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Influence of seasonality and biological activity on infection by helminths in Cantabrian bear

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ABSTRACT

This study aimed to investigate the variations of parasites in the feces of brown bears *Ursus arctos* inhabiting the Cantabrian Mountains (NW Spain). A total of 248 bear fecal samples were collected throughout one year, spanning from August 2018 to September 2019, at an approximate frequency of 20 samples per month. The results were analyzed in relation to both the season and the biological activity of the brown bears, i.e., hibernation, mating and hyperphagia. Among the examined samples, eggs of *Dicrocoelium dendriticum* (32.2%; 95% Confidence Interval: 26.4–38.1), *Baylisascaris* sp. (44.8%; 38.5–50.9), ancylostomatids (probably belonging to *Uncinaria* spp.) (16.5%; 11.9–21.1) and *Trichuris* sp. (1.2%; 0–2.6) were observed. Significant seasonal differences were noted for *Baylisascaris* and ancylostomatids ($\chi^2 = 21.02$, $P = 0.001$ and $\chi^2 = 34.41$, $P = 0.001$, respectively). Furthermore, the presence of helminth eggs was correlated with the activity phase of the brown bears. *Dicrocoelium* attained the highest prevalence during the mating phase, while *Baylisascaris* and ancylostomatids were more frequent during hyperphagia. Notably, the highest egg-output counts for *Dicrocoelium* and *Baylisascaris* sp. were recorded during the mating phase and hibernation, respectively, whereas ancylostomatids eggs peaked during hyperphagia. Additionally, variations in egg-output counts were significant for all helminths concerning the season, with the exception of *Trichuris* sp., and for *Dicrocoelium* and *Baylisascaris* sp. According to bear activity. It is concluded that infection by gastrointestinal helminths depends on the season and the biological activity of the bears from the Cantabrian Mountains, and their health status could result influenced.

1. Introduction

The brown bear (*Ursus arctos*) is a terrestrial mammal belonging to the Ursidae family, and it has a wide distribution across northern Europe, Asia, and North America, with several small and isolated populations occurring in southern and western Europe. Notable examples of these populations include the Alpine and Abruzzo in Italy, the Cantabrian in Spain, and the Pyrenean shared between Spain and France (Swenson et al., 2021). Specifically, the Cantabrian Mountains in Spain host one of these small populations of brown bears. According to the most recent results of the genetic census, approximately 370 individuals were detected in this area. Among them, the western subpopulation comprises about 250 bears, which is a higher number compared to the eastern subpopulation with approximately 120 bears.

It is well known that certain parasites can profoundly impact the health and welfare of wild animals (Mackenstedt et al., 2015). Furthermore, they have been identified as potential vectors of several veterinary and zoonotic diseases, capable of infecting both animals and

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humans (Paoletti et al., 2017; Swenson et al., 2021). Despite the significant implications of parasites, there remains a scarcity of information regarding their distribution in wild European brown bear populations. Previous studies have reported infections by various parasites, including flukes, tapeworms and nematodes (primarily roundworms and hookworms) (Borecka et al., 2013 Aghazadeh et al., 2015; Orosová et al., 2016; Borka-Vitális et al., 2017; Paoletti et al., 2017). Specifically, studies on Cantabrian brown bears are limited to (1) the analysis of 38 fecal samples, which revealed the presence of protozoa (*Giardia* spp. And different coccidia), cestodes (*Diphyllobothrium latum*, *Taenia* spp) and nematodes (ancylostomids and ascarids) (Martín et al., 2008); and to (2) the observation of eggs of small liver trematodes (*Dicrocoelium dendriticum*) and *Trichuris* sp. in 18 samples of brown bear feces collected during May–August (Costa et al., 2022).

The primary objective of this research was to enhance the understanding of brown bear parasite infection within the small and endangered population of the Cantabrian Mountains throughout the entire year. By collecting and analyzing fecal samples over an annual cycle, the study aimed to identify seasonal variations in parasite prevalence and intensity among the bears. Such information is crucial for understanding the health risks posed to the bear population and devising appropriate conservation strategies to safeguard their well-being and the health of the surrounding ecosystem.

