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ORIGINAL RESEARCH

Genetic identification of gastrointestinal parasites in the world's most endangered ungulate, the hirola (*Beatragus hunteri*)

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Abstract

Background: The hirola (*Beatragus hunteri*) is the world's most critically endangered antelope. Its parasitic fauna has never been genetically characterised, raising questions about its possible role in the species' population decline. This study aimed to assess the presence of gastrointestinal parasites in hirolas and their transmission pathways.

Methods: Thirty-one hirola faecal samples were examined using coprological methods. The identification of trematode and nematode species was performed by PCR amplification of the internal transcribed spacer 2 (ITS-2) and 18S ribosomal RNA (18S rRNA) genes.

Results: Trematode and nematode eggs were detected in five (16.1%) and 23 (74.2%) samples, respectively. The trematode sequence was identical to *Cotylophoron cotylophorum*, while the nematode sequence showed a 99.3% identity to *Chabaudstrongylus ninhae* (18S rRNA) and 99.4% identity to *Cooperia curticei* (ITS-2).

Limitations: The sample size is relatively small; however, it represents a large portion of the extant hirola population at the year of sampling.

Conclusions: A high percentage of the animals were infected by parasites whose presence may be associated with co-occurrence between hirolas and other ungulate species. Monitoring the parasite burden in local livestock may therefore be crucial for hirola conservation.

KEYWORDS
endangered species, gastrointestinal parasites, Paramphistomidae, Trichostrongylidae

INTRODUCTION

Gastrointestinal parasite infections in ungulates are economically and medically significant worldwide.[1] Among them, those caused by trematodes (Paramphistomidae and Fasciolidae), nematodes (i.e., Trichostrongylidae) and some protozoa are of most concern for both domestic and wild ungulates,[2] with several of these parasite species causing serious diseases,[3] of which some are zoonotic.[4]

Some of these parasites have an indirect lifecycle where both intermediate and definitive hosts are necessary for the infection to occur. Consequently, all of the hosts play an important role in pathogen survival, proliferation and transmission, which are affected by several biotic and abiotic factors, including urban encroachment, global warming and faunal reshuffling.[2] Indeed, the free-living environmental stages of these parasites are significantly influenced by climate, with soil moisture being essential for the development and survival of the parasites as well as their intermediate and paratenic hosts. Therefore, while rising temperatures may accelerate the development of certain parasite larval stages,[5] rainfall and vegetation could increase host exposure to parasites and affect their infection patterns.[6] In addition, while some gastrointestinal parasites are host specific, others are generalists that can be transmitted between different hosts.[7,8]

In recent years, parasitic infections have come to represent an emerging threat to several wild ungulates, posing a challenge to their management and ongoing conservation efforts.[9] For instance, helminth parasitic burden is deemed to be one of the leading factors behind the drastic decline of some species,[10] with bleak forecasts of climate warming-driven parasitic infections and associated disease outbreaks in certain regions.[11] In this context, characterising the gastrointestinal parasite communities in co-occurring livestock and wild ungulate species is crucial.[12]

The hirola (*Beatragus hunteri*) (Figure 1), first described in 1888,[13] is a critically endangered medium-sized antelope with a narrow distribution currently restricted to northeastern Kenya but formerly encompassing bordering Somalia. This region is also inhabited by other ungulates, such as the oryx (*Oryx* spp.), the Grant's gazelle (*Nanger granti*), the Burchell's zebra (*Equus quagga burchellii*) and the coastal topi (*Damaliscus lunatus*). In addition, although hirolas avoid direct association with livestock, some individuals roam areas that have been previously grazed by cattle.[14] To date, knowledge about hirola parasites is still limited; therefore, it is not known if these could have played a role behind its decline, which has been historically attributed to rinderpest virus transmission from other ruminants[15] and, more recently, to habitat degradation and predation.[16,17] Habitat restoration and protection are ongoing conservation efforts to enhance the recovery of the hirola, but characterising the diversity of parasite species infecting this antelope across its narrow range is crucial for understanding potential health threats and enhancing conservation management.

For this reason, the main objective of the present study was to assess the presence of gastrointestinal parasites in the hirola and to molecularly identify them. The information obtained will help to elucidate infection pathways from other wild or domestic ungulates and facilitate the development of strategies to reduce this phenomenon.

