THESIS

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BENZIMIDAZOLE RESISTANCE IN *HAEMONCHUS CONTORTUS* IN DOMESTICATED AND WILD RUMINANT POPULATIONS IN SOUTHWESTERN HUNGARY

Kaposvár
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ABBREVIATIONS

AR	anthelmintic resistance
BZ	benzimidazole
ABZ	albendazole
ABZSO	albendazole-sulphoxide
DNA	deoxyribonucleic acid
EHT	egg hatch test
FECRT	faecal egg count reduction test
FEN	fenbendazole
GIN	gastrointestinal nematode
H	Shannon diversity index
H. contortus	*Haemonchus contortus*
I	importance index
IVM	ivermectin
L1	first stage nematode larva
L2	second stage nematode larva
L3	third stage nematode larva
L4	fourth stage nematode larva
L5	fifth stage nematode larva
MBZ	mebendazole
PCR	polymerase chain reaction
R	resistant

RFLP-PCR	restriction fragment length polymorphism-polymerase chain reaction
RR	homozygous resistant
RS	heterozygous
S	susceptible
SC	Sorensen coefficient
SNP	single nucleotide polymorphism
SS	homozygous suscptiblet
OXF	oxfendazole
TBZ	thiabendazole

1. REVIEW OF THE LITERATURE

1.1. *Haemonchus contortus* and haemonchosis in small ruminants

1.1.1. Taxonomy and life cycle

Haemonchus contortus (Phylum: Nematoda; Order: Strongylida; Superfamily: Trichostrongyloidea; Family: Trichostrongylidae; Subfamily: Haemonchinae; Genus: *Haemonchus*) is a blood-feeding and highly pathogenic parasite live on the abomasal mucosa. They can infect a wide range of hosts, such as Bovidae, Cervidae, Camelidae, Giraffidae and Antilocapridae (Anderson, 2000; Kassai, 2003; Hoberg et al., 2004).

H. contortus, as known as barber's pole worm in English literature, has a direct life cycle. The adult worms live inside the abomasum of the host, where the fecund females produce a large number of eggs. The morulated eggs excrete via faeces and in optimal environmental condition can hatch within 1 day. After 2 moults the hatched L1 larvae develop to the infective L3. The specimens of this stage can move horizontally and vertically on the vegetation and the ground. The L3s ingested by the host, and in its abomasum moult to L4. In this phase, the larvae development could continue in two different manners. The first is a continuous development to L5, subadult and adult stages, while the worms become fertile and after copulation, the females lay eggs. In this case, the prepatent period is 3-4 weeks (Figure 1).

The second manner is hypobiosis. Hypobiosis is the most useful life cycle adaptation to ensure persistence and has been reported in most of the Trichostrongylid nematodes parasitizing small ruminants. It facilitates the synchronization of the nematode life cycle to changing host and unfavourable environmental conditions. It allows *H. contortus* (barber's pole worm) to survive until more favourable conditions resume. (Gibbs, 1986; Kassai, 2003; Taylor et al., 2016). In favourable environmental circumstances, the development of hypobiotic larvae proceeds as it was shown above.

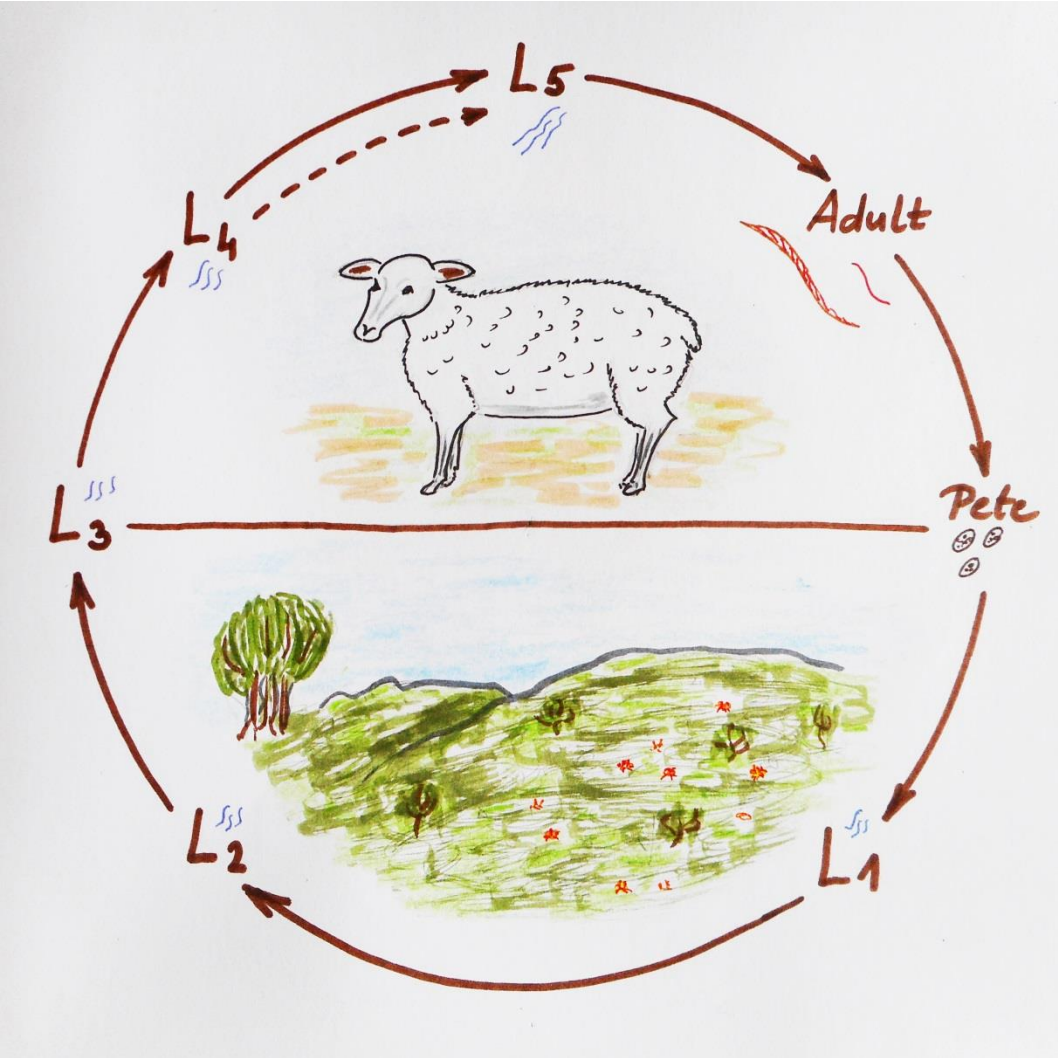

Figure 1. The direct life cycle of *H. contortus* (barber's pole worm)

1.1.2. Geographical distribution

H. contortus is originated in the sub-Saharan region of Africa. The domestication of artiodactyls and the very intensive sheep *(Ovis aries)*, goat *(Capra aegagrus hircus)*, cattle *(Bos taurus)* translocation since the 1500s, were the core associations for the global distribution of the helminth (Hoeberg et al., 2004). Although the presence of the worm is more considerable in tropical and subtropical climatic zones in both hemispheres, the species has a remarkably adaptive strategy over a colder and dryer environment. Due to the high biotic potential, the *H. contortus* is able to successfully conform to the colder, dryer climates, where just a short, favourable period exists for the development of its free-living stages (Waller and Chandrawathani, 2005; O'Connor et al., 2006).

This adaptive mechanism is well confirmed by Troel et al. (2006) and Falzon et al. (2014). These results suggested that infective (L3) *H. contortus* larvae could be able to over-

winter on pasture in the temperate and cold climate zones of the northern hemisphere. This phenotypic trait was regardless of tropical or cold zone origin of the larvae. Although the surviving rate was lower than other important parasite genera such as *Teladorsagia* spp., *Nematodirus* spp., and *Trichostrongylus* spp., this cold tolerant trait may contribute to the continuous infection.

The increasing presence and common occurrence of *H. contortus* in various temperate and sub-polar climate zones, the hypobiosis or arrested development is the keystone of continuous perpetuance (Waller et al., 2004).

1.1.3. Environmental factors affecting the free-living stages

The zonal distribution and seasonal epidemiological characteristics of *H. contortus* reflect on the major climatic variables affecting the development and survival of free-living stages. The main determinants of developmental success are undoubtedly temperature and moisture, and of course, some other factors (e.g. vegetation, light intensity) might also have importance (O'Connor et al., 2006). The optimal development requirements in the four free-living stages differ (Table 1).

Table 1: Effects of environmental factors on the free-living stages of *Haemonchus contortus* under controlled conditions (adapted from Gasser and von Samson-Himmelstjerna, 2016)

Target of investigation	Environmental factor	Optimal condition	Limiting condition
Development and survival of unembryonated eggs	moisture	relative humidity 100% at 20-35°C	relative humidity <85% at 20-35°C
Development and survival of embryonated eggs	moisture	relative humidity 100% at 25-35°C	relative humidity <88% at 20°C
Survival of unembryonated eggs	low temperature	0-4°C (<10 days)	<0°C
Survival of embryonated eggs	low temperature	0-4°C (<2 months)	<0°C
Development of eggs to infective larvae	temperature (no moisture restriction)	15°C: 4-12 days 22-25°C: 3-7 days 35-37°C: 3 days	<8 °C: no development 10 °C: 2-4 weeks 40 °C: no development
Survival of infective larvae	moisture	relative humidity 60-90% at 20 °C: 8-36 weeks	relative humidity <50% at 20 °C: few days to 3 weeks
Survival of infective larvae	temperature	5-10 °C: >12 months 15-20°C: 32-56 weeks 25-30°C: 17-36 weeks	<0°C: <20 weeks 35-40°C: 1-2 weeks >40°C: few days

1.1.4. Clinical symptoms, diagnosis

Among the gastrointestinal nematodes of small ruminants, *Haemonchus contortus* has almost the most overwhelming importance. Owing to the fecundity, genetic diversity, and the very adaptive phenotypic plasticity, this abomasal bloodsucking worm may be the most important invader parasite in sheep and goats. This species causes enormous economic losses, health problems and frequently increases the mortality rate in the host populations. *H. contortus* has shown a great ability to develop anthelmintic resistance (AR) which has emerged in all countries of the world that produce small ruminants (Waller and Chandrawathani, 2005; Papadopulos et al., 2012; Barrere et al., 2013; Roeber et al., 2013).

The symptoms of *H. contortus* infection relate almost entirely to the blood-feeding activities of adult and/or L4, L5 larval stages (Taylor et al., 2016). Depending on the worm/larvae number, the haemonchosis can be categorised as a hyperacute, acute, and chronic infection. In hyperacute form, the symptoms are a massive blood loss, haemorrhagic gastritis, and terminal anemia. In such a case, the parasite number exceeds 25-30 000 specimens. The acute haemonchosis develops ingesting numerous L3 after 4-6 weeks post-infection. The worm burdens of 2000-20 000 worms per host. It is characterised by severe anaemia, variable degrees of oedema, of which the submandibular form (‘bottle jaw’), and ascites are the most easily recognised symptoms, while lethargy, dark-coloured faeces, and falling wool might be also detected. In the temperate climatic regions, the most prevalent form is a chronic infection. Chronic haemonchosis is associated with progressive weight loss, weakness, neither severe anaemia nor gross oedema. The performance (weight gain, milk production) decreases and manifests similarly as malnutrition (Gasser and von Samson-Himmelstjerna, 2016; Taylor et al, 2016).

1.2. Benzimidazoles as anthelmintics

1.2.1. Overview of benzimidazoles

In the grazing livestock, agriculture has been depending heavily on the routine application of broad-spectrum anthelmintics. Nowadays, just four broad-spectrum anthelmintic classes can be found, namely the benzimidazoles (BZs), the imidazothiazoles, the macrocyclic lactones and the monepantel (Kaplan, 2004; Kaminsky et al., 2008). Due to

the intensive use of these drugs resulted in the rapid appearance of AR in small ruminants (Table 2).

Table 2. Broad spectrum athelmintic classes and appearance of resistance in sheep. (Adapted from Kaplan (2004).

Drug	Year of approval	First published report of resistance
Benzimidazoles Thiabendazole	1961	1964
Imidothiazoles Levamisole	1970	1979
Avermectin–milbemycins Ivermectin Moxidectin	 1981 1991	 1988 1995
Amino-acetonitrile derivative Monepantel	2008	2013

In veterinary practice, one of the most preferred drug groups is BZs, due to their advantageous properties; such as high therapeutic index, the absence of toxic residuals in milk and meat and economical availability (Tiwari et al., 2006). The usage of albenzimidazole (ABZ) is surpassing in the sheep farming practice, although the group contains several drugs (Figure 2).

The primary effect of BZs is disruption of the tubulin microtubule-equilibrium. Microtubules have a fundamental role in the maintenance of cellular homeostasis in eukaryotic cells. Through binding of BZ molecules to the β-tubulin monomer, the proliferation of polymeric microtubules by addition of α-/β-tubulin heterodimers is inhibited, which can cause energy deficiency (von Samson-Himmelstjerna et al., 2007).

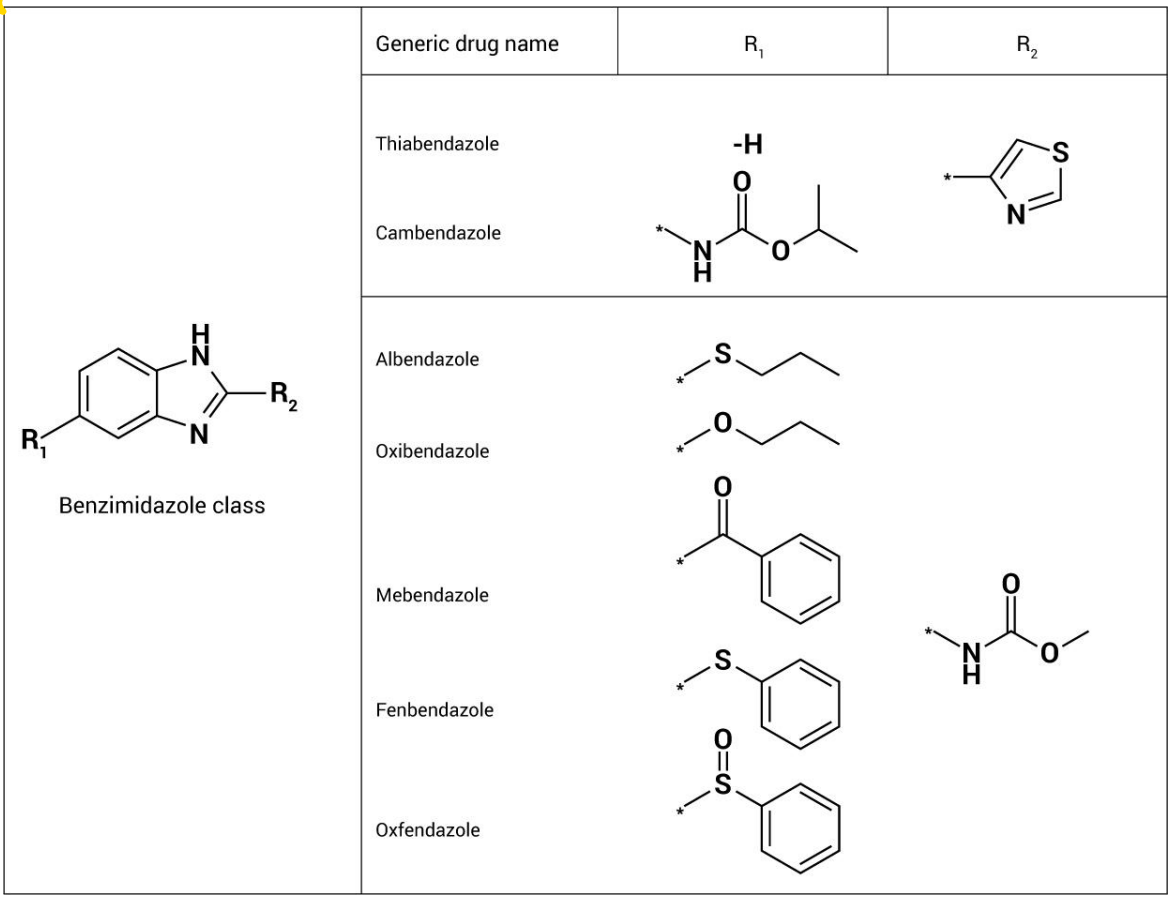

Figure 2. Representatives of BZ class (Adopted from: Anonymous, 2007)

1.2.2. Resistance to benzimidazoles

It is accepted, that AR is a pre-adaptive heritable phenomenon coded in a gene or genes. It could be present within the parasite population even prior to the drug being used for the first time. The appearance of drug resistance facilitates an increase in the frequency of individuals able to tolerate elevated drug doses relative to a normal population, therefore reflects changes in the composition of a parasite population gene pool (Beech et al., 1994). In the last decades, one of the most threatening factors for grazing, especially in small ruminants is AR (Rose et al., 2015). For this reason, the level of BZ resistance in *H. contortus* all over the word is a living problem (Jabbar et al., 2006) (Table 3).

