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Article

Epidemiological tools to assess the spread of *Fascioloides magna*

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ABSTRACT – The giant liver fluke (*Fascioloides magna*) has four continuously expanding focus endemics in Europe. The parasite is considered an invasive species in our continent and could affect the local host populations or, after host switching, it can infect other potential host species. Therefore it is important to track this alien species' presence. The authors compared the gold standard test with the effectiveness of two potential screening tests, the conventional sedimentation method and an illustrated guideline-based approach, which could be applicable in the field. The gold standard test was based on the necropsy detection of adult flukes in the liver tissue of hunted animals (N=319). Besides applying the linear regression, the sensitivity and specificity were determined in both approaches. The analysis showed that the shed egg number was moderately associated with the fluke burden (R^2=0.5679; p<0.0001) and the flukes' dry mass (R^2=0.6016; p<0.0001). The final results of sensitivity (100%; CI95%: 97.2 – 100) and specificity (96.3%; CI95%: 92.5 – 98.5) confirmed that the illustrated guideline-based approach is a capable method for monitoring the *F. magna* expansion in endemic areas.

Keywords: *Fascioloides magna*, giant liver fluke, red deer, expansion, southwestern Hungary

INTRODUCTION

The European distribution of *F. magna* is well known. So far as we know, the presence of two independent phylogenetic lineages is verified, the Italian population and a second one containing the fluke populations of the other endemics, namely the Czech-Poland (CZ-PL) focus and the Danube floodplain forests (DFF). Although the giant liver fluke dispersion is well confirmed in CZ-PL and DFF foci, DFF seems to be the only continuously expanding focus in our continent (*Králová-Hromadová* et al., 2016). The characteristics of these abutting

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habitats provide optimal conditions for intermediate and final hosts of the giant liver fluke as well (*Malcicka*, 2015).

The knowledge about the parasite expansion in Hungary is a bit incomplete because the first studies show the spread as a large-scale process. Studies conducted in DFF suggested the continuous spread of this parasite in the adjacent countries (*Novobilský* et al., 2007; *Florijančić* et al., 2010). The appearance of *F. magna* ensued at the beginning of the 1990s. The worm dispersion was verified after almost 15 years and approximately 300 km farther (*Majoros* and *Sztojkov*, 1994; *Giczi,* 2008). A subsequent study revealed that giant liver fluke reached the River Drava and moved towards the west and north within southwestern Transdanubia (*Nagy* et al. 2018).

The giant liver fluke is considered an invasive species in Europe. The parasite was transported via different deer species at least twice from the American continent (*Králová-Hromadová* et al., 2011). In the indirect life cycle *F. magna*, few hosts can be involved. The definitive hosts are usually deer species in Europe, mainly red deer (*Cervus elaphus*) and fallow deer (*Dama dama*), while the main intermediate hosts belong to the lymnaeid snails (*Malcicka*, 2015). Due to the quick expansion in our continent, the fluke appeared in new habitats, and its spread seems being continuous (*Nagy* et al. 2018). Introducing an emerging parasite into new geographic territories could affect the local host populations or, after host switching, it can infect other potential host species. Any situation can threaten the stability of the concerned host species and cause severe economic losses in wild and domesticated ungulates (*Laaksonen* et al., 2009; *Lymbery* et al., 2014; *Sattmann* et al. 2014). For this reason, it is important to systematically track this alien species' presence and range expansion (*Hulme*, 2014).

The Hungarian hunting regulation does not allow the continuous shooting of red deer. The hunting season lasts from the 1st of September to the end of the next February. Therefore the monitoring, based on necropsies, does not suitable for continuous surveying. For this reason, this study aimed to compare the effectiveness of two different methods, which could be applicable all year round. We compared the conventional sedimentation method and an illustrated guideline-based approach to evaluate the *F. magna* infection in red deer populations living in Hungary's new Transdanubian endemic area.

MATERIAL AND METHODS

Animals

Our study involved the investigation of red deer populations from southwestern Hungary between September 2020 and January 2021. The animals were

shot for hunting purposes, and none of them was killed for accomplishing the research.