MATERIALS AND METHODS

Study area and samples

A total of 39 hirola faecal samples from semi-captive herds within the predator-free fenced sanctuary in Garissa County (SANT; $n = 27$) and from wild herds at Ishaqbini Community Conservancy (CONS; $n = 5$) were collected from the ground between December 2017 and February 2018 (Figure 2). No metadata were recorded for seven samples. All the samples were individually labelled and preserved in plastic vials filled with 70% ethanol.

The faecal samples were genotyped to identify the individuals they came from, as described in a previous study.[18] Eight of the 39 samples had non-unique genotypes, indicating that the same individuals had been sampled more than once, and these were therefore excluded from downstream analyses. The number of individuals identified per sampling area varied between 20 (SANT) and five (CONS), but for six individuals, we could associate no locality information. The final dataset consisted of faecal samples from 31 individuals, representing a large portion (around 25%) of the extant hirola population at the time of the survey.

Coprological techniques

Faecal samples were examined using two classical copromicroscopic methods: sedimentation[20,21] and flotation.[22] For both techniques, 3 g of faeces was homogenised in 42 mL of water and filtered using a 150 µm mesh. Two 12 mL crystal tubes were filled with this faecal solution, and the remaining solution was preserved in labelled plastic tubes stored at 4°C. These crystal tubes were then centrifuged at 680×*g* for 5 minutes and the supernatant was discarded.

Trematode eggs were detected by diluting the sediment contained in a single 12 mL crystal tube in 1000 mL of tap water. The suspension was allowed to settle for 20 minutes in a sedimentation cone; afterwards, the supernatant was discarded, and the sediment was resuspended in 500 mL of tap water. After another 20 minutes of sedimentation and the subsequent decantation, the solid phase was recovered into a 100 mL sedimentation cone that was filled with tap water. Subsequently, all the sediment obtained was used to fill a McMaster chamber, and this was directly observed under a microscope (Olympus CX43, Olympus Corporation). For the detection

FIGURE 1 Adult hirola (*Beatragus hunteri*)

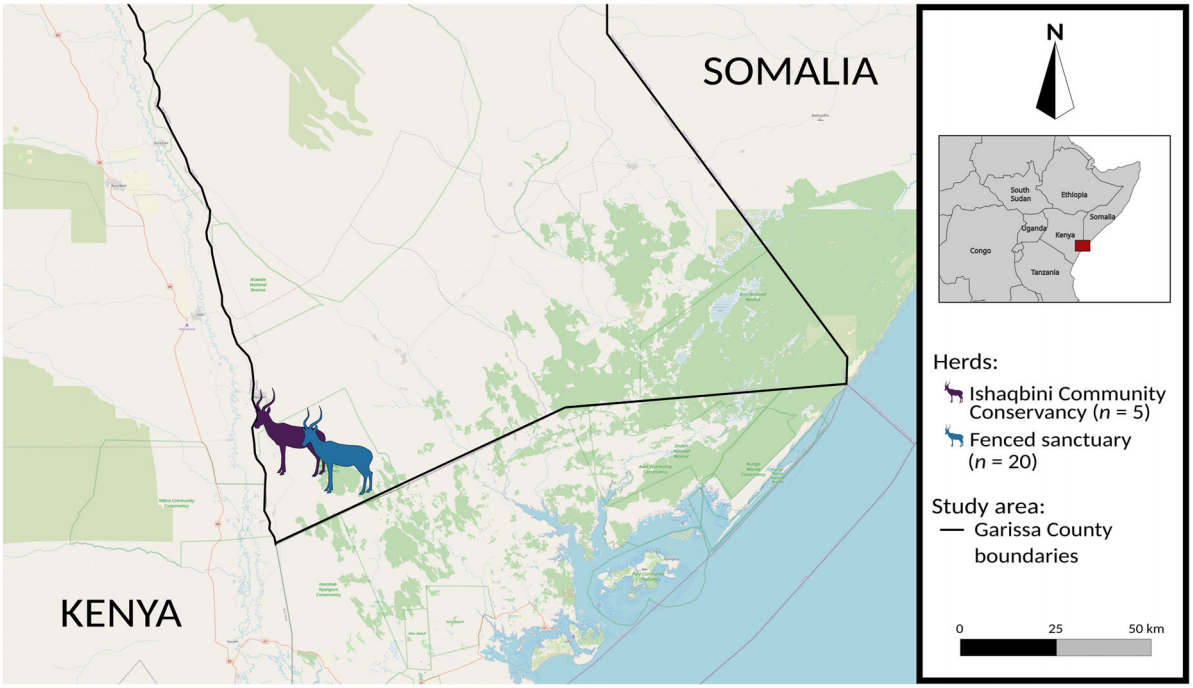

FIGURE 2 Collection sites of hirola faecal samples. Modified from OpenStreetMap[19]

of nematode eggs, the sediment obtained from the other crystal tube was processed using flotation in Sheather's sucrose solution[22] (specific gravity: 1.27) and observed under a microscope.