Table 3. Resistance to BZs in *H. contortus* reported in different parts of the world (Adopted from Jabbar et al., 2006)

Drug	Country	Date of publication
TBZ	Australia	1979
	United Kingdom	1983
	United States of America	1970
BZ	Australia	1981
	Belgium	1993
	France	1986
	Germany	1987
	India	1992
	Kenya	1997
	Malaysia	1994
	Netherlands	1997
	New Zealand	1990
	South Africa	1997
	Southern Latin America, Brazil	1996
	United Kingdom	1991
	Zimbabwe	1997
FEN	Australia	1985
	India	1993
	Kenya	1997
	United Kingdom	1989
	United States of America	1992
	Zimbabwe	1997
OXF	Australia	1992
	Netherlands	1997
	Pakistan	2003

BZ = Benzimidazole; MBZ = Mebendazole; FEN = Fenbendazole; TBZ = Thiabendazole; OXF = Oxfendazole

1.2.3. Methods for detection of benzimidazoles resistance

The most common, practical, *in vivo* BZ resistance detection method is the faecal egg count reduction test (FECRT), which compares the egg count before and after treatment with the anthelmintic drug. The egg hatch test (EHT) is an in vitro test, which is proper only for the detection of BZ resistance. Its usage can assess the ovicidal activity of this molecule group. Both of these tests have a considerable disadvantage, the sensitivity. FECRT and the EHT are capable to detect resistance only when at least 25% of the worm population carries resistance genes (Martin et al., 1989). Although there are some other time consuming and labour intensive *in vitro* tests, as larval development test, adult development test, adult motility test, their application is confined, because of cost, applicability, interpretation or reproducibility of findings (Jabbar et al., 2006).

It is generally accepted that mutations in the β-tubulin genes are the major determinants of BZ resistance. Two genes, isotypes 1 and 2, were isolated and correlated with resistance. To our present knowledge, it seems that 3 different SNPs (Single Nucleotide Polymorphism: i.e., Phe200Tyr, Phe167Tyr or Phe167His, and Glu198Ala) in the isotype-1 β-tubulin can be responsible mostly for BZ resistance. A thymine-to-adenine transversion causes a substitution of phenylalanine with tyrosine at codon 200 of the isotype 1 protein (Kwa et al., 1994). This mutation is widespread all over the world, therefore (Coles et al., 2006). At position 167, one allele is being associated with BZ resistance in *H. contortus* Prichard, 2001). This polymorphism involves a substitution of phenylalanine with tyrosine, but also substitution of phenylalanine with histidine. The last detected mutation in isotype 1 can also contribute to BZ resistance in *H. contortus* described by Ghisi et al. (2007); it is known as an adenine-to-cytosine transversion that leads to a glutamate-to-alanine polymorphism at codon 198 in BZ-resistant populations from Australia and South Africa.

Several PCR protocols have been developed, which provide high accuracy and sensitivity when used to investigate the AR. These methods are able to detect 1% of resistant individuals within a susceptible worm population, which is a tremendous improvement comparing to *in vivo* and *in vitro* tests (Roos et al., 1995). By using a representative number of sample, the allele-specific PCR, real-time PCR, restriction fragment length polymorphism-PCR and sequencing can provide reliable results about the resistance status of a given worm population.

1.3. Nematode cross-transmission between domesticated and wild ruminants

Disease transmission between wild and domesticated ruminants has many important aspects. Its incidence can affect the population dynamics of wild games and sometimes can cause serious economic problems in animal husbandry (Martin et al., 2011). The agents of these diseases are predominantly microparasites (viz prions, viruses, and bacteria); and a merely smaller amount is caused by macroparasites in the wildlife-livestock interface (Wiethoelter et al., 2015). Although the nematodes of domestic ruminants have very exiguous importance in human parasitic infections; but on occasion, those could emerge in well confinable areas (Holló et al., 1970; Vilimszky and Szigethiné, 1971; Sato et al., 2011). The economic losses derived from clinical and subclinical gastrointestinal nematode infection are enormous problems all over the world. The induced damages have been derived mainly by

decreasing meat and milk production, fertility rate, and increased susceptibility to other bacterial or viral diseases. In wild ruminant populations, parasites have pronounced effects on individual productivity; which could affect population dynamics (Gunn and Irvine, 2003; Roeber et al., 2013; Walker and Morgan, 2014).

In grazing systems shared between wild and domesticated ruminants, the cross-infection could occur in both directions (Zaffaroni et al., 2004; VanderWaal et al., 2014; Walker and Morgan, 2014). Preston et al. (1979) infected Thomson's gazelle *(Eudorcas thomsonii)* by sheep-derived mixed gastrointestinal nematode (GIN) larvae. After 4 weeks, *Haemonchus contortus* was the only species recovered during necropsy. Similar results were observed in an experiment when the authors infected white-tailed deer with *H. contortus* recovered from sheep. The established worms affected significantly the haematological parameters (packed cell volume, haemoglobin, and total serum protein) and caused severe infection in fawns (McGhee et al.,1981). In Tapia-Escárate et al's investigation (2015), red deer *(Cervus elaphus)* calves were infected with mixed, sheep-originated gastro-intestinal nematode (GIN) larvae. After 4 weeks, the animals were euthanized and dissected to collect GIN from their alimentary tracts. They concluded that *Haemonchus, contortus*, *Teladorsagia circumcincta*, *Cooperia curticei*, *Trichostrongylus axei* and *Oesophagostomum venulosum* were able to establish infection. The results of the abovementioned studies may suggest that only some generalist nematodes (eg *Haemonchus contortus*) could establish and configure stable metapopulation in wild hosts.

On the other hand, wild species could also infect domestic ones in different circumstances. In field conditions, a serious setariosis was observed between 2003 and 2005 in Finnish, semi-domesticated reindeer (Laaksonen et al., 2009). The clinical symptoms (peritonitis) were caused by *Setaria tundra* derived from free-range roe deer *(Capreolus capreolus)* and reindeer *(Rangifer tarandus)* populations.

A similar direction of infection was detected in Poland by molecular diagnostic tools. The transmission of *Ashworthius sidemi* infection from wildlife to domestic animals has confirmed on a natural pasture shared by cattle, sheep and wild ruminants (Moskwa et al., 2015). The results suggested the worm presence in cattle herds and verified the transfer from wild ruminants to domesticated ones.

In the experiment of Handeland et al. (2000), *Elaphostrongylus cervi* L3 larvae were used to infect small ruminants. As a consequence of larvae migration, inflammation and

necrosis were observed in the central nervous system and other organs, although adult nematodes and patent infections were not observed.

An experimental infection of sheep using nematode larvae recovered from the faeces of naturally infected endangered Swayne's Hartebeest *(Alcelaphus buselaphus swaynei)* was carried out by Desta et al. (2014). After necropsy; from the abomasums, *Haemonchus placei* while from the large intestine, *Oespophagostomum venulosum* and *Trichuris* spp. were identified; and the authors demonstrated that transmission of helminths between Swayne's hartebeest and sheep is experimentally possible. Analogous results were found in a roe deer-sheep-cattle model by Chintoan-uta et al. (2014) when roe deer-derived GIN larvae were used to infect a calf and it has resulted in a successful establishment and infection. After 21 days, faeces from the calf was collected and used for larval culture and extraction to infect a single lamb. The experimental animals were slaughtered at the end of the study and abomasal nematodes were collected. The authors recovered *H. contortus*, *Spiculopteragia spiculoptera* and *Ostertagia leptospicularis* from the calf and *H. contortus* and *Ostertagia leptospicularis* from the sheep. It was concluded that wild roe deer have the potential to acquire GIN (mainly *H. contortus*) from cattle and sheep.

As the abovementioned shows it, the helminthic cross-infection is an existing phenomenon between different host species and may play a role in the spread of resistance allele also in a habitat overlapped by domesticated and wild ruminants.

2. CONCLUSIONS DRAWN FROM THE DATA OF THE LITERATURE

Arguably, the presence of *Haemonchus contortus* populations is one of the most important threatening factors in the small ruminant sector. Due to its high pathogenicity and the unique phenotypic plasticity driven by a considerable genetic diversity, this parasite causes huge production losses and socio-economic problems globally, each year. Although this bloodsucking helminth is originated as a tropical species, today, however, its spatial distribution covers all of the climatic zones excepting the polar regions. The climate change, raising mean temperature and other weather anomalies can provide more and more capable habitats for surviving of free-living stages.

Decrease the losses caused by GIN, anthelmintic treatments and chemo-prophylaxis could be efficacious tools. Until now, just a few broad-spectrum drug classes are available for control. The oldest one, benzimidazoles, was introduced almost 60 years ago, while the youngest one, amino-acetonitrile derivative, is accessible since 2008. Unfortunately, these relatively inexpensive drugs became the only weapon in the battle against the endoparasites. Their solely and frequent application resulted in the occurrence and spread of AR within a few years in every class. Nevertheless, these obvious facts are well-known in several regions of the World; the level of AR in Hungarian sheep sector is absolutely unidentified. The lack of this information can cause not just economic and animal health problem in the sector but hamstrings the efficient GIN control.

The molecular diagnostic methods are very useful tools for a rapid and accurate approach of the routine diagnosis of GIN infections in the sheep farming practice. They could combine with conventional tests (e.g. FECRT, in vitro assays) to recognise the early appearance and dispersion of AR in the worm populations.

The grazing lands are fundamental resources of the small ruminant production systems. Due to their naturality, the included habitats are shared many times between wild and domestic ungulate species. This mutual exploitation can generate the potential for transmission of parasitic nematodes between host populations. Studies of GIN transmission at the wildlife/livestock interface rarely discussed; although they could induce strong effects in health, production, and population dynamics. Some studies verified the possibility of *H. contortus* cross-infection between different types of ruminants in natural circumstances. Confirmed by genotyping (Cerutti et al., 2010), *H. contortus* strains were well mixed between

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wild and domestic ungulate hosts, and it was concluded this transmission occurs in shared pastures regularly.

To date, few studies have been accomplished to confirm the potential role of cervids in carrying and spreading of AR nematodes. AR *H. contortus* were found in free-living, untreated roe deer and successfully transmitted experimentally to sheep and cattle. These results indicating, that deer could contribute to the spread of AR in natural circumstances.

3. ANTECEDENTS AND OBJECTIVES OF THE DISSERTATION

Hungarian sheep farming has a very important socio-economic role, mainly in the less-developed rural region. For sufficient incomes, the farmers should maintain well-managed flocks. In this process, the parasite control has a core role. Unfortunately, parasitological status and the level of AR in Hungarian sheep sector are absolutely unidentified. The lack of this information can cause not only economic and animal health problem in the sector but hamstrings the efficient GIN control.

The Game Management Landscape Centre of the Kaposvár University has one of the most important red deer stocks in Middle-Europe. In 1300 hectare, more than 1500 red deer are managed for venison and hunting purposes. This animal density is much higher than in the neighbouring natural habitats. Without drug application against micro and macroparasites, the economic aims proposed by the management are unimaginable.

Our co-operation with the SEFAG Forest Management and Wood Industry Share Co. dates back to 2007. In its framework, we have been monitoring the stock of game managed by the firm. This surveillance covers all of the hunting areas and their wild boar *(Sus scrofa)*, red deer *(Cervus elaphus)*, fallow deer *(Dama dama)*, roe deer *(Capreolus capreolus)*, mouflon *(Ovis aries)*, red fox *(Vulpes vulpes)*, and golden jackal *(Canis aureus)* populations. Due to this activity, we picked up information about several pathogens are existing in the concerned areas. As the headquarter has a One Health approach in wildlife management, a study was planned to clarify whether parasitic diseases are existing in the wild populations and could infect the domesticated animals and humans. (*One World – One Health – One Medicine* (briefly *One Health*) approach that amalgamates skills of both epidemiology and ecology serves additional information. Application of ecological methods during investigation on a parasitic or an infectious disease is more useful than simple diagnostic investigation of reservoir species and statistical evaluation of prevalence and intensity data.) In the frame of this cooperation, the *Haemonchus contortus* populations were studied in red and roe deer.

Our principal objective was to determine the frequency of benzimidazole resistance in different ruminant species. For this reason, a parasitological and a molecular diagnostic method (RFLP-PCR) were conducted in *Haemonchus contortus* specimens derived from sheep, farmed and free-ranging red deer and roe deer flocks.

An additional objective was to evaluate the role of free-ranging deer species in the spread of anthelmintic resistance.

4. METHODOLOGICAL SUMMARY OF THE DISSERTATION

In our work, we studied the level of resistance in *H. contortus* isolated in different host populations and assessed the possible role of wild ruminants in the spread of resistance. For this reason, we collected and dissected sheep, red deer, and roe deer abomasa in the southwestern part of Hungary. For implementing the aims, four studies were conducted. All procedures, such as sample collection, worm identification, molecular diagnostics, and statistical evaluation were carried out by the author.

In Study 1 and Study 2, domesticated ruminant flocks were surveyed. In the third investigation, free-ranging cervids, and sheep flocks were compared to determine the AR level in their *H. contortus* populations. In the last study, the possible role of roe deer was assessed in the spread of AR. The connections and relevant information between the research elements are shown in Figure 3.

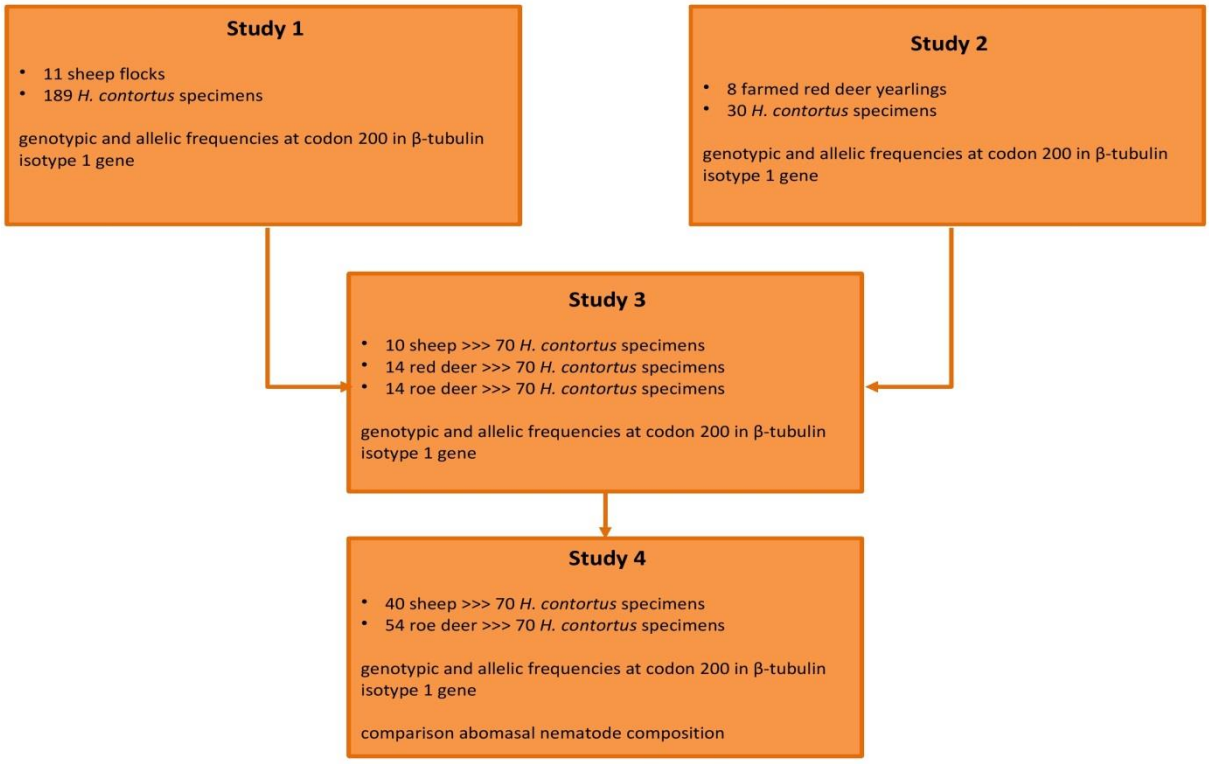

Figure 3. Flowchart of the studies

4.1. Study sites

The sample collection was carried out from three counties (Somogy, Baranya, Tolna) of the region. In the case of sheep, altogether 11 farms were involved in the study (Figure 4). The size of the flocks ranged 20-1200 animals. Their management is characterized by conventional systems, including one lambing period annually, grazing-based feeding with moderate concentrate and hay supplementation in lactation and winter time.