Parasitological examination

The participant hunting estates covered about 768 km2 area from Somogy County (*Figure 1*).

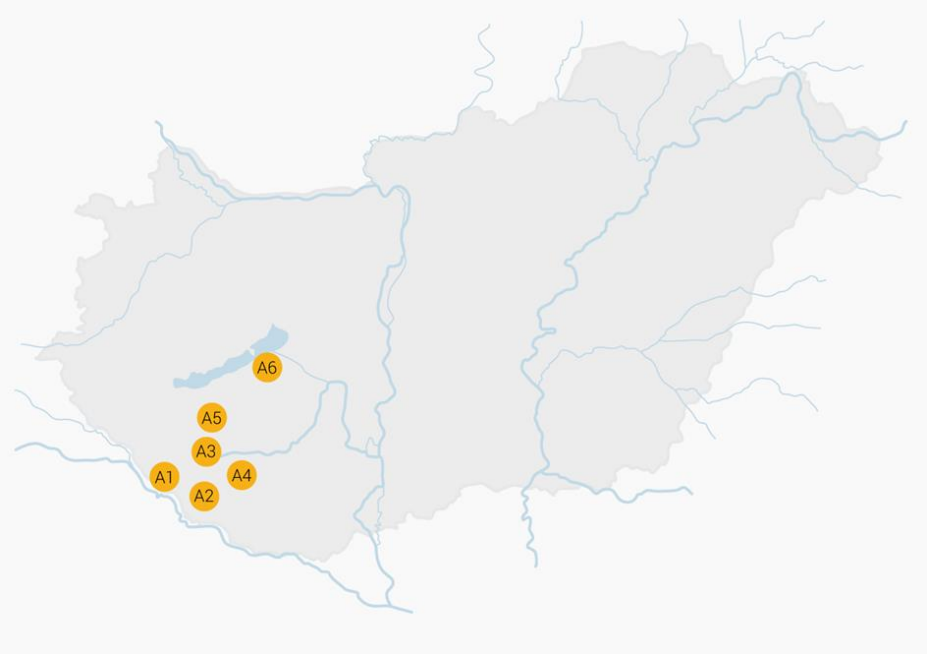


Figure 1. Location of sampling areas of the present study (Yellow circles named A1-A6 indicated the involved hunting areas.)

We involved areas where the worm's presence was not confirmed until the beginning of this study. For this reason, we prepared an illustrated guideline (IG) for hunters to confirm the presence of giant liver fluke infection (*Appendix 1*). Based on IG, after evisceration, the hunters identified the infected animals. Liver and fecal pellets were collected from every animal to confirm the sensitivity and specificity of IG and the conventional sedimentation method. The organs were cut into 1.5 cm slices, and after gentle washing, the collected *F. magna* specimens were counted (FM). Using the fecal material, we performed a sedimentation method to determine the egg number in one gram (EPG) faeces (*Zajac* and *Conboy*, 2012). The gathered worms were dried at 65°C until

constant weight and measured with 0.0001 g accuracy to determine the total dried fluke matter (TDFM).

Statistical analyses

We evaluated the associations between EPG and the other variables, viz. FM and TDFM. Linear regressions were performed using the SPSS statistical software version 27.0 after logarithmic transformation to stabilize the variance. In the case of FM and EPG, we added 1 to each count before taking the log10 transformation (*Alexander*, 2012). Only those deer samples were involved in the linear regression analyses, which were characterized by fluke burden after evaluating the surface of the organs (N=137; 45 stag and 92 hind samples).

The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy (with 95% confidence interval; CI95%) of IG and fecala sedimentation methods were calculated using MedCalc online software version 20.110. (*https://www.medcalc.org/calc/diagnostic_test.php*). Liver fluke detection by necropsy was considered a gold standard test. For determining true positives (TP), false positives (FP), true negatives (TN), and false negatives (FN), we followed the general epidemiological rules by *Trevethan* (2017). The method was presented in *Table 1*.