DNA extraction

Samples with excessive faecal material hindering egg collection were diluted and cleared with tap water. Gastrointestinal trematode and nematode eggs were individually collected from the faecal solution with a micropipette and stored in RNAse- and DNAse-free plastic tubes at 4ºC. DNA extraction was carried out in batches of four to five trematode or nematode eggs using the High Pure PCR Template Preparation Kit (Roche Diagnostics), following the manufacturer's instructions.

Parasite molecular detection

Trematode identification was performed using a previously described PCR protocol,[23] which targets an

TABLE 1 Primers used for Strongylidae species detection and identification

Gene target	Name	Primer sequence 5′–3′	Fragment size	Nt	T_m (°C)	CG (%)	Self-dimers	Cross-primer dimers
18S rRNA	StrongF	AAGGGTGCAATTATTAGAGCA	650 bp	21	60.6	38.1	–	–
	StrongR	GAACCGAAGTCCTCTTTTATTAT		23	58.7	34.8	–	–

Abbreviations: CG, cytosine–guanine content; Nt, number of nucleotides; T_m, melting temperature.

approximately 400 bp-long fragment of the internal transcribed spacer 2 (ITS-2) gene. In each amplification reaction, nuclease-free water was included as a negative control, and rumen fluke (*Calicophoron daubneyi*) DNA obtained from a cow (*Bos taurus*) was included as a positive control.

For identifying gastrointestinal nematode parasites, a new primer set (StrongF/StrongR) was designed to target approximately 650 bp of the 18S ribosomal RNA (18S rRNA) gene of the species belonging to the family Strongylidae. Nucleotide sequence variation across different genera, as inferred from their available GenBank records (Table S1), was used for designing the primers after aligning the sequences with the online CLUSTALW tool GenomeNet Multiple Sequence Alignment (www.genome.jp/tools-bin/clustalw). Primer compatibility and melting temperatures were assessed using the online tool Multiple Primer Analyser (ThermoFisher Scientific) (Table 1), while Primer-Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to assess their target sequence identity and amplificability. The PCR reaction mix consisted of 1.75 µL of 3.5 mM $MgCl_2$, 2.5 µL of 200 µM dNTPs, 1.5 µL (0.6 µM) of each primer, 0.5 units of NZYTaq II DNA polymerase (NZYTech) and 3 µL of template DNA in a final volume of 25 µL. Amplifications were carried out in a T100 Thermal Cycler (Bio-Rad Laboratories). The cycling conditions were as follows: initial denaturation of 5 minutes at 94°C, 40 cycles of 45 seconds at 94°C, 45 seconds at 59°C and 1 minute at 72°C followed by a final elongation of 7 minutes at 72°C. For further identification of the gastrointestinal nematodes, a PCR protocol previously described,[24] targeting an approximately 300 bp fragment of the ITS-2 gene, was carried out. In each amplification reaction, nuclease-free water was included as a negative control, and *Trichostrongylus colubriformis* DNA obtained from a sheep (*Ovis aries*) was included as a positive control.

Successful amplification was assessed using electrophoresis on 1.5% agarose gels stained with RedSafe (iNtRON Biotechnology) and then visualised using a Fluor-S MultiImager (Bio-Rad Laboratories). PCR products were subsequently purified and sequenced bidirectionally at the Sequencing and Fragment Analysis Unit of Santiago de Compostela University. The sequences were manually edited using ChromasPro (Technelysium) and compared with those deposited in the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The unique partial sequences identified in this study were deposited in GenBank under accession numbers PQ639947, PQ639948 and PQ640031.