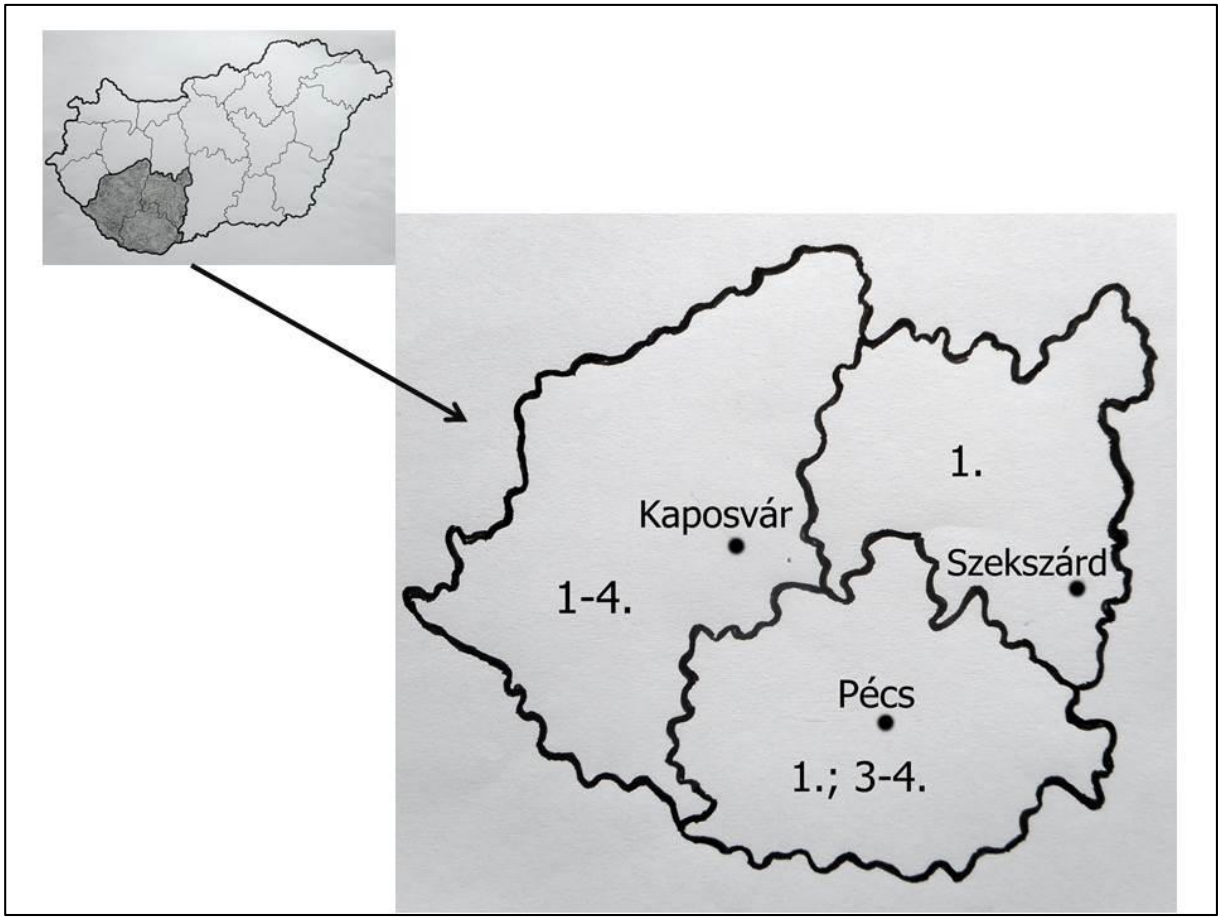

Figure 4: Localization of study sites (numbers identify the certain studies described in Figure 3)

The free-range cervid samples were collected from a hunting area managed by SEFAG Forest Management and Wood Industry Share Co. This study site was a contiguous area without any natural or man-built isolating elements that impede the movements of animals (Figure 5). The territory (approximately 30 km^2) is characterized by 145-276 m altitude above sea level, sub-Mediterranean climate, with some submontaneous habitat patches, with 10°C annual mean temperature and 630-800 mm annual precipitation.

The proportion of forests and agricultural areas on the study site is about 55% and 45%, respectively. In the core of the site, a 6000-hectare monoblock forest exists with the domination of oak (*Quercus* spp.), lime (*Tilia* spp.), hornbeam (*Carpinus betulus*) and European beech (*Fagus sylvatica*). The agricultural areas (viz. grasslands, pastures, arable lands, and old orchards) are the most typical on the periphery of the study site and they provide a heavily fragmented landscape structure. The average density of red deer, roe deer, and sheep are 1.71, 0.84 and 0.17 animal/km^2, respectively. These data are based on hunting statistics (hunted deer specimen/km^2); while in the case of sheep, it was derived from the official registry of the Hungarian Sheep and Goat Breeders Association.

The farmed deer samples were collected from a farm (centre of the farm: N 46° 13' 43.20", K 17° 50' 47.84") funded more than 20 years ago and have been managed by the Kaposvár University. The farm was developed from arable lands and hunting areas. The individuals of the breeding herd were captured from the surrounding areas. The breeding herd consists of 230 hinds, 10 stags, 70 yearlings, and 140 calves. The sampled stock is strictly separated from the free-range area by 2.6 m height wired fence. In this farming system, the animals could not mingle with any other ruminant species; and it has been never renewed by individuals derived from other farms or free-range area. In the rutting seasons, own stags take part in mating, exclusively.

4.2. Parasitological procedure

During the study, in the interval from September of 2013 till December of 2016, I collected sheep, red deer, and roe deer abomasa. In the case of sheep, the collection was accomplished both in a regional abattoir that perished animals from the farms and in Kaposvár diagnostic veterinary institute during diagnostic necropsies. The organs of deer were collected from hunting bags during regular hunting events. After evisceration, each abomasum was placed separately into a plastic bag immediately and dissected within few hours after death. If the dissection was postponed, the organs were stored at –18 °C until examination.

In order to isolate worm, I cut the organs alongside the big curvature and placed in a plastic bucket filled with saline solution. The worm collection was performed in a veterinary laboratory, where we washed thoroughly the abomasum mucosa and left the content to consolidate. After 5 minutes, the supernatant was decanted. This process was repeated until

the supernatant had become lucid. For the molecular diagnostic procedure, *Haemonchus contortus* males were picked randomly from each organ. The worms were collected by their "barber pole" characteristics (Figure 6). The species identification was performed by a light microscope with 100× magnification. The process was based on the length of the left and right spicule barbs and the length of the cuticular ridges (Lichtenfels et al., 1994). Until genotyping, the isolated parasites were kept in 96% alcohol.

Figure 5: Female *Haemonchus contortus*

In the comparative abomasal faunistic study, the worm collection was very similar as described above. The abomasal content was placed into a plastic jar and the mucosa was washed thoroughly. After this procedure the suspension was filled up to 2 L level and was left for 10 minutes to configure sediment; then thoroughly homogenized before a 200 mL subsample was scooped out. I counted all of the nematodes from this subsample (10 % of the whole amount), thus we multiplied the observed number by 10 to obtain the calculated worm count. The isolated nematodes were placed into 96% alcohol until further examination. During species identification, I used light microscopy at 40× and 100× magnification and the

process was based on Lichtenfels and Pillit's (1983), Lichtenfels and Hoeberg's (1993), Lichtenfels et al.'s (1994), Drózdz's (1995) and Rehbein's (2010) work. The members of Haemonchinae subfamily were identified by characteristics of both genders. In the case of Ostertagiinae subfamily, the determination of species was based on the morphology of male nematodes, excepting *Teladorsagia* spp. In this case, both sexes were proper for identification I did not make a distinction between major and minor morphotypes, thus I represented *Teladorsagia circumcincta*/*Teladorsagia trifurcata*/*Teladorsagia davtiani*; *Ostertagia ostertagi*/*Ostertagia lyrata*; *Spiculopteragia sipculoptera*/*Spiculopteragia mathevossiani*; *Spiculopteragia asymmetrica*/*Spiculopteragia quadrispiculata* and *Ostertagia leptospicularis*/*Ostartagia kolchida* as the same species.

4.3. DNA extraction and molecular diagnostic procedure

In order to genotype the collected worms, we used the detection of Phe200Tyr SNP on codon 200 of β-tubulin gene isotype 1, which is the most common molecular marker conferring BZ resistance in trichostrongyles of small ruminants (Coles et al., 2006). DNA lysates were made separately from the adult male worms. The applied RFLP-PCR method and the primer sequences (AvikaF: 5'-CTA CCCTTTCCGTCCATCAA -3' and AvikaR: 5'-TGAAGACGAGGGAATGGAAC -3') were detailed by Tiwari et al. (2006). PCR reactions were performed in a total volume of 10 µl, containing 200 µM of each dNTP, 0.2 µM primers, 10 × PCR buffer, 0.5 unit Dynazyme DNA polymerase (Finnzymes Oy, Espoo, Finland) and 100 ng genomic DNA. The PCR cycling profile consisted of denaturation at 94°C for 3 min, 45 cycles of denaturation at 94°C (for 30 s), annealing at 56 °C (for 30 s), and extension at 72°C (for 30 s), followed by a final extension at 72°C for 5 minutes. After amplification, 1 µl TaaI restriction endonuclease (5 U/µl; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), and 1.22 µl 10× digestion buffer were added to the total PCR volume. Digestion was carried out at 65 °C, overnight. Digested fragments were resolved on 2% agarose gel stained with GRGreen Nucleic Acid Gel Stain and visualized under UV light. Genotype determination was based on the fragment lengths such as 305 bp for S allele and 257 bp for R allele.

4.4. Statistical methods

In the molecular diagnostic survey, we followed the nomenclature of genotypes detailed by Pierce (2012). Allele frequencies were determined by GenAlEx software 6.502 version (Peakall and Smouse, 2012), while the true prevalence of a certain genotype counted with 95% confidence interval was determined by Reiczigel et al's method (2010). For comparison of genotype and allele frequency in the ruminant populations, chi-squared test with a significance level of 0.05, was performed with Bonferroni correction using R statistical software (version i386 3.3.0).

In order to characterize the abomasal nematode fauna of each host, we calculated the importance index (I), the Shannon diversity index (H) and the Sorensen coefficient (SC) of similarity (Thul et al., 1985; Legendre and Legendre, 1998) by using ComEcoPac software (Drozd, 2010).

All of the applied statistical softwares were freely available at the authors' websites.

5. CHAPTERS

5.1. CHAPTER 1

Situation of benzimidazole resistance in *Haemonchus contortus* in southwestern Hungary

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Situation of benzimidazole resistance in *Haemonchus contortus* in southwestern Hungary

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ABSTRACT

Among the gastrointestinal nematodes of small ruminants Haemonchus contortus *has almost the most overwhelming importance. This abomasal bloodsucking parasite has been presented all over the world, and it causes enormous economic and health problems in the sheep sector. A total of 189 adult male* H. contortus *worms were collected from sheep, bred southwestern Transdanubian region of Hungary, for monitoring whether the long-term usage of benzimidazoles could affect their effectiveness. The summarised allele frequencies, analyzed by RFLP-PCR, were 36.24% and 63.76% in case of susceptible and resistant ones, respectively. The proportion of homozygous susceptible (23.28%) and heterozygous (25.93%) worms were similar and the portion of homozygous resistant was about twice as much (50.79%). The correlation was pronouncedly significant between resistance allele frequency and the usage of benzimidazoles. According to our results, it seems the BZ resistance has appeared and extended within* Haemonchus contortus *in Hungarian sheep flocks.*

(Keywords: *Haemonchus contortus*, Hungary, sheep, benzimidazole resistance)

INTRODUCTION

Among the gastrointestinal nematodes of small ruminants *Haemonchus contortus* has almost the most overwhelming importance. This abomasal bloodsucking parasite has been presented all over the world, including in Hungary; and it causes enormous economic and health problems in the sheep sector (*Waller and Chandrawathani*, 2005).

The treatment of gastrointestinal nematode infections could be feasible by broad-spectrum anthelmintics, which can be divided into three groups such as benzimidazoles (BZ), macrocyclic lactones (ML), imidazothiazoles (IT). In the recent years, it has seemed that the effectiveness of these drugs, mainly BZ and ML, reduced in many Hungarian sheep and goat flocks.

BZs are the most majorly used anthelmintics, due to their advantageous properties; such as high therapeutic index, the absence of toxic residuals in milk and meat and economical availability (*Tiwari et al.*, 2006). BZ resistance in *H. contortus* is associated with single-nucleotide polymorphisms (SNP) on codon 167, 198, and 200 of β-tubulin isotype 1 gene (*Mottier and Prichard*, 2008). The most relevant diagnostic tool is the detection of Phe200Tyr SNP on codon 200 (*Coles et al.*, 2006). The main advantages of molecular diagnostics are sensitivity and accuracy; therefore, even a low frequency of resistant alleles can be detected. On the other hand, comprehensive application of these methods is impeded by expenses.

Many factors could facilitate the occurrence and spread of anthelmintic resistance (AR) in worm populations. However; probably the most important one is the inadequate usage of drugs. The frequent usage of anthelmintics may result the development of AR (*Waller*, 1997).

Rigid defensive strategies, wherein the number of treatments may consist of 5 or more occasions, and a very strong selective pressure could modify the worm populations. The frequent drug usage supports the surviving of such parasites; which possesses resistance allele. By the continuous selection in the nematode population, the occurrence of resistance allele (R) could be dominant; and the susceptible allele (S) could be restricted, thus the given anthelmintics may lose their effectiveness.

The sub-optimal dosage may also play a role in AR development (*Smith*, 1990). The underdosing promotes the survival of not just homozygous resistant (RR) but heterozygous (RS) parasites. These fault treatments eliminate just homozygous susceptible (SS) specimens and result a domination of the R allele.

The long-term use of anthelmintics could contribute the increasing of AR level. The continuous usage of a given drug creates a selection against S, as it was shown in the case of frequent treatments. Some authors interpreted that permanent usage of BZ and ML without rotation has resulted AR in *H. contorus* in South Africa (*van Wyk et al*., 1988).

In Hungarian veterinary practice, one of the most preferred drug groups is BZ, which has been presenting in the market for several decades. The aim of our study was, whether the long-term usage of these anthelmintics could affect their effectiveness of worm control in the Hungarian small ruminant sector.

MATERIAL AND METHODS

Collection of parasites

Adult male *H. contortus* worms were collected from sheep flocks in the southwestern Transdanubian region of Hungary (*Figure 1*).