Table 1
Determination of true positives (TP), false positives (FP), true negatives (TN), and false negatives (FN)

	Gold standard test (necropsy) results	
	+	**-**
New screening test +	TP	FP
New screening test -	FN	TN

Sensitivity (SENS), specificity (SPEC), positive predictive value (PPV), and negative predictive value (NPV) were calculated by the following formulas (*Trevethan*, 2017):

$$SENS(\%) = \frac{TP}{TP + FN} \times 100$$

$$SPEC(\%) = \frac{TN}{TN + FP} \times 100$$

$$PPV(\%) = \frac{TP}{TP + FP} \times 100$$

$$NPV(\%) = \frac{TN}{TN + FN} \times 100$$

The test accuracy (ACC) viz overall probability that a new screening test result correctly demonstrates the gold standard test result was also calculated by the MedCalc online software version 20.110. as follows:

$$ACC(\%) = SENS \times prevalence + SPEC \times (1 - prevalence)$$

RESULTS

The regression analysis showed that the EPG was associated with both other variables, the FM and TDFM. The most definite relationship was observed between TDFM and EPG (R^2=0.6016; p<0.0001), while fluke burden showed a moderate connection with EPG (R^2=0.5722; p<0.0001) (*Figure 2*).

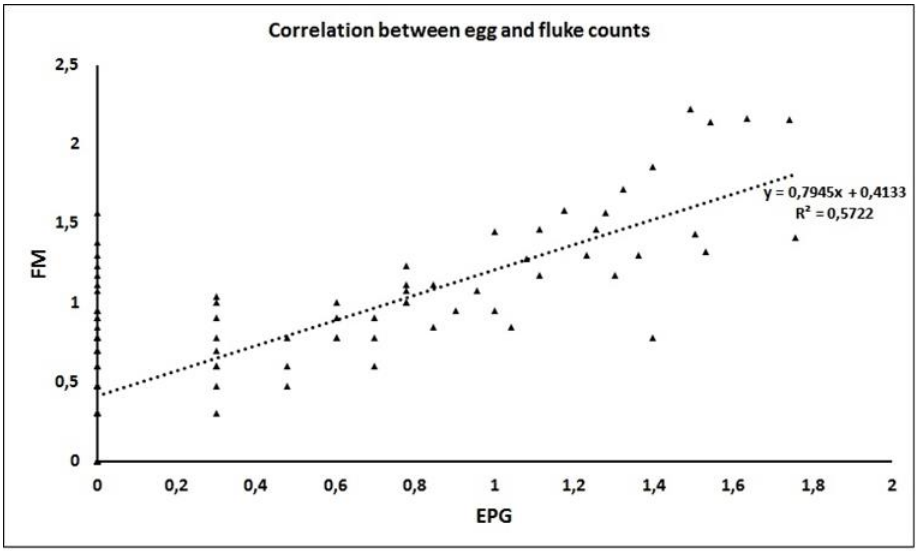
Using the IG, hunters confirmed the fluke presence in 137 animals, but after necropsies, flukes were collected only from 130 organs. We found eggs in 50 fecal materials from the 137 IG-confirmed animals during the sedimentation. The true and false positive and negative values used for sensitivity and specificity calculation showed in *Table 2.* The sensitivity, specificity and characteristics differed between the two methods (*Table 3*).

Table 2
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

Table 3.
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)



