, centrados principalmente na falta de medidas preventivas.

O control dos parasitos non se pode apoiar soamente na desparasitación, debido a que certos parasitos, como tricostronxílidos ou estrónxilos desenvólvense no chan a partir dos ovos que elimínanse nas feces dos animais infectados, ata as fases de larvas de primeiro estadio (L1) que saen e mudan en L2 e finalmente en L3 ou fases infectantes. Por todo isto, considerouse o emprego dunha ferramenta eficaz para previr a infección entre herbívoros cautivos nun parque zoolóxico, que se avaliou en especies silvestres de ruminantes e équidos, articulándose nunha estratexia que comprendeu a utilización dalgunhas cepas de fungos saprófitos do chan illadas previamente a partir de mostras de terra e feces de diferentes animais do citado zoolóxico. Concretamente, utilizouse unha mestura de dous fungos, *Mucor circinelloides*, capaz de adherirse á cuberta, penetrar e tomar o contido interior dos ovos ou quistes (ovicida), e *Duddingtonia flagrans*, que elabora trampas no micelio onde se atrapan as larvas dos nematodos e logo toman o contido do seu interior.

Tendo en conta os datos recollidos anteriormente en animais domésticos, a idea central do presente estudo virou ao redor das posibilidades prácticas de contribuír ao control integrado dos parasitos entre os herbívoros cautivos nun xardín zoolóxico. Para conquerilo, levóuse a cabo a administración oral dunha mestura de esporas, considerando que en investigacións anteriores demostrorase que algúns SSF eran capaces de sobrevivir ao paso polo tracto gastrointestinal e chegar ao exterior coas feces sen perder a súa actividade biolóxica, que neste caso consistía nun efecto antagónico contra os estadios (ooquistes, ovos, larvas) de certos parasitos. No **primeiro ensaio**, o obxectivo foi esclarecer qué formulación era máis adecuada para poder fornecer unha mestura de clamidosporas dos dous SSF mencionados aos bóvidos silvestres (wapitíes) (*Cervus canadensis*) mantidos nunha parcela onde poden pastar todo o día. Dado que no inicio estes ruminantes eliminaban ovos de tricostronxílidos nas feces, viñan recibindo periodicamente fenbendazol, pero á vista dos elevados recontos de ovos por gramo de feces (OPG) que se alcanzaroan varios meses despois do tratamento, a dirección veterinaria do zoo tivo en conta outras estratexias que implicasen a prevención de novas infeccións, cara a limitar o risco de parasitación. Neste senso, formulouse unha mestura de clamidosporas de *M. circinelloides* e *D. flagrans* en dúas presentacións orais, en forma de pellets nutricionais pulverizados xusto antes de fornecerllelos aos wapitíes cada dous días, ou en forma de penso peletizado enriquecido durante a elaboración industrial nunha fábrica comercial, de xeito que o produto final levase 10^4-10^5 esporas de cada un / kg de alimento. Ambos os procedementos avaliáronse sobre mostras fecais tomadas directamente do chan, debido á imposibilidade de recollelas individualmente do recto. Outro punto importante foi que non é posible observar un grupo de control non tratado con idénticas características. As feces analizáronse mediante as probas coprolóxicas de flotación (McMaster) e sedimentación, e os resultados expresáronse como recontos de OPG de tricostronxílidos. Con estes datos estimouse o FECR (porcentaxe de redución do reconto de ovos en feces), considerando como testigos os valores de OPG obtidos en wapitíes que recibiron no primeiro ano só fenbendazol, durante 10 meses (sen clamidosporas). Ademais, prestouse especial atención aos posibles efectos secundarios no tracto dixestivo, aparello respiratorio e á pel. A ausencia de diferenzas significativas en canto aos parámetros climáticos ao longo do estudo (2014 a 2018) levou á consideración conxunta dos datos obtidos en forma dun patrón climático único.