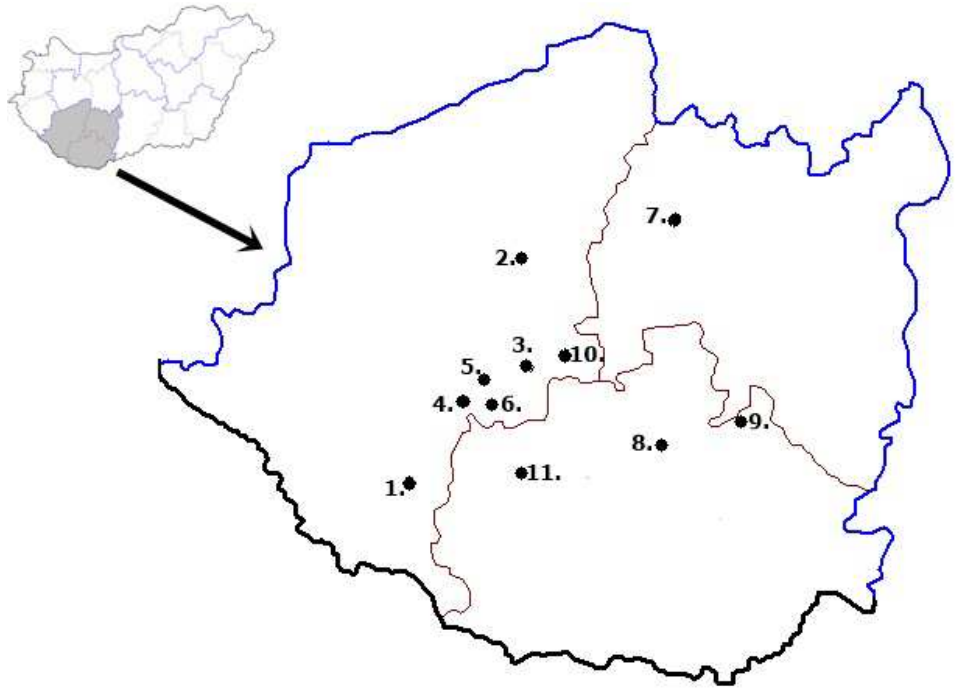

Figure 1.

Localization of sheep flocks

The 189 specimens were isolated either from a regional abattoir, perished animals from the farms or during diagnostical necropsies from the diagnostic veterinary institute. In every case, an abomasal dissection was carried out as soon as possible after death, when we cut the organ

alongside the big curvature and placed in a plastic bucket filled saline solution. The worm collection was performed in a veterinary laboratory, where we washed thoroughly the abomasum mucosa and left the content to consolidate. After 5 minutes, the supernatant was decantated. This process was repeated till the supernatant had become lucid.

The worms were collected by their "barber pole" characteristics. The species identification was performed by a light microscope with 100X magnification, using the work of *Lichtenfels et al*. (1994). Until genotyping, the isolated parasites were kept in 96% alcohol.

DNA extraction and genotyping

The genotypic analysis was carried out by Restriction Fragment Length Polymorphism-Polymerase Chain Reaction (PCR). The applied primer sequences were as follows: AvikaF : 5'- CTA CCCTTTCCGTCCATCAA -3' and AvikaR: 5'- TGAAGACGAGGGAATGGAAC -3' (*Tiwari et al*., 2006). Primers were designed to amplify a 303 bp fragment using DNA sequence of β-tubulin isotype 1 gene. PCR reactions were performed in a total volume of 10 µl, containing 200 µM of each dNTP, 0.2 µM primers, 10 × PCR buffer, 0.5 unit Dynazyme DNA polymerase and 100 ng genomic DNA. The PCR cycling profile consisted of denaturation at 94°C for 3 min, 45 cycles of denaturation at 94°C (for 30 sec), annealing at 56 °C (for 30 min), and extension at 72°C (for 30 min), followed by a final extension at 72°C for 5 minutes. Digested fragments (by TaaI endonuclease) were resolved in 4% agarose gel stained with SYBR® Green II Nucleic Acid Gel Stain and visualised under UV light. Genotypes were determined based on the fragment lengths such as 305 bp S allele and 257 bp for R allele.

Data collection and statistical analysis

The genotypic and allelic frequencies were determined by GenAlEx software 6.502 version (*Peakall and Smouse*, 2012) separately in every flock and all together.

In order to determine the linear correlation between BZ usage and R allele frequency (RALL), we have had a questionnaire was filled by farmers or their veterinaries. Information was collected about the average annual frequency of treatments in the past 3 years (BZAT) and since when the farmers have been using BZ (SBZU). The correlation was determined between variables by R statistical software, version 3.3.0 (https://www.r-project.org/).

RESULTS AND DISCUSSION

We examined a total of 189 male *H. contortus* derived from 11 different, southern Transdanubian sheep flocks. The BZ resistance was detected at codon 200 in β-tubulin isotype 1 gene. The occurrence of the three genotypes and the allele frequency showed a wide variety among the flocks (*Table 1*). The summarised allele frequencies were 36.24% (S) and 63.76% (R), respectively. The occurrence of SS and RS was similar (23.28% and 25.93%, respectively), and the proportion of RR was about twice as much (50.79%).

The homozygous susceptible genotype was observed in 5 flocks (2 flocks were SS in 100%). We found just a flock where all the collected worms were homozygous resistant. The correlation coefficients were very similar between the variables (RALL and BZAT: 0.7674; RALL and SBZU: 0.7789) and both connections proved to pronouncedly significant also (RALL and BZAT: p=0.0058; RALL and SBZU: 0.0047).

Table 1
Occurrence of different genotypic and the frequency of resistant and susceptible alleles in flocks

Flock	Sample size	Genotipic frequency (%)			Allele frequency (%)	
		SS	RS	RR	S	R
No.1	15	0	30	70	16.7	83.3
No.2	15	0	26.7	73.3	13.3	86.7
No.3	15	0	20	80	10	90
No.4	15	0	26.7	73.3	13.3	86.7
No.5	17	11.8	58.8	29,4	41.2	58.8
No.6	18	0	27.8	72.2	13.9	86.1
No.7	20	100	0	0	100	0
No.8	17	11.76	58.83	29.41	41.2	58.8
No.9	20	15	40	45	35	65
No.10	17	100	0	0	100	0
No.11	20	0	0	100	0	100
Sum	189	23.28	25.93	50.79	36.24	63.76

The emerging of AR in several nematodes of ruminant species is known all over the world, including Europe (*Kaplan*, 2004; *Ihler*, 2010; *Papadopoulos et al*., 2012). However; till now there was not any information on BZ resistance in Hungary, though our study showed its presence. One of the most influential factors in the occurrence of AR is the usage method of anthelmintics. It is well known that the intensive chemical treatments exclusively could not assist a long-term protection against worms. The continuous drug application, without any rotation, could facilitate the increasing of resistance level in helminth populations (*Waller*, 1997; *Jabbar et al*., 2006). The results of our genetic and statistical analysis confirmed a strong linear correlation between R allele frequency and the treatment frequency and the length of BZ usage. In a study, *Calvete et al*. (2010) analysed the management and environmental factors related to benzimidazole resistance, in Northeast Spain. Applying a principal component analysis, the authors suggested, that frequency of deworming was the single management variable that increased the BZ resistance level in the worm populations.

By our result, we suggest the farmers, practitioners, experts, and veterinaries to change their approach in connection with anthelmintic strategies. They should form novel, integrated, complex and sustainable methods, which contain more actions to fight against worms, for instance resistance breeding, environmental and immunological control, improved pasture and nutritional management, target selective treatment and the refugia management (*van Wyk*, 2001; *van Wyk et al*., 2006, *Kenyon et al*., 2009, *Bath*, 2014).

CONCLUSION

According to our results, it seems the BZ resistance has appeared and extended within *Haemonchus contortus* in Hungarian sheep flocks. We hypothesise, that long-term usage and the recurrent anthelmintic treatments could be in the background of pronounced proportion of resistant allele. Therefore; we strongly recommend the farmers, practitioners, experts, and veterinaries, to change their own approaches to chemical protection. They need to apply a more complex and integrated defending strategy against gastrointestinal nematodes in order to prepare an effective parasite control management.

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5.2. CHAPTER 2

Benzimidazole resistance in *Haemonchus contortus* recovered from farmed red deer

Pa tol Res
D 10.1007/s00436-016-5155-6

SHORT COMMUNICATION

Benzimidazole resistance in *Haemonchus contortus* recovered from farmed red deer

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Received: 27 March 2016 / Accepted: 24 May 2016

Abstract Thirty *Haemonchus contortus* male worms were collected from farmed red deer yearlings in order to determine whether routine administration of albendazole for a long-term period (17 years) could select anthelmintic resistance. PCR–RFLP method based on single-nucleotide polymorphism of codon 200 in isotype 1 ß-tubulin gene (Phe200Tyr) was applied. The results showed a significant frequency of either the resistant allele (85 %) or the homozygous resistant genotype (70 %). By chi-square test, Hardy–Weinberg equilibrium of the population was accepted ($p = 0.334$, power of test 0.01). True prevalence of the resistant genotype (RR) was estimated to be 46.5–87.2 % (confidence interval 95 %) calculated by Sterne's exact method. These results confirmed that long-term use of benzimidazoles could change the relative allele frequency of genes associated with drug resistance and may cause a large-scale spread of the resistant allele. To our knowledge, this study supported benzimidazole resistance in *Haemonchus contortus* in red deer for the first time.

Keywords Red deer · *Haemonchus contortus* · Albendazole · Resistance

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Introduction

Anthelmintic resistance (AR) is one of the most threatening and economically important factors in grazing ruminant production systems all over the world (Kaplan and Vidyashankar 2012). Its development depends on many factors, and there are several keystones, including frequency of treatment, rotation and dosage of anthelmintics (Silvestre et al. 2002; Falzon et al. 2014).

One method of delaying emergence of AR is the rotation of drugs. Waller et al. (1989) studied AR status in different management methods, principally slow and rapid alternations of drug groups. Authors concluded that slow rotation (annually) between drugs could slow the development of resistance in the case of *Haemonchus contortus*. On the other hand, rapid change of anthelmintic groups (from treatment to treatment) hastens AR. In the case of benzimidazoles (BZ), they observed a considerable resistance, which seemed invariant for several years after the last application. However, some simulation models (Barnes et al. 1995; Dobson et al., 2011) suggest that rapid rotation and use of mixing anthelmintics could be more effective to delay AR. Despite these diverse opinions, most experts agree that prolonged usage with high treatment frequency of anthelmintics from the same chemical group leads to the occurrence of AR and selection for resistant alleles (Jabbar et al. 2006).

It is generally accepted that underdosing (i.e. a subtherapeutic dose rate) is one of the most important factors contributing to development of AR (Smith et al. 1999; Jabbar et al. 2006). In addition, the true therapeutic dose rate may differ between species of animals (including farmed and wild animals), breeds and individuals due to differences in drug metabolism. Prichard (1985) reviewed connection between host physiology and efficacy of drugs and highlighted the role of the liver in drug metabolism. This organ, among

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ther factors, affects the rate of metabolism and the active level of drug within the host organism. Albendazole sulphoxide (ABZSO) is the main anthelmintically active metabolic product found systemically in ruminants after albendazole (ABZ) administration (Delatour et al. 1991; Virkel et al. 2004). Velík et al. (2005) studied ABZ biotransformation in ruminants, and a few monogastric species. Their results show that ABZ transformation to (+) and (−) ABZSO enantiomers depends on the liver microsomal enzymes (e.g. cytochrome P450). Overall amount of these products and the ratio of these enantiomers are responsible for anthelmintic effects of this drug. In sheep, the quantity of ABZSO was more than twofold greater than that in the red deer, and the ratio of (+) ABZSO and (−) ABZSO was 3.17. They found that liver microsomal enzymes of deer species produce less ABZSO, and the +/− ratio in red deer was 0.67. They concluded that effective dosage of ABZ in deer species cannot be inferred from data collected from domestic ruminants.

BZ resistance in *H. contortus* is associated with single-nucleotide polymorphisms (SNP) on codon 167, 198 and 200 of β-tubulin isotype 1 (Mottier and Prichard 2008). The most relevant diagnostic tool is the detection of Phe200Tyr SNP on codon 200 (Coles et al. 2006). The main advantages of molecular diagnostics are sensitivity and accuracy; therefore, even a low frequency of resistant alleles can be detected. On the other hand, comprehensive application of these methods is impeded by expenses.

The current study was undertaken, using PCR–RFLP detection of Phe200Tyr SNP, to test the hypothesis that 17 years of continuous ABZ usage, together with the innate effects of liver metabolism of ABZ in red deer, could have induced ABZ resistance in *H. contortus*.

Materials and methods

Deer farm

The red deer farm in this study (N 46° 13′ 43.20″, K 17° 50′ 47.84″) is located in the Trans-Danubian region of Hungary and was funded more than 20 years ago. The farm was developed from arable lands and hunting areas. The individuals of breeding herd were captured from the surrounding areas. The breeding herd consists of 230 hinds, 10 stags, 70 yearlings and 140 calves. The sampled stock is strictly separated from free range area by 2.6-m-height wired fence. In this farming system, the animals could not mingle with any other ruminant species, and it has been never renewed by individuals derived from other farms or free range area. In rutting seasons, own farmed deer receive in mating, exclusively.

The managers use two anthelmintic groups, benzimidazoles and macrocyclic lactones. The use of oral ABZ (Vermitan 10 % szuszpenzió AUV, CEVA-Phylaxia Veterinary Biologicals Co. Ltd., dose rate: 10 mg/body weig... (BW) kg; dosed to the weight of the heaviest animal in the group) carried out in a rigid regime. In the first 10 months of age, four drenching actions were applied against the worms. The first was at the time of ear tagging (2 months of age), the second at weaning (6 months of age), the third in the middle of winter (8 months of age) and the last one at yearlings' grouping (10 months of age). After the first year, animals were treated with a macrocyclic lactone (Dectomax inj. AUV, Pfizer PGM, dose rate: 0.3 mg/ BW kg; dosed to the weight of the heaviest animal in the group) in the rest of their life. This regime has been continued for more than 17 years, and in this period, there was not any test of efficacy on this anthelmintic strategy.

Worm collection

We collected 30 *H. contortus* males from eight red deer yearlings culled 7 months after the previous ABZ treatment. The abomasum was opened immediately after evisceration, and the content was washed into a plastic jar. For species identification, we followed Lichtenfels et al. (1994) morphological keys. After species identification, we collected all *H. contortus* individuals into a common plastic jar, then chose 30 worms randomly and placed each one into a separate 2-ml Eppendorf tube filled with ethanol (96 %). Prior to the PCR procedure, they were stored at 4 °C.

The number of analysed worms was determined by the following formula used in veterinary epidemiology (Pfeiffer 2002):

$$n = (\log(1-\beta))/\left[\log\left(1-\frac{d}{N}\right)\right],$$

where n = sample size, ß = level of confidence, d = number of diseased and N = population size.

By this calculation, if there is no homozygous resistant (RR) worm among the sampled 30 *H. contortus*, we could assume with 95 % confidence interval that BZ resistance must be fewer than 10 % within the studied nematode population.

DNA extraction and restriction fragment length polymorphism–PCR

The applied primer sequences were as follows: AvikaF : 5′-CTA CCCTTTCCGTCCATCAA -3′ and AvikaR: 5′-TGAAGACGAGGGAATGGAAC -3′ (Tiwari et al. 2006). Primers were designed to amplify a 303-bp fragment using DNA sequence of β-tubulin isotype 1 gene (Genbank Accession Number x67489). PCR reactions were performed in a total volume of 10 μl, containing 200 μM of each dNTP, 0.2-μM primers, 10× PCR buffer, 0.5 unit Dynazyme DNA polymerase and 100-ng genomic DNA. The PCR cycling

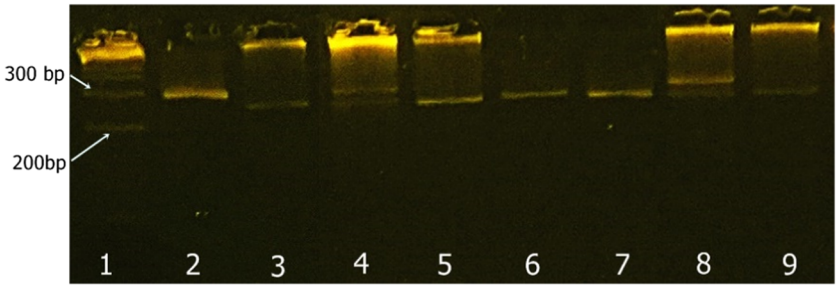

Fig. 1 Result of gel electrophoresis of adult male *Haemonchus contortus* collected from red deer yearlings; lane 1: 100-bp molecule ladder (TrackItTM 1-kb Plus DNA Ladder); lane 2: undigested PCR product (the length is 305 bp); lane 3, 5, 6, 7 and 9: homozygous RR sample (length of digested product is 257 bp); lane 4 and 8: heterozygous RS sample (length of digested products are 305 and 257 bp)

profile consisted of denaturation at 94 °C for 3 min, 45 cycles of denaturation at 94 °C (for 30 s), annealing at 56 °C (for 30 s), and extension at 72 °C (for 30 s), followed by a final extension at 72 °C for 5 min. After amplification, 1 μl TaaI restriction endonuclease (5 U/μl) and 1.22 μl 10× digestion buffer were added to the total PCR volume. Digestion was carried out at 65 °C, overnight. Digested fragments were resolved in 4 % agarose gel (Lonza Rockland, Inc.) stained with GRGreen nucleic acid gel stain and visualised under UV light. Genotype determination was based on the fragment lengths such as 305 bp for S allele and 257 bp for R allele.

