

Theses of Doctoral Dissertation

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**Occurrence of the large American liver fluke
(*Fascioloides magna*) in South Transdanubia**

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1. PRELIMINARIES AND OBJECTIVES

1.1. Introduction

The large American liver fluke (*Fascioloides magna*) is a parasite living in the liver of deer. It causes significant damage to liver tissue (Majoros and Stoykov, 1994), as a result of which the metabolism, general health, and thus nutritional status and performance of the infected individual may be impaired (Marinkovic et al., 2013; Simonji et al., 2022; Sommer et al., 2022). At the population level, deterioration of performance (e.g. reproduction and trophy quality) and a decrease in the number of individuals in the population can be observed, mainly in its new distribution areas (Marinkulic et al., 2002; Shury et al., 2019). The parasite is considered an invasive species in Europe. This liver fluke first appeared at the end of the nineteenth century and is believed to have been introduced with infected cervids imported from North America. The parasite appeared in the Danube catchment area in the 1990s and began to spread along the river (Rajsky et al., 1994).

F. magna has an indirect lifecycle: mud snails belonging to the family Lymnaeidae play the role of intermediate host in its development cycle. Wetlands housing intermediate host snails are crucial to the parasites' appearance, survival, and spread (Lockyer et al., 2004). The floodplain forests surrounding the Danube River provide excellent habitat for both permanent and intermediate host species, so researchers expected the parasite to spread downstream already when it appeared in the catchment area (Hörweg et al., 2011; Juhász and Majoros, 2023). It is assumed that the settlement of intermediate hosts drifting away with tidal waves in lower reaches causes the spread along the river (Majoros and Stoykov, 1994), while definitive deer hosts spread the parasite on their home territory, forming local endemics such as those observed in Szigetköz and Gemenc (Majoros and Stoykov, 1994; Toth, 2012).

The first occurrence of the great American liver fluke was confirmed in 2016 in southern Transdanubia, far from the Danube (Nagy et al., 2018). According to the previous expectation, the parasite can spread naturally in the Danube Valley in Hungary, while its appearance further away from the river is mainly due to artificial introductions (Majoros and Sztojkov, 1994). Already,

epidemiological studies following the first case not related to the Danube River showed that both artificial and natural processes may contribute to the spread of the parasite in South Transdanubia (Nagy et al., 2018). However, the *F. magna* occurrence in 2016 in Zselic, in the middle of Southern Transdanubia, happened about 100 km from the Danube. The appearance of the parasite in new areas, due to the damage it has experienced in previous epidemics, is considered a significant wildlife management risk. The most important goal of our studies was to clarify which host species can play a role in maintaining the infection in the newly discovered range, how the epidemics can be followed at the border of the endemic, and where and at what speed the infection spreads.

1.2. Objectives

Our study aimed to assess the extent of the Zselic endemic and the risks of its spread. We planned to achieve the goals through the following studies:

1. Investigating parasite occurrence in red and roe deer by necropsy of individuals hunted on individual and social hunts.
2. Examine the effectiveness of the sedimentation technique by comparing the liver dissection findings (worm count, worm weight) with the number of eggs detectable in faeces.
3. Involve hunters in the surveillance of parasite spread. We developed an illustrated guideline to help the eviscerator identify liver lesions. We evaluated the dissection results and the classification of livers assessed as positive or negative by hunters. Finally, we determined and compared both methods' sensitivity, specificity, and accuracy.
4. Clarification of the role of roe deer in maintaining infection: post-mortem and histopathological examination of specimens hunted in the new range, comparison of lesions with those seen in red deer. Hatching of eggs that can

be obtained from red and roe livers, comparison of survivability in the case of eggs from the two host species.

5. Monitoring of distribution area changes during the four years of research.

2. MATERIALS AND METHODS

Our research aimed to explore the epidemiological peculiarities of the large American liver fluke (*F. magna*) endemic discovered in South Transdanubia in 2016. During the four years of investigation, we continuously examined hunter-harvested red deer and roe deer specimens to determine annual period prevalence and to follow-up the trends of the endemic.