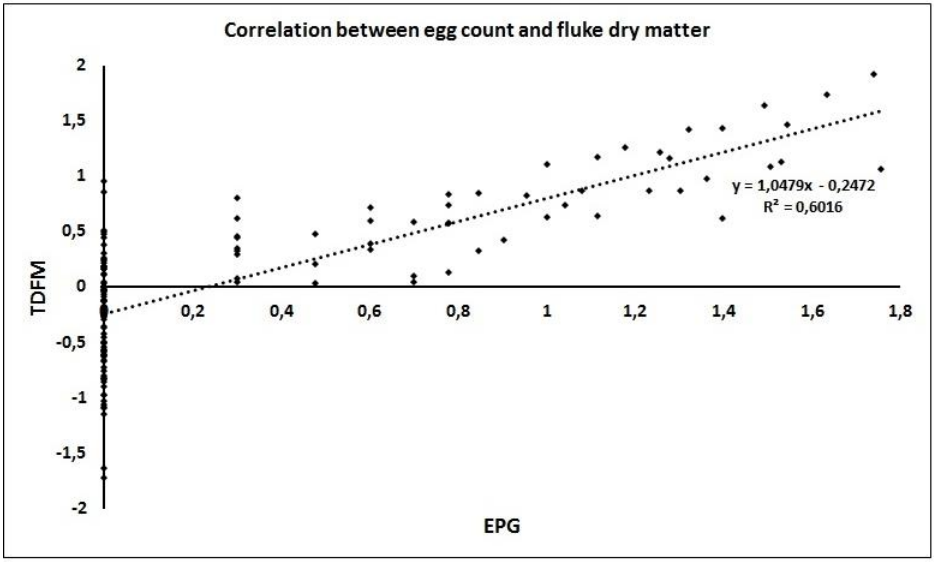


Figure 2. Linear regressions between egg count (EPG), fluke count (FM) and total dried fluke matter (TDFM).

DISCUSSION

The study aimed to develop a simple, cost-effective field method for monitoring the occurrence and spread of *F. magna* at the borders of an endemic area. For this reason, we compared two new screening methods with the gold standard, necropsy detection of adult *F. magna* parasites in the liver tissue. The first screening method was based on a photo-illustrated guideline, which was provided to the hunters who worked in the study area. The hunters evaluated all hunted animals by comparing the livers to photos of characteristic lesions caused by *F. magna* infection.

The second method was developed by fecal egg count determination in all hunter-evaluated and necropsied carcasses. By this double comparison, we could investigate whether visual-only evaluation by hunting personnel or counting fecal egg numbers could be a more efficient method for investigating epidemiological trends of *F. magna* on a hot spot.

In comparison to the gold standard, screening by hunting personnel proved to have a high sensitivity; whereas fecal egg number counting had high specificity and a very weak sensitivity. The diagrams of *Figure 1* explained this phenomenon as few flukes and/or a small amount of fluke dry matter can produce very different numbers of eggs, indeed no eggs at all. In the case of a more severe infection, the fecal egg count can represent the scale of infection moderately. Despite the fair average correlation, field-collected fecal samples cannot provide appropriate information about the epidemiological situation. Especially on the borders of an endemic area where plenty of animals should be mildly infected.

Visual-only evaluation of the eviscerated livers resulted in 100% sensitivity and 96.3% specificity. The hunting personnel claimed 137 liver samples to be infected following the photo-illustrated guideline. Though only 130 of the samples proved to be positive by necropsy detection of adult flukes. The difference originated in the gold standard test chosen for comparison. In the early stages of infection, characteristic lesions can confirm the presence of *F. magna*; though adult worms cannot be detected. In these cases, hunters seemed to evaluate the samples falsely positive. If we had chosen molecular confirmation of *F. magna* DNA in the livers as the gold standard test, false positives might have proved true positives. In the future, it is worth investigating how the initial lesions are characteristic for F. magna and how different they are from those caused by *Fasciola hepatica*.

Positive and negative predictive values (PPV & NPV) could better represent the two tests' usefulness. *Table 3* demonstrated that in IG test, the probability

of true positive results (PPV) was 94.9%; while for negative results, this value proved 100% (NPV). On the other hand, PPV and NPV in the sedimentation test were 100% and 70.3%, respectively. This means that in the IG test, both the positive and negative results appropriately showed the true condition of the investigated samples. In the case of the sedimentation test, the positive results were correct, while the negative results would have needed additional tests to confirm the disease-free statement. This phenomenon could be demonstrated by the accuracy of the two investigated screening tests, as IG test showed much better accuracy than the sedimentation test, 97.8%, and 74.9%, respectively.

Comparing the two potential screening tests, hunters' evaluation of eviscerated livers (IG) proved appropriate for monitoring the epidemiological trends of *F. magna* within and on the borders of an endemic area. Though we attempted to develop a method that can be applied outside of the hunting seasons, fecal egg count, which can be measured in field-collected samples, could not fulfill the expectations. On the other hand, the photo-illustrated guideline is a very simple tool to involve hunters in the monitoring activity. This method has some disadvantages. It cannot run all year round and it needs contributors who are trained in neither parasitology nor pathology. This monitoring requires close cooperation between the stakeholders. Before the initiation of the fieldwork, all contributing hunters must go through practical training on the use of the photo-illustrated guideline and theoretical training on the importance of *F. magna* infection in deer. Without the recognition of *F. magna* as potential harm to the managed deer population, any efforts to teach monitoring skills will remain useless.