2. Materials and methods

2.1. Study area

The area of study is located in north-western Spain and encompasses approximately 3300 km^2. It includes the Principality of Asturias and the northern part of the province of León (Fig. 1). The climate in the region is characterized as oceanic, with a predominantly continental influence. The southern slopes of the area tend to be drier, while the northern ones have a temperate and humid climate. The landscape of the study area is primarily composed by forests, shrublands (such as broom *Cytisus* sp. And heather (*Erica* sp., *Calluna* sp.) and farmland. The forests on the southern slopes are mainly comprised of semi-deciduous and evergreen oak species (*Quercus* sp.), while the northern slopes feature deciduous forests with species like *Fagus sylvatica*, *Q. robur*, *Q. petraea* and *Betula* sp., which are more abundant. Above the tree line, berry shrubs such as bilberries (*Vaccinium myrtillus*) can be found in the region (Pato and Obeso 2012).

2.2. Sample collection

Between August 2018 and September 2019, a total of 248 brown bear fecal samples were taken in the western core of the Cantabrian Mountains (Fig. 1). The collection of fecal samples was carried out by experienced researchers and agents of the Bear Patrol (Patrulla Oso) of the Principality of Asturias during their regular working days, e.g., during bear monitoring and assessment of bear damages. The samples were recognized by their distinctive size and shape. Additionally, some of the samples were collected by the authors of the study. For each fecal sample, a 100 mL plastic container was used for preservation, the containers were labeled with the date of collection, UTM coordinates of the place, and the name of the collector. The samples were stored at a temperature of 5 °C until they were shipped for coprological analysis, conducted at the laboratory of the COPAR Research Group (Faculty of Veterinary, University of Santiago de Compostela, Spain). Following the examination, the samples were stored at a temperature of −20 °C for future reference and potential additional investigations.

2.3. Coprological examinations

The fecal samples were analyzed by flotation, sedimentation and larval migration tests, and the results were expressed as egg counts per gram of feces (EPG) and larvae per gram of feces (LPG). For the detection of protozoan oocysts and eggs of cestodes and gastrointestinal nematodes, the flotation technique consisted of homogenizing 4 g of feces in 41 mL of water and then the solution passed through a 150-μm pore diameter sieve. The filtrate was divided into two 15 mL test tubes and centrifuged at 2000 rpm for 10 min. After removing the supernatant, the sediment was resuspended in NaCl solution ($\rho = 1.20$) for egg counting in a McMaster chamber under an optical microscope at 10x magnification (Voinot et al., 2020, 2021). The sedimentation technique was applied for the observation of trematode eggs, and involved that 3 g of feces were homogenized in water and passed through a 150 μm pore diameter mesh. The fecal material was then decanted successively, and the analysis was conducted in a McMaster chamber (Voinot et al., 2020). Finally, the larval migration technique was used for the detection of broncopulmonary larvae (Carrau et al., 2021); a total of 10 g of feces were placed on filter paper in a funnel connected to a test tube. Water was added to submerge the feces, and after 24 h, the contents were collected in tubes and centrifuged at 2000 rpm for 10 min. The supernatant was removed using a vacuum tube, and 2 mL of the remaining sediment were deposited in a Favati chamber for larval counting (Voinot et al., 2021).

2.4. Data management

The results obtained from the fecal analyses were presented in terms of the average numbers of eggs per gram ± SD. Prevalence values were expressed as percentages along with the corresponding 95% Confidence Interval (95% CI). Data distribution was first examined by the

