Cross-transmission of resistant gastrointestinal nematodes between wildlife and transhumant sheep

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ABSTRACT

Wild and domestic ungulates can be infected with the same species of gastrointestinal parasitic nematodes. These parasites have a free leaving stage in the environment that contributes to the ease of transmission among different host species. In addition, gastrointestinal nematodes have developed resistance to anthelmintics which is now considered a major problem for the livestock sector. In a context where wild and domestic ungulates share the same pastures, the maintenance and circulation of resistant gastrointestinal nematodes between species have rarely been explored.

In the European Alps, domestic sheep are driven in the high-altitude summer pastures leaving in sympatry with wild ungulates for several months. In this study we investigated the nemabiome of domestic sheep and Alpine ibex, *Capra ibex*, in three different areas of the French Alps to evaluate the parasites circulation between the two host species. The Alpine ibex is a protected mountain ungulate that is phylogenetically related to sheep and hosts nematode species common to sheep.

Using internal transcribed spacer 2 (ITS-2) nemabiome metabarcoding, we found sheep and ibex sharing similar gastrointestinal nematodes, except for a few species, such as *Marshallagia marshalli* and *Trichostrongylus axei*. This suggests that the long-term co-occurrence of sheep and ibex on mountain pastures has promoted the exchange of gastrointestinal nematodes between the two hosts. Based on the sequencing of the isotype 1 of the beta tubulin gene, associated with benzimidazole resistance, we found resistant nematodes in all sheep farms and in all Ibex populations. Our results demonstrated that Ibex can host and shed resistant strains before transhumant sheep arrive on pastures, and then can act as a reservoir or refugia for resistant gastrointestinal nematodes. The relative role of ibex to the nemabiome and in particular to the maintenance and circulation of resistant strains in sheep remain to be determined.

Keywords: ITS-2 rDNA, benzimidazole resistance; isotype-1 β-tubulin; livestock; nemabiome metabarcoding; wild ungulates, transhumant sheep, Alpine ibex

Introduction

Parasites represent a large proportion of animal diversity and are key components of food webs (Hudson et al., 2006) and are essential determinants of the health, fitness, population dynamics and community composition of their hosts (Tompkins et al., 2011). The parasites of the Nematodes class infect a wide range of species worldwide, including animals and plants (Blaxter and Koutsovoulos, 2015). In animals, the gastrointestinal nematode parasites are of major concern for livestock productivity and security as they can impact animal health implying reduced animal productions and economical losses (Charlier et al., 2020; Roeber et al., 2013).

To limit the parasite load and its impact on livestock health, the use of anthelmintics to treat livestock against gastrointestinal nematodes is a common and cost effective practice (Vercruysse et al., 2018). Nonetheless, the repeated use of anthelmintics has led to the selection of anthelmintic-resistant strains of gastrointestinal nematodes. Within the gastrointestinal nematodes, resistances to several families of anthelmintics (e.g., benzimidazole, macrocyclic lactones and levamisole) has been observed, and resistance to several drugs is increasing (Bordes et al., 2020; Kaplan and Vidyashankar, 2012; Rose et al., 2015; Rose Vineer et al., 2020). The first case of resistance to benzimidazole was reported in 1964 in lambs from central Kentucky (Drudge et al., 1964).

Today, resistance to benzimidazoles is widespread throughout the world (Kaplan and Vidyashankar, 2012), and is particularly common on sheep farms in Europe (Papadopoulos et al., 2012; Rose Vineer et al., 2020). The mechanisms of resistance to benzimidazole are well known and documented (Whittaker et al., 2017). The anthelmintic effect of benzimidazoles relies on the fixation of the molecule to the parasite β-tubulin isotype-1, resulting in the disruption of tubulin–microtubule equilibrium (Whittaker et al., 2017). In resistant nematodes, specific mutations of the β-tubulin isotype-1 gene have been correlated with the resistance to benzimidazole in several gastrointestinal nematode species (Charlier et al., 2022). These mutations were associated with alteration of the β-tubulin isotype-1 structure, decreasing the affinity of the protein with the benzimidazole and subsequently, activity of benzimidazole (Whittaker et al., 2017).

Some generalist gastrointestinal nematodes can infect several host species (Walker and Morgan, 2014), including both domestic and wild ungulates (e.g.,

Beaumelle et al., 2022; Cerutti et al., 2010). The transmission of gastrointestinal nematodes among hosts, even if they do not simultaneously occupy the same pastures, is possible thanks to their free-living infective larval stage that may rely active until several months in the environment (Carlsson et al., 2013; Fiel et al., 2012; Walker and Morgan, 2014). Transmitted parasites can also include gastrointestinal nematodes resistant to anthelmintics. For instance, benzimidazole-resistant nematodes have been detected in free-living populations of roe deer living in sympatry with livestock (Chintoan-Uta et al., 2014; Nagy et al., 2017). To date, the role of wild ungulates in the epidemiology of resistant nematodes remains to be determined, but it has been suggested that wildlife may act as a reservoir of resistant nematodes for livestock (Brown et al., 2022; Chintoan-Uta et al., 2014; Francis and Šlapeta, 2023; Laca Megyesi et al., 2019; Walker and Morgan, 2014). Yet, we need to investigate the presence of resistant nematodes between co-grazing wild and domestic ungulates in different contexts, i.e., for different host species, in different landscape, and under different climatic conditions, to accurately evaluate the potential role of wildlife as reservoir for anthelmintic resistant gastrointestinal nematodes.

Transhumant pastoralism is a common practice in the European Alps and consists of the seasonal movement of grazing livestock from lowland areas to mountain meadows in summer which provide fresh pasture for domestic ungulates, i.e., mainly sheep, but also cows or goats (Biber, 2010). These mountainous areas are inhabited year-round by wild ungulates, particularly those living at high altitude in the European Alps, like Alpine ibex (*Capra ibex*), or Northern chamois (*Rupicapra rupicapra*). While wild ungulates tend to avoid domestic herds spatially or temporarily during the summer (Acevedo et al., 2008), certain factors may contribute to the use of the same pastures by both wild and domestic ungulates.

Spatial segregation between wild and domestic ungulates is usually observed once livestock arrive on pasture (Brivio et al., 2022; Ryser-Degiorgis et al., 2002). Livestock are generally released onto the best grazing areas during the summer season due to their high productivity and accessibility of water for animals, and salt licks provides essential mineral nutrients in relatively nutrient-poor alpine ecosystems (Chirichella et al., 2014; Richomme et al., 2006; Ryser-Degiorgis et al., 2002). However, prior to the arrival of livestock, mountain ungulates have been observed using preferentially the same grazing areas as those used by livestock on their arrival

(Brivio et al., 2022; Ryser-Degiorgis et al., 2002) and ibex, for example, have been observed to return to the sheep grazing area immediately after the sheep have left (Ryser-Degiorgis et al., 2002). The presence of wild and domestic ungulates in attracting zones such as salt licks, even if not simultaneous, offers good opportunities for parasites transmission, and these areas are therefore considered hotspots for parasites infection (Richomme et al., 2006; Ryser-Degiorgis et al., 2002; Utaaker et al., 2023).