O exame das feces pola sedimentación foi negativo, e coa flotación observáronse ovos de tricostronxílidos nas mostras fecais. Mediante a realización de coprocultivos, as larvas clasificáronse e ordenaron nos xéneros *Cooperia*, *Nematodirus* e *Trichostrongylus*. Debido a que se atoparon de xeito intermitente ooquistes de protozoos (*Eimeria* spp.) e ovos de *Trichuris* sp., estes datos non foran incluídos na presente investigación. Os valores de eliminación de ovos de tricostronxílidos foron superiores a 300 OPG ao comezo do ensaio, e a desparasitación con fenbendazol tivo unha eficacia do 99%. O número de ovos aumentou nas feces ata maio do seguinte ano por riba de 350 OPG. A administración de flubendazol no segundo ano resultou eficaz (FECR= 100%), e malia a administración de pellets pulverizados con clamidosporas dos fungos, os ovos de nematodos volveron aparecer nas feces dos wapitíes dous meses despois do tratamento, manténdose entre 50 e 100 OPG ata o final do ensaio (10 meses). No terceiro ano, a administración de fenbendazol en setembro foi un éxito de novo (FECR= 100%); durante este ano, cando fornecéronse pellets enriquecidos con clamidosporas aos wapitíes, non se observaron ovos de tricostronxílidos ata o cuarto mes despois do tratamento, e obtivéronse recontos inferiores a 50 OPG ata o final do ensaio (xuño). Amosáronse diferenzas significativas entre a produción de ovos de tricostronxílidos no primeiro ano (testigo) e nos dous restantes mediante a proba non paramétrica de Friedman (χ^2= 53,063, P= 0,001). A análise combinada dos resultados obtidos demostrou unha redución do 30-100% (media: 69%) dos recontos de ovos de tricostronxílidos no segundo ano respecto ao primeiro, cun valor medio do 69%; no terceiro ano rexistrouse unha redución do 36-100% (media: 71%). Hai que subliñar a ausencia de consecuencias adversas que afectasen ó tracto dixestivo, ao aparello respiratorio ou á pel mentres se administraban esporas dos fungos parasiticidas aos wapitíes, quen non rexeitaron tomar os pellets nutricionais asperxidos nin os pellets enriquecidos. O fracaso das medidas útiles e prácticas para limitar o risco de infección por tricostronxílidos téntase solucionar aumentando a frecuencia de desparasitación, pero esta estratexia podería evolucionar a eficacias inferiores ás esperadas, e mesmo promover a selección de cepas de parasitos resistentes a antihelmínticos específicos, que poderían ser capace de transmitila. Os resultados recolleitos no presente ensaio mostraron que a administración dunha mestura de clamidosporas de *M. circinelloides* e *D. flagrans* proporciona un elevado efecto beneficioso na diminución da infección por tricostronxílidos en wapitíes mantidos nunha parcela de herba. Compre salientar que as parcelas de pasto son consideradas para que os herbívoros poidan alimentarse, socializar e tamén interrelacionarse co medio, pero nelas concorren as condicións idóneas para que certos nematodos gastrointestinais poidan desenvolver facilmente a fase externa do seu ciclo vital, o que sinala a necesidade de tomar medidas adecuadas para limitar o risco de infección dos herbívoros en pastoreo. No medio, existe un equilibrio natural no que interveñen os hospedadores (mamíferos, principalmente herbívoros), patóxenos e certos microorganismos do solo que actúan coma antagonistas dos patóxenos. Cando o equilibrio altérase (hóspedes inmunodeprimidos, redución de antagonistas), os patóxenos poderían exacerbarse. Os resultados obtidos no presente estudo reforzan a hipótese de que as esporas de *M. circinelloides* e *D. flagrans* reducen o número de estadios infectantes (larvas de terceiro estadio) dos tricostronxílidos no solo, co que diminúe o risco de contaminación na parcela. Compre subliñar que rexistrouse unha eficacia similar mediante as dúas formulacións ensaiadas, aínda que a administración de pellets nutricionais enriquecidos coas clamidosporas fai máis doada a súa distribución entre os herbívoros cautivos, e non se engaden tarefas adicionais aos coidadores dos animais.

Unha vez amosado que a mellor formulación era a dos pellets nutricionais enriquecidos con clamidosporas de ámbolos SSF durante a fabricación industrial (10^4-10^5 esporas de cada un / kg de concentrado), realizouse un **segundo ensaio** en bóvidos salvaxes pertencentes ás subfamilias Antilopinae (*Antilope cervicapra*, *Gazelle*

Cuvieri), Caprinae (*Capra aegagrus hircus*, *Ovis orientalis musimon*), Bovinae (*Bison bison*, *Kobus kob*) e Reduncinae (*Tragelaphus spekii*), mantidas en diferentes parcelas dun zoológico, nas que alimentabanse de trevo vermello (*Trifolium pratense*), raigrás perenne (*Lolium perenne*) e dáctilo (*Dactylis glomerata*). O deseño incluíu a desparasitación dos herbívoros, baseada na presenza de ovos de tricostronxílidos nas feces. Con tal motivo, cando se superaba un limiar de 300 OPG administrábase de novo tratamento antihelmíntico. Tamén se considerou unha medida preventiva, consistente na administración de pellets enriquecidos con clamidosporas, cada dous días durante 3,5 anos.