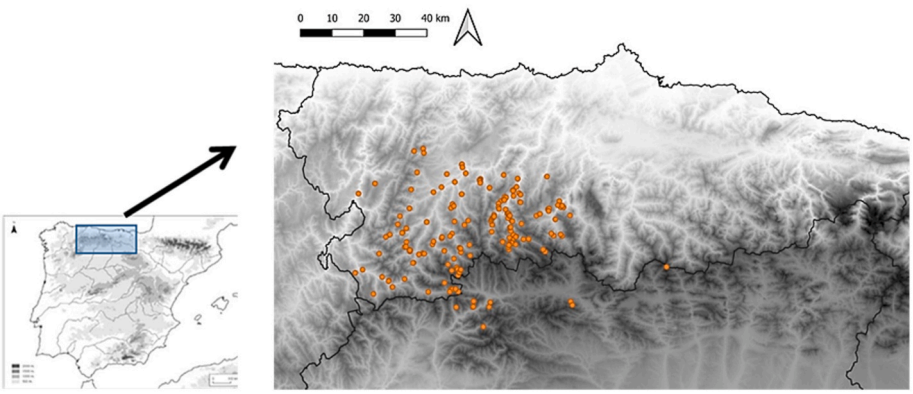

Fig. 1. Distribution of the sampling of brown bear feces (n = 248) in the western part of the Cantabrian Mountains (Asturias and León provinces, Spain). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Kolmogorov-Smirnov test, and because they did not follow a normal distribution (Z values < 0.05), non-parametric statistical tests were used for the analysis. The Kruskal-Wallis and the Mann-Whitney U-tests were performed at a significance level of $P < 0.05$. All tests were carried out using the statistical package SPSS, version 20 (IBM SPSS Inc., Chicago, IL, USA).

The data collected from the fecal analyses were organized and examined based on the season (Spring, Summer, Autumn, and Winter) and the specific bear activity periods, which were defined as hibernation (January to mid-April), mating (mid-April to June) and hyperphagia (July to December) (Martínez Cano et al., 2016).

3. Results

3.1. Parasitological analysis

The overall percentage of fecal samples that tested positive for the presence of parasites in the brown bear population was 64.1% (95% Confidence Interval = 58.1–70.1). Among the identified parasites, *D. dendriticum* was found in 32.2% of the samples (95% CI = 26.4–38.1), *Baylisascaris* sp. in 44.8% (95% CI = 38.5–50.9), ancylostomatids (most probably belonging to *Uncinaria* spp.) in 16.5% (95% CI = 11.9–21.1), and *Trichuris* sp. in 1.2% (95% CI = 0–2.6). Oocysts of *Eimeria* spp. were rarely observed and thus not considered in the analysis.

Regarding the association of parasites, 30.8% of the samples presented eggs of both groups of helminths (95% CI = 25.3–36.8), 19.5% of the fecal samples contained trematodes only (95% CI = 14.4–24.3), and 49.7% had nematodes only (95% CI = 43.4–55.8).

3.2. Seasonal variations

The prevalence of fecal samples positive to the coprological tests varied across the seasons. The lowest prevalence was observed in spring at 55.5% (95% CI = 39.5–71.8), and the highest prevalence was recorded in autumn at 75.4% (95% CI = 64.9–85.8). However, the difference in prevalence among seasons was not significant ($\chi^2 = 5.26$, $P = 0.15$). The prevalence of eggs of *Dicrocoelium* in feces increased from winter to spring, and then decreased, but this trend was not significant ($\chi^2 = 3.694$, $P = 0.296$) (Fig. 2). On the other hand, the percentages of fecal samples containing eggs of *Baylisascaris* sp. Or ancylostomatids decreased between winter and spring but significantly increased to reach the highest values in autumn ($\chi^2 = 21.029$, $P = 0.001$ and $\chi^2 = 34.410$, $P = 0.001$, respectively). Eggs of *Trichuris* sp. were observed in low percentages of feces (≈3%) in spring and autumn only, and the difference in prevalence between seasons was not significant ($\chi^2 = 2.736$, $P = 0.43$).

The fecal egg counts for *Dicrocoelium* EPG remained consistently low (<150 EPG) during all four seasons (Fig. 3). However, there was a significant increase in the EPG values from summer to spring ($\chi^2 = 8.840$, $P = 0.032$). The numbers of *Baylisascaris* sp. EPG increased during summer and peaked in winter, but then decreased in spring ($\chi^2 = 18.79$, $P = 0.001$). The highest counts of eggs of ancylostomatids were observed in the fall and subsequently decreased ($\chi^2 = 31.280$, $P = 0.001$). On the other hand, low levels of *Trichuris* sp. EPG were recorded throughout the study, without significant differences related to the seasons ($\chi^2 = 2.050$, $P = 0.560$).