Consequently, transhumant pastoralism represents a risk of pathogens transmission between wild and domestic ungulates in mountain areas (Rossi et al., 2019). Pathogen exchanges at the interface of wild and domestic ungulates have already been well documented. The Alpine ibex has been identified as the wildlife reservoir of brucellosis (*Brucella melitensis*) which was transmitted to cattle and human in the Bargy massif in northern French Alps (Marchand et al., 2017). In addition, sheep have been confirmed as the domestic reservoir of the Border disease, which induced a major virus outbreak in Southern chamois (*Rupicapra pyrenaica*) population in the Pyrenees (Luzzago et al., 2016). The transmission of gastrointestinal nematodes has already been described between wild ungulates and transhumant domestic ungulates in mountainous areas (Cerutti et al., 2010; Citterio et al., 2006; Khanyari et al., 2022; Zaffaroni et al., 2000). However, no study has yet investigated the transmission of anthelmintic-resistant nematode in a transhumant pastoral system.

In this study, we investigated the community of gastrointestinal nematodes infecting Alpine ibex and domestic sheep (*Ovis aries*) and the prevalence of resistance to benzimidazole, in three different regions of the French Alps. The Alpine ibex was close to extinction at the beginning of the 19th century but the reinforcement of its populations by several reintroductions in different part of the Alps has increased the species' overall abundance and range (Brambilla et al., 2022). Today,the Alpine ibex species is estimated at 52 000 individuals in Europe (Brambilla et al., 2020).

Given that the co-occurrence of sheep and ibex on the same pastures is limited to the summer period and since sheep, and that ibex usually host species-specific gastrointestinal nematodes (Walker and Morgan, 2014), we expect the nemabiome to be highly differentiated between the two species in the three mountain areas (H1). We expect sheep to host benzimidazole-resistant strains of gastrointestinal nematode, in line with the general pattern observed for sheep in France

(Papadopoulos et al., 2012; Rose Vineer et al., 2020). With the implementation of reintroduction programs in the second half of the 20th century, ibex have colonized pastures traditionally grazed by sheep. We therefore expect that ibex will also host benzimidazole-resistant gastrointestinal nematodes but to a lesser extent, as resistance do not represent a selective advantage for nematodes in the ibex environment (Hahnel et al., 2018) (H2). Because there are very few documented ibex dispersal events among the 3 ibex populations ((Brambilla, 2020), R. Papet, C. Toïgo and E. Vannard, personal communication), we should observe genetic differences among nematodes species/community or strains (ASV) among the populations of ibex due to genetic drift (H3).

Materials and Methods

Study area

Samples of sheep and ibex feces were collected in the French Alps in 3 different mountain areas (Figure 1). The Belledonne mountain is located in the western part of the Alps in southeast France. The Cerces and Champsaur mountains are in the north and the south parts of the Ecrins National Park, respectively (Figure 1).

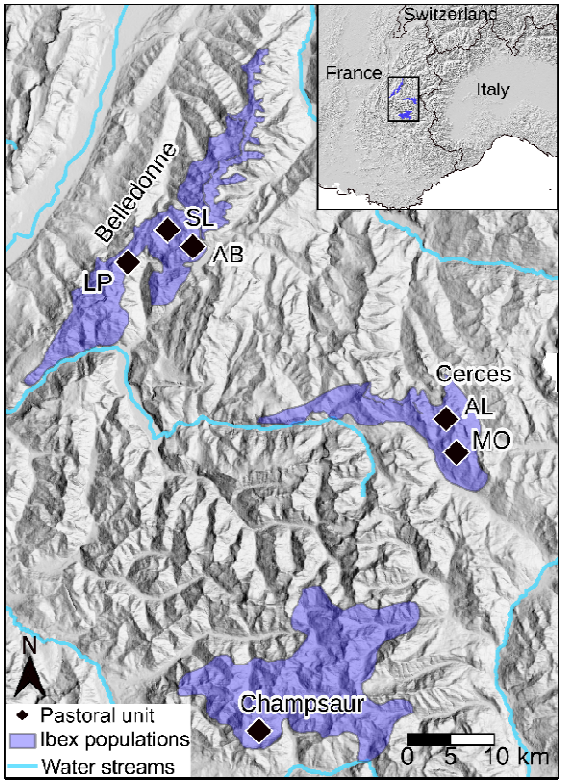

Figure 1 : Sampling locations in the French Alps were sheep and ibex feces were collected. Pastoral unit: area where both ibex and sheep have been sampled. Cerces : AL: Aiguillette de Lauzet, and MO: Montagne de l'Oule ; Belledonne : AB: Ane Buyant, LP : La Pesée and SL : Sept Laux.

The 3 study areas are characterized by the presence of steep slopes, high peaks (>2500m) and agropastoral activities. Climatic conditions are harsh in these mountains with a mean temperature in winter (December-March) 2015-2019 of 0°C in Belledonne (alt:1785m), 1°C in Champsaur (alt:1620m) and 1.5°C in Cerces (alt:1553m). During summer (June-September), the mean temperature is 13°C in Belledonne, 14°C in Champsaur and 16°C in the Cerces (réseau d'observation météo du massif alpin). Champsaur is the southern study area and has a Mediterranean influence. Consequently, rainfall is less important in this area compared to Cerces and Belledonne. The vegetation is distributed along an elevation gradient from coniferous woodland (*Abies alba* and *Picea abies* in Belledonne and *Larix decidua* and *Pinus sylvestris* in Cerces and Champsaur) in the lower range of ibex, to a landscape dominated by heathland with *Rhododendron ferrugineum*,

Vaccinium spp. and *Juniperus communis*, and grassland (*Carex* spp. *Festuca* spp.) above the tree line (Ozenda, 1985).

The ibex populations were established in Belledonne, Cerces and Champsaur, in 1983 with the introduction of 20 ibex, in 1959-1961 with the introduction of 6 ibex and in 1994-1995 with the introduction of 30 ibex, respectively.

The distribution of ibex in Belledonne, Cerces and Champsaur range between 630m and 2860m on 200km², between 1410m and 3100m on 120km² and between 1320m and 3550m on 280km², respectively. Census populations sizes are estimated to 800, 320 and 420 individuals in Belledonne, Cerces and Champsaur, respectively.

Traditional pastoral activity is practiced in all massifs, where sheep flocks arrive early summer to graze mountain pastures coming from the plain on foot or by truck. In Belledonne, the size of the herds are 750 ewes followed by their lambs in La Pesée, 900 ewes in Sept Laux, and 1600 ewes in Ane Buyant. A dozen of rams are also present within the La Pesée and Sept Laux herds, as well as some goats in La Pesée. Each sheep herd from Belledonne belongs to one farmer while several farmers grouped their sheep herds in Cerces and Champsaur. In Champsaur, the herd included 4 breeding farms for a total of 1070 sheep and 5 goats. In Cerces, the herd located in the West (Aiguillette du Lauzet) included 3 breeding farms for a total of 800 sheep and the herd located in the East (Montagne de l'Oule) included 4 breeding farms for a total of 940 sheep.

Sample collection

We collected sheep feces up to 15 days after arrival on pasture to ensure that we collected nematode species representative of sheep at the time of arrival on pastures and to avoid detecting nematodes ingested secondarily on alpine pastures. Similarly, ibex feces were collected prior to the arrival sheep and until 15 days after arrival to ensure that the nematode community was not yet influenced by the arrival of domestic livestock. Fresh ibex feces were mostly collected directly on the ground and, in Belledonne, also during captures as part of the long-term monitoring program conducted by the French Office for Biodiversity. Where possible, feces were collected immediately after observation of ibex to avoid collecting of feces from the same individual. Samples were stored in plastic bags, sealed after air removal, and

analyzed within 48h upon receipt maximum 15 days after field work (mean: 2.5 [min: 0 - max: 15] days) in the parasitology laboratory of the National Veterinary School of Lyon (ENVL, Marcy-l'Étoile, France). In total, we sampled 167 fecal samples from ibex and 90 fecal samples from 6 sheep herds, distributed over four study sites, i.e., Belledonne, Champsaur, Aiguillette du Lauzet, Cerces and Montagne de l'Oule, Cerces (Table 1, Figure S1).