A avaliación das medidas de control integrado realizouse mediante as análises coprolóxicas das mostras recollidas da parte superior das feces directamente do chan en cada parcela, xa que, como é evidente, a recollida individual é imposible. As feces analizáronse mediante as probas de sedimentación [sensibilidade (Se)= 30 ovos / ooquistes por gramo de feces] e de flotación (Se= 30 ovos / ooquistes por gramo de feces), e os resultados describíronse coma o número de ovos / ooquistes por gramo de feces. Tras realizar os coprocultivos, identificouse o xénero dos nematodos segundo as claves morfolóxicas, preparando *pools* de 10 g de feces tomados ao principio do estudo, que incubáronse a 25°C durante 18 días. O efecto da desparasitación avaliouse examinando as feces 14 días despois do tratamento e calculando a porcentaxe de redución do reconto de ovos en feces (FECR). Tamén se estimou este parámetro ao longo do ensaio, así como o período en que as feces non representaban un risco de contaminación do chan (NRFP, *non-risky feces period*), definido coma o intervalo de tempo (meses) que transcorre dende a última administración dun antihelmíntico eficaz ata a aparición de novo de ovos nas feces. Ademais, examinouse a posibilidade de que se producisen danos no aparato dixestivo ou respiratorio e na pel.

Mediante a proba de McMaster (flotación), observáronse ovos de tricostronxílidos; de cando en cando detectáronse ooquistes de *Eimeria* spp. e ovos de tricúridos (*Trichuris* spp.) ao longo do ensaio, polo que estes datos non se incluíron nas análises. O exame dos coprocultivos indicou a presenza de larvas de terceiro estadio (L3) dos xéneros *Trichostrongylus*, *Nematodirus*, *Chabertia* e *Haemonchus*.

A eficacia da desparasitación administrada ao longo do ensaio oscilou entre o 97 e o 100% sen observar diferenzas significativas. Entre marzo e agosto do primeiro ano, os valores de NRFP foron de 0 a 1 mes para os antílopes (*Antilope cervicapra*), e de 1 mes para as gacelas (*G. cuvieri*), cabras (*Capra aegagrus hircus*), muflóns (*Ovis orientalis musimon*), bisontes (*Bison bison*), sitatungas (*T. spekii*) e cobos de auga (*Kobus kob*). Desde novembro, estes valores aumentaron a 1-2 meses para antílopes e cabras, e a 3 meses para os demais ruminantes. Ata novembro do primeiro ano, detectáronse valores de OPG de tricostronxílidos nos Antelopinae (antílopes e gacelas) superiores a 300 OPG en catro ocasións (antílopes) e tres (gacelas). Entre novembro e marzo, atopáronse recontos superiores a 300 OPG en dúas ocasións (antílopes) e nunha (gacelas). A partir dese mes, rexistráronse recontos de ovos de tricostronxílidos darredor de 100-150 OPG. Nos ruminantes da subfamilia Caprinae, o punto de corte de desparasitación (300 OPG) superouse unha vez en cabras e dúas veces en muflóns ata novembro. Desde decembro do primeiro ano ata o final do ensaio, os valores fecais de OPGs mantivéronse en 80-120. Por último, nos Bovinae e Reduncinae superouse o corte en tres ocasiones ata novembro no primeiro ano, manténdose despois entre 50 e 110 ata o final do estudo. Observáronse diferenzas significativas en todas as especies en catividade, principalmente durante os dous primeiros anos. Non se detectaron efectos adversos no aparato dixestivo ou respiratorio nos bóvidos salvaxes que recibiron pellets enriquecidos con esporas fúnxicas. Tamén se confirmou a ausencia de lesións cutáneas, mentres que nunca se observou rexeitamento para tomar os pellets. Estes resultados poñen de manifesto o efecto beneficioso que pode obterse integrando dúas medidas eficaces, a desparasitación eficaz xunto coa administración dunha mestura de dous SSF con actividade complementaria. Tamén se conclúe que, en ausencia de medidas preventivas, os estadios infectantes (L3) que se desenvolven no solo garanten un risco case infinito de infección durante todo o ano, especialmente en zonas con climas suaves.