In Hungary, the endemic area of *F. magna* is continuously extending. The epidemiological role of the River Danube (*Králová-Hromadová* et al. 2016) and the River Drava is known (*Nagy* et al., 2018). On the other hand, the northward spread in Transdanubia needs ongoing monitoring. This activity should focus on the frontlines, the northern borders of the endemic area where newly infected animals are expected to be found. In these conditions, those methods could be suitable to detect the early stages of the infection. The hunters' visual-only evaluation of eviscerated livers is a very sensitive and specific method of monitoring even initially infected populations. Unfortunately, this method can be carried out exclusively during the hunting season in cooperation with non-professional stakeholders. For this reason, it needs strict organization and interdependent collaboration between the parasitologist and the hunting personnel.

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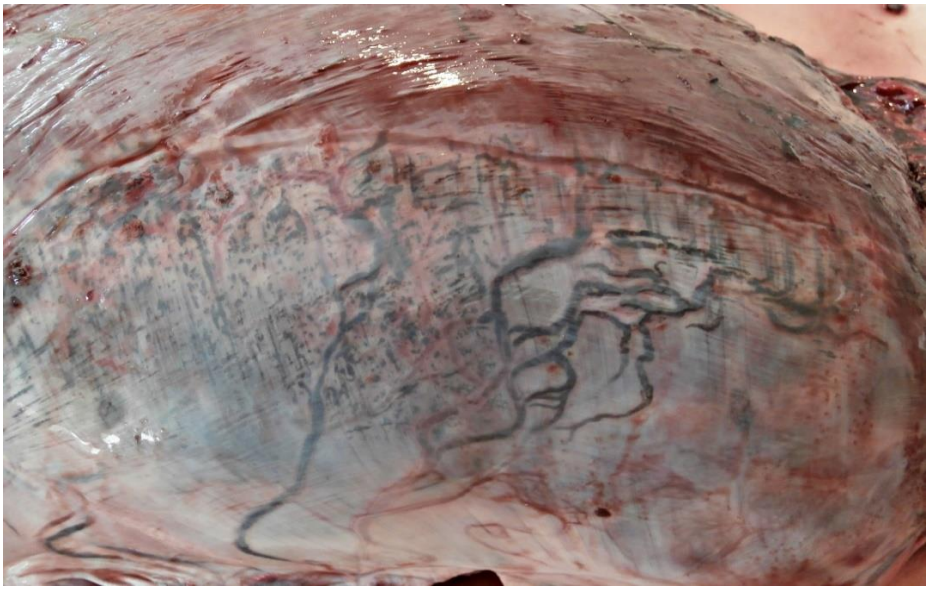
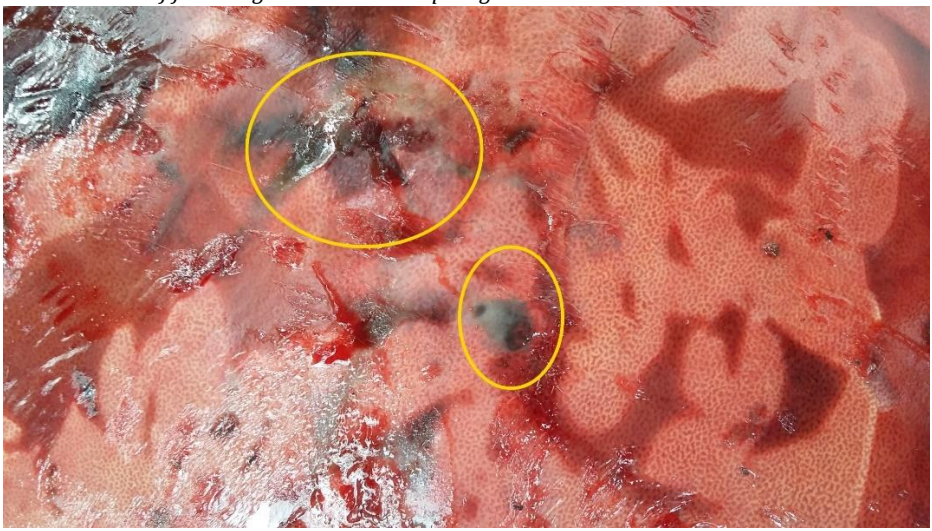
Appendix