Table 1 : Location, period and number of samples collected in the French alps. Coproscopic results of the gastro-intestinal (GI) nematodes (strongyles) eggs are also reported (med[min-max]). Cerces : AL: Aiguillette de Lauzet, and MO: Montagne de l'Oule ; Belledonne : AB: Ane Buyant, LP : La Pesée and SL : Sept Laux.

	Sampling date	N	GI nematodes epg
Cerces mountain			
Sheep	June 2019	AL: 15	AL: 7.5 [0-60]
		MO: 15	MO: 7.5 [0-225]
Ibex	AL: May-June 2019	AL: 29	AL: 7.5 [0-30]
	MO: May 2018-2019	MO: 18	MO: 7.5 [0-60]
Champsaur mountain			
Sheep	June 2019	15	7.5 [0-30]
Ibex	May 2019	40	7.5 [7.5-105]
Belledonne mountain			
Sheep	July 2019	AB: 15	AB: 30 [0-90]
		LP: 15	LP: 7.5 [0-165]
		SL: 15	SL: 0 [0-30]
Ibex	July 2018 and May-June 2019	80	15 [0-525]

Parasitological analyses

The number of gastro-intestinal nematodes eggs per gram of feces (epg) was counted following a modified McMaster protocol (Raynaud et al., 1970). In order that strongyles reach the L3 stage, coprocultures of feces were done at 24 ± 1 °C during 12-15 days with regular mixing and moistening. After the collection of L3 in tape water with a Baermann apparatus, we evaluated the success of the coproculture by counting the number of L3 in each sample. We extracted gastrointestinal nematodes DNA from samples for which there were at least 20 L3 and we limited the extraction to ~200 L3. DNA was extracted using extraction kit (Qiagen DNeasy® PowerSoil) following the manufacturer's instruction with an elution volume of 50 µl of water. We

extracted twice the DNA of 30 randomly chosen samples as internal extraction controls. We quantified DNA concentration for all samples using Qubit 2.0 fluorometer (Life Technologies) and we homogenized DNA samples to a DNA concentration of 1ng/µl (DNA samples were not diluted if the DNA concentration was <1 ng/µl).

High throughput sequencing analyses

To determine the nemabiome of sheep and ibex we used a modified version of the protocol developed by Avramenko et al., (2015). The ITS2 region of the nuclear rDNA was amplified using the primer pair NC1 (Forward - 5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2 (Reverse- 5'-TTAGTTTCTTTTCCTCCGCT-3') with the following PCR conditions: 10µl of Applied Biosystems™ Master Mix AmpliTaq Gold™ 360, 5,84µl of molecular biology grade water, 0,16µl of Bovine Serum Albumin, 2µL of 5 µM mixed F and R primers form, 2 µL of DNA lysate. The PCR was performed under the following conditions: 10 min initial denaturation at 95°C; 35 cycles of denaturation (30 s at 95°C), annealing (30 s at 54°C), and extension (1 min at 72°C); a final extension at 72°C for 7 min. The thermocycling parameters were choose identically to Avramenko et al., (2015). To detect the mutations responsible of the resistance of gastrointestinal nematode to benzimidazole, we used a modified protocol of Avramenko et al., (2019). We amplified the isotype-1 β-tubulin fragment comprising the codon at position 167, 198 and 200 with two pairs of primers in two independent PCR.

The PCR conditions were: 10µl of Applied Biosystems™ Master Mix AmpliTaq Gold™ 360, 5.84 µl of molecular biology grade water, 0.16 µl of Bovine Serum Albumin, 2 µL of 5 µM mixed forward and reverse primers mix, 2 µL of DNA lysate. The PCR was performed under the following conditions: 10 min initial denaturation at 95°C; 40 cycles of denaturation (30 s at 95°C), annealing (30 s at 65°C), and extension (30 s at 72°C); a final extension at 72°C for 7 min. We targeted *Teladorsagia circumcincta* and *Trichostrongylus* spp. (Forward:5'-CGCATTCWCTTGGAGGAGG-3' and Reverse: 5'-GTGAGYTTCAAWGTGCGGAAG-3') and *Haemonchus contortus* (Forward:5'-CGCATTCYTTGGGAGGAGG-3' and Reverse: 5'-GTGAGTTTYAAGGTGCGGAAG-

3') with the primers described by Avramenko et al., (2019). All forward and reverse primers were tagged at 5' in order that each sample had a unique combination of tagged primers.

In all PCRs, we added positive PCR controls (i.e., *Haemonchus contortus* and *Teladorsagia circumcincta* DNA extracts), negative PCR controls (distilled H_2O) and negative DNA extraction controls. All samples (including controls) were independently amplified 4 times to ensure reliability of the sequencing, in 96-well plates including 209 samples, 17 PCR positive controls, 13 PCR negative controls, 7 extraction negative controls, 30 DNA extraction controls, as well as 12 empty wells in each 96-well plates to quantify tag jumping during PCR and sequencing steps (Figure S2) (De Barba et al., 2014; Taberlet et al., 2018).

All PCR products of the ITS2 and the two β-tubulin isotype 1 sets were purified using QIAquick® Spin Columns (QIAquick® PCR Purification KitQIAGEN) and quantified using a Qubit 2.0 fluorometer (Life Technologies). Next, we pooled the 3 purified DNA pools (ITS2, two β-tubulin isotype 1) based on their initial concentration and in proportion according to the following ratio: ITS2 50%, β-tubulin isotype 1 25% for each. According to preliminary tests, we expect to achieve a sequencing depth of 20 000 reads per ITS2 DNA sample and 5 000 reads per β-tubulin isotype 1 DNA sample. Sequencing was performed with pair-end sequencing technology on the Illumina platform (2*250 bp Miseq) at Fasteris, Geneva, Switzerland.

Sequence analysis and taxon assignation

The sequence reads were first analyzed with the OBITOOLS package (Boyer et al., 2016). Forward and reverse reads were assembled with the *alignpairedend* function, and we kept only sequences with a good score of alignment (rnorm>0.8). Sequences were attributed to their samples with the *ngsfilter* function with default parameters. Subsequently, assigned sequences were analyzed with the dada2 package (Callahan et al., 2016) following the pipeline available in www.nemabiome.ca. The *dada2* pipeline returns Amplicon Sequence Variant (ASV) which are sequence variants differing by as little as one nucleotide (Callahan et al., 2017). Following Beaumelle et al., (2021), the gastrointestinal nematodes were identified with three different methods of assignation: BLASTn (Altschul et al., 1990) against the NCBI

database (November 2022), and AssignTaxonomy (Callahan et al., 2016; Wang et al., 2007) and IDTaxa (Murali et al., 2018) against the nematode ITS2 rDNA database 1.1.0 (Workentine et al., 2020) or the nematode β-tubulin isotype 1 DNA reference sequence supplied in Avramenko et al., (2019). We chose to attribute a confidence level to taxonomic identification at species level: high or moderate confidence if the three or two methods of assignation, respectively, were congruent. We also adjusted the sequence filtering based on an adapted procedure of Calderón-Sanou et al., (2020). We kept only ASVs present in at least 2 replicates of the same samples and removed ASVs that were not assigned to genus level for the ITS2 and species level for β-tubulin isotype 1. We removed potential contaminants (reagent contaminants and cross-contaminations) following the procedure detailed in Calderón-Sanou et al., (2020). For each sample, we sum the reads of the two replicates with the highest similarity and if this similarity is higher than the mean similarity among all replicates. At the end, we removed samples if they have <1000 reads of ITS2 and <500 reads of β-tubulin isotype 1 (Figure S3).