In the first phase, we tried to clarify the epidemiological role of the deer populations living in the area. To this end, a comparative study of roe and red deer harvested in the endemic area was carried out: evaluated the pathological and histopathological characteristics of the lesions observed in hosts, counted worms in each pseudocyst, evaluated the egg production of flukes by microscopic examination, hatched eggs isolated from cysts containing one and two flukes and observed embryonic development, and survival.

To follow-up the epidemic changes in the South Transdanubian distribution area of *F. magna*, we planned to develop a simple surveillance method, which is appropriate to carry out large-scale investigation. Based on the dissection of shot red deer, we tested two methods of surveillance. On the one hand, by using the sedimentation technique for shaded egg collection, we determined the effectiveness of faecal egg count (FEC) to assess the infection in hosts. On the other hand, we took a series of photographs of the livers damaged to varying degrees, with the help of which we compiled a guide for the evisceration hunting personnel so that they could recognize the presence of the *F. magna* in the livers. The positive and negative organs certified by the specialist staff were subjected to a detailed post-mortem examination to determine the qualification's effectiveness.

Parasitological examination of red deer killed at different distances from the northern boundary of the previously known endemic determined the fact of spread and its distance from the previously known northernmost case.

2.1. Study areas

The samples on which the studies are based were typically collected from hunting areas located in Somogy County and, to a lesser extent, in Baranya

County (Figure 1). During the sample collection, we looked for large American liver fluke infections in deer populations living in these areas.

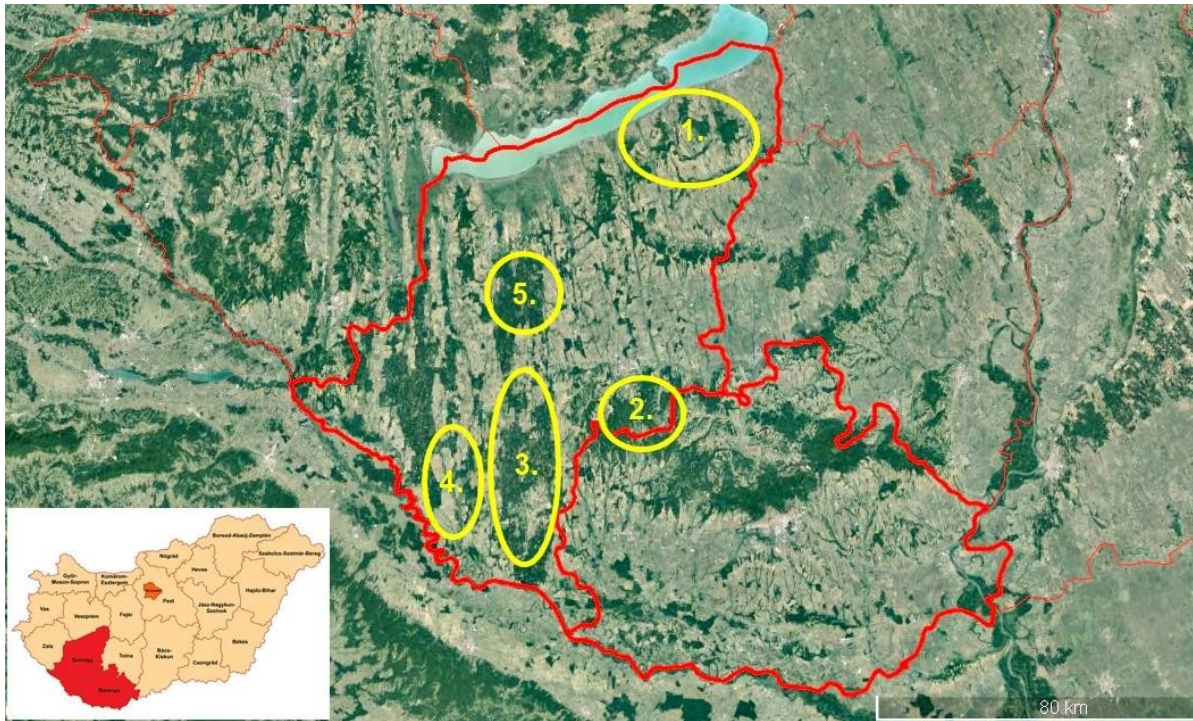


Figure 1. Location of investigated areas. (Note: red line=boundaries of counties, yellow line=hunting areas providing samples, 1. SEFAG Ltd. Szántód Forestry, 2. SEFAG Ltd. Zselic and Kaposvár Forestry, 3. SEFAG Ltd. Lábod Hunting Forestry, 4. SEFAG Ltd. Iharos Forestry (Zsitfapuszta), 5. South Transdanubian Fauna Hunting Party)