Co galo de obter información sobre a utilidade de integrar dúas medidas, a desparasitación eficaz e a prevención da infección, nun programa de control de parasitos en équidos salvaxes cativos, desenvolveuse un **terceiro ensaio** con individuos de *Equus quagga*, *E. asinus* e *E. africanus asinus* que elminaban ovos de estrónxilos nas feces. Mentres que as cebras e os asnos europeos aliméntanse nunha pradería, os asnos salvaxes africanos mantivéronse nunha zona arenosa, onde a herba só está presente nas esquinas. A estratexia consistiu en que os équidos foron tratados con ivermectina + praziquantel ao principio do ensaio, e os équidos recibiron pellets nutricionais cunha mestura de 10^4 - 10^5 clamidosporas de cada fungo / kg de concentrado, cada dous días durante tres anos. Como nos ensaios previos, recolléronse mostras fecais periodicamente, directamente do chan en cada parcela, á primeira hora da mañá, antes da chegada dos visitantes. As mostras analizáronse mediante as probas de sedimentación e flotación (McMaster), e os datos obtidos expresáronse como recontos de ovos de estrónxilos por gramo de feces (OPG). O efecto da estratexia de control dos parasitos avaliouse mediante o cálculo da redución dos OPG e dos cabalos positivos (PHR). Ademais, tamén se estimou o período de reaparición dos ovos (ERP) e o tempo transcorrido dende a desparasitación anterior (TPD).

As análises fecais mostraron a presenza de ovos de estrónxilos, e de cando en cando atopáronse ovos de *Trichuris*, sen evidencia de coccidios, trematodos, cestodos ou vermes pulmonares. Dez días despois da primeira administración de antihelmínticos, os valores de FECR foron do 100% para os burros europeos, as cebras e os asnos salvaxes africanos, e os PHR tamén foron do 100% (ningún dos équidos eliminaba ovos nas feces). Rexistrouse un ERP de oito semanas para cebras e asnos europeos, e de 12 semanas para os asnos africanos. A cinética inicial de ovos nas feces chegou ao seu punto máximo en decembro do segundo ano, cando se alcanzaron valores superiores a 400 OPG e, por tanto, volveuse administrar o tratamento antihelmíntico, con plena eficacia (FECR= 100%; PHR= 100%). O ERP foi de 12 semanas para as cebras e asnos europeos, e de 16 semanas para os asnos africanos. A pesar de que os recontos diminuíron significativamente, rexistrouse un novo incremento, cando se observaron números superiores a 400 OPG nas feces de todos os équidos, polo que se considerou a necesidade dun novo tratamento (15 meses despois da última desparasitación), con resultados exitosos como o demostra que só un individuo dos burros europeos e outro dos africanos permaneceron eliminando ovos de estrónxilos polas feces, e o ERP en todos os équidos foi de 24 semanas. Finalmente, administráronse antihelmínticos 18 meses despois da última desparasitación, sendo totalmente efectivos, xa que un dos burros europeos e un dos asnos africanos foron positivos, cun ERP de 28 semanas en todos os équidos cautivos. Considerando as fases entre a aplicación da desparasitación, os valores medios de OPG reducíronse nun 13-30% no segundo período e nun 34-48% no terceiro. A inxestión de pellets foi completamente aceptada, e os équidos tomaron todo o alimento fornecido.

A través da formulación e realización de tres ensaios en herbívoros silvestres cautivos nun zoolóxico, no presente estudo infírese que o control parasitario conleva unha problemática idéntica á que vénse denunciando no manexo das especies domésticas dende hai décadas, e que basicamente se podería resumir nunha excesiva dependencia da administración de antiparasitarios, xunto cunhas medidas preventivas pouco efectivas e prácticas que non son factibles. En canto á desparasitación, sería desexable apoiala en datos colleitados na análise de diferentes mostras, inda que isto presenta varios atrancos.