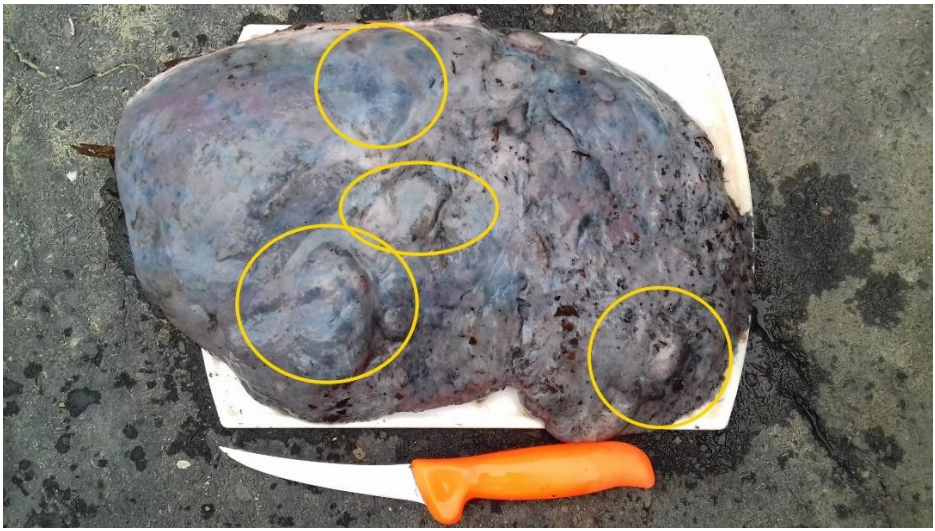


Mételyek vándorlásának nyoma a rekeszizom ín lemezében.
Characteristic of fluke migration on the diaphragma.

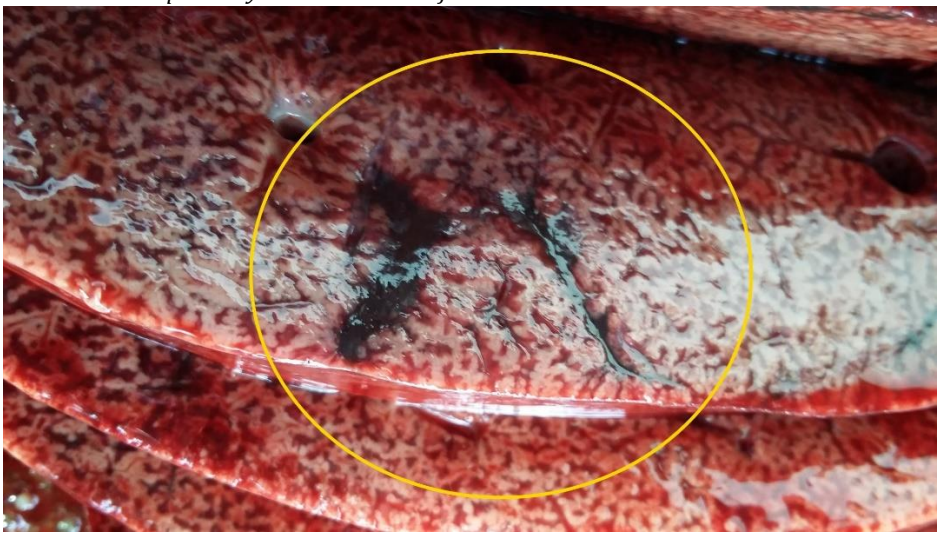


Mételyek vándorlásának nyoma a máj felszínén.