2.2. Parasitological methods

2.2.1. Necropsy

For parasitological studies, livers of red and roe deer hunted during individual and group hunt events were used. After evisceration of the animals, the organs were separated and processed as soon as possible. The liver tissue was destroyed, and the flukes released from tissue debris were collected. The isolated parasites are primarily characterized by their morphological features (body formation, body length and width), for which the work of Králová-Hromadová et al. (2016) was used.

2.2.2. Sedimentation

During the method, the number of eggs excreted with faeces was determined. A known number of corpses were used for the study, thoroughly washed with tap water. The faecal suspension was allowed to stand, and the supernatant was decanted and poured again with water. This operation was carried out until the water was clear enough for microscopic detection of eggs. The egg count was determined per gram of faeces.

2.2.3. Molecular identification

To confirm the spread of the large American liver fluke, in addition to morphological identification, a molecular method was also used. We randomly selected parasites from each infected host shot in different areas. DNA extraction was performed from about 10 mg of its tissue, lysed in 100 µl of 5% Chelex 100 suspension. Species-specific primers were used for identification, which allowed *Fascioloides magna* (forward: 5'-ACCAGTTATCGTTGTGTTG-3'; reverse: 5'-CCGTCTTTAAACAACAG-3') and *Fasciola hepatica* (forward: 5'-CTTATGATTTCTGGGATAATT-3'; reverse: 5'-CCGTCGCTATATGAAAA-3') to be clearly distinguished from each other (Houszka et al., 2016). Products obtained during a PCR reaction (*Fascioloides magna* 152 bp; *Fasciola hepatica* 112 bp) were evaluated by gel electrophoresis (90 mV, one h) on a 2% agarose gel.

2.2.4. Egg hatching

For the study, infected roe and deer livers were dissected as already described. Pseudocysts containing flukes were immediately collected with a syringe and cleaned of their contents by repeated rinses with distilled water. The cavern content originated from one and two fluke cysts collected separately for the *in vitro* hatching procedure. During hatching, the larvae's development was constantly monitored. The works of Swales (1935) and Campbell (1961) were used to evaluate the different stages of development.

2.3. Statistical methods

During the hatching experiment, the prevalence and average intensity of parasitic infestation in the tested deer population and the number of pseudocysts containing one, two and three flukes in the two host species were

determined and compared. During hatching, the proportion of eggs at different development stages was compared between and within host species. For this, the Kaplan-Meier survival analysis was used. Statistical calculations were performed using SPSS 27.0 software.

In the next phase of the study, our goal was to develop a quick-to-perform method for detecting the spread of the parasite. In this case, autopsy testing was chosen as the gold standard method. We compared this to the number of eggs excreted via faeces and the use of an illustrated guide (IG) that helps the eviscerator recognize liver changes that can be seen during the fluke infection. The study also determined the sensitivity and specificity of these two possible methods. SPSS 27.0 and MedCalc online software (https://www.medcalc.org/calc/diagnostic_test.php) were used for statistical analysis.

While investigating the spread of the parasite in South Transdanubia, we looked for signs of infection in red deer populations. The presence of worms in the killed animals confirmed how far the fluke had dispersed since the 2017-2018 survey. The spread was compared to the site of the northernmost occurrence detection in Inner Somogy.

3. RESULTS

3.1. The epidemiological role of roe deer in the Fascioloides magna spread

A total of 19 red deer and 60 roe deer livers were examined in the study. The infection rate was 68.4% (CI95%=44.6-85.3%) and 36.7% (CI95%=24.8-50.0%), respectively. The mean intensity was similar between the two species, with ten worms in infected red deer (CI95%=4.9–22.6) and 7.59 in roe deer (CI95%=2.7–24.2). These two indices characterizing infection differed significantly in the first case, while in the latter, they did not. The number of cysts containing one fluke was 17, two flukes were 46, and three were 7. In roe deer, far fewer pseudocysts were observed, one containing a fluke and two parasites being isolated from two. A total of 662 eggs were used for hatching (Table 1).

