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Experimental infection with *T. canis* and *T. leonina* in farm mink (*Neovison vison*)

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Abstract

Introduction: Farm mink (*Neovison vison*) can be naturally exposed to *T. canis* and *T. leonina* pathogens on the farm. If mink were hosts, it would imply some veterinary public health as well as animal welfare issues. For this reason, the aim of the study was to determine whether mink might be definitive or paratenic hosts of these parasites. **Material and Methods:** Four groups of mink were infected with both parasite species using larvated eggs or feed containing mouse tissue previously infected with the parasites. Following inoculation, the infections were monitored *in vivo* by faecal examination for 14 weeks p.i., and then western blotting and ELISA were performed. **Results:** Coprology did not reveal any canine roundworm eggs, neither were nematodes found in mink intestines during *post mortem* examination. The specific IgG antibodies recognising excretory/secretory (ES) antigens of both parasite species were identified in mink sera. Single *T. leonina* tissue larvae were found in digested organs. **Conclusions:** Our results confirm that farm mink may contribute both *T. canis* and *T. leonina* infections. It was proved that farm mink were not their definitive hosts, and therefore mink faeces need not be considered a source of canine roundworm eggs in any soil it fertilises. Nonetheless, as farm mink may be a paratenic host for both parasite species, this may have some impact on the health and welfare of infected animals.

Keywords: farm mink (*Neovison vison*), *Toxocara canis*, *Toxascaris leonina*, definitive/paratenic host.

Introduction

There are several concerns about welfare and veterinary public health issues in farm mink. One of these is a possible link between mink and human toxocarosis. As long as human toxocarosis still remains a serious problem from a veterinary public health point of view, it implies that continuous attempts must be made to control the infection, especially in pet carnivores (19).

The infection in humans as paratenic hosts is mainly due to the oral-faecal route by contaminated soil or vegetation. The eggs of *T. canis* and *T. leonina* can remain infective (under optimal conditions) for years outside in soil, solely due to a resistant outer shell composed of ascarosides. That acellular layer enables canine roundworm eggs to be resistant to various harsh chemicals, extreme temperature changes, and various degrees of moisture (2).

The mink industry in Poland has been intensively modernised and developed for the last two decades and a lot of attempts are being made to enhance the welfare of farm mink. There are currently around 8.5 million mink furs from Poland sold on the international market (18). It means that the breeding population of female mink is about 2.1 million. It results in large amounts of mink manure, which must be appropriately disposed of or utilised. The amount of mink manure (calculated using different methods) varies from over 100 to 238 thousand tons yearly (10). The composition of mink

manure is similar to some nitrogen-based artificial fertilisers. For that reason it was recognised as a valuable source of organic matter, and became widely used to fertilise soil in fields for crop cultivation as well as in small gardens (10). It is approved in Poland as category 2 byproduct – an organic (natural) fertiliser for agriculture. Sometimes it is also used as a raw material for biogas (4). Due to its special beneficial content dehydrated mink manure was even experimentally used as a protein source for growing pigs (24).

Previously there was no data concerning *T. canis* and *T. leonina* development in farm or wild mink. Subsequently, the question has arisen whether the mink could have a definitive host role for these nematodes, and if the manure could be possibly a source of toxocarosis. It was assumed that doubts ought to be definitively elucidated concerning safety of the farm mink manure usage in agriculture. Seen from that perspective mink manure would indirectly provide a potential risk factor for humans particularly involved in agriculture or gardening.

Therefore, the aim of the study was to experimentally infect farm mink with *T. canis* and *T. leonina* and resolve doubts concerning possible involvement of these animals in the epidemiology of these parasites. If mink were found to be the definitive or a paratenic host of both canine parasites, the course of possible pathological lesions should be determined in order to maintain health and welfare standards in farm mink.

Material and Methods

Animals – farm mink (*Neovison vison*, formerly known as *Mustela vison*). A total of 50 mink originating from the same farm were used in the study. Prior to the experiment the mink were preventively vaccinated against infectious diseases according to the routine protocol used on the farm (Febrivac DIST and Febrivac 3+, Impfstoffwerk Dessau-Tornau GmbH). Next, animals were transferred and kept in special facilities for mink (wire cages) on the Obory farm of Warsaw University of Life Sciences. Mink were randomly divided into four experimental groups (G1–G4; n = 10) and one control group (n = 10). Each group contained an equal number of females and males (5/5). Mink were kept individually in boxes, given commercial feed at a standard dose, and *ad libitum* drinking water was available. Prior to the start of the experiment faeces of all mink were individually collected and examined with the flotation and McMaster methods to exclude possible helminth infections. During the study mink were regularly inspected and no specific symptoms were noticed.

Preparation of the infective material. Two methods of mink infection were applied in the study – an oral inoculation of larvated eggs, and ingestion of tissue larvae present in roundworm-infected mice.