RESULTS

A total of 23 individuals (74.2%; 95% confidence interval [CI]: 55.1–87.5%) tested parasite positive using at least one coprological technique. Specifically, trematode eggs were detected through sedimentation in five of the 31 individuals (16.1%; 95% CI: 6.1–34.5%), while gastrointestinal nematode eggs were detected through sucrose flotation in 23 individuals (74.2%; 95% CI: 55.1–87.5%) (Figure 3). No other parasitic forms were detected in the samples analysed. Coinfections with nematodes were observed in all of five the animals positive for trematodes. The overall prevalence of gastrointestinal parasites varied across localities, with CONS showing the highest prevalence (5/5; 100%; 95% CI: 46.3–100%), followed by SANT (16/20; 80%; 95% CI: 55.7–93.4%) and individuals of uncertain provenance (2/6; 33.3%; 95% CI: 6.0–75.9%). The gastrointestinal nematode prevalence was the same as the total prevalence for each sampling area, being 100%, 80% and 33.3% in individuals sampled in CONS, SANT and uncertain provenance, respectively. Meanwhile, trematode prevalence was similar in CONS (1/5; 20%; 95% CI: 1.1–70.1%) and SANT (4/20; 20%; 95% CI: 6.6–44.3%), but no trematodes were detected in individuals of uncertain provenance.

The Paramphistomidae trematode ITS-2 sequence (381 bp) obtained was 100% identical to *Cotylophoron cotylophorum* (GenBank record KX668959) isolated in cattle from Kenya[25] and showed a high similarity (99.5–99.7%) to *Cotylophoron* sp. (GenBank records KX668913, KX668970 and KX668975) isolated in cattle and sheep from Kenya.[25]

Although a few nematode eggs were collected and five DNA extractions were carried out, only one sample tested positive using the PCR protocol targeting the Strongylidae 18S rRNA gene. This sample showed 99.3% identity to *Chabaudstrongylus ninhae*, syn. *Cooperia ninhae* (GenBank record LC415111), which was isolated from a Reeves's muntjac (*Muntiacus reevesi*) in Japan.[26] This newly amplified sequence also showed a 98.4% similarity to *Trichostrongylus colubriformis* (GenBank record AJ920350) obtained from sheep (*O. aries*)[27] and a 98.0% similarity to *Haemonchus contortus* (GenBank records EU086374 and EU086375) obtained from a captive giraffe (*Giraffa camelopardalis*) from Florida,[28] an *H. contortus* laboratory strain (GenBank records LS997563

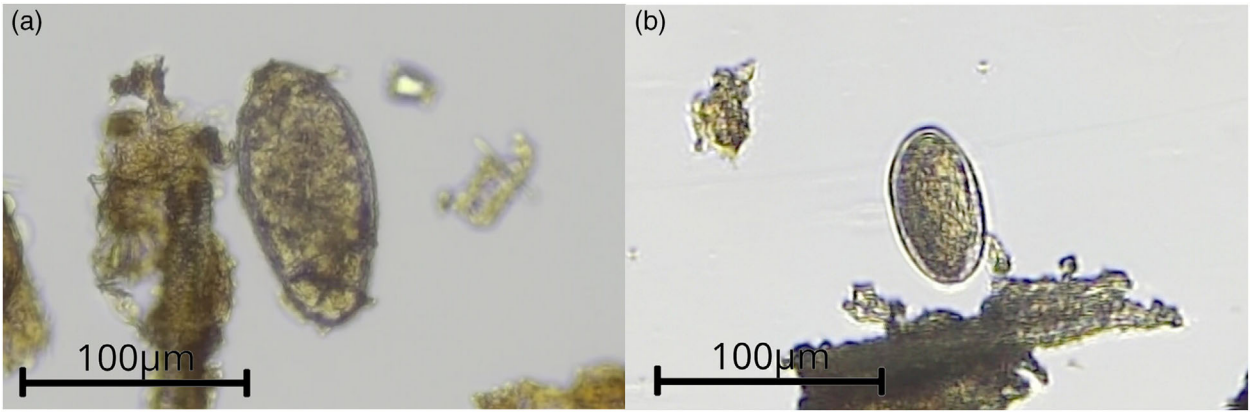

FIGURE 3 Examples of (a) trematode and (b) nematode eggs observed in the faecal samples

and LS997564)[29] and *Graphidioides affinis* (GenBank records OR611714 and OR611716) obtained from a Patagonian mara (*Dolichotis patagonum*) from Bulgaria (unpublished). The ITS-2 sequence (325 bp) obtained for this sample showed a 98.2–99.4% identity to the previously deposited *Cooperia curticei* sequences (GenBank records KC998734–KC998739) isolated in sheep from New Zealand,[30] and a 97.2% identity to *Cooperia oncophora* sequences (GenBank records KC998742, KC998743 and AB534601) obtained from cattle in New Zealand[30] and Japan (unpublished). This sequence also showed a 96.6% similarity to the *C. ninhae* sequence (GenBank record LC415111) isolated from a Reeves's muntjac in Japan.[26]