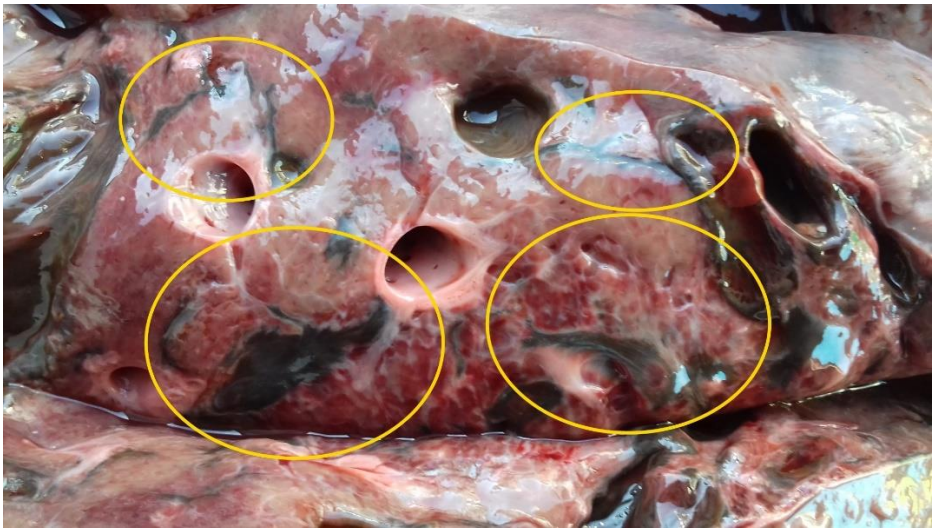
Characteristic of fluke migration ont he liver surface.



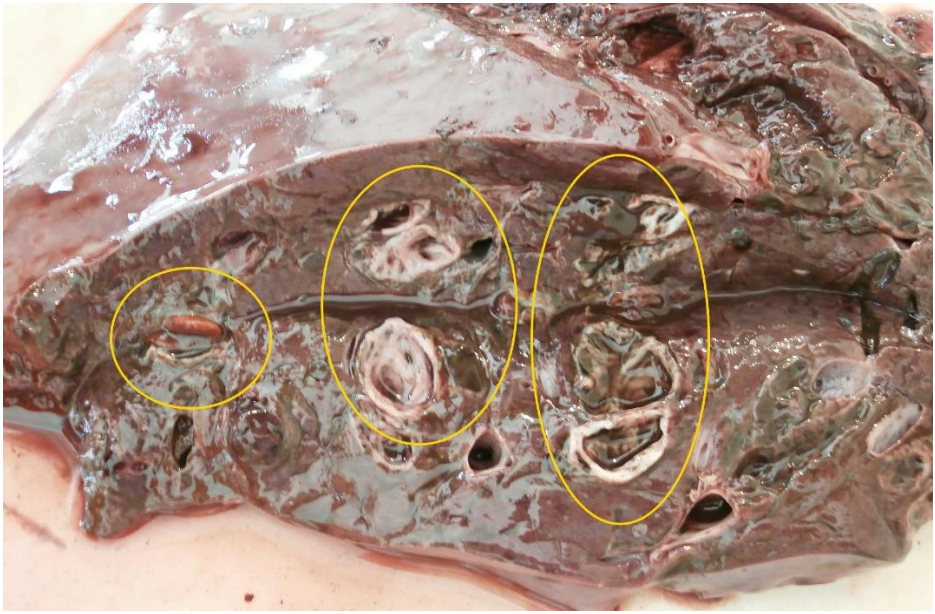
Mételyeket tartalmazó pszeudociszták a máf felszínén.
Fluke contained pseudocysts on the liver surface.



Métely vándorlás nyoma a máj metszéslapjá.
Characteristic of fluke migration on cutting surface of the liver.



Métely vándorlás nyoma a máj metszéslapjá.
Characteristic of fluke migration on cutting surface of the liver.



Pszeudociszták a máj metszéslapján.
Pseudocysts on cutting surface of the liver.

Kifejlett amerikai májmételyek (*F. magna*) és közönséges májmétely (*Fasciola hepatica*).
Adult giant liver flukes (F. magna) and common liver fluke (Fasciola hepatica)