Table 1. Percentage of the different developmental stages of one-fluked and two-fluked pseudocyst in both hosts.

	total egg	embryo-nated	eye-spotted	pre-hatching	hatching
RED-PC1*	212	80.2% (170)**	64.2% (136)	64.2% (136)	52.8% (112)
RED-PC2	232	84.5% (196)	70.3% (163)	70.3% (163)	60.3% (140)
ROE-PC1	84	36.9% (31)	14.3% (12)	14.3% (12)	4.8% (4)
ROE-PC2	134	44.8% (60)	20.1% (27)	20.1% (27)	9.7% (13)

*RED-PC1: red deer one-fluked pseudocyst, RED-PC2: red deer two-fluked pseudocyst, ROE-PC1: roe deer one-fluked pseudocyst, ROE-PC2: roe deer two-fluked pseudocyst

** Percentage of the developmental category to the total egg count (number of eggs).

During the incubation of the two host species, significant differences were observed in the hatching of miracidia (Figure 2).

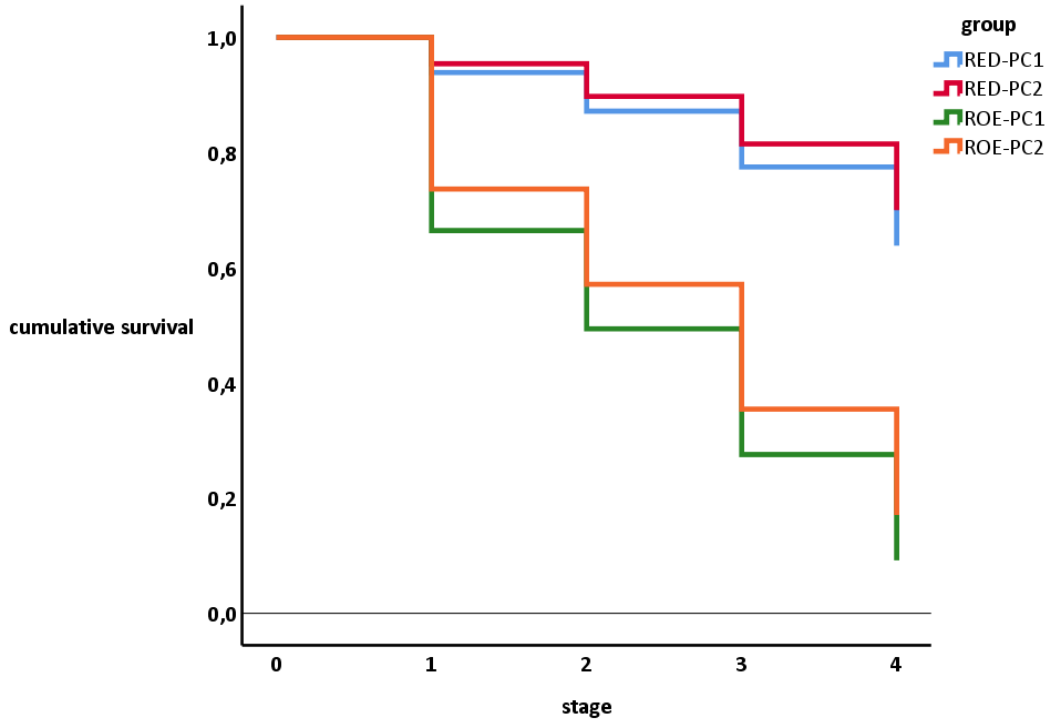


Figure 2. Kaplan-Meier survival curves of *F. magna* eggs originated from different types of hosts and pseudocysts. (Note: stage 0: fresh, 1: embryonated, 2: eye-spotted, 3, pre-hatching, 4: hatching)

The red deer-originated eggs derived from pseudocysts containing one and two flukes were hatched in a significantly higher proportion than roe deer ones. No statistical differences between single and double pseudocysts were observed within the species (Table 2).

Table 2. Comparison of the hatching ability of different egg types.

