MOLECULAR IDENTIFICATION OF *TRICHOSTRONGYLUS AXEI* ON EUROPEAN BROWN HARE (*LEPUS EUROPAEUS*) IN WESTERN ROMANIA – CASE REPORT

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Abstract
Trichostrongylosis is a cosmopolitan parasitic disease affecting domestic and wild ruminants, equines, and last but not least, leporids. Three species of strongyles commonly parasitize the digestive tracts of leporids, the most prevalent being *Trichostrongylus retortaeformis*. This paper describes the first case of *Trichostrongylus axei* infestation in a wild hare in western Romania. A female wild hare carcass found in Timis County was examined at the Parasiology Department of the Faculty of Veterinary Medicine in Timisoara. A clinical, post-mortem and PCR examination was performed to establish a diagnosis, with molecular analysis confirming the presence of the nematode *Trichostrongylus axei* in European brown hare.

Introduction
The European brown hare (*Lepus europaeus*) is a species native to Europe and parts of Asia [1]. It can be regarded as one of the most important game animals in Europe [2]. In Europe, the brown hare is a major representative of small game and is widespread throughout our country, from the Black Sea basin to mountainous areas. In Europe since the late 1960s, a decline in the European hare population has been observed and has been the subject of numerous studies focusing on the role of habitat [3-5], agriculture [5-8], predation [9] and disease [10-13]. This unfavourable development has been reported in several European countries such as France [14], Denmark [9], Slovakia [15], Poland [16], Serbia [17] and Germany [18].
Apart from common rabbit diseases with a significant impact on rabbit mortality, parasitic diseases are also considered to be a factor in rabbit population reduction [19]. Various parasites, mainly *Eimeria* spp., *Trichostrongylus* spp. and occasionally *Dicrocoelium* spp. and *Linguatula serrata* have been identified in European brown hares [10, 12, 15, 20-23].
The aim of the present study was to identify via molecular analysis the *Trichostrongylus* species present in the European brown hare.

Experimental
Clinical examination
The carcass of a female European brown hare of almost 2 years of age (Figure 1) was found in a forest area in the vicinity of a village in the north of Timis County and was examined in the Parasitic Diseases Clinic of the Faculty of Veterinary Medicine in Timisoara.

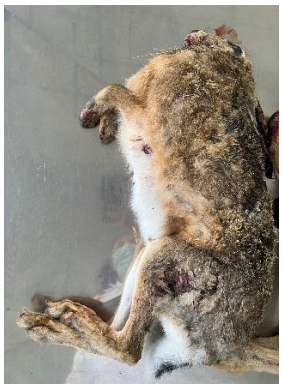

Figure 1. European brown hare

After the clinical examination, where no changes were observed, the animal was necropsied. The heart, lungs, liver, spleen and mesenteric lymph nodes were removed and examined separately. Smaller organs were placed in a Petri dish with distilled water and examined under a microscope; the liver and lungs were sectioned into slices of approximately 5 mm to 1 cm and examined; and the intestine was sectioned longitudinally and examined under a stereomicroscope. Parasites found were isolated and preserved in 70% ethanol.

DNA extraction and molecular analysis

The PCR reaction was performed according to the technique described by Yong et al. in 2007 [24]. Amplification was performed by classical PCR and was based on amplification of a ~450 bp sequence for Trichostrongylus spp. modified for the requirements of the mixture.

According to the protocol, the following primers were used: JHTSP (5′-TTATGTGCCACAAATGAAGA-3′ forward primer) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′ reverse primer). A MyTaqTM Red Mix Master Mix (BIOLINE®) was used for the reaction. The final volume of the PCR reaction was 25 µl, of which 12.5 µl MyTaqTM Red Mix (BIOLINE®), 1 µl primer 1 Forward, 1 µl primer 2 Reverse (diluted to a concentration of 10 pmol/µl according to the protocol described by the manufacturer), DNA extracted from the sample to be analysed and ultrapure water. The amplification program was carried out with the My Cycler thermocycler (BioRad®). This program included the steps of DNA denaturation at 95°C for 1 minute; 32 cycles of: denaturation at 95°C for 30 seconds, hybridization at 50°C for 30 seconds and extension at 72°C for 30 seconds; followed by incubation at 4°C. Amplicon analysis and control was performed by horizontal electrophoresis in a 1.5% agarose gel electrophoresis submersion system with the addition of MidoriGreen flourescent dye (Nippon Genetics® Europe) at 120 V and 90 mA for 60 minutes. After migration of the samples into the agarose gel, the image of the gel with migrated DNA fragments was captured using a UV photodocumentation system (UVP®).