Identification of non-synonymous mutations in codons 167, 198 and 200

For each nematode species, all β-tubulin isotype 1 ASVs were aligned to one of the β-tubulin isotype 1 consensus sequences of the reference database (Avramenko et al., 2019) using the *AlignSeqs* function of the DECIPHER package (Wright, 2016). We examined each β-tubulin isotype 1 ASV at codon 167,198 and 200 positions to record whether the codon is associated with a non-synonymous mutation. However, we ignored the other polymorphism sites of the Exon.

Statistical analyses on measures of nemabiomes

To measure the differences of nemabiome among the two host species (sheep and ibex) and the 4 study sites (Aiguillette de Lauzet, Montagne de l'Oule, Champsaur and Belledonne), we considered two measures of diversity, i.e., the alpha diversity and the beta diversity. The alpha diversity was measured with the Shannon index that considers richness and evenness of communities and the beta diversity was measured with the weighted UniFrac index estimated using the R phyloseq package

(McMurdie and Holmes, 2013). The weighted UniFrac distance is a phylogenetic distance between the set of ASV from each nemabiome weighted by the transformed abundance of each ASV (Lozupone and Knight, 2005). The phylogenetic distances were computed from a phylogenetic tree which was conducted using a maximum likelihood tree with the GTR+G+I model according to the *ModelTest* function (Posada and Crandall, 1998; Schliep, 2011). The exact count of ITS2 reads were transformed with the Hellinger transformation (e.g., square root on relative frequencies) to account for the high number of zero in community table and to decrease the influence of rare ASVs in statistical analyses (Legendre and Legendre, 2012).

We tested the effects of host species and site, including their interaction, on alpha diversity with linear models, and on beta diversity using perMANOVA (*adonis2*, vegan R package (Oksanen et al., 2020)). All possible models including the null model were computed. For perMANOVA models, we used a custom function to compute Akaike's information criterion corrected for small sample size (AICc) based on residual sums of squares (Dyson, 2018). In a model selection approach, for both alpha and beta diversity, all possible models were ranked using the AICc and we selected the model with the lowest AICc value. Models with ΔAICc ≤ 2 were considered equivalent (Burnham and Anderson, 2002), and in this case, we considered the most parsimonious one, i.e., the model with the lowest degree of freedom.

All analyses were carried out using R 3.6 (R Core team, 2020. https://www.R-project.org/).

Statistical analyses on measures of resistant nematode strains

To compare the importance of benzimidazole resistance of gastrointestinal nematodes between ibex and sheep and among the 3 sites, we tested if the host species, the site and the gastrointestinal nematode species influence the relative abundance of ASVs with a resistant allele. For this purpose, we used a generalized linear model with a binomial family and a model selection approach such as described above.

We used *AlignSeqs* (Wright, 2016) to generate multi-sequence aligned β-tubulin isotype 1 haplotype data. For each gastrointestinal species, we removed short ASVs,

e.g., ASVs with a sequence length <10% comparing to the median ASVs length. PopART v1.7 (Leigh and Bryant, 2015) was used to draw the median joining networks based on each gastrointestinal nematode haplotypes data.

Results

Parasite material

The median number of eggs of strongyles per gram of feces were lower in sheep ($7.5[0,148]_{95\%IQR}$; n = 90) than in ibex ($15[0,163]_{95\%IQR}$; n = 167) feces (Mann–Whitney U test; W = 9043.5, P = 0.006). As a result of the low level of infestation in some samples, the number of L3 hatched from eggs were not sufficient (<20) for 48 ibex or sheep samples. These samples were not used for subsequent genetic investigations. Specifically, all samples from the Sept Laux sheep herd (n=15, Belledonne) were discarded. Therefore, the nemabiome was determined based on the ITS2 for 196 (n= 55 sheep and n=141 ibex) out of 209 samples for which DNA was extracted (Figure S1).

Diversity of gastrointestinal nematodes in sheep and ibex

In total, we detected 408 ASVs corresponding to 13 gastrointestinal nematode species (Table 2, Figure S4). Eight ASVs were assigned to the genus level (i.e., *Marshallagia* spp., *Nematodirus* spp. and *Trichostrongylus* spp.) due to non-identical assignation among taxonomic methods. An ASV corresponding to the lungworm *Cystocaulus ocreatus* were discarded for the statistical analyses because we only focus on gastrointestinal nematodes.

Teladorsagia circumcincta was the most prevalent nematode species and was detected in 85% of samples (90%, n=127/141 ibex and 71%, n=39/56 sheep), followed by *Trichostrongylus vitrinus,* 63% (73%, n=103/141 ibex and 36%, n=20/55 sheep) and *Haemonchus contortus,* 56% (70%, n=98/141 ibex and 22%, n=12/55 sheep) (Table 2, Figure S4). *Nematodirus* spp. and *Ostertagia leptospicularis* were the rarest species and were detected in only 2 samples and 1 sample, respectively and with a very small relative frequency (<0.1%).

The Nematodirus were not considered for the following results as our protocol is not appropriate for this species (Hoberg et al., 2001)

Table 2 : Number of ASV, reads and samples for each nematode taxon, including the results from the ITS2 (nemabiome) and the β-tubulin isotype 1 (resistance to benzimidazole). N= number and percentage of samples in which the taxa was detected. Host species and study sites are mixed here.

	ITS2			β-tubulin isotype 1		
	ASV	**Reads**	**N (%)**	**ASV**	**Reads**	**N (%)**
Bunostomum trigonocephalum	8	61 631	12 (6%)	-	-	-
Chabertia ovina	19	95 585	62 (32%)	-	-	-
Cooperia curticei	4	803	6 (3%)	-	-	-
Cooperia fuelleborni	2	358	5 (3%)	-	-	-
Haemonchus contortus	47	1 937 587	110 (56%)	38	392 110	96 (62%)
Marshallagia marshalli	23	338 064	57 (29%)	-	-	-
Marshallagia spp.	2	404	2 (1%)	-	-	-
Oesophagostomum venulosum	13	311 530	82 (42%)	-	-	-
Ostertagia leptospicularis	1	5	1 (1%)	-	-	-
Ostertagia ostertagi	2	269	2 (1%)	-	-	-
Teladorsagia circumcincta	235	3 311 094	166 (85%)	310	343 551	145 (94%)
Trichostrongylus axei	26	462 936	103 (53%)	33	68 414	104 (68%)
Trichostrongylus colubriformis	12	411 776	102 (52%)	8	144 643	112 (73%)
Trichostrongylus vitrinus	9	521 817	123 (63%)	44	92 834	107 (69%)
Trichostrongylus spp.	5	71 124	70 (36%)	-	-	-

According to the model selection approach, the host species was the only factor that explained the alpha diversity (Table S1). The model indicates that sheep had a lower alpha diversity compared to ibex ($\beta = -0.42 \pm 0.07$, $P < 0.001$, R^2 of the model=0.15) (Figure 2b). The site was not retained in the model selected to explain the alpha diversity (Table S1).