3.3. Influence of bear activity on parasites

The percentages of bear feces testing positive for the presence of helminths showed only slight variations across the different bear activity phases. During hibernation, 56.5% of the samples were positive for helminths, while during the mating period, 57.1% of samples were positive, and in the hyperphagia phase, 66% of samples were positive. However, these differences were not significant ($\chi^2 = 1.46$, $P = 0.48$; Fig. 4). During hibernation, the percentages of bear feces positive for *Dicrocoelium*, *Baylisascaris*, and ancylostomatids were all lower than 30%. In the mating phase, half of the samples had eggs of small liver flukes, whereas *Baylisascaris* or ancylostomatids were detected in less than 15% fecal samples. During the hyperphagia period, half of the samples contained eggs of *Baylisascaris* sp., while the numbers of samples with *Dicrocoelium* reduced, and those with ancylostomatids increased slightly. Eggs of *Trichuris* sp. were identified in this period only. Significant differences were observed for the *Baylisascaris* sp. EPG across the bear activity phases ($\chi^2 = 15.23$, $P = 0.001$).

The analysis of feces related to the hibernation period revealed the highest counts of *Baylisascaris* sp. EPG throughout the study (Fig. 5), whereas levels lower than 200 EPG were observed for *D. dendriticum* and ancylostomatids.

During the mating phase, a slight increase in the counts of *Dicrocoelium* EPG was recorded, while a reduction was observed in those of *Baylisascaris* sp. And ancylostomatids. Additionally, the levels of *Baylisascaris* sp. (reaching values near to 500) and ancylostomatids (≈176) EPG increased during the hyperphagia period, with values around 2 ± 6 EPG *Trichuris* sp. Significant differences were observed for the numbers of EPG of *Dicrocoelium* ($\chi^2 = 8.74$, $P = 0.01$) and *Baylisascaris* sp. ($\chi^2 = 14.49$, $P = 0.001$).

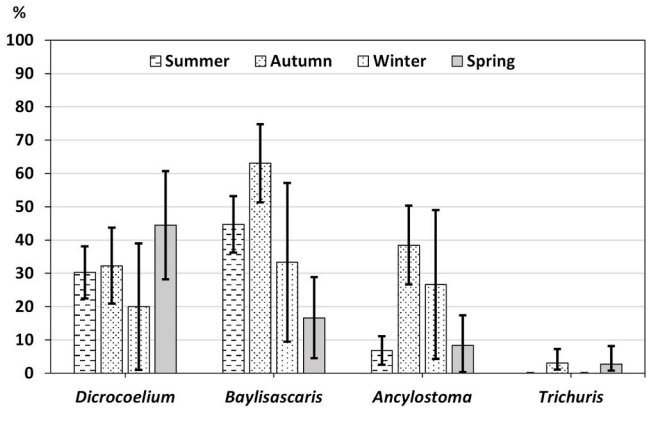

Fig. 2. Seasonal variations in the prevalence of helminth infection in feces of brown bears ($n = 248$) from the western part of the Cantabrian Mountains (Asturias and León provinces, Spain). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

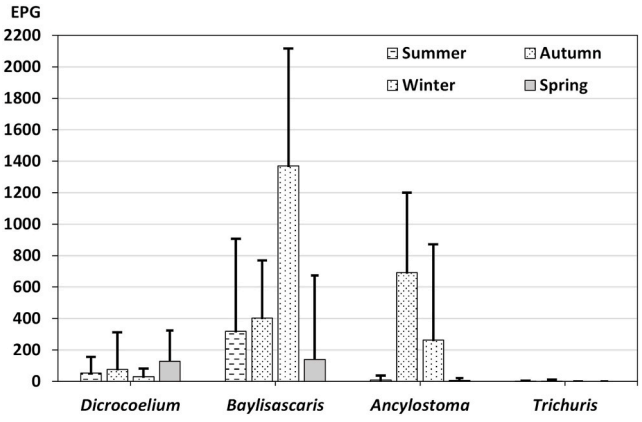

Fig. 3. Seasonal dynamics of helminths egg-output in Cantabrian brown bears ($n = 248$) (Asturias and León provinces, Spain). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