	RED-PC2	ROE-PC1	ROE-PC2
RED-PC1*	0.059**	<0.0001	<0.0001
RED-PC2		<0.0001	<0.0001
ROE-PC1			0.056

*RED-PC1: red deer one-fluked pseudocyst, RED-PC2: red deer two-fluked pseudocyst, ROE-PC1: roe deer one-fluked pseudocyst, ROE-PC2: roe deer two-fluked pseudocyst

**p-value

3.2. Field surveillance method

We found a weak, medium relationship between worm infestation in 45 red deer stags and 92 hinds, and the number of eggs excreted. The linear relationship between egg count and isolated fluke mass was somewhat more pronounced ($R=0.6016$; $p<0.0001$). The relationship between egg count and number of flukes was weaker ($R=0.5722$; $p<0.0001$).

When examining the illustrated guide (IG), a total of 319 animal livers were evaluated by the involved hunters. Of these, 137 cases were claimed to be infected using the depicted material. During autopsies, a total of 130 animals infected with flukes were found, and eggs were isolated from 50 samples during the sedimentation examination of the corpses. The true and false positives used to compare the sensitivity and specificity of the methods are given in Table 3.

Table 3. The true positive (TP), false positive (FP), true negative (TN), and false negative (FN) values in IG (illustrated guideline) and sedimentation methods.

	Gold standard test (necropsy) results		Total
	+	-	
IG +	130 (TP)	7 (FP)	137
IG -	0 (FN)	182 (TN)	182
Sedimentation +	50 (TP)	0 (FP)	50
Sedimentation -	80 (FN)	189 (TN)	269
Total	130	189	319

The sensitivity of the two methods differed markedly. For specificity, the difference was not found to be significant (Table 4). Overall, with the illustrated guide, hunters could recognize infected animals much more accurately than we could have detected in the laboratory by applying the sedimentation technique.

Table 4. Sensitivity (SENS), specificity (SPEC), positive predictive value (PPV), negative predictive value (NPV), and accuracy (ACC) values for IG (illustrated guideline) and sedimentation methods (screening tests).

	IG test	Sedimentation test
SENS	100% (CI95%: 97.2 – 100)	38.5% (CI95%: 30.1 – 47.4)
SPEC	96.3% (CI95%: 92.5 – 98.5)	100% (CI95%: 98.1 – 100)
PPV	94.9% (CI95%: 90.0 – 97.5)	100%
NPV	100%	70.3% (CI95%: 67.3 – 73.0)
ACC	97.8% (CI95%: 95.5 – 99.1)	74.9% (CI95%: 69.8 – 79.6)

3.3. Confirmation of *Fascioloides magna* spread in the southwestern Transdanubian region

During parasitological examinations, a large American liver fluke infection with varying intensity was detected in four animals. At the same time, only characteristic traces of tissue lesions caused by the worm were found in one young stag. Eggs in the liver were found only in the hind, from which 29 worms were isolated. Morphological and molecular diagnostic testing of isolated worms confirmed infection with *F. magna* in red deer (Figure 3).

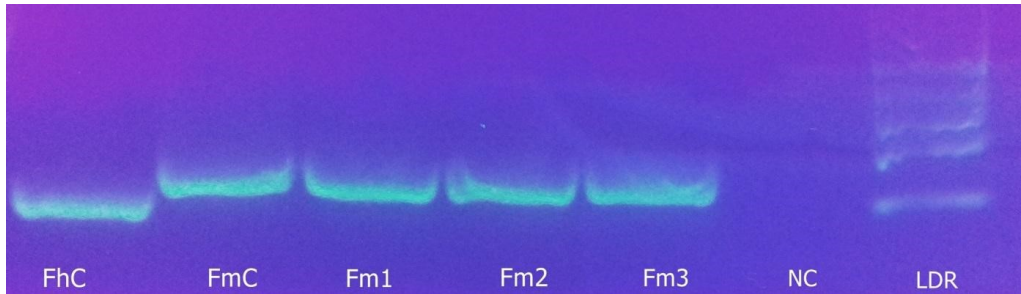


Figure 3. Results by gel electrophoresis of some isolated helminths. (FhC: *Fasciola hepatica* control, 112 bp; FmC: *Fascioloides magna* control, 152 bp; Fm1, Fm2, Fm3: isolated *F. magna*; NC: negative control; LDR: 100 bp ladder).