Beta diversity is best explained by both factors, host species ($F_{1,188} = 27.69$, $P = 0.001$) and site ($F_{3,188} = 16.39$, $P = 0.001$) and their interaction ($F_{3,188} = 25.61$, $P =$

0.001) according to our model selection (Table 3, Table S2). Some gastrointestinal nematodes were mostly (*Trichostrongylus axei*: mean reads relative abundance (RRA) of 19% [11%; 26%]$_{95CI}$ in sheep feces and 2% [1%;2%]$_{95CI}$ in ibex feces) or only (*Bunostomum trigonocephalum, Cooperia* spp.) found in in sheep feces or only in ibex feces (*Marshallagia* spp., *Ostertagia* spp.; Figure 2a). *Trichostrongylus colubriformis* was more frequent in the Ecrins national park (mean RRA: 10% [6%; 13%]$_{95CI}$) than in Belledonne (mean RRA: 4% [2%; 6%]$_{95CI}$). Likewise, *Marshallagia* spp. was more frequent in ibex feces in Cerces and Champsaur mountains (mean RRA: 11% [6%;15%]$_{95CI}$) than in ibex feces in Belledonne (0.4% [-0.2%; 1%]$_{95CI}$). The distribution of *Haemonchus contortus* in host species and sites had a particular pattern. This parasite was more frequent in ibex feces compared to sheep feces in Belledonne (mean RRA: 0.004% in sheep; 48% in ibex) and Champsaur (mean RRA: 0% in sheep; 41% in ibex), while the opposite was observed in the Cerces (Aiguillette du Lauzet: mean RRA of 7% in sheep and 0.9% in ibex; Montagne de l'Oule: mean RRA of 30% in sheep and 0% in ibex).

Table 3 : Parameters estimated from the best PerMANOVA model explaining the beta diversity in ibex and sheep. The effect of host species (ibex or sheep) and site (Aiguillette de Lauzet, Montagne de l'Oule, Champsaur or Belledonne) and the interaction between the two factors are reported. Partial R^2 are reported with the corresponding *F*-value and *p value* (P).

Diversity index	Best model selected	Variables	partial R^2	F-value	P
Weighted UniFrac	β ~ Site x Host species	Residuals	0.55	-	-
		Site	0.14	16.39	0.001
		Host species	0.08	27.69	0.001
		Site: Host species	0.22	25.61	0.001

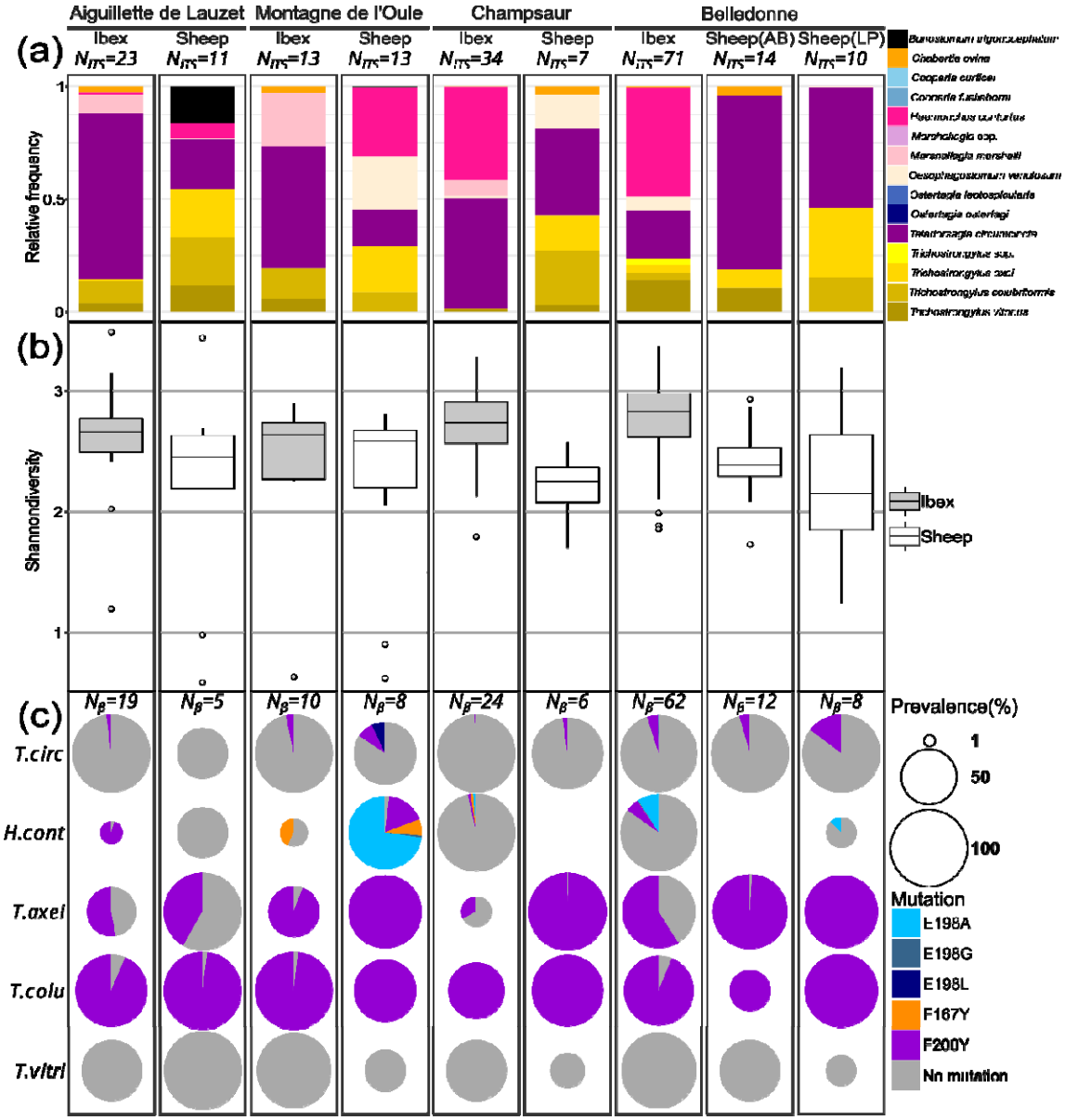

Figure 2 : (a) Mean relative frequencies of gastrointestinal nematodes species and (b) Shannon diversity of ITS2 ASVs and (c) prevalence and mean relative frequencies of β-tubulin isotype 1. For each host species (sheep or ibex) and each site (Cerces: Montagne de l'Oule and Aiguillette de Lauzet; Champsaur and Belledonne). The sample size for ITS2 ASVs (N_{ITS}; panel (a)) and for β-tubulin isotype 1 (N_β; panels (b)) is given for each population. On the panel (c), the size of the pie chart corresponds to the prevalence of the corresponding gastrointestinal nematode species in the population, and the size of each sector to the mean proportion of each allele.

Anthelmintic resistance

We found 433 different β-tubulin isotype 1 ASVs in 154 (n= 39 sheep and n=115 ibex) out of 209 samples for which DNA was extracted. Among the 5 gastrointestinal nematode species targeted by primers, we detected, *H. contortus* in 96 samples, *Teladorsagia circumcincta* in 145 samples, *Trichostrongylus axei* in 104 samples, *Trichostrongylus vitrinus* in 107 samples, or *Trichostrongylus colubriformis* in 112 samples (Table 2, Figure S5). No resistance mutation has been detected for *T. vitrinus.* Therefore, *T. vitrinus* was not included in the model explaining the relative abundance of resistant reads.