Statistical analysis

We determined whether genotype frequencies of the worm population were in Hardy–Weinberg equilibrium using R-statistics software version 3.1.1 Package 'HardyWeinberg' version 1.5.4. (Graffelman 2010). True prevalence of a certain genotype in the population was calculated by Sterne's exact method (confidence interval 95 %) (Reiczigel 2003).

Results

Analysis of 30 *H. contortus* specimens was carried out to detect BZ resistance at codon 200 (Fig. 1). Among the worms, 21 were genotyped as homozygous resistant and nine as heterozygous (RS). We found that proportion of RR worms and the frequency of resistant (R) allele were very high in the worm population (Table 1).

Statistics based on the values of Table 1 showed that the studied worm population is in Hardy–Weinberg equilibrium ($p = 0.334$; power of test 0.01). True prevalence of RR genotype was proved to be 46.5–87.2 % (confidence interval 95 %) calculated by Sterne's exact method. Calculated by this method, SS genotype and S allele showed 0.0–11.2 and 7.8–26.5 % true prevalence, respectively.

Discussion

Resistance, to the main broad-spectrum anthelmintics in major domesticated ruminant species, is widely extended all over the world (Kaplan and Vidyashankar 2012). In the case of deer, which are members of the minor food-producing species, we have less information about the status of resistance in their nematodes.

As we know; our results verified firstly the genetic evidence of BZ resistance in *H. contortus* in the red deer host. Effect of ABZ and ivermectin were studied formerly in the helminthosis of fallow deer (Mylrea et al. 1991). The authors suggested that those anthelmintics work insufficiently in that species. They hypothesised that restricted effect of anthelmintic treatments was affected by some factors such as metabolism of active substance, failure of absorption, worm burden, malnutrition, sub-optimal dose rate or parasite resistance. Mackintosh et al. (2014) demonstrated anthelmintic resistance to moxidectin and abamectin, in relation to *Ostertagia*-type species, and concluded that faecal egg reduction test (FECRT) overestimates efficacy of these anthelmintics in the red deer. Chintoan-Uta et al. (2014) tested thiabendazole on *H. contortus* collected from fallow, red and roe deer. Two types of methods (egg hatch test and molecular test) provided evidence for the presence of BZ resistance in the worms derived from roe deer.

Table 1 Genotypic allele frequency in male *Haemonchus contortus* (*S* = susceptible, *R* = resistant); *N* = 30

Genotypic frequency (%)			Allele frequency (%)	
Homozygous resistant (RR)	Heterozygous (RS)	Homozygous susceptible (SS)	Resistant (R)	Susceptible (S)
70	30	0	85	15

We found high proportion of R allele (85 %) and RR genotype (70 %). These results clearly demonstrated BZ resistance in *H. contortus* on the studied deer farm. There has not been any rotation of unrelated anthelmintics in this herd to delay AR (Dobson et al. 2012), and it is likely that routine use of ABZ for 17 years has favoured survival of the resistant genotype, thus inducing spread of R allele in this *H. contortus* population. The worm population was in Hardy–Weinberg equilibrium, although the power of test was not strong. This suggests that anthelmintic treatments cannot kill all the heterozygous worms; hereby, sensitive allele can subsist, and between anthelmintic treatments, genotypes can enter into balance.

Many factors, such as nutrition, gender, age, gestation, other disease and/or medication and differences between species, contribute to transformation of ABZ (Prichard 1985; Křížová-Forstová et al. 2011). Metabolism of ABZ differs between species among ruminants. This characteristic of microsomal liver metabolism in deer strongly suggests that these species require a higher dose rate than sheep and cattle to attain optimal efficacy rates against susceptible parasites. Nevertheless, the inadequate dosing accelerates the dominance of resistant allele, and it contributes to the selection of a resistant worm population (Smith et al. 1999; Jabbar et al. 2006).

In Hungarian veterinary practice, one of the most preferred drug groups is BZs. Veterinarians usually follow the producer's dosage recommendations for the two main target species (sheep and cattle). However, in practice, these drugs are used not just in these animals, but also others, especially goats and red deer, which are likely to be given sub-optimal dose rates, due to their different hepatic metabolism of drugs. Therefore, some authors suggest that effective anthelmintic treatment must be based on a higher dose in goats than in sheep (Hennessy et al. 1993; Hoste et al. 2000; Várady et al. 2011). According to the similarity of the goat and the red deer efficacious working of cytochrome P450, it should be recommended to apply a higher dose of anthelmintics in the red deer than in sheep.

Our result showed that routine, long-term administration of the same drug could contribute to the appearance of AR in *H. contortus* in the red deer. Moreover, use of cattle dose rates in red deer was likely to contribute to the development of AR. Therefore, we recommend that further research is essential to determine species-specific dose rates of anthelmintics in cervids.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competi interests.

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5.3. CHAPTER 3

Benzimidazole resistance within red deer, roe deer and sheep populations within a joint habitat in Hungary

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Benzimidazole resistance within red deer, roe deer and sheep populations within a joint habitat in Hungary

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ABSTRACT

The anthelmintic resistance of gastrointestinal nematodes is one of the most important, economic risk factors in grazing ruminant systems, all over the world. We have infinitesimal information about the resistance status of nematodes in deer species. Our aim was to determine the presence of BZ resistance in the generalist worm, *Haemonchus contortus* in pastured sheep and free ranging red and roe deer by RFLP-PCR method based on the detection Phe200Tyr single nucleotide polymorphism. By investigation of 70 worms from each host species, the homozygous susceptible genotype was the most representative in the red deer (100%), the homozygous resistant genotype was most prevalent in the sheep (68.6%) and moderate in the roe deer (17.1%), while the heterozygous genotype was observed in equal proportion in the sheep and roe deer (28.6%). Our results suggest that overlapping habitats of sheep flocks and roe deer could contribute to the occurrence and spread of resistant allele within wildlife.

1. Introduction

The anthelmintic resistance (AR) of gastrointestinal nematodes is one of the most important economic risk factors in grazing ruminant systems all over the world (Sutherland and Leathwick, 2011; Papadopoulos et al., 2012; Rose et al., 2015). The first appearing of resistance against broad spectrum drugs dates back to the 1960's and its spreading could be observed in every class (FAO, 2004; Kaplan, 2004).

Although, the phenomenon of AR is well known in major domesticated ruminant species, as in the case of sheep (Barrere et al., 2013; Peña-Espinoza et al., 2014; Chaudhry et al., 2015); within the minor food-producing species (eg. deer species), we have much less information about the AR status of their nematodes. In a study (Mackintosh et al., 2014), the resistance to moxidectin and abamectin was demonstrated by faecal egg count reduction test (FECRT) in red deer. The authors suggested that AR of *Ostertagia*-type species was in the background of drug ineffectiveness. A similar result was showed in the case of albendazole and ivermectin (Mylrea et al., 1991) in fallow deer. Both studies were carried out under farm circumstances. It was hypothesized that failure of treatments was affected by several factors including the AR of gastrointestinal nematodes. Chintoan-Uta et al. (2014) tested and confirmed the cross transmission of *Haemonchus contortus* and its benzimidazole (BZ) resistance between the fallow deer, red deer, roe deer, cattle, and sheep. Both of their resistance detection methods (egg hatch test and molecular test) demonstrated the presence of AR in the worms derived from roe deer. Three single nucleotide polymorphisms (SNPs) in the β-tubulin gene isotype 1 have been associated with benzimidazole resistance in *H. contortus*. The Phe200Tyr mutation has been found in every country at a relatively high frequency. The SNPs at codons 167 and 198 have also been reported in multiple countries but have a more variable occurrence and are generally present at a lower frequency than the Phe200Tyr mutation (Chaudhry et al., 2016). Since the most common molecular mechanism that confers BZ resistance in trichostrongyles in small ruminants involves a phenylalanine to tyrosine mutation at codon 200 of β-tubulin gene isotype 1; therefore, molecular diagnostic methods based on the detection of this SNP are used most frequently to identify BZ resistance in *H. contortus* (Coles et al., 2006).

A Hungarian study (Nagy et al., 2016) demonstrated a high homozygous resistant genotype (70%) and resistance allele (85%) proportion in farmed red deer. The results of a molecular diagnostic method showed that routine, long-term administration of the

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drug could contribute to the appearance of AR in *H. contortus* in the red deer.

Recently, a new approach, refugia theory, arose during the investigation of AR in natural environment. It hypothesizes that the presence of wild ruminants on pastures of livestock provides a significant percentage of homozygous sensitive ones among the total amount of infectious larvae. Regular infection by this mixed population impedes the unlimited spread of resistance within a worm population infecting a livestock flock concerned. (Van Wyk, 2001).

In this study, we aimed to determine the presence of BZ resistance in the generalist worm, *Haemonchus contortus* in a microregion, where the resources partly shared by sheep and free ranging red and roe deer.

2. Materials and methods

2.1. Study site

Our investigation was carried out in southwestern Hungary, within a contiguous area without any natural or man-built isolating elements that impede movements of animals (Fig. 1). The study site (approximately 30 km^2) characterized by 145–276 m altitude above sea level, sub-Mediterranean climate, with some submontaneous habitat patches, with 10 °C annual mean temperature and 630–800 mm annual precipitation. The proportion of forests and agricultural areas on study site are about 55% and 45%, respectively. In the core of the site, a 6000-ha monoblock forest exists with the domination of oaks (*Quercus* spp.), limes (*Tilia* spp.), hornbeam (*Carpinus betulus*) and European beech (*Fagus sylvatica*). The agricultural areas (viz. grasslands, pastures, arable lands, and old orchards) are the most typical on the periphery of the study site and they provide a heavily fragmented landscape structure. The average density of red deer, roe deer, and sheep are 1.71, 0.84 and 0.17 animal/km^2, respectively. Our data were based on hunting statistics (hunted deer specimen/km^2); while in the case of sheep, it was calculated by the official registry of the Hungarian Sheep and Goat Breeders Association.

2.2. Worm collection and molecular diagnostic procedure

During the interval from September of 2013 till August of 2015, we collected 38 abomasi from red deer (N = 14), roe deer (N = 14) and sheep (N = 10 from 5 different flocks). The organs of deer were collected from hunting bags in regular hunting seasons, while sheep were sampled at a regional abattoir. After evisceration, each abomasum was placed separately into a plastic bag immediately, and each was stored at −18 °C until examination. After melting, the organs were opened alongside the big curvature, and the content was placed into a plastic jar, while the mucosa was washed thoroughly. In the case of deer species, 5 *Haemonchus contortus* males were picked randomly from each organ, while 7 specimens were collected from every single sheep abomasum. For morphological identification, we used Lichtenfels's et al. (1994) work. That method is based on the length of the left and right spicule barbs.

In order to genotype the collected worms, we used the detection of Phe200Tyr single-nucleotide polymorphisms on codon 200 of β-tubulin gene isotype 1, which is the most common molecular mechanism conferring BZ resistance in trichostrongyles of small ruminants (Coles et al., 2006). DNA lysates were made separately from 210 adult male worms. The applied Restriction Fragment Length Polymorphism-PCR method and the primer sequences (AvikaF: 5′-CTA CCCTTTCCGTCCATCAA -3′ and AvikaR: 5′- TGAA-GACGAGGGAATGGAAC -3′) were previously detailed by Tiwari et al. (2006). PCR reactions were performed in a total volume of 10 μl, containing 200 μM of each dNTP, 0.2 μM primers, 10 × PCR buffer, 0.5 unit Dynazyme DNA polymerase (Finnzymes Oy, Finland) and 100 ng genomic DNA. The PCR cycling profile consisted of denaturation at 94 °C for 3 min, 45 cycles of denaturation at 94 °C (for 30 s), annealing at 56 °C (for 30 s), and extension at 72 °C (for 30 s), followed by a final extension at 72 °C for 5 min. After amplification, 1 μl TaaI restriction endonuclease (5 U/μl; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), and 1.22 μl 10 × digestion buffer were added to the total PCR volume. Digestion was carried out at 65 °C, overnight. Digested fragments were resolved on 4% agarose gel stained with GRGreen Nucleic Acid Gel Stain and visualized under UV light. Genotype determination was based on the fragment lengths such as 305 bp for S allele and 257 bp for R allele.

2.3. Statistical methods

In this survey, the nomenclature of genotype and allele frequency, we followed, were detailed by Pierce (2012). The proportions were determined with 95% confidence interval (CI95%) (Reiczigel et al., 2010). For comparison of genotype and allele frequency in the ruminant populations, chi-square test with significance level of 0.05 was performed with Bonferroni correction using R statistical software i386 3.3.0 version.

3. Results

In this study, we examined altogether 210 male worms, which were identified as *Haemonchus contortus,* to determine the proportion of Phe200Tyr SNP on codon 200 of β-tubulin gene isotype 1 within three different ruminant species. Among the worms, 110 were genotyped as homozygous susceptible (SS), 40 as heterozygous (RS) and 60 as homozygous resistant (RR), thus the total frequency of SS, RS and RR was 52.4% (CI95% = 45.5–59.1%), 19% (CI95% = 14.1–24.9%) and 28.6% (CI95% = 22.7–35.2%), respectively. Considering the alleles, the proportion of susceptible (S) and resistant (R) allele was 61.9% (CI95% = 57.2–66.5%) and 38.1% (CI95% = 33.5–42.8%), respectively. Distribution of the different genotype was showed a wide variety in hosts.The SS was the most representative in the red deer, where all of the worms belonged to this genotype group. The RR was most prevalent in the sheep and it was moderate in the roe deer, while the RS was observed in equal proportion in these two hosts (Table 1). Difference of allele frequencies between the host populations was confirmed significant by chi-square test.

4. Discussion

Although several studies were concerned with AR in small ruminants (Papadopoulos et al., 2012; Rose et al., 2015), our knowledge is very deficient in connection with wild ruminants (Chintoan-Uta et al., 2014; Mackintosh et al., 2014) We investigated the BZ resistance status of *H. contortus,* collected from sheep and two sympatric deer species and our results confirmed considerable divergence between the hosts. In Hungarian veterinary practice, one of the most preferred drug groups is BZs; therefore, their usage in parasite management is considered very general. Our results suggest that the high level of R allele proportion in sheep in this region is derived from the high treatment frequency and the long-term unrotated drug application; as our previous work confirmed it (under publication). On the other hand, in Hungary, the usage of anthelmintics and other prescription medicines for hunting parties are restricted by the food safety law, because the consumption of the therapeutic dose cannot be ensured and the withdrawal period cannot be controlled in wildlife. In consequence, the hunting parties of the studied area have never applied any anthelmintics. Based on these

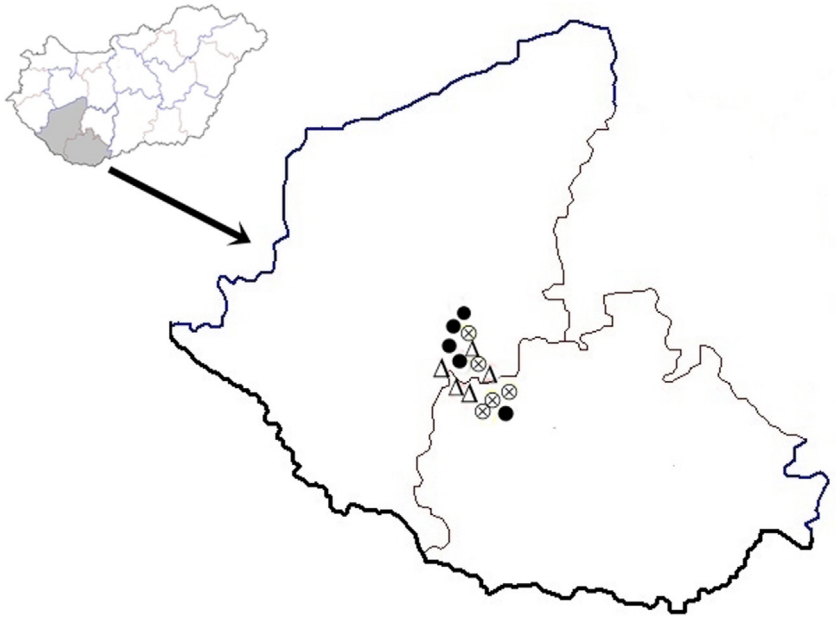

Fig. 1. Localization of collected *Haemonchus contorus* from different hosts (circles with x = red deer; triangles = roe deer; black dots = sheep). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Genotype and allele frequencies (CI95%) in *Haemonchus contortus* (S = susceptible, R = resistant) collected from different ruminant hosts.