PCR results were sequenced at Macrogen Europe® Company (Amsterdam, The Netherlands) and compared with those available in the GenBank database using BLAST alignment.

Results and discussion

Parasites found (n=21) were examined using a stereomicroscope (17 females and 4 males) (Figures 2 and 3). After analysis, two adults were preserved in 90% ethanol for molecular analysis.

Figure 2 Posterior extremity of a *Trichostrongylus axei* female

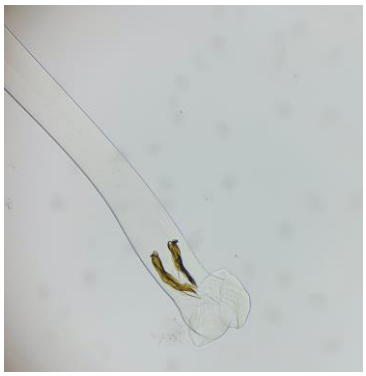

Figure 3. Posterior extremity of a *Trichostrongylus axei* male illustrating the copulatory bursa and the spicules

Samples examined by the PCR method were successfully processed. The first well contains the molecular marker at 100 bp and well 2 contains the positive DNA sample amplified from *Trichostrongylus* spp. adults (Figure 4).
Samples were checked in GenBank, then compared with available sequences using BLAST. The sequencing results of the samples can be seen in Table 1.

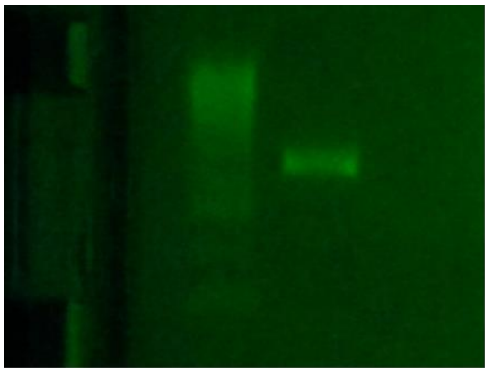

Figure 4. Image of 1.5% agarose gel electrophoresis of amplicons resulting from amplification of extracted DNA with specific primers

Table 1

Results of sequenced samples

Crt. no.	Sample name	Similar sequence	Species
1.	434	ON677948.1	*Trichostrongylus axei*
2.	435	AY439026.1	*Trichostrongylus axei*

Trichostrongylus axei is a ruminant-specific parasite and has never been found in naturally infected hares in western Romania. Experimental infections with *Trichostrongylus axei* and *Trichostrongylus colubriformis* have been performed in hares. Female hares have been shown to be more susceptible to infection with *T. axei* than males, the latter being more susceptible than females to *T. colubriformis* [25].
Natural transmission of some nematode species present in the digestive tracts of ruminants is possible but uncommon in domestic or wild hares. For example, Saulai and Cabaret (1998) reported ruminant-specific parasites, namely *T. colubriformis* and *T. capricola* in *Oryctolagus cuniculus* and *Lepus capensis*, respectively [26]. However, these infections were sporadic despite the large number of ruminant species. In Italy, *T. colubriformis, T. axei, T. vitrinus* and *Ostertagia ostertagi* have been reported in *O. cuniculus* in Sicily [27], while in Sardinia, *T.*

vitrinus and *T. colubriformis* have been reported in wild hares [28]. Regarding *T. circumcincta*, experimental infection of rabbits with larvae has been found to be difficult but reasonable in *L. europaeus*, and not in *O. cuniculus* [29].