Resistance mutations were highly frequent (93.5%; n=144/154) with only 10 ibex feces (3 from Belledonne and 7 from Champsaur) in which no resistant mutation has been detected.

Based on the best model for resistant RRA, the frequency of resistant nematodes depended on gastrointestinal nematode species and the interaction between host species and the study site (Table 4, Table S3). *Teladorsagia circumcincta* was the species with the lowest resistant RRA and the nematode species with the higher resistant RRA was *Trichostrongylus colubriformis* (Table 4). The mean observed RRA of resistant nematodes differed between GIN species (*H. contortus:* 19% $[13;25]_{95CI}$*; T. circumcincta:* 4% $[3;6]_{95CI}$; *T. axei:* 70% $[63;78]_{95CI}$; *T. colubriformis:* 96% $[93;99]_{95CI}$). The resistant RRA is generally lower in ibex compared to sheep ($\beta = -2.21 \pm 0.66$, $P < 0.001$). Resistant RRA were the lowest in the Aiguillette de Lauzet but this is also the only site were ibex had significantly higher resistant RRA than sheep (Table 4).

The most frequent resistance mutation is the F200Y present in the 4 gastrointestinal nematode species and 141 feces samples, follow by the E198A (46 samples, 2 nematodes species: *H. contortus* and *T. circumcincta*), the F167Y (17 samples, 2 nematodes species: *H. contortus* and *T. circumcincta*), the E198L (7 samples, *T. circumcincta*) and the E198G (4 samples, *H. contortus*) (Figure 2c).

Table 4 : Parameter estimates for the best generalized linear model explaining the resistant reads relative abundance (RRA) in ibex and sheep. The effect of host species (sheep as reference), study sites (Belledonne as reference), and their interaction, in addition to the nematode species (*Teladorsagia circumcincta* as

reference) are reported. Parameter estimates with standard error (SE) are reported with the corresponding *z-value (z*-val*)* and *p value* (*P*). AL: Aiguillette de Lauzet; MO: Montagne de l'Oule; Ch: Champsaur; Hc : *Haemonchus contortus*; Ta: *Trichostrongylus axei*; Tcol: *Trichostrongylus colubriformis*.

Best model selected	Variables	Parameter estimate ± SE	z-val	*P*
Resistant RRA ~ host species x study sites + nematode species	Intercept	-2.02 ± 0.59	-3.45	5e-04
	Species	-2.21 ± 0.66	-3.35	8e-04
	Mountain(AL)	-2.89 ± 0.99	-2.93	0.003
	Mountain(MO)	-1.37 ± 0.89	1.54	0.123
	Mountain(Ch)	-0.10 ± 1.18	-0.08	0.935
	Nematode(Hc)	2.52 ± 0.62	4.05	5e-05
	Nematode(Ta)	4.68 ± 0.59	7.95	1e-15
	Nematode(Tcol)	7.38 ± 0.75	9.79	<2e-16
	Species:Site(AL)	2.90 ± 1.19	2.44	0.015
	Species:Site(MO)	0.04 ± 1.28	0.03	0.973
	Species:Site(Ch)	-0.75 ± 1.32	-0.56	0.574

Sheep and ibex shared 164 (38%) β-tubulin isotype 1 haplotypes and 238 (55%) β-tubulin isotype 1 haplotypes was only found in ibex samples (Figure 3). Most of the resistant haplotypes of *T. circumcincta* and *H. contortus*, e.g., containing a non-synonym mutation to the codon 167, 198 or 200, were genetic variants of a common sensitive haplotype shared by ibex and sheep (Figure 3). The resistant haplotypes of *T. axei* and *T. colubriformis* were more common than the sensitive haplotypes and the most similar sensitive haplotypes are found either in both sheep and ibex samples, or only in ibex samples. Both, *T. colubriformis* and *T. vitrinus* showed two distinct lineages, separated by ≥10 mutations. One of the lineages of *T. vitrinus* was only found in ibex from Belledonne and an ibex from Champsaur (Figure 3) while the ASVs of the second lineage are found both in ibex and sheep. One of the lineages of the *T. circumcincta* was more diverse (Figure 3).

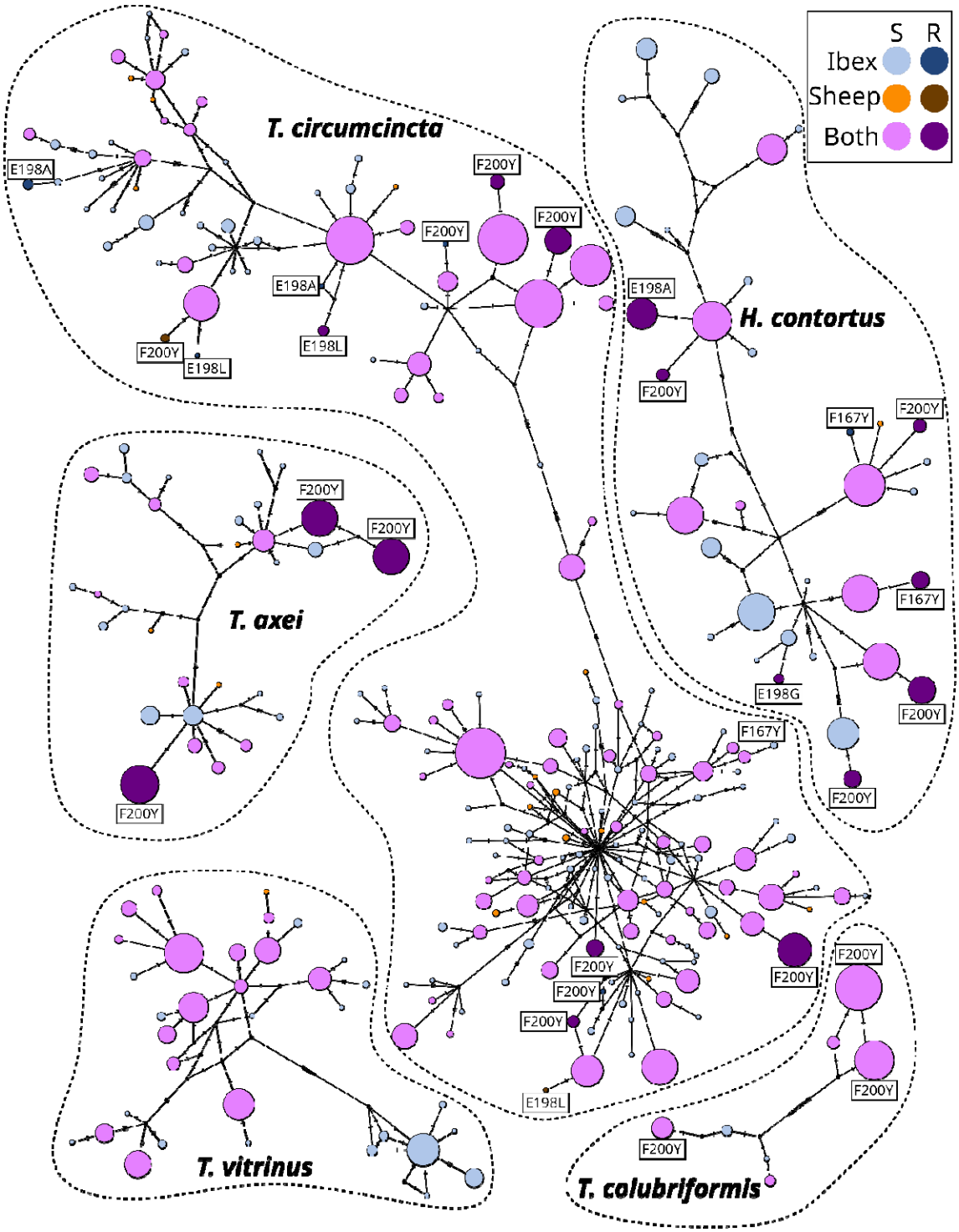

Figure 3 : Median joining network of β-tubulin isotype 1 haplotypes. Each point represents a unique haplotype, and the colors correspond to the host species in which the haplotype was detected. The size of the points is proportional to the number of samples in which the haplotype was found. S: sensitive haplotype, R:

resistant haplotype. The tag above the points indicated the name of the mutation, based on the codon position and the substitution of the amino acid.

Discussion

Because transhumant sheep and resident Alpine ibex use the same pastures during the summer, we sought to assess the extent of nematode parasites sharing between these two host species. Specifically, we investigated the presence of anthelmintic-resistant nematode strains in sheep and ibex to determine the role of transhumant sheep in contaminating alpine pastures, and whether ibex may play a role in the maintenance and circulation of anthelmintic resistant nematodes. We used a metabarcoding approach based the sequencing of ITS2 and the β-tubulin to demonstrate that both sheep and ibex were infected by the same gastrointestinal nematode species and shared anthelmintic-resistant strains, despite the absence of sheep on alpine pastures for much of the year and therefore a narrow temporal window of contamination.

In line with other studies investigating the gastrointestinal nematodes of sheep and ibex (Burgess et al., 2012; Gruner et al., 2006; Redman et al., 2019; Zaffaroni et al., 2000), the most prevalent and abundant species in both host species was *Teladorsagia circumcincta*. Next, *Trichostrongylus vitrinus* was moderately prevalent, but not abundant in sheep and ibex nemabiome. In accordance with the climatic conditions of ibex environment, these two nematode species, as well as *Marshallagia* spp., are better adapted to cold temperatures than the other nematode species detected in this study (O'Connor et al., 2006; Zaffaroni et al., 2000). The sheep flocks originate from French plains and/or the south of France and are driven into mountain areas in early summer. Consequently, their nemabiome at the time of sampling is representative of the gastrointestinal nematode communities present in sheep on the farm, i.e., prior to transhumance. In a similar context, Gruner et al., (2006), observed a high prevalence of *T. circumcincta* in two of three transhumant sheep flocks at the beginning of the grazing season in the mountains south of the Alps. Furthermore, transhumant sheep flocks appear to ingest mainly *T. circumcincta* when grazing in the mountains, as this parasite remains the dominant species identified in feces and tracer lambs during the summer (Gruner et al., 2006). Pastoral activity in

mountainous areas of France could therefore favor nematode species more adapted to cool and wet environmental conditions, such as *T. circumcincta* (O'Connor et al., 2006), compared with sheep grazing on the plains all year round. To confirm this hypothesis, a study of the variation in the nemabiome of transhumant sheep during the summer should be undertaken.

High relative frequency (>30%) of *H. contortus* was detected in ibex in Belledonne and Champsaur. In contrast, almost no *H. contortus* was observed in sheep flock droved in these mountains, raising the possibility that ibex may be contributing to the reinfection of sheep with *H. contortus*. To our knowledge, this is the first time that such relative abundance of *H. contortus* is reported in Alpine ibex (see previous studies based on morphological identification: Carcereri et al., 2021; Marreros et al., 2012; Zaffaroni et al., 2000). In addition, it should be noted that Alpine ibex were sampled before a potential contamination by domestic sheep could be detected, i.e., before the end of the pre-patent period: time between the infestation and the first eggs production, and at the end of spring – early summer, i.e., at the start of the epidemiological period at risk for *Haemonchus contortus* in a high-altitude mountain area. We can therefore expect higher levels of contaminations in late summer, when domestic sheep leave mountain pastures. In addition, we cannot exclude that some laboratory issues might have reduced the apparent prevalence and abundance of *H. contortus* as some samples from sheep have been kept in the fridge at 4°C during 2 to 3 days (including e.g., the sheep samples without *H. contortus* from Belledonne and Champsaur) which can reduce the proportion of *H. contortus* eggs hatching (McKenna, 1998).

The detection of *H. contortus* raises conservation issues for Alpine ibex as this nematode species is known to be highly pathogenic to sheep (Taylor et al., 2015). Infection of a phylogenetically related species, the Pyrenean ibex (*Capra pyrenaica pyrenaica*), with a few thousand of *H. contortus* resulted in severe clinical signs, including extremely low weight and hemorrhagic anemia (Lavín et al., 1997). In addition, *H. contortus* may have been involved, along with pneumonia, in the collapse of the Northern Chamois in the province of Lecco, Italy from November 2000 to March 2001 (Citterio et al., 2006). As gastrointestinal nematodes can have an impact on the demographic dynamics of the host population (Acerini et al., 2022; Albery et al., 2021; Albon et al., 2002), they are suspected of being involved in the low natality rates observed in the French Alpine ibex populations (Brambilla et al., 2020). While

Alpine ibex appears to be fairly resilient to parasite infections (Marreros et al., 2012), further investigations should be carried out to assess the consequences of gastrointestinal nematodes infections for ibex at both individual and population level.

We detected anthelmintic resistant alleles in 4 out of the 5 nematodes species, namely *H. contortus*, *T. circumcincta*, *Trichostrongylus axei*, *Trichostrongylus colubriformis*, but not *T. vitrinus*, for which the β-tubulin amplicons have been sequenced. Both sheep and ibex hosted resistant strains of the 4 nematode species and only 10 out of 116 ibex did not have resistant strains to any of the nematode species studied. The benzimidazole resistance was therefore very common in the studied sheep flocks, in agreement with the situation of sheep farms in Europe (Rose et al., 2015; Rose Vineer et al., 2020). The presence of anthelmintic resistant nematodes in ibex is most likely explained by the indirect transmission of resistant nematodes from sheep to ibex through the environment. The large number of shared β-tubulin ASVs between sheep and ibex and the high overlap between their nemabiome confirm this scenario (Figure 2c, Figure 3). This is in accordance with other studies investigating the share of nematode parasites at the interface of wild and domestic ungulates (Beaumelle et al., 2022; Cerutti et al., 2010; Laca Megyesi et al., 2019).

It is worth noting that feces of ibex were sampled before the arrival of sheep on pastures. This important result demonstrates that anthelmintic resistant nematodes can be maintained in mountainous areas from year to year in wild populations of ibex despite harsh winter environmental conditions, and in the absence of the main source of parasites during most of the year, i.e., the domestic sheep. The shedding of eggs from resistant nematodes by ibex prior to the arrival of domestic sheep suggests the potential role of ibex as a reservoir of anthelmintic resistant nematodes for other susceptible domestic and wild ungulates. In addition, the position of resistant mutant strains detected in ibex at the periphery of haplotypes networks (Figure 3) supported the relatively recent selection of benzimidazole resistance and the lack of benzimidazole resistant reversion since the resistant strains have been transmitted to ibex.