Host	Genotype frequency (%)			Allele frequency (%)	
	homozygous susceptible (SS)	heterozygous (RS)	homozygous resistant (RR)	susceptible (S)	resistant (R)
red deer (N = 70)	100 (94.9–100)	0 (0–5.1)	0 (0–5.1)	100 (97.4–100)	0 (0–2.6)
roe deer (N = 70)	54.3 (42.4–65.9)	28.6 (18.9–40.6)	17.1 (9.7–27.6)	68.6 (60.4–75.9)	31.4 (24.18–39.6)
sheep (N = 70)	2.90 (0.5–9.5)	28.6 (18.9–40.6)	68.6 (56.5–78.9)	17.1 (11.6–24.1)	82.9 (75.9–88.4)

facts, we suggested, the resistant *H. contortus* recovered from roe deer might derive rather from cross infection from sheep, than from the failure of hunting management.

Our observation could not explain exactly the absence of R allele in the red deer. We hypothesized that the presence of sheep on pastures indicated avoidance behaviour in the red deer. Some free range studies (Osborne 1984; DeGabriel et al., 2011) showed the absence of sheep in a given habitat induced a higher red deer density. According to Cuartas et al. (2000), this phenomenon was attached to the disturbance effect of sheep husbandry rather than any direct or indirect competition between the two species. During our investigation, many sheep flocks used regularly the pastures of the study area, thus their presence might be affected the red deer aggregation; however, this study did not cover detailed field observation of all host species. The result of a Norwegian study (Mysterud et al., 1999) suggested that roe deer reserved its territory by a trade-off mechanism between shelter and forage. In conclusions, the authors found that there was no any evidence for avoidance between the two ruminants.

The home range of roe deer can vary widely in scale (Jeppesen, 1990; Maillard et al., 2002) and it can be affected by habitat resources. Some studies confirm that the roe deer adjust home range size in response to landscape structure. Fragmented landscapes can provide abundant, high-quality food, which increases deer habitat carrying capacity (Reimoser, 2003; Saïd and Servanty 2005). These valuable, fragmented landscapes can also be utilizable for sheep grazing; as it was observed in the study site. We supposed that the overlap in habitat use between sheep flocks and roe deer could contribute to the occurrence and spread of resistant allele within wildlife. Our results raised an issue about the role of roe deer in AR; whether roe deer is a potential transmitter of the problem or the potential solution for it, as a source of SS genotyped worms supporting refugia formation on pastures (Van Wyk, 2001). To answer this question, we need to accomplish more regulated and complex studies to know the exact effects of this host species.

Our study was based on the molecular diagnostic detection of Phe200Tyr SNP on codon 200 of β-tubulin gene isotype 1; while SNPs on codon 167 and 198 were not investigated. Therefore; the true prevalence of BZ resistance within the studied *H. contortus* population might be underestimated; notwithstanding, in all three host species; thereby those were comparable. The sample collection period was rather long because deer for post-mortem examination could be reached during their hunting seasons and in a limited count. This fact could affect our results through the annual circle of worm burden in certain hosts. Sheep samples could be taken all year round; therefore, these data represent mostly the average prevalence of resistance. Roe deer samples were collected mainly in spring and summer when the intensity of this parasite is generally rather high in ruminants. On the other hand, red deer were harvested during autumn and winter when the level of infection is largely moderate or low. Considering these facts, our deer data cannot describe the statement of resistance accurately. The remarkable difference between the two deer species can be explained even by different sampling periods. Roe deer were sam-

in spring and summer when the sheep with lambs at their feet are on pastures and shed a lot of eggs. Under these circumstances, worms that infect the roe deer might originate partly from sheep. On the other hand, the sampling of red deer was carried out after the main worm burden of sheep, when few larvae can be ingested by grazing. During this period, the researcher can meet a naturally selected population of worms; which were affected by a lot of environmental factors, except anthelmintics, in a wild ruminant stomach. Considering the above, our results also support the refugia hypothesis if we assume that abomasal circumstances of wild ruminants evolve a sort of selection pressure to parasites. Among these conditions, the selective-advantage attributed to AR might prove to be useless; and those worms, which carry R allele, will perish in larger amount under transferred selection pressure. As Leathwick (2013) confirmed in his study, worms carrying anthelmintic resistance mechanism are likely to be less fit. For scientific justification of this hypothesis, further investigations will be needed.

Conflict of interest

The authors declare no conflict of interest.

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5.4. CHAPTER 4

Shared pastures and anthelmintic resistance in wildlife and livestock

Shared Pastures and Anthelmintic Resistance in Wildlife and Livestock

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Summary

Parasitic diseases are an important threat to grazing livestock. Until recently, the most accepted control methods were regular, herd-level deworming regime and grazing on "clean" or "safe" pasture. Presence of wild ruminants on pastures was considered as the main risk of parasitic infection. In the last decades, the failure of these conventional attitude was suspected. This study was carried out in Hungary, where springtime, whole-herd deworming is still in practice. Our hypotheses were that the above-mentioned strategy led to high prevalence of anthelmintic resistance; on the other hand, wildlife could not contribute to deleterious parasitosis of livestock. For this, we accomplished an investigation in the close surroundings of typical sheep herds. The aims were to determine the species structure and anthelminthic resistance in the parasite community of the sheep herds and the adjacent roe deer population. As a result, we found that in the roe deer (N=53), a more diverse parasite community exists and the most devastating worm species, *Haemonchus contortus* plays a less important role in it; than in the sheep (N=40). Prevalence of benzimidazole resistance in *H. contortus* was 17.1% and 68.6% in the roe deer and sheep, respectively. Our findings suggest that routine deworming cannot succeed; while presence of roe deer is rather useful, as its parasites attenuate the simplistic, anthelmintic resistant pasture community.

Key words

abomasal nematode fauna, roe deer, sheep, anthelmintic resistance

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Introduction

Farming on pastures is a nature friendly, low-input production system; which proposes the least animal welfare issues, if nutrition and animal health is well-managed. In the last decades, the most threatening factors for grazing, especially in small ruminants, are gastrointestinal nematodes (GIN) and their anthelmintic resistance (AR) (Rose et al., 2015). Until recently, the most accepted control methods were regular dose-and-move regime. This meant that, mainly in springtime, the whole herd was treated by an antiparasitic medicine and after a few days awaiting time, the "parasite-free" animals were driven to a "clean" pasture, which has not been grazed for a long time and considered as quasi free from parasites (Michel, 1985; Boa et al, 2001).

Nowadays, the failure of this method is confirmed. After a mass deworming, a part of the parasite community survives; and in this part, genes of AR can occur. If the farmer regularly repeats this practice; the prevalence of AR increases time and time again. Treated animals will excrete a selected, mostly anthelmintic resistant, worm community onto the "clean" pasture; and AR will be general in the close surroundings of a regularly dewormed livestock herd. In these conditions, the presence of wild ruminants could be even advantageous; as they are never treated by anthelmintics, therefore, their less human influenced parasite community can serve a kind of buffer within the parasite pool of the pasture. In this context, the alimentary tract of wild ruminants and the mixed helminth fauna of a natural pasture should be considered as refugia for anthelmintic sensitive worms (van Wyk, 2001; Nagy et al., 2017). On the other hand, wild ruminants can even transmit AR alleles between livestock herds, and further research is needed to assess their exact effect (Chintoan-Uta et al., 2014).

In our study, we investigated the close surroundings of regularly dewormed sheep herds. Based on the examination of abomasa from both sheep and roe deer; we carried out a faunistic analysis and a determination of AR prevalence in the isolated *Haemonchus contortus* population. Our hypothesis were that worm fauna of the two species should be different, and the level of resistance should be lower in roe deer. By confirmation of these facts; we would have liked to support the refugia hypothesis and attempt to assess the role of roe deer in AR transmision or fighting against it.

Materials and methods

Our investigation was conducted in southwestern Hungary between April of 2014 and December of 2016. The study site was characterized by a central, 6000 ha forest monoblock with agricultural lands; mostly pastures around it. We examined abomasa of roe deer (N=53) and sheep (N=40) in order to determine the differences and similarities of abomasal nematode fauna of the two species in the same habitat. The organs of deer were collected from hunting bags, while sheep were sampled at a regional slaughterhouse and on farms. For species identification, we used Lichtenfels et al.'s (1994), Drózdz's (1995) and Rehbein's (2010) works.

For a finer characterization, the genotypic analysis was carried out on codon 200 of β-tubulin gene isotype 1 by Restriction Fragment Length Polymorphism-Polymerase Chain Reaction described by Tiwari's et al. (2006). We used chi-squere test by GenAlEx software 6.502 version (Peakall and Smouse, 2012) to compare 140 *H. contortus* males (70 from each host) to determine the genotypic and allelic frequencies.

In order to characterize the abomasal nematode fauna of both hosts, we calculated the importance index (I), the Shannon diversity index (H) and the Sørensen coefficient (SC) of similarity (Thul et al., 1985; Legendre and Legendre, 1998) by using ComEcoPac software (Drozd, 2010).

Results

The roe deer had more diverse abomasal fauna (9 species), than the sheep had (2 species). Seven of the worms were proved to be a dominant or codominant species; the rests belonged to subordinate ones (Table 1). We found just two common worm species but just *H. contortus* was considered as a dominant in both ruminant populations. For all parasites, species diversity as reflected by Shannon's diversity index was 1.79 in roe deer, whilst in sheep it was 1. The Sørensen coefficients of similarity between hosts were low (SC=0.36).

Table 1. Importance values (I) of nematode species by hosts

Nematode	Roe deer	Sheep
Ashworthius sidemi	0.13^{CD}	0
Haemonchus contortus	21.67^{D}	43.99^{D}
Teladorsagia circumcincta/T. trifurcata	0.001>	43.94^{D}
Spiculopteragia spiculoptera/S. mathevossiani	17.21^{D}	0
Spiculopteragia asymmetrica/S. quadrispiculata	0.17^{CD}	0
Ostertagia leptospicularis/O. kolchida	34.3^{D}	0
Ostertagia ostertagi/O. lyrata	0.009	0
Trichostrongylus axei	0.004	0
Nematodirus oiratianus subsp. *interruptus*	0.004	0

$^{(D)}$ indicate dominant species, while $^{(CD)}$ does codominant ones

The homozygous susceptible (SS) genotype was the more representative in the roe deer (54.3%), than in sheep (2.9%). On the other hand, the homozygous resistant (RR) worms were most prevalent in the sheep (68.6%) and were moderate in the roe deer (17.1%), while the heterozygous (RS) genotype was observed in equal proportion in both hosts (28.6%). Difference of allele frequencies (roe deer: susceptible allele = 68.6%; resistant allele = 31.4%; sheep: susceptible allele = 17.1%; resistant allele = 82.9%) between the host populations was confirmed as significant ($p<0.05$).

Discussion

In this study, we carried out a faunistic analysis of parasite community in sheep herds and the sympatric roe deer population; and moreover we compared the AR prevalence of the isolated *H. contortus* population. As a general result, we ascertained that roe deer carries a more diverse helminth population, than sheep; and the AR level also differs significantly in the two species.

In the sheep, only two species, *H contortus* and *T. circumcincta/T. trifurcata* were detected, with very similar importance values. Comparing this finding with those obtained during investigation of naturally kept ruminants; the most conspicuous difference is the extinction of competitively superior species (e.g. *T. axei*) for the favour of *H. contortus*. In the lack of regular anthelmintic treatment, *H. contortus* plays an inferior role in a helminth infracommunity (in the abomasum of a host individual). As a competitively inferior parasite species, *H. contortus* has a better capacity to survive in the environment, but cannot invade the host such aggressively as superior ones. It is probable that in the environment of a regularly medicated livestock herd, a lot of surviving larvae accumulate on the pasture, and supersede less environment resistant competitors (Diez-Baños et al., 1981).

This drift in the structure of the helminth fauna is not necessarily due to AR. Most of the anthelmintics have no real ovicide effect; therefore, after deworming, a lot of viable eggs and larvae are excreted to the pasture. In these conditions, the most environment resistant species should reach the most dense population in the environmental pool. Principally; competitively superior species are affected during deworming of the host. This should cause the rising of *H. contortus*, a naturally satellite member of a parasite community.

It is interesting that among the nine parasite species of roe deer, *H. contortus* was confirmed to be dominant. Its importance is not so remarkable as in the sheep, but it is not a satellite species at all. Moreover; the most important, most numerous parasite species of roe deer, *O. leptospicularis/O. kolchida* could not be detected in the studied sheep herds. These findings suggest that rather the sheep farming affects the roe deer habitat, than vice versa.

The comparison of AR prevalence in the two hosts also supports the superiority of human influence. Within the studied habitat, antiparasitic treatment of wildlife has never been in practice; as the authors know. In spite of this; AR is present in the parasite community of the roe deer; though its level is much lower than in the sheep.

Notwithstanding; AR transmitting role of roe deer cannot be excluded by this study, our results support the hypothesis, that habitat overlapping between sheep and roe deer means rather an advantage than a real risk for antiparasitic strategies. Worm community excreted by wild ruminants contains less AR individuals and more competitively superior, non blood-sucking species, which has got a stimulating effect on the host's immune system. These two features behave like a buffer in the environmental pool of parasites; and the effect depends on its portion.

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6. GENERAL DISCUSSION

The aims of our survey -expanded from 2013 till 2016- were to determine the occurrence of the BZ resistance in *Haemonchus contortus* infested domesticated and wild ruminant populations, and the role of free-ranging cervids, as carriers, in its spread.

Currently available methods to assess BZ resistance are in vivo (e.g.FECRT), in vitro (e.g. EHA) and molecular tests. The sensitivity of them differs. The generally accepted molecular diagnostic methods are the most sensitive, they provide an efficient assessment of the resistance level in a worm population (von Samson-Himmelstjerna et al., 2007). In Europe, the most prevalent SNP, which is associated with BZ resistance, is on codon 200 of β-tubulin gene isotype 1 (Ramücke et al., 2016). Based on these facts I used RFLP-PCR method (Tiwari et al., 2006) for ascertaining BZ resistance.

It is accepted that resistant gene or genes exist as rare alleles within a natural population prior to the first exposure of a drug. Therefore AR arises from a selection within the normal phenotypic range, (Beech et al., 1994). The development and spread of resistant alleles in a population could depend on several factors which can be classified as genetic, biological or operational. In a survey, Silvestre et al. (2002) assess the relevance of five factors could be associated with AR. The authors suggested that AR spread is not a consequence of high selection pressure by intensive anthelmintic treatment frequency. It is concluded, by their review, the most three pivotal factors are 1.) the introduction of resistant worms through sheep/goat purchased or the pastures shared by several farms; 2.) repeated use of one anthelmintic and 3.) a large contribution of worms that survive treatment, while just a few infective larvae are available on pasture. The effect of frequent treatment and under-dosing has a marginal role. This conclusion is partly controversial to others. The reason may be a subjective opinion. The cited survey is out of any statistical approach, therefore a bit questionable.