Outra cuestión é que, malia que aínda non se verificou, suxeriuse a posibilidade de que os ruminantes salvaxes poidan metabolizar e excretar certos benzimidazoles máis rapidamente que os domésticos. A avaliación do efecto da desparasitación realízase a través da Redución do Reconto de Ovos en Materia Fecal (FECR), que permite tamén sospeitar da posibilidade de resistencia aos antihelmínticos. Baseándose no mantemento de grupos testigo separados é practicamente imposible, a eficacia debe ser calculada en comparación coa media de OPG antes do tratamento. É posible que, nos parques zoolóxicos moi grandes, poderíase observar un grupo testigo no mesmo ecosistema/área e mantido illado do grupo tratado; con todo, deberían ser necesarios outros requisitos coma valores de OPG estatísticamente comparables, o que complica inda máis o problema. Unha cuestión pendente refírese á falta de directrices exhaustivas para a avaliación de antiparasitarios nos herbívoros silvestres en cautividade, como as dispoñibles para os animais domésticos.

O control dos parasitos entre os herbívoros silvestres cautivos en zoos debe implementar estratexias que comprendan programas de control que inclúan a integración dunha desparasitación eficaz (cando sexa necesaria) e medidas adecuadas e útiles para previr a infección. No transcurso da presente investigación demostrouse que a administración oral dunha mestura de SSF con actividade complementaria sobre os parasitos no medio (ovicida e larvicida) constitúe unha ferramenta moi interesante e útil para evitar que as parcelas con vexetación onde se manteñen os herbívoros silvestres nun parque zoolóxico poidan alcanzar un elevado nivel de contaminación por estadios infectantes, o que implicaría que os animais fosen desparasitados con frecuencia para asegurar o seu adecuado estado sanitario. A correcta distribución de esporas fúnxicas pode converterse nunha seria dificultade, debido á inconveniencia de manter un estreito contacto cos coidadores.

Compre prestar especial atención á superficie da zona de pastoreo onde se alimentan os herbívoros, xa que isto determinará o efecto sobre os estadios parasitarios de vida libre, así como o tempo necesario para conseguir os resultados agardados. Os ensaios realizados neste estudo leváronse a cabo en parcelas de entre 300 e 10000 m^2, con herba, e recolléronse resultados satisfactorios a partir dun ano como mínimo. Polo momento non se dispón de datos sobre a posibilidade de aplicar esta estratexia a parques zoolóxicos nos que participen moitas especies de animais salvaxes que compartan pradarías de maior tamaño, polo que os resultados obtidos na presente investigación non poden extrapolarse directamente. A extensión da zona de pastoreo tamén afecta á forma de administrar os SSF, xa que, segundo a experiencia actual, os herbívoros silvestres en cautividade reciben un suplemento nutricional cada dous días, o que favorece a administración das clamidosporas. É necesario realizar máis investigacións para tratar de adaptarse a condicións de cría diferentes ás do presente estudo.

A partir dos resultados obtidos, pódese concluír que a administración de clamidosporas de *M. circinelloides* e *D. flagrans* cada dous días a herbívoros silvestres en cautividade proporciona un procedemento seguro e san para conquerir un efecto útil e duradeiro co obxecto de evitar a infección por nematodos gastrointestinais. Os datos presentados demostran que esta estratexia pode apoiarse en dúas formulacións orais, en forma de gránulos nos que se pulverizaron previamente as esporas, ou en forma de gránulos fabricados industrialmente cos fungos. Esta última parece a formulación máis axeitada para garantir a administración da dose adecuada, e proporciona unha ferramenta nova e sostible para o desenvolvemento de programas de control integrado de estrónxilos que inclúan tamén a desparasitación con antihelmínticos, pero tendo en conta a falta dun produto comercial dispoñible para estas especies, pódense conseguir efectos moi prácticos e de similar éxito pulverizando as esporas sobre os pellets xusto antes de alimentar aos animais. Finalmente, conclúese que un programa que integra a desparasitación con antihelmínticos (bencimidazoles ou lactonas macrocíclicas), xunto coa administración periódica de clamidosporas dos fungos saprofitos *M. circinelloides* e *D. flagrans*, supón unha contribución nova e útil para o control de parasitos que afectan a herbívoros silvestres en parques zoolóxicos.

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The possibilities of developing the integrated control of digestive helminths in captive herbivores was investigated in a study divided into three field trials conducted in the Marcelle Natureza Zoological Park (Outeiro de Rei, Lugo, Spain). For the purpose to prevent their infection by certain helminths, chlamydospores of two soil saprophytic fungi (SSF) were administered by using different edible formulations. From the results obtained, it was concluded that a program that integrates deworming with anthelmintics (benzimidazoles or macrocyclic lactones), together with the periodic administration of chlamydospores of the saprophytic fungi M. circinelloides and D. flagrans, offers a novel and useful contribution to the control of parasites affecting wild herbivores in zoos.