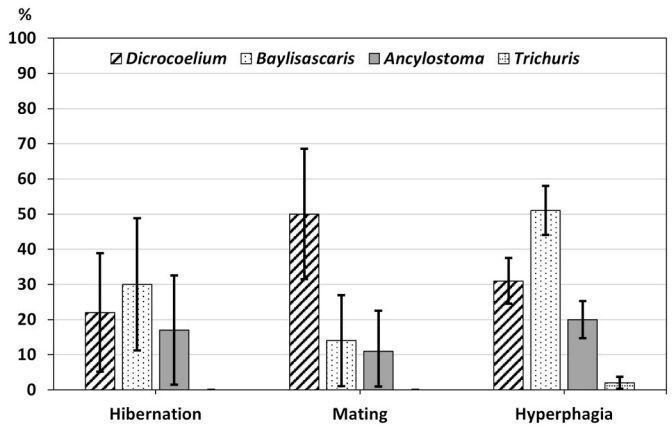

Fig. 4. Seasonal variations in the prevalence of helminth infection in feces of brown bears (n = 248) from Cantabrian Mountains (Spain) according to their activity periods. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

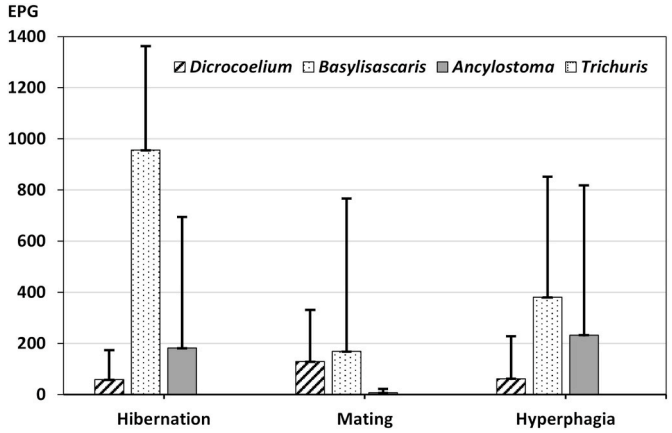

Fig. 5. Seasonal kinetics of helminths egg-output in Cantabrian brown bears (n = 248) according to their activity periods. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

The analysis of feces from Cantabrian brown bears in the current study revealed the presence of both hepatic and intestinal endoparasites. Specifically, the identified parasites included *Dicrocoelium dendriticum*, *Baylisascaris* sp., ancylostomatids, and *Trichuris* sp. Comparing the findings of this study to prior research, it seems that similar endoparasites have been detected in brown bears from other regions. Previous studies based on a limited number of samples (feces or intestinal content) from brown bears in Croatia, Poland and Slovakia reported infections by *Baylisascaris* sp., *Toxascaris (= Baylisascaris) transfuga*, *Ancylostoma* sp. And *Capillaria* sp. (Goldová et al., 2003; Borka-Vitális et al., 2017; Aghazadeh et al., 2015; Orosová et al., 2016; Borka-Vitális et al., 2017; Štrkolcová et al., 2018). In a recent coprological survey conducted in spring involving 14 samples of bears from the Cantabrian Mountains, the presence of eggs of *D. dendriticum* and *Trichuris* sp. was detected (Costa et al., 2022).