DISCUSSION

The hirola—listed as critically endangered in the IUCN Red List of Threatened Species and still experiencing a decreasing demographic trend[31]—is referred to as the most threatened antelope in the world. Its dramatic global population decline has been attributed to multiple causes, among which the 1980s rinderpest virus outbreak, resulting in mass mortality of the ruminants in eastern Kenya,[32] stands out. While the recovery of the hirola started only after the eradication of this virus, which points to its leading role in the plight faced by this rare antelope, several other factors, such as habitat loss and degradation, poaching and competition with livestock, have also been involved.[17,33,34] Although some authors have suggested that the limited recovery of hirola global populations is likely attributable to their low genetic diversity,[15] the only pertinent molecular investigation carried out until now revealed no evidence of inbreeding.[18]

The present study is, to the authors' knowledge, the first to assess the presence of gastrointestinal parasites in the hirola through sequencing, thus advancing our knowledge on the ecology of this rare species. The results obtained indicate that more than half of the studied animals were infected with gastrointestinal parasites. These findings agree with those reported for other wild ungulates from Kenya, where more than 50% of the surveyed individuals carried at least one helminth species,[35] suggesting a high exposure to these parasites. Prevalences up to 100% were reported in Burchell's zebras[35,36] and several African antelopes,[35,37,38] including hirolas, while lower, but still high, values were reported in zebuine cattle (*Bos indicus*) (29%) and Cape buffalo (*Syncerus caffer*) (47–87%).[35,37] On the other hand, helminths were only found in 9% of the examined giraffes.[35]

Two taxa of gastrointestinal parasites were detected by coprological analysis of the samples collected for this study: Paramphistomidae trematodes and Trichostrongylidae nematodes. Previous studies in hirolas have reported gastrointesinal protozoa (Coccidia) together with Trichostrongylidae, Strongylidae and Ascarididae species, bronchopulmonary nematodes and trematodes.[38,39] However, other nematodes (Trichuridae, Spiruridae, Oxyuridae) and cestodes (Anoplocephalidae) detected in other wild ungulates from Kenya[35,38–40] have not been identified in this species. We cannot rule out that the low parasite diversity seen in the present study could be due to the sample preservation method rather than reflecting an intrinsically poor gastrointestinal parasite community. Nevertheless, low parasite diversity has been reported in other wild ungulates from the same area.[37,40] According to previous observations,[41] this outcome may be due to the animals' limited contact with different habitats and parasite hosts, which is in line with the narrow distribution range of the hirola. Concordantly, it has been proposed that migration and transhumance could lead to higher parasite diversity[35]; therefore, the low parasite diversity observed in this species could also be associated with absence of seasonal movements of the sampled herds at the time of sample collection.

Gastrointestinal nematodes (74.2%) were more frequent than trematodes (16.1%), which is consistent with previous studies carried out on hirolas, which found prevalences of around 54% and 24% for these taxa, respectively.[38,39] These results are also consistent with those obtained for other African antelopes[35,37,40–42] and Cape buffaloes.[37]

As far as the high prevalence of Trichostrongylidae nematodes is concerned, it is important to note their ability to survive in dry conditions.[43,44] In addition,

it has been reported that some ungulates share certain gastrointestinal nematodes species,[35,45,46] which increases egg environmental contamination and the probability of interspecies transfer. By contrast, Paramphistomidae trematodes require wet environments to survive, since they need an amphibious or aquatic gastropod to complete their lifecycle.[44] These parasites have been detected in a high percentage of water buffaloes (*Bubalus bubalis*),[35,37] which inhabit marshy areas where Paramphistomidae intermediate hosts[47] thrive. In addition, species of this family have also been detected in up to 90% of the domestic cattle, sheep and goats from Kenya,[25,40] which inhabit watered natural habitats where the intermediate host is present.[25]