Conclusion

This is the first study to confirm the presence of the nematode *Trichostrongylus axei* in the digestive tract of the European brown hare (*Lepus europaeus*) in western Romania.

References

[1] Mori, E., Carbone, R., Viviano, A., Calosi, M., Fattorini, N., Factors affecting spatiotemporal behaviour in the European brown hare *Lepus europaeus*: A meta-analysis. Mamm. Rev. 2022, 52, 454–470.
[2] Tsokana, C.N., Sokos, C., Giannakopoulos, A., Birtsas, P., Valiakos, G., Spyrou, V., Athanasiou, L.V., Burriel, A., Billinis, C., European brown hare (*Lepus europaeus*) as a source of emerging and re-emerging pathogens of public health importance: a review. Veterinary Medicine and Science, 2020, 6(3).
[3] Pavliska, P.L., Riegert, J., Grill, S., Šálek, M., The effect of landscape heterogeneity on population density and habitat preferences of the European hare (*Lepus europaeus*) in contrasting farmlands. Mamm. Biol. 2018, 88, 8–15.
[4] Pépin, D., Angibault, J.M., Selection of resting sites by the European hare as related to habitat characteristics during agricultural changes. Eur. J. Wildl. Res. 2007, 53, 183–189.
[5] Jennings, N., Smith, R.K., Hackländer, K., Harris, S., White, P.C.L., Variation in demography, condition and dietary quality of hares *Lepus europaeus* from high-density and low-density populations. Wildlife Biol. 2006, 12, 179–189.
[6] Panek, M., Habitat factors associated with the decline in brown hare abundance in Poland in the beginning of the 21st century. Ecol. Indic. 2018, 85, 915–920.
[7] Smith, R.K., Vaughan Jennings, N., Harris, S., A quantitative analysis of the abundance and demography of European hares *Lepus europaeus* in relation to habitat type, intensity of agriculture and climate. Mamm. Rev. 2005, 35, 1–24.
[8] Tapper, S.C., Barnes, R.F.W., Influence of Farming Practice on the Ecology of the Brown Hare (*Lepus europaeus*). J. Appl. Ecol. 1986, 23, 39–52.
[9] Schmidt, N.M., Asferg, T., Forchhammer, M.C., Long-term patterns in European brown hare population dynamics in Denmark: effects of agriculture, predation and climate. BMC Ecology. 2004, 4(1).
[10] Brustenga, L., Franciosini, M.P., Diaferia, M., Rigamonti, G., Musa, L., Russomanno, B.L., Veronesi, F., Parasitological Survey in European Brown Hare (*Lepus europaeus* Pallas, 1778) Breeding Facilities in Southern Italy. Pathogens. 2023, 12, 208.
[11] Faehndrich, M., Klink, J.C., Roller, M., Wohlsein, P., Raue, K., Strube, C., Prenger-Berninghoff, E., Ewers, C., Capucci, L., Lavazza, A., Status of Infectious Diseases in Free-Ranging European Brown Hares (*Lepus europaeus*) Found Dead between 2017 and 2020 in Schleswig-Holstein, Germany. Pathogens 2023, 12, 239.
[12] Panayotova-Pencheva, M.S., Endoparasites of the European brown hare (*Lepus europaeus* Pallas, 1778 L.) (Lagomorpha: *Leporidae*) from Bulgaria. Ann Parasitol. 2022; 68(3): 553-562.
[13] Wibbelt, G., Frölich, K., Infectious Diseases in European Brown Hare (*Lepus europaeus*). Wildl. Biol. Pract. 2005, 1, 86–93.
[14] Marboutin, E., Bray, Y., Peroux, R., Mauvy, B., Lartiges A., Population dynamics in European hare: breeding parameters and sustainable harvest rates. Journal of Applied Ecology. 2003, 40(3): 580–591.