During the distance measurement, it was possible to establish the distances between the northernmost occurrence of the parasite in Inner Somogy, known in 2018, and the current points of occurrence, which were between 21.0 and 37.8 km (Table 4).

Table 4. Parasitological and epidemiological characteristics of *F. magna* infection are described in this study.

date	sex	age (year)	intensity	coordinates	distance from previous finding (km)^{§*}
2019.10.01.	stag	1,5	1	46° 24' 46,40" N 17° 32' 40,17" E	31.26
2019.10.06.	stag	5	1	46° 28' 28,16" N 17° 29' 41,08" E	37.78
2019.10.07.	hind	4	29	46° 24' 21,22" N 17° 29' 49,38" E	30.16
2019.10.07.	stag	1,5	only macroscopic lesion	46° 19' 23,77" N 17° 29' 14,71" E	20.96
2019.10.15.	hind	1,5	10	46° 22' 7,96" N 17° 33' 40,00" E	26.69
2020.01.16.	hind	4	2	46° 06' 45,86" N 17° 15' 07,28" E	26.5

§For distance measurement, 46° 8'5.25"N is determined from our previous test; A reference point of 17°29'4.22"E and 45° 58'49,16"N; 17°31'06,96"E were used.

*Distance measurements: with Google Earth Pro software (Google Ireland Limited, Gordon House, Barrow Street, Dublin 4, Ireland).

4. CONCLUSIONS AND PROPOSALS

The series of studies conducted between 2019 and 2023 aimed to identify the epidemiological peculiarities of the large American liver fluke endemic area discovered in the central and western areas of South Transdanubia. We wanted: 1.) to clarify the role of red and roe deer in maintaining and spreading the parasite; 2) to lay the foundation for developing a surveillance method to help wildlife managers detect the appearance and spread of the parasite; 3.) to verify the spread of the *F. magna* by determining its direction.

The main disadvantage of our surveillance method - involving hunting personnel in the field assessment of wild animals with the help of a guide compiled using photographs of lesions - is that it allows monitoring only during hunting seasons. As a result, it does not allow continuous year-round non-invasive testing without disturbing the animals. The field faecal collection and sedimentation test from the samples, which could have satisfied the latter requirement, needed to be sufficiently sensitive. It gave reliable results only in individuals infected with at least five adult flukes. Because of this, it can be suggested that by molecular examination of faeces obtained from shot animals with varying degrees of infection, it would be worthwhile to look for genetic markers that indicate the presence of infection, even in case of a lower degree or recent infection. The development of this method went beyond the scope of this work. Still, it is necessary to follow this research direction in the future to determine more precisely the appearance and spread of certain populations of cervids.

Based on the positive experiences of surveillance in the illustrated guide, it is recommended to involve a wider range of game managers in the investigations, even within the framework of further training courses organized by the National Hungarian Hunting Chamber. Based on the direction of spread established by the present study, it is expected that the extent of the endemic area will reach the most important red deer habitats of Zala County within a few years, the consequences of which for wildlife management are difficult to estimate for the time being. The experience gained in Zselic and Inner Somogy, which did not demonstrate significant damage, differs somewhat from the adverse effect on trophy quality observed in Gemenc (Majoros and Stoykov, 1994).

Experience from studies of the interaction between roe deer and the large American liver fluke highlights the importance of genetic testing in host and parasite populations. To assess the resistance of host populations, it is recommended to study the main histocompatibility gene complex (MHC). Identifying host allelic variations that may play a role in developing resistance or resilience to fluke infection could contribute to a better knowledge of the *F. magna* dynamics in endemic or newly invaded areas. In the case of the parasite, identifying bottleneck effects on the population and determining the genetic diversity of populations living in different endemic areas identifiable in Hungary can also be recommended.