Although trematode eggs had been previously detected in hirola faecal samples,[38] no species identification had been carried out. It is worth mentioning that *Cotylophoron* spp. had been previously detected in a wide range of domestic and wild African ruminants.[25,48,49] Given that *C. cotylophorum* is known for its broad host range and is frequently found in African ungulates,[49] its identification in the hirola is not unexpected, especially given the co-occurrence of this antelope with other wild and domestic ruminants,[14,50] which could facilitate cross-infection between species. This is in line with previous studies, which suggested that the infection risk with broad host-range parasites increases in communities with co-occurring hosts.[37,46] This cross-species contact is of particular concern for endangered species that are the object of intensive conservation efforts.[37] However, previous studies have suggested that deworming domestic species could be an effective way to reduce the transmission between livestock and threatened wild ungulates.[46,51]

The characterisation of the 18S rRNA gene revealed that the Trichostrongylidae species detected in the present study was phylogenetically close to a *C. ninhae* specimen obtained from a Reeves's muntjac from Japan.[26] The genus *Chabaudstrongylus* has been previously identified in some deer (Cervidae) and mouse deer (Tragulidae) species,[52–54] suggesting that the eggs found in the hirola faecal samples might therefore belong to the same or a highly related genus. However, the characterisation of the partial sequence obtained for the ITS-2 gene found the highest similarity to be with *C. curticei*, a species commonly detected in sheep and goats,[44] with a sequence identity of lower than 97% for the closest *C. ninhae* sequence.

Since the hirola is the only representative of the genus *Beatragus*,[55,56] the detected nematode could be host specific; however, information about the molecular characterisation of Strongylidae species in African antelopes is still scarce. A previous study carried out in the hirola[38] identified other Trichostrongylidae genera, such as *Trichostrongylus* spp., *Oesophagostomum* spp., *Bunostomum* spp., *Haemonchus* spp., *Cooperia* spp. and *Ostertagia* spp.; however, there is no information about the procedures used for such identification, more so as nematode eggs of those genera are morphologically indistinguishable. In view of these considerations and the limited number of eggs that could be collected and analysed in this study, it is conceivable that other Trichostrongylidae species could be co-circulating in the same hirola population. Moreover, we cannot rule out that the partial sequences obtained belong to two different species. Several *Oesophagostomum*, *Trichostrongylus*, *Cooperia*, *Haemonchus* and *Teladorsagia* species have been molecularly identified in African ruminants,[40,57,58] which calls for further studies to assess the systematic placement of hirola Trichostrongylidae as well as to unravel their epidemiology and host range.

Finally, it is important to state that the samples processed in the present investigation were retrieved from the environment and preserved with the purpose of carrying out a genetic diversity study of the extant hirola populations, not to perform coprological analysis. Ethanol preservation is a good method for conserving faecal samples over time if DNA retrieval is the main objective,[59] but the best protocol for studies such as this one is to refrigerate and process them as soon as possible to prevent the death, destruction and morphological alteration of the parasitic forms.[60,61] For this reason, the presence of bronchopulmonary nematodes was not assessed in the present investigation, since their detection technique relies on the existence of motile larvae.[62] In addition, although sedimentation is a quantitative technique, it was only used in a qualitative way, since the preservation method could have affected parasite counts, resulting in an underestimation of the intensity of egg shedding. Nevertheless, we deem our results to still hold a special interest from an ecological point of view, since data on gastrointestinal helminths affecting the hirola are still scant.

In conclusion, our study highlights the presence of two gastrointestinal helminth groups, Paramphistomidae and Trichostrongylidae, in the critically endangered hirola antelope. These findings underscore the need for further research to understand the pathogenicity and epidemiology of these helminths in hirola populations, which is essential for effective conservation.

AUTHOR CONTRIBUTIONS

Susana Remesar designed and carried out the analyses and wrote the manuscript. David García-Dios carried out analyses and reviewed the manuscript. Giovanni Forcina performed data curation and writing—review and editing. Abdullahi H. Ali and Mathew Ndunda performed sampling and writing—review and editing. Michael J. Jowers conceived the study and reviewed and edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The authors will make the data available upon request.

ETHICS STATEMENT

This study does not involve animal experimentation, so no ethical approval was deemed necessary.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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