In a meta-analysis, a risk assessment was modelled using 30 scientific articles (Falzon et al., 2014). The frequency of anthelmintic treatment seems a high-risk factor and the authors concluded this coefficient is the most important contributors to the development of AR. It is significantly proved, that flocks that are treated more frequently have higher odds of having resistance, compared to those farms that treated less often. Mixed-species grazing (cattle and sheep) has long been hypothesized as a protective factor for AR because cattle generally do

not become infected by sheep GINs, and could use to reduce L3 number and pasture contamination. However, some studies included in meta-analysis suggest that on shared grassland, cattle may accelerate the development of AR, as it may result in a reduction in the number of L3 on pasture. Although mixed-species grazing as a factor has high odds, this estimate was not statistically significant.

Dose-and-move is a common practice in several countries as well as in Hungary. In this approach the farmers move their animals immediately after anthelmintic treatment to clean pastures, thus reducing GIN re-infection. However, this routine hastens the development of resistance, because the resistant parasites, which survive anthelmintic treatment (mostly RS or RR) have a selective advantage resulting in the multiplication of R allele on clean pastures (Abott et al., 2012). However, the dose-and-move practice is positively associated with AR (a four-times higher odds).

The Study 1 revealed that the BZ resistance level in *H. contours* in sheep as high as (63.76%) is existing in several European countries (Anonymous, 2007; Ihler, 2010; Papadopulos et al., 2012). The results showed that the RR genotype proportion has a strong connection with the treatment frequency and the long-term use of BZ. This result proved that the intensive suppressive chemical control strategies may not be a successful approach any longer.

A similar prominent resistance level (70%) was observed in the farmed red population, too. It is the first genetic evidence of BZ resistance in *H. contortus* in the red deer. There has not been any rotation of unrelated anthelmintics in this herd to delay AR. It is likely that routine use of ABZ for many years has favoured survival of the resistant genotype, thus inducing spread of R allele in this *H. contortus* population. The long-term application of an anthelmintic, as a selection driver, can mitigate the S allele frequency and without any rotation, could facilitate the increase of resistance level in helminth populations. This has been reported as the reason for the fast development of resistance in *H. contortus* in South Africa and New Zealand (van Wyk et al., 1989; Shoop, 1993).

In the case of red deer, I hypothesize, that the speed of applied ABZ metabolism contributed to the high resistance. Metabolism of xenobiotics as ABZ differs between ruminant species. This characteristic of microsomal liver metabolism in deer strongly suggests that these species require a higher dose rate than sheep and cattle to attain optimal efficacy rates against susceptible parasites. Prichard (1985) reviewed the connection between

host physiology and efficacy of drugs and highlighted the role of the liver in drug metabolism. This organ, among other factors, affects the rate of metabolism and the active level of drug within the host organism. Albendazole sulphoxide (ABZSO) is the main anthelmintically active metabolic product found systemically in ruminants after ABZ administration (Delatour et al., 1991; Virkel et al., 2004). Velík et al. (2005) studied ABZ biotransformation in ruminants and a few monogastric species. Their results show that ABZ transformation to (+) and (−) ABZSO enantiomers depend on the liver microsomal enzymes (e.g. cytochrome P450). The overall amount of these products and the ratio of these enantiomers are responsible for anthelmintic effects of this drug.

In sheep, the quantity of ABZSO was more than twofold greater than that in the red deer, and the ratio of (+) ABZSO and (−) ABZSO was 3.17. They found that liver microsomal enzymes of deer species produce less ABZSO, and the +/− ratio in red deer was 0.67. They concluded that effective dosage of ABZ in deer species cannot be inferred from data collected from domestic ruminants. Nevertheless, the inadequate dosing accelerates the dominance of resistant allele, and it contributes to the selection of a resistant worm population (Smith et al., 1999; Jabbar et al., 2006).

This metabolic hypothesis was confirmed in an Australian study (Mylrea et al., 1991) One hundred and fifty enclosed, weaned fallow deer were kept on intensive pastures. One-third of the animals showed clinical symptoms, like weight loss, diarrhoea and some of the animals died. For this reason all of the animals treated twice with ABZ, but despite drug application ill-thrift and deaths continued. The necropsies confirmed gross damages on the abomasal mucosa because of the presence of significant numbers of parasites, namely *Spiculopteragia asymmetrica* (75%), *Ostertagia ostertagi* (13%), *Skrjabinagia kolchida* (8%) and *Haemonchus contortus* (4%). After this drug failure, a FECRT was conducted in four groups by using two concentration of ABZ and one concentration of ivermectine (IVM) and it was revealed the ineffectiveness of both drugs. The conclusion was that deer are able to metabolise and excrete benzimidazole compounds more quickly than sheep and cattle.

Although several studies were concerned with AR in small ruminants (Papadopoulos et al., 2012; Rose et al., 2015), our knowledge is deficient about wild ruminants. In a study, Chintoan-Uta et al. (2014) tested and confirmed the cross-transmission of *H. contortus* and its BZ resistance between the fallow deer, red deer, roe deer, cattle, and sheep. Both of their resistance detection methods (EHA and molecular test) demonstrated the presence of AR in

the worms derived from roe deer. It was concluded that AR nematodes may spread between farms by wild deer, which could act as vectors.

The third and fourth study showed, that in a natural environment, where sympatric cervids and sheep share the feeding places, wild ungulates could contribute to AR flowing. The molecular test confirmed a moderate frequency (31.4%) and absence of R allele in roe deer and red deer, respectively. As the home-range of roe deer can vary widely in scale (Jeppesen, 1990; Maillard et al., 2002) and it can be affected by habitat resources. Some studies suggest that the roe deer adjust home-range size in response to landscape structure. Fragmented landscapes can provide abundant, high-quality food, which increases deer habitat carrying capacity (Reimoser, 2003; Saïd and Servanty, 2005). These valuable, fragmented landscapes can also be utilizable for sheep grazing; as it was observed in the study site. I supposed that the overlap in habitat use between sheep flocks and roe deer could hasten of AR spread within wildlife and may be between domesticated flocks.

The absence of R allele in the red deer could not be explained exactly. I hypothesized that the presence of sheep on pastures indicated avoidance behaviour in the red deer. Some free-range studies (Osborne, 1984; DeGabriel et al., 2011) showed that the absence of sheep in a given habitat induced a higher red deer density. According to Cuartas et al. (2000), this phenomenon was attached to the disturbance effect of sheep husbandry rather than any direct or indirect competition between the two species. During our survey, many sheep flocks used regularly the pastures of the study area, thus their presence might have affected the red deer aggregation and might have influenced the absence of R allele in this host. Because of this, we suggest, the red deer might have a marginal role in dynamics of BZ resistance in the studied habitats.

Based on the results of Study 3, a finer assessment was conducted to shape the role of shared grassland as refugia in AR spread in a natural habitat. In the frame of this work, parasitological and molecular diagnostic methods were carried out. The results showed that roe deer carries a more diverse abomasal helminth population than sheep; and the AR level also differs significantly in the two species.

In the sheep, only two species, *H contortus* and *T circumcincta/ T. trifurcata* were detected, with very similar importance values. While in the roe deer, a more diverse helminth infracomunity was found. In which, *H. contortus*, *S. spiculoptera/S. mathevossiani* and *O.*

leptospicularis/O. kolchida played a dominant role, while codominant species were confirmed to be *A. sidemi* and *S. asymmetrica/S. quadrispiculata*.

Interestingly, *T. axei*, which proved to be an adaptively superior competitor in other studies (Holmes, 1973; Diez-Baños, 1992), was found solely in one roe deer individual within the studied area. Therefore, among these circumstances; *T. axei* was considered subordinate. On the other hand, *T. circumcincta* and *H. contortus* were promoted dominant in sheep; though the above-mentioned studies confirmed its role as a satellite species within the abomasal helminth fauna. The dominance of *T. axei* was verified in natural conditions. Normally, *T. axei* has got similar environmental requirements for development to those of *T. circumcincta* (Bailey et al., 2009); whereas high soil temperature (29 ^{o}C<) and low soil relative humidity (98.5%>) limit survival of both (Callinan, 1978). In the alimentary tract of a host, both have the ability to inhibit the establishment of *H. contorus* (Emery et al., 2016). During the last decade, the majority of studies mention *T. axei* as a less common parasite species (Bailey et al., 2009; Palcy et al., 2010; Melville et al., 2016). Based on these data, it should be hypothesized that marginalisation of this species in domestic ruminants is a human-induced evolutionary mechanism. In the lack of regular anthelmintic treatment, parasite species should replace each other within a host as predicted by the rock-paper-scissors model, which is based on a dynamic balance of interspecific interactions and regulated mainly by the immune system of the host (Diez-Baños et al., 1992; Bashey, 2015).

Within a human-influenced environment in parallel with global warming, in a sheep herd, a less diverse parasite community developed. Regular medication against parasites favoured *T. circumcincta* and *H. contortus*; and made them dominant in sheep (Callinan, 1978; Emery et al., 2016). Having regard to the highest mean intensity values in this host species, it is assumable that human effect confounded the dynamic balance between the host and its parasite community. Losing the heterogeneity in the competitive environment eventuated in decreased diversity, and through that, a modified competition of parasites. During competitive coexistence, each species has to limit its own growth for the sake of sustainability; and the main drive of regulation is the host's resiliency determined by its genotype (Bashey, 2015). In the case of a sheep herd with a specified breeding purpose, genetic diversity of the host population is limited, which should cause an additional effect on parasite diversity, thereby interspecific interaction within the host.

On the other hand, *S. spiculoptera/ S. mathevossiani*, *S. assymmetrica/ S. quadrispiculata* and *O. leptospicularis/ O. colchida* were confirmed to be characteristic for

the roe deer. These species proved to be dominant or codominant; and reached a recognizable prevalence and mean intensity in the hosts. In our study, within the roe deer; these parasites seemed superior competitors of sheep-specific ones and lived in a stable competitive coexistence with those.

As far as the author know; on the study site, anthelmintic treatment of game animals has never been in practice. Comparing our findings with previous experiences, the dominance of these species is interpretable by the lack of direct regular human influence. In these conditions, competitive interactions between parasites and the regulatory role of the hosts' immune system could shape the structure of the parasite community; predominantly (Diez-Baños et al., 1992; Bashey, 2015). This structure is a result of a rock-paper-scissors dynamic (Hoffman et al., 2015), inasmuch as infection of hosts is a stochastic process, which originates from the environmental pool.

Among these circumstances, momentary competitive success of helminths, both outside and inside of the host, depends on a lot of interacting factors. In the case of regular anthelmintic medication, the competitive environment becomes simplistic; whereas the effect of the chemical was advanced to the main determinant of evolutionary success (Diez-Baños et al., 1992; Budischak et al., 2016).

Though, restricted importance of *T. axei* should mean that neither cervid hosts are free from human influence. It is very probable that through local sheep herds, farmers can influence the structure of environmental pool of parasites. In accordance with others' studies (Holmes, 1973; Diez-Baños et al., 1992), our results also support that *T. axei* might be extremely sensitive to even the indirect effect of anthelmintics; which is possibly intensified even by climate warming (Callinan, 1978; Emery et al., 2016).

This human influence was confirmed by the fact that the studied roe deer population carried the two sheep specific helminthes. Moreover, *H. contortus* was confirmed to be dominant in this small cervid species. This phenomenon can be explained by the lifestyle of the cervid. This small ruminant is a strongly territorial, less migrating one (Mysterud, 1998). Our sample collecting period was during their breeding season when males patrol their territories and females also stay within stable ranges to give birth to their kids and to mate. These home-ranges, on the studied site, overlap sheep pastures. This continual contact with sheep herds allows of changing parasites between the two host species. In the lack of direct encroachment, this mild influence cannot provide an over-proportioned evolutionary advance

for *H. contortus*; therefore, a more or less balanced, diverse parasite community with medium intensity evolves in the roe deer.

The presence of *A sidemi* in roe deer is a very interesting phenomenon. Both *H. contortus* and *A. sidemi* belong to the same subfamily of Haemonchinae (Ferté et al., 2000). As close relative blood-sucking abomasal nematodes, these two species are expected to occupy the same niche and competitively exclude each other. *A sidemi* is generally found in cool climatic conditions; while *H. contortus* is a rather tropical parasite species (Hoberg et al., 2002; Hoberg et al., 2004). The temperate climate of Hungary is barely suitable for both of them. Among these circumstances, both need a special niche to survive. In the case of *H. contortus*, the competition-colonization trade-off mechanism should be its evolutionary strategy. In natural populations, *H. contortus* is better at free-living survival but slower to recover or grow within the host. In these conditions, the species remains a competitively inferior one; and only the human effect makes it competitively superior. *A sidemi* is different. This parasite can cause serious problems also in natural wildlife populations. In the European bison *(Bison bonasus)* population of Białowieża Primeval Forest, a severe *A sidemi* infection emerged and reached 100% prevalence in a relatively short time; causing a considerably deleterious effect on the host population. After years of coexistence, the balance was struck between the host and the parasite (Kołodziej-Sobocinska et al., 2016). On the studied site, this phenomenon was not experienced; or at most a very long time ago. During this study, prevalence and intensity data of *A. sidemi* suggested a delicate ecological balance in the host-parasite system.

Białowieża experiences suggest that the evolutionary strategy of *A. sidemi* is based on rather a quick invasion of the host than good survival in the environment. Aggressive spread in dense, non-medicated bison herds also supports this hypothesis (Kołodziej-Sobocinska et al., 2016). And these are in consonance with our findings, as in the non-medicated, quasi-natural roe deer population, the *A. sidemi* proved to be a codominant species of the diverse parasite community. On the other hand, human-influenced sheep herds of the same habitat were not infected with *A. sidemi*, but with *H. contortus*. Our findings suggest that these blood-sucking nematodes; though those are close relatives, do not exclude each other directly during evolutionary competition. For *H. contortus*, human-induced diversity-loss is favourable, when the environmental survival capacity of the species provides an inconquerable advance. For *A. sidemi* an immune-regulated competitive environment is advantageous, where the less

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environment-resistant helminth need not fight against a lot of other parasites for survival out of the host.

The complete absence of human influence within agricultural areas and in the adjacent buffer zones is not obtainable. In the surroundings of pastured livestock, numerous eggs and larvae of artificially selected parasite strains exist. In these conditions, the environmental pool, from which hosts get infection, is imbalanced; as more environment-resistant, naturally inferior competitor parasite eggs and larvae predominate around domestic animals. Wild ruminants feed also on these pastures and pick up infectious materials. The 'pioneer' parasite infracommunities in sympatric wild ungulates consist of both wildlife originated and directly human-influenced part. This mixed community in the abomasum of a wild ungulate meets the immune system, and a special combination of parasite species develops. During the life-span of the individual, the within-host parasite infracommunity gets a replacement from the environment; both from the natural and the human-influenced part. Without direct anthelminthic treatment; within the host, the natural regulation effaces less invasive, slowly recovering species. Notwithstanding; the larger amount of livestock originated parasites the wild ungulate can meet, the more livestock specific species are parts of its parasite infracommunity.

The limited cross-infection between wildlife and livestock is supported by the moderate or low value of Sorensen coefficient of similarity among wild and domesticated hosts. Only a generalist nematode, *H. contortus*, was observed to play a minor role in cross-infection. This parasite occurs in a very wide range of hosts, which are phylogenetically very different (Nilsson, 1972; Ezenwa, 2003; Cerutti et al., 2010; Rinaldi et al., 2015).

Our results confirmed GIN transmission between wildlife and livestock within the study area. Though this transmission seems discreet by observing epidemiological data; change in parasite community structure calls the attention to the ecological risks. Extinction of *T. axei* and rising of *H. contortus* might indicate human encroachment on natural habitat in parallel with climatic change. The comparison of AR prevalence in the two hosts also supports the superiority of human influence. Within the studied habitat, antiparasitic treatment of wildlife has never been in practice. In spite of this, AR is present in the parasite community of the roe deer, though its level is much lower compared to the sheep.