The 5 nematodes species for which we have studied the resistances seemed to have different selection dynamics which may reflect the life history traits of species (Redman et al., 2015). In fact, we detected no resistant allele in *T. vitrinus* and conversely, the proportion of benzimidazole resistant strains of *T. axei* and *T.*

colubriformis were high in sheep and in a lesser extent in ibex (Figure 2c). The resistance proportions of *T. circumcincta* and *H. contortus* were lower compared to *T. axei* and *T. colubriformis*, excepted for the *H. contortus* of the sheep flock of the Montagne de l'Oule. In this study area, the proportion of resistant strains of *H. contortus* was very high.

Consistent with our study, benzimidazole-resistant strains of *T. vitrinus* were rare in other studies of sheep farms (in the UK, Avramenko et al., 2019, and in Canada, Queiroz et al., 2020). In contrast, high frequencies of benzimidazole resistance in *T. axei* and *T. colubriformis* (between 40% and 100%) were already reported in sheep; in UK, *T. axei*: 26-27% and *T. colubriformis:* 53-62% (Avramenko et al., 2019); in Austria, *T. colubriformis:* 77%-100%, (Hinney et al., 2020); in France, *T. axei*: 63%, (Palcy et al., 2010). In contrast, Hinney et al., (2020), observed in transhumant sheep flocks in Austria Alps, a higher mean frequency of the F200Y resistance allele in *T. circumcincta*: 32.4 ± 6.8% (mean ± standard error of the mean) and *H. contortus:* 91.9 ± 3.7%, compared to the sheep flocks of this study (*T. circumcincta*, 6.6 ± 3.5% and *H. contortus,* 69.9 ± 14.4%, all resistant alleles combined).

Several factors are suspected to contribute to interspecific differences in the selection of resistance strains between nematode species, including specific reproductive rate, seasonal dynamics, climatic conditions in the location of sheep farms, anthelmintic strategies, e.g., treatment molecules, timing and rate of anthelmintic treatments, grazing management and the cost of benzimidazole resistance (Hodgkinson et al., 2019; Redman et al., 2015). However, the links between traits of parasites and interspecific variation of resistant acquisition by gastrointestinal nematodes have not been tested yet (Morgan et al., 2019). Our results suggested a few clues in relation to the ecology of nematode species.

Firstly, nematode species have different abilities to practice hypobiosis, i.e., the ability to halt embryonic development under environmental constraints (Gibbs, 1986). *H. contortus* and *T. circumcincta* are known to arrest development more frequently than *Trichostrongylus* spp. (Langrová et al., 2008), and hypobiotic larvae have been shown to be less sensitive to drug (Sargison et al., 2007). Secondly, among the *Trichostrongylus* spp., *T. vitrinus* may have a higher proportion of overwintering larvae in pastures as this species is more resistant to cold temperature compared to *T. axei* and *T. colubriformis* (O'Connor et al., 2006). As parasites on pasture are not

subject to selection pressure by anthelmintics, they are a source of susceptible strains.

As the proportion of resistant strains is generally lower in ibex compared to sheep, ibex may have contributed to a dilution effect of resistant strains, i.e., by hosting susceptible nematodes. However, the role of ibex in the maintenance of a refugia needs to be investigated by considering the relative number of susceptible strains deposited by ibex on pasture compared with sheep. Furthermore, it seems that the role of ibex in the maintenance of a refugia vary according to nematode species. For example, ibex excrete a lower proportion of *T. axei* eggs than sheep (Figure 2a), while resistant strains are also dominant in ibex (Figure 2c). In contrast, *T. circumcincta* and *H. contortus* in ibex were more frequently susceptible and genetically diverse (higher number of ITS2 and β-tubulin ASVs) compared with *T. axei* and *T. colubriformis* ibex (Table 2, Figure 3). As *T. circumcincta* was dominant in ibex, a refuge of susceptible *T. circumcincta* strains may have been maintained within ibex and may contribute to limit the spread of resistance in sheep farms. The results concerning *H. contortus* were variable according to the massifs since ibex from the two Cerces sites had a low amount of *H. contortus* compared to the two other populations, i.e., Belledonne and Champsaur.

The divergence between massifs can be observed at the community and genetic level among sheep flocks and ibex populations. Indeed, it was expected that some differences in nemabiome composition would be observed between the massifs and sheep flocks, given that sheep and ibex from different areas never meet (R. Papet, C. Toïgo, E. Vannard, Pers. communication). Furthermore, the sheep flocks come from different locations and have been subjected to different anthelmintic strategies. For the ibex, differences in the original population of translocated animals (Gauthier and Villaret, 1990; Kessler et al., 2022) and potential founder effect – not all parasites present in the source population were present in the newly established sink population - may have had a long term impact on the composition of the nemabiome. For example, the distinct population of *T. vitrinus*, found mainly in ibex in Belledonne, may had been inherited from the founding ibex population. This highlights that various reintroductions of ibex in the study area can also influence the composition of the parasite community. This distinct population *of T. vitrinus* was absent in sheep grazing in Belledonne, which hosted other strains of *T. vitrinus* and despite several summers of co-occurrence of sheep and ibex in Belledonne. In is possible that sheep

never graze on pastures contaminated by these strains, but this observation may also be due to a sampling bias as the number of sheep sampled remains low. Within the same mountain area, few differences have, however, been observed among the nemabiome of ibex, e.g., between the ibex of the Aiguillette de Lauzet and those of the Montagne de l'Oule, whereas the sheep flocks host distinct nemabiome communities.

In conclusion, transmissions of gastrointestinal nematodes species, including resistant nematodes strains, have occurred between sheep and ibex even though the contact between the two species is limited to the summer period. In this study, we demonstrated more specifically that ibex can maintain and shed eggs of resistant gastrointestinal nematodes despite the absence of sheep on pastures for several months, suggesting a potential role of ibex as a reservoir for these nematodes. However, the extent to which each host species can influence the nematode community of the other during the transhumant period remains to be determined. To this end, it would be useful to analyze the nemabiome of sheep and ibex before and after the transhumant period. The lower proportion of resistance allele in ibex nemabiome compared with that of sheep underlines the possibility that ibex could contribute to the maintenance and circulation of susceptible strains in sheep. Based on our results, it seems that ibex have helped to limit the spread of anthelmintic resistance of *T. circumcincta* and *H. contortus* in sheep flocks, by maintaining parasite refugia not exposed to anthelmintic pressure. As with sheep and roe deer (Beaumelle et al., 2022), domestic sheep contributes to the modification of the nemabiome of ibex. This raises concerns about the conservation of the ibex, and the consequences of strongyles infection in ibex should be investigated. Indeed, ibex is characterized by low genetic diversity due to the strong demographic decline of this species, followed by multiple re-introduction (Grossen et al., 2018) and a high genetic structure of immunity-related loci among populations (Kessler et al., 2022) whereas both neutral and adaptive genetic diversity are known to have an influence parasite resistance in ungulates (Portanier et al., 2019).

Appendices

Supplementary data to this article can be found online in MendeleyData (DOI: 10.17632/cm97cg87d6.1).

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Conflict of interest disclosure

The authors declare that they have no conflict of interest.

Data, scripts, and supplementary information availability

The bioinformatic pipeline, the ASV analysed during the current study and the R script of statistic analyses are available in MendeleyData (DOI: 10.17632/cm97cg87d6.1).

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