The present work did not include the study of intermediate host populations. At the same time, literature data has highlighted the epidemiological risk of habitat change due to climate change and human activity. Slowing water flow due to water management activities on rivers, eutrophication, and invasive plant and snail species may also contribute to the parasite's greater spread and damage (Grabner et al., 2014; Schols et al., 2021). One of the most prominent examples is the snail species *Pseudosuccinea columella*, spreading in Europe (Pointier et al., 2007; Vignoles et al., 2018). The species has significant invasive potential and has been shown to provide particularly favourable conditions for developing *F. magna* in infection experiments (Pankrác et al., 2016; Rondelaud et al., 2014). There is already evidence of its occurrence in Hungary (Vignoles et al., 2018; Varga and Lökkös, 2021). However, the common occurrence of snails and parasites in their natural habitat has not yet been studied in Europe. The mapping of the occurrence and spread of the snail in Hungary could significantly contribute to clarifying epidemiological risk assessment.

Elucidating the optimal environmental requirements of the indigenous intermediate host snail species *G. truncatula* and *R. labiata/peregra* and the reasons for the significant population increase may provide additional data for determining the risk of infection of host species living in a given area.

Post-mortem monitoring of the wild boar populations in the endemic area may also be recommended. Studies carried out in Croatian endemic areas raised the possibility that the epidemiological role of wild boar may change due to modification of host-parasite interaction, thus becoming a definitive host that maintains the infection (Konjevic et al., 2017). Pathological and histopathological examination of wild boar liver samples and genetic testing of mature egg-producing flukes may provide further information on the degree of adaptability of the parasite, a phenomenon that has accompanied the entire evolutionary history of the species (Cshivincsik et al., 2023).

5. NEW SCIENTIFIC RESULTS

1. A view-only surveillance method has been developed to determine the prevalence of the large American liver fluke (*Fascioloides magna*) during cervid evisceration, with a sensitivity of 100% (CI95%: 97.2 – 100%) and a specificity of 96.3% (CI95%: 92.5 – 98.5%) compared to the gold standard autopsy study.

2. We found a weak relationship between the egg excretion of infected hosts and the degree of infection. Thus, the sensitivity of the surveillance method based on faecal sedimentation testing was only 38.5% (CI95%: 30.1 – 47.4%), while its specificity was 100% (CI95%: 98.1 – 100%) compared to the gold standard.

3. In the test area, there is clear evidence that *F. magna* infestation is spreading intensively upstream in the catchment area of the river Drava both westwards and northwards.

4. Viable miracidia were hatched from eggs extracted from the liver of the host species deer (*Capreolus capreolus*), thus proving the deer's potential ability to sustain infection.

5. The survival ability of eggs recovered from the liver of the host species red deer (*Cervus elaphus*) was significantly better than that of deer livers.

6. The ability of *F. magna* to self-fertilize was demonstrated in solitary flukes from red deer and roe deer host species. The viability of eggs resulting from self-fertilization did not show any significant difference from the viability of eggs of flukes living in groups, i.e. presumably sexually reproducing.

6. RELEVANT PUBLICATIONS

PEER-REVIEWED SCIENTIFIC PUBLICATIONS IN THE TOPIC OF THE THESIS (IN FOREIGN LANGUAGE)

Halász, T., Tari, T., Nagy, E., Nagy, G., Csivincsik, Á., 2023. Hatchability of *Fascioloides magna* eggs in cervids. *Pathogens*. 12(5), 741. <https://doi.org/10.3390/pathogens12050741>

Csivincsik, Á., Halász, T., Nagy, G., 2023. The large American liver fluke (*Fascioloides magna*): A survivor's journey through a constantly changing world. *Parasitologia*. 3, 300-326. <https://doi.org/10.3390/parasitologia3040031>

Halász, T., Nagy, G., Tari, T., Csányi, E., Vicze, D., Németh, S., Csivincsik, Á., 2023. Epidemiological tools to assess the spread of *Fascioloides magna*. *Acta Agrar. Kaposvariensis*. 27(1), 27(1-2), 67-76. <https://doi.org/10.31914/aak.3004>

PEER-REVIEWED SCIENTIFIC PUBLICATIONS IN THE TOPIC OF THE THESIS (IN HUNGARIAN)

Halász, T., Nagy, G., Csányi, E., Németh, S., Csivincsik, Á., 2020. Az amerikai májmételey (*Fascioloides magna*) expanziója Délnyugat-Magyarországon – Előzetes közlemény. *Acta Agrar. Kaposváriensis*. 24(2), 27-37. <https://doi.org/10.31914/aak.2444>

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