The liver fluke *D. dendriticum*, which is not commonly observed in feces of large carnivores, was found to be relatively abundant in the brown bear population of the Cantabrian Mountains, as evidenced by both the present study (32% of prevalence, with the highest values in spring) and Costa et al. (2022; 71% of prevalence in spring). In another study conducted on brown bears in Croatia, gall bladder examination revealed that trematodes were present in 60% of the bears (n = 136), with significantly higher percentages observed in autumn and lower percentages in spring (Reljić et al., 2017). The eggs of the trematode *D. dendriticum* released in the feces can resist freezing temperatures, but dryness and high temperatures are responsible for their destruction (Manga-González et al., 2007; Sandoval et al., 2013). The transmission involves two intermediate hosts: firstly, the eggs of the trematode are ingested by land snails, where they develop into cercariae. The cercariae are then taken by ants, which serve as the second intermediate host, and the cercariae transform into metacercariae. Infection occurs when ants harboring metacercariae are ingested, completing the life cycle of *D. dendriticum* (Manga-González et al., 2007). The seasonal availability of ants as an important food source for brown bears, along with their hibernation behavior in winter, may play a significant role in determining the risk of brown bear infection by the liver fluke *D. dendriticum*. Ants are known to provide high nutritional value to bears and are likely to be an attractive food source when available. However, during winter, when ants typically hibernate, the risk of bear infection should be limited as there are fewer opportunities for bears to consume infected ants. The current study's findings showing maximal levels of *D. dendriticum* during spring could be explained by bears ingested infected ants in early spring, when ants become active again, and in winter. Due to ants hibernate and are less active during winter, one possibility could rely on they did not hibernate, or more likely that the bears took ants from their nests, but these hypotheses need further investigation. This information is crucial for understanding the dynamics of *D. dendriticum* infections in brown bears because, to our knowledge, there are still limited data on bear infections by this trematode. Most of the existing research has been conducted on small ruminants (such as sheep), with a prepatent period (the time between infection and egg output) of 2–4 months. These studies have reported maximal egg-output in winter which is a period of low temperatures when the development and survival of the parasite's eggs are favored, and the lowest egg-output in summer (Manga-González, 1987).

In the present study, eggs of *Baylisascaris* sp. were detected in 45% of the samples, and ancylostomatids in 41% of the samples, while *Trichuris* was found in lower levels, which is consistent with the findings reported by Costa et al. (2022). Prior studies conducted on European bear populations have also reported infections by *Baylisascaris* spp. (Major et al., 2009; Orosová et al., 2016; Štrkolcová et al., 2018), which increases from spring to autumn (Molnár et al., 2020).

Indeed, all the nematodes identified in the feces of brown bears, i.e., *Baylisascaris* sp., *B. transfuga*, ancylostomatids, *Capillaria* sp. And *Trichuris* sp., have free-living phases that develop in the soil until they reach their infective stages. Since these endoparasite eggs have been detected in bear feces in all seasons, it suggests that bears can potentially become infected with these nematodes throughout most of the year.

Baylisascaris spp and ancylostomatids are nematodes that can release thousands of eggs per gram of feces, leading to high levels of soil contamination. These nematodes have different life cycles that contribute to their ability to persist in the environment and remain infective for extended periods. For *Bayliscaris* spp, the eggs are highly resistant to unfavorable conditions, allowing them to remain infective in the soil for years. The eggs contain second-stage larvae (L2) inside, and individuals can become infected by ingesting eggs containing these L2 larvae. The high resistance of *Bayliscaris* eggs to environmental conditions enables them to persist as a potential source of infection for hosts, including brown bears (Bauer, 2012). Similarly, ancylostomatids also contribute to soil contamination with its high egg output. Non-embryoanted eggs of ancylostomatids are passed in feces and embryonate in the soil. Then the first-stage larva (L1) hatches and moults to L2, which develop to L3 stage, the infective form (Catalano et al., 2015; Kilinç et al., 2015). The seasonal variations in the prevalence of feces with eggs of these nematodes, with the lowest occurrence in winter and increasing from spring to autumn, are consistent with observations in Slovakia (Orosová et al., 2016; Molnár et al., 2020). It is important to

note that in the current investigation, helminths were detected in feces of bears by means of the McMaster flotation test, a procedure with known weaknesses as sensitivity or species-identification) (Bugmyrin et al., 2017). Accordingly, molecular techniques are indicated to accurately identify the species, such as *B. transfuga* (De Ambrogi et al., 2011; Sapp et al., 2017). These techniques would be useful to differentiate the different species belonging to *Dicrocoelium* genus also (Manga-González and Ferreras, 2019).