[15] Dubinsky, P., Vasilková, Z., Hurníková, Z., Miterpáková, M., Slamečka, J., Jurčík, R., Parasitic infections of the European brown hare (*Lepus europaeus* Pallas, 1778) in south-western Slovakia. Helminthologia. 2010, 47(4): 219–225.
[16] Kornaś, S., Wierzbowska, I.A., Wajdzik, M., Kowal, J., Basiaga, M., Nosal, P., Endoparasites of European brown hare (*Lepus europaeus*) from southern Poland based on necropsy. Annals of Animal Science. 2014, 14(2): 297–305.
[17] Ilić, T., Petrović, T., Dimitrijević, S., Parasitic infections of wild rabbits and hares. Veterinarski Glasnik. 2014, 68(3–4): 241–250.
[18] Sliwinski, K., Ronnenberg, K., Jung, K., Straub, E., Siebert, U., Habitat requirements of the European brown hare (*Lepus europaeus* Pallas 1778) in an intensively used agriculture region (Lower Saxony, Germany). BMC Ecology. 2019, 19(1).
[19] Alzaga, V., Tizzani, P., Acevedo, P., Ruiz-Fons, F., Vicente, J., Gortazar, C., Deviance partitioning of host factors affecting parasitization in the European brown hare (*Lepus europaeus*). Naturwissenschaften. 2009, 96: 1157–1168.
[20] Jitea, B.A.-M., Imre, M., Florea, T., Sîrbu, C.B., Luca, I., Stancu, A., Ciresan, A.C., Darabus, Characterization of the Tongue Worm, *Linguatula serrata* (Pentastomida), Identified from Hares (*Lepus europaeus*) in Romania. Int. J. Mol. Sci. 2023, 24, 12927.
[21] Jitea (Sîrbu), B.A.M., Sîrbu, C.B., Florea, T., Hotea, I., Dărăbuș, Gh., Endoparasites found in *Lepus europaeus* hunted In Western Romania, 22nd International Multidisciplinary Scientific GeoConference SGEM 2022, 691 – 697.
[22] Sergi, V., Romeo, G., Serafini, M., Torretta, E., Macchioni, F., Endoparasites of the European Hare (*Lepus Europaeus*) (Pallas, 1778) in Central Italy. Helminthologia. 2018, 55(2):127-133.
[23] Hora, F., Mederle, N., Badea, C., Tilibaşa, E, Ilie, M., Dărăbus, G., Digestive parasite fauna in hare (*Lepus europaeus*) in Western Romania. Scientific Works. Series C. Veterinary Medicine. 2015, 61, 138 - 141.
[24] Yong, T.S., Lee, J,H., Sim, S., Lee, J., Min, D.Y., Chai, J.Y., Eom, K.S., Sohn, W.M., Lee, S.H., Rim, H.J., Differential diagnosis of *Trichostrongylus* and hookworm eggs via PCR using ITS-1 sequence, Korean Journal of Parasitology. 2007, 45 (1): 69 – 74.
[25] Ciordia, H., Bizzell, W.E., Porter, D.A., Dixon, C.F., The effect of culture temperature and age on the infectivity of the larvae of *Trichostrongylus axei* and *Tricostrongylus colubriformis* in rabbits and guinea pigs. The Journal of Parasitology, 1966, 52, 5, 866 – 870.
[26] Saulai, M., Cabaret, J., Limited role of lagomorphs (*Oryctolagus cuniculus* and *Lepus capensis*) in the dispersion of parasite nematodes of ruminants. Vet. Parasitol. 1998, 77: 301 – 304.
[27] Giannetto, S., Virga, A., Baccarani, E. M., Canestri Trotti, G., Elminti intestinali in conigli selvatici (*Oryctolagus cuniculus*). Atti SISVet., 1998, 52: 165 – 166.
[28] Lai, M., Arru, E., Trichostrongylidae del coniglio selvatico (*Oryctolagus cuniculus*). Parassitologia. 1969, 11: 97 – 99
[29] Stott, P., O'callaghan, M., Phillips, P., Verbyla, A., The experimental establishment of ruminant nematodes in European hares (*Lepus europaeus*). Vet. Parasitol., 2009, 159: 82 – 85.