Our results support the hypothesis, that habitat overlapping between sheep and roe deer means rather an advantage than a real risk for antiparasitic strategies. Worm community

excreted by wild ruminants contains fewer AR individuals and more competitively superior, non-blood-sucking species, which has got a stimulating effect on the host's immune system. These two features behave like a buffer in the environmental pool of parasites, and the effect depends on its portion. These facts supported our hypothesis that wild ungulates serve as refugia for worms within a pasture ecosystem. During deworming events, this part of the worm community escapes from anthelminthic effect, therefore it avoids selection pressure of drugs. Inside the sheep, which pick up these drug-sensitive larvae from the pasture, therefore resistant alleles will be diluted by these refugia originated infection. This dilution effect prevails most considerably in the spring, when small amount of infective larvae reside on the pastures; and most of them originate from wild ruminants, therefore they carry resistant alleles with very low frequency. When the sheep enter the pasture after the wintering period, the animals meet a very diverse, less human-influenced parasite community, which carries homozygote sensitive genotype with very high prevalence.

In the abomasa of wild ruminants, a nearly natural competition takes place, by which anthelmintic resistance should not be considered as an evolutionary advantage for worm individuals. Therefore, this part of the worm community shifts to lower anthelmintic resistance, and the spread of resistant alleles decelerates within the whole supracommunity of the shared habitat.

This phenomenon needs to be studied in order to determine the exact drivers of parasite transmission within the wildlife-livestock interface. Expected results might provide additional methods to future antiparasitic strategies, which should be based on a holistic approach, instead of the routine application of anthelmintics.

As a result of our studies, the obligate use of anthelmintics against abomasal nematodes revealed to be an insufficient strategy. Frequent and long-lasting application of chemotherapy creates an evolutionary pressure on parasitic worm populations in the synanthropic environment. This will change the survival ability of certain genotypes, and by this the allele frequency will shift to predomination of resistance.

Antiparasitic methods that do not enhance the selection for anthelmintic resistance should be alternatives or ancillary tools in defence strategies. These holistic strategies should contain accurate nutrition, application of plant secondary metabolites, well-planned husbandry systems, pasture management, and regular health-check of individual animals.

In the case of farmed cervidae, a complete supervision of the recent husbandry system is needed. Our hypothesis is, that the physiological demands of these species are inevitable to recognize before strategy planning. Special nutritional requirements, effects of stress induced by farm conditions, and pharmacokinetics of drugs should be studied.

One Health approach, which investigates a health problem with its complete environment and all connections, should be an advantageous way to support strategic planning of antiparasitic defence. Investigations on ecological demands of different parasites can provide information about the maintenance and spread of parasites outside the hosts. Immunological research should supply useful data on the survival of worms within the hosts. Multidisciplinary analysis of the concerned ecosystem should provide further data, which should help to identify the driving forces of infection through all the susceptible hosts and the habitat they share.

Holistic approach is suggested to investigate the factors that may affect natural or human-influenced lifecycle of parasites in a semi-natural pasture ecosystem. Further studies are needed to determine the possible role of One Health, and the usefulness of multidisciplinary working groups during analysing the function of a parasite community within a habitat.

7. CONCLUSIONS

The Study 1 revealed that the presence of homozygous BZ resistant *H. contours* is high (50.79%) The frequency showed a strong correlation with the treatment frequency (R=0.7674; p=0.0058) and the long-term use (R=0.7789; p=0.0047) of BZ.

In Study 2, a similarly prominent resistance level (70%) was also observed in a farmed red deer population. It is the first genetic evidence of BZ resistance in *H. contortus* in the red deer. There has not been any rotation of unrelated anthelmintics in this herd to delay AR. It is likely that routine use of ABZ for many years has favoured the survival of the resistant genotype, thus inducing spread of R allele in this *H. contortus* population. In this case, the speed of applied ABZ metabolism could contribute to the high resistance level. The characteristic of microsomal liver metabolism in deer strongly suggests that these species require a higher dose rate than sheep and cattle to attain optimal efficacy rates against susceptible parasites. This organ, among other factors, affects the rate of metabolism and the active level of drug within the host organism. Owing to the liver microsomal enzymes (e.g. cytochrome P450), the red deer detoxicates its organism much more effectively than sheep. Therefore the correct sheep or cattle dosing is inadequate to eliminate the nematodes from deer. It causes a suboptimal-dosage and accelerates the dominance of resistant allele, and contributes to the selection of a resistant worm population.

The molecular test of Study 3 confirmed a moderate frequency (31.4%) and absence of R allele in roe deer and red deer, respectively. By these results, it seems that in the spread of BZ resistance, the roe deer could play a role. This theory is proved by the parasitological examination of Study 4. In this case, only the *H. contortus* was the dominant species in both hosts. Notwithstanding, its importance was not so remarkable in the cervids (21.67) as in the sheep (43.99). These findings suggest that rather the sheep farming affects the roe deer habitat, than vice versa. The comparison of AR prevalence in the two hosts also supports the superiority of human influence. Within the studied habitat, antiparasitic treatment of wildlife has never been in practice. In spite of this, AR is present in the parasite community of the roe deer; though its level is much lower than in the sheep. These results support the hypothesis, that habitat overlapping between sheep and roe deer in this area means rather an advantage than a real risk for antiparasitic strategies. Worm community excreted by wild ruminants contains fewer AR individuals and more competitively superior, non-blood-sucking species.

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The helminths they shed behave like a buffer in the refugia and may dilute the R allele frequency in the shared pastures.

8. NEW SCIENTIFIC RESULTS

1. We have found high benzimidazole resistant allele frequency (63.76%) in *Haemonchus contortus* in sheep flocks. This incidence shows a strong linear correlation with treatment frequency and length of anthelmintic usage.

2. We have found high benzimidazole resistant allele frequency (85%) in *Haemonchus contortus* in a farmed red deer flock.

3. We have found a high (82.9%) a moderate (31.4%) and a lack of benzimidazole resistant allele frequency in *Haemonchus contortus* in sheep flocks and free-living roe and red deer populations, respectively, in a natural habitat shared by them.

4. *Haemonchus contortus* was considered as a dominant nematode species in free-living roe deer and the sympatric sheep populations.

5. We have confirmed the presence of *Ashworthius sidemi* and *Nematodirus oiratianus* subsp. *interruptus* in Hungary.

9. SUMMARY

The elemental aim objective was to confirm the presence and frequency of benzimidazole resistance in different ruminant populations. For this reason, a parasitological and a molecular diagnostic method conducted in *Haemonchus contortus* specimens derived from sheep, farmed and free-ranging red deer and roe deer. An ancillary objective was to assess the role of free-ranging deer species in the anthelmintic resistance spread in natural circumstances.

The sample collection was carried out from the southern part of Transdanubia. For restriction fragment length polymorphism-polymerase chain reaction, we dissected abomasa of the hosts and *Haemonchus contortus* males were picked up from organs.

In the first study, altogether 189 worms were analysed from 11 sheep flocks. The result revealed that the BZ resistance level in *H. contours* in sheep as high as are being in several European countries. The correlation between RR genotype proportion and both treatment frequency and long-term use of anthelmintic was strong. This result confirms that this intensive chemical control strategy could enhance AR development.

In Study 2, we have found genetic evidence of BZ resistance in *H. contortus* derived from red deer. It was hypothesized that routine use of ABZ for a long-term period has favoured the spread of R allele. We supposed some differences in working of liver microsomal enzymes may hasten the problem too. The red deer are able to metabolise and excrete benzimidazole compounds more quickly than sheep and cattle, thus the recommended drug level of domesticated ruminant could cause under-dosage in cervids.

The applied molecular test in Study 3 confirmed a moderate frequency and absence of BZ resistance in free-ranging roe deer and red deer populations, respectively. By these results, we suggest that free-ranging roe deer could have a pronounced role, while red deer might have only a marginal role in the spread of BZ resistance within the studied habitats. This hypothesis was strengthened by the abomasal nematode community analysis in sheep and roe deer. The results showed that roe deer carries a more diverse abomasal helminth population than sheep. We found just two common worm species but just *H. contortus* was considered as a dominant in both ruminant populations. The other nematode, *Teladorsagia circumcincta* was dominant in sheep and had satellite role in roe deer.

Summarizing all of the results, we can conclude, the level of BZ resistance in *H. contortus* can be high in sheep and farmed red deer, while can be low in the roe deer population in southwestern Hungary. In the case of two domesticated species, especially the inadequate anthelmintic application could cause this problem. Our results support the hypothesis, that habitat overlapping between sheep and roe deer means rather an advantage than a real risk for antiparasitic strategies. We suppose that wild ungulates serve as refugia for worms within a pasture ecosystem and ‘dilute’ the R allele frequency.

10.ÖSSZEFOGLALÁS

Dolgozatom elsődleges célja a benzimidazollal (BZ) szembeni rezisztencia jelenlétének megerősítése és mértékének meghatározása a vizsgálati területek kérődző-populációit fertőző *Haemonchus contortus* parazitapopulációkban. Ezért parazitológiai és molekuláris diagnosztikai vizsgálatokat végeztünk juhokból *(Ovis aries)*, szabad területi és farm körülmények között tartott gímszarvasokból és szabad területi őzekből *(Capreolus capreolus)* származó *H. contortus* mintákon. Vizsgálatom másodlagos célja annak meghatározása volt, hogy a szabad területi szarvasféléknek milyen szerepe lehet az anthelmintikumokkal szembeni rezisztencia terjesztésében.

A mintagyűjtést a Dél-Dunántúl Régióban végeztük. A molekuláris diagnosztikai vizsgálatokhoz (RFLP-PCR: restriction fragment length polymorphism – polymerase chain reaction) a gazdafajokból származó oltógyomor-minták boncolásával gyűjtöttünk *H. contortus* hím férgeket.

Az első vizsgálatban összesen 189 féregmintát vizsgáltunk meg 11 juhállományból. Az eredmények megerősítették, hogy vizsgált területen a BZ-rezisztencia mértéke ugyanolyan jelentős, ahogy azt korábbi kutatások nyomán tapasztalták több európai országban is. Kérdőíves felméréssel bizonyítottuk, hogy az anthelmintikum-használat gyakorisága és a folyamatos alkalmazás időtartama, valamint az RR (homozigóta rezisztens) genotípus gyakorisága erősen korrelál egymással. Ez az eredmény megerősíti, hogy az intenzív kémiai védekezésen alapuló féregellenes stratégia elősegíti az anthelmintikum-rezisztencia terjedését.

A második vizsgálatban a gímszarvasokból *(Cervus elaphus)* származó *H. contortusok* esetében sikerült molekuláris genetikai vizsgálattal bizonyítani a BZ-rezisztencia jelenlétét. Hipotézisünk az volt, hogy a hosszú ideje tartó BZ-használat kedvez az R allél terjedésének. Feltevésünk szerint ezt a folyamatot jelentősen gyorsítja a szarvasfélék májában a mikroszomális enzimrendszer aktívabb működése is. A szakirodalmi adatok szerint, a szarvasok a házi kérődzőknél gyorsabban metabolizálják a BZ-t, ezáltal valószínűsíthető, hogy hatékony kezelésükhöz magasabb dózisokra van szükség, illetve a házi kérődzőkre törzskönyvezett adagokkal történő kezelés során aluldozírozás történik, amely szintén hozzájárul a BZ-rezisztencia terjedéshez.

A harmadik vizsgálat során elvégzett molekuláris vizsgálat a szabad területi őzben mérsékelt mértékű BZ-rezisztenciát mutatott ki, míg a szabad területi gímben nem mutatta ki az R-allél jelenlétét. Az eredmények alapján valószínűsíthető, hogy az őznek lehet szerepe a BZ-rezisztencia terjedésében, míg a gímszarvas szerepe elhanyagolható a vizsgált ökoszisztémában. Ezt a feltevésünket az adott területen élő juhokból s őzekből származó oltógyomrok faunisztikai vizsgálata is megerősítette. Két olyan parazitafajt azonosítottunk, amely mindkét gazdafajban előfordult, de csupán a *H. contortus* bizonyult mindkét gazdában domináns fauna-alkotónak. A másik közös parazitafaj, a *Teladorsagia circumcincta* a juhban domináns volt, ám az őzben csupán másodlagos faunaelemként volt jelen.

Az eredményeinket összegezve megállapítható, hogy a BZ-rezisztencia a juhokban és a zárttéri gímszarvasokban jelentős mértékű, míg a szabad területi őzben csak nagyon alacsony szintű a vizsgált élőhelyeken, a Dél-Dunántúlon. A farm körülmények között tartott két gazdafaj esetében, vizsgálataink nyomán, egyértelműen azonosítható a nem megfelelő anthelmintikum- használat oktani szerepe. Eredményeink alapján megerősítést nyert az a feltételezésünk, hogy az őz és a juhállományok közös élőhely-használata inkább kedvező hatású a paraziták elleni védekezés során, mintsem kockázati tényezőként szerepelne. Úgy véljük, hogy a vadon élő kérődzők refugiaként (a parazita-közösségnek a féregellenes szerek által el nem ért részeként) viselkednek egy legelői ökoszisztémában, ahol képesek hígítani az R-allél előfordulási gyakoriságát.

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13.PUBLICATIONS AND PRESENTATIONS

SCIENTIFIC PAPERS AND LECTURES ON THE SUBJECT OF THE DISSERTATION

Peer-reviewed papers published in scientific journals

Nagy, G., Zsolnai, A., Csivincsik, Á., Sugár, L. Detection of benzimidazole resistance by PCR-PFLP in *Haemonchu contortus* recovered from sheep. Magyar Állatorvosok Lapja. 2015.137(3):167-172.

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Nagy, G., Csivincsik, Á., Sugár, L., Zsolnai, A. Benzimidazole resistance within red deer, roe deer and sheep populations within a joint habitat in Hungary. 2017. Small Ruminant Research. 149:172-175.

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Oral presentations

Nagy, G., Ács K., Csivincsik, Á., Sugár, L. Occurrence of *Haemonchus contortus* in south Transdanubian ruminants. MTA Állatorvostudományok Biztottsága, Akadémiai beszámoló. Budapest, Hungary. 29 January 2014.

Nagy, G., Zsolnai, A., Csivincsik, Á., Sugár, L. Occurrence of nucleotide-polymoprphism causes benzimiazole resistance in south Transdanubian ruminants. MTA Állatorvostudományok Biztottsága, Akadémiai beszámoló. Budapest, Hungary. 28 January 2015.

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SCIENTIFIC PAPERS AND LECTURES NOT RELATED TO THE SUBJECT OF THE DISSERTATION

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Nagy, G., Ács, K. Csivincsik Á., Varga, Gy., Sugár L. The occurrence of thorny-headed worm *Macracanthorhynchus hirudinaceus* in Transdanubian wild boar populations in relation to certain environmental factors. Erdészettudományi Közlemények. 2014. 4(1):197-206.

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Csivincsik Á., Rónai Zs., Szabó, Sz., Balog, T., Nagy G. Two sides of the golden jackal: its controversial role in southwestern Hungary. Book of Proceedings VIII International Scientific Agriculture Symposium Agrosym. Sarajevo, Bosznia-Hercegovina: University of East Sarajevo, Faculty of Agriculture. 2017. 2140-2144.

Oral presentations

Nagy, G., Csivincsik, Á., Rózsa, D., Tossenberger, J. Effects of a tannin contained supplement on gastrointestinal nematodes and performance of weaned lambs. Magyar Buiatrikus Társaság XXVI. Nemzetközi Kongresszusa, Budapest, Hungary. 9-12 October 2016.

14.CURRICULUM VITAE

Gábor Nagy was born in Kecskemét on 30 August 1972. He absolved the primary and secondary schools in his hometown.

In 1993, he obtained his animal husbandry engineer bachelor degree.
In the periods of **1997-2006** and **2011-2013,** he was employed as veterinary assistant in a private praxis. He dealt with mainly parasitology of wild and domesticated species, environmental epidemiology and One Health approach in veterinary and animal husbandry practices. He worked for a caritative foundation between **2006 and 2010**.

He followed his studies during **2011-2013** to obtain a master's degree.
From December 2013, he participated in the research activities at the Department of Animal Nutrition, Faculty of Agricultural and Environmental Sciences of Kaposvár University. His research interest focuses on the application of secondary plant materials as anthelmintics.
Between 2013 and 2016, he was a full-time student at the Doctoral School of Animal Science of Kaposvár University.
Since 2018 he has been the member of the Hungarian Society of Parasitologists.