Seasonal differences in the presence of helminths in bear feces have been linked to their physiological activity, with a higher prevalence of endoparasite infection recorded during the hyperphagia phase (summer and fall). This increase in prevalence during hyperphagia is attributed to the bears' heightened ingestion, which can be 2–3 times more than during other periods (Orosová et al., 2016). The lower prevalence of endoparasites during the hibernation period is mainly attributed to adult stages being eliminated before this phase (Gau, 1999; Goldová et al., 2003; Molnár et al., 2020), or to the possibility that adult parasites die during denning and are eliminated in the first spring feces (Finnegan, 2009). This possibility could explain the seasonal variations in nematodes, where prevalence and egg dynamics increase starting from spring, peaking in winter for *Baylisascaris* sp. Or autumn for ancylostomatids. Conversely, trematodes reduced from spring to winter. These differences in helminth species might be explained based on the location of adult stages in the final host. Ascarids, hookworms and whipworms localize in the intestine and, therefore, seem to be easily expelled with the feces into the environment, contrary to what is expected for small liver flukes inhabiting the hepatic bile ducts (Hendrix and Robinson, 2012). Additionally, the highest prevalence of endoparasites occurs in winter when hibernation does not take place or extends over a short period of time, as bears continue to take food during that period (Orosová et al., 2016).

The phenomenon of "self-curation" has been observed in some livestock species, characterized by a decrease in the fecal egg counts of certain nematodes, mainly trichostrongylids, before the spontaneous elimination of adults from the gut and after the ingestion of new infective stages during a heavy challenge infection (Foster & Elsheikha, 2012; Dever et al., 2015). However, the exact mechanisms underlying this phenomenon are not fully understood. It is unclear whether self-curation is primarily due to innate immunity or involves specific immunity processes. Research has shown that multiple mechanisms may be implicated in the self-curation process. These mechanisms include an increase in peripheral eosinophilia (elevated eosinophils in the blood), an increment in degranulation of mast cell proteases in the small intestine, and an upregulation in the expression of Th2 cytokine genes, which are involved in immune responses. Interestingly, antibodies have not been found to play a significant role in this process (Garza, 2014).

The potential role of bears in the transmission of parasites to humans has been discussed, with trichinellosis being the most well-known concern related to brown bears (Swenson et al., 2021). However, the data acquired from the present coprological survey indicate a very low risk of brown bears transmitting parasites to humans. Although all *Baylisascaris* species are potentially etiological agents of larva migrans syndrome, the ones predominantly associated with wild carnivores are those that affect raccoons, such as *B. procyonis* and *B. columnaris* (Molnár et al., 2020). Infection by *B. transfuga* in brown bears is often asymptomatic, but heavy infection could cause illness or even death, or severe larva migrans syndrome among accidental or paratenic hosts (Testini et al., 2011). Similarly, hookworms can cause several clinical signs in mammals, such as bloody feces, anorexia and weight loss. In accidental hosts, hookworms can also lead to larva migrans syndrome (Kilinç et al., 2015; Catalano et al., 2015). Overall, the risk of brown bears transmitting parasites to humans is considered to be low based on the findings of the coprological survey. However, it is essential to remain vigilant and take appropriate precautions when interacting with wildlife to minimize any potential risks of zoonotic infections. It is concluded that infection by gastrointestinal helminths depends on the season and the biological activity of the bears from the Cantabrian Mountains, and their health status could result influenced.

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Author contributions

Esther Valderrábano Cano: Conceptualization, Data curation, Investigation, Writing - Original Draft; **Vincenzo Penteriani:** Conceptualization, Formal analysis, Writing - Review & Editing, Project administration, Funding acquisition; **Iris Vega:** Resources, Validation, Investigation, Writing - Review & Editing; **María del Mar Delgado:** Visualization, Validation, Writing - Review & Editing; **Enrique González-Bernardo:** Methodology, Visualization, Writing - Review & Editing; **Giulia Bombieri:** Visualization, Supervision, Writing - Review & Editing; **Alejandra Zarzo-Arias:** Methodology, Visualization, Writing - Review & Editing; **Rita Sánchez-Andrade:** Conceptualization, Resources, Supervision, Writing - Review & Editing; **Adolfo Paz-Silva:** Conceptualization, Formal analysis, Writing - Review & Editing, Funding acquisition.

Conflicts of interest

No conflicts of interest.

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