Isolation and cultivation of roundworm eggs. Live adult *T. canis* and *T. leonina* were isolated from naturally infected dogs. Eggs were obtained from uteri excised from gravid females. Then eggs were cultured in 0.1 N H_2SO_4 (for 3 weeks) for embryonation at room temperature (RT) according to Oakes and Kayes (25). Fully embryonated eggs (containing L3 larvae) were cleaned several times in 0.9% NaCl by suspension and centrifugation (300×*g*, 10 min). Finally, infective doses were prepared to infect mice in order to obtain tissue larvae and later to infect mink directly. Each infective dose contained approximately 500 eggs in 200 µL of saline (0.9% NaCl), collected by pipetting under microscope control.

Infection of the BALB/c mouse paratenic host. BALB/c mice (n = 20) were orally infected with a single infective dose of approximately 500 *T. canis* (n = 10) and *T. leonina* eggs (n = 10). Three weeks post infection the mice were euthanised with CO_2. Serum samples were collected and mouse bodies were skinned and minced. Homogenised tissues of mice were pooled, mixed, and equal portions of approximately 25–30 g were prepared to infect mink. In order to confirm that roundworm infection developed in infected mice, western blotting using excretory/secretory (ES) antigens and mouse sera was performed prior to mink infection.

Infection of mink. Mink feeding was restricted for 24 h prior to the infection. All infective doses (eggs or tissues) were placed in small pieces of the commercial feed and given orally to mink. All mink ingested their infective portions by themselves. Control mink were fed the commercial feed only.

Table 1. The schedule of mink infection

Group of mink	Number of individuals	Means of infection – portion of feed containing:
G1	10	*T. canis* – larvated eggs in saline
G2	10	*T. canis* – L3s in homogenised tissues of paratenic host
G3	10	*T. leonina* – larvated eggs in saline
G4	9*	*T. leonina* – L3s in homogenised tissues of paratenic host
Control	10	not infected

*one mink died before inoculation

Faecal examinations. Faecal samples were regularly collected at 2-week intervals beginning from the 4th, and continuing until the 14th weeks post infection. Faeces were taken from the bottom of the boxes, put into plastic containers and kept at 4°C. Each sample was examined with the flotation method with super-saturated NaCl according to Fülleborn, as modified by Willis and Schlaff (11). Subsequently, all samples were tested using the McMaster method (11).

Parasitological autopsy of mink. Mink from all groups were euthanatized with CO_2 at 16 weeks post infection. Following euthanasia serum samples were collected and all mink were autopsied. As a first step, the removed gastrointestinal tracts were examined to detect worms within the lumen. Then samples of parenchymal organs (intestinal lymph nodes, kidneys, liver, lungs, small intestines, and spleen) and samples of striated muscle (heart, skeletal and tongue) were collected and stored at −20°C for further larval investigation.

Detection of larvae in mink tissues. All samples were processed according to the procedure stipulated in Commission Regulation No.: 2075/2005 for detection of *Trichinella spiralis* in meat, with the authors' own modifications. After 10 weeks of storage, frozen tissues (approximately 30 g of parenchymal organs and skeletal muscle) of each mink were thawed prior to the examination and homogenised with a meat blender. Then mink tissue samples were separately digested in 0.2% HCl with pepsin 2000 FIP/g (10 g/L), at 44–48°C for 30 min on a magnetic stirrer. Afterwards, the mixture was left for 30 min for sedimentation and 50 mL of the sediment was collected into Falcon tubes. After the next 30 min 40 mL of the supernatant was discarded and the remaining sediment was placed on plastic dishes and examined carefully using an SMZ-10A binocular stereoscope (Nikon, Japan). Pictures of tissue larvae were taken and stored with NIS-Elements D 3.00 SP7 software (Laboratory Imaging, Czechia).

Preparation of *T. canis* and *T. leonina* ES antigens. Roundworm ES antigens were collected according to the method previously described by Długosz *et al.* (3). Briefly, the suspension of hatched larvae in Minimal Essential Medium (MEM, Sigma-Aldrich, Poland) supplemented with penicillin, streptomycin, and amphotericin B (100 U/mL, 100 µg/mL, and 2.5 µg/mL, respectively) was placed in a Baermann apparatus and incubated for 24 h at 37ºC with 5% CO_2 atmosphere. Viable larvae were collected and cultured *in vitro*. MEM was aseptically collected every three days and replaced with a fresh portion of sterile medium. The spent medium was pooled, dialysed into PBS buffer and concentrated in Amicon Ultra centrifugal filters (Merck Millipore, Ireland). The antigen concentration was measured using a BCA Protein Assay Kit (Pierce, USA).

Western blotting. *T. canis* and *T. leonina* ES antigens (7µg/well) were separated on 12.5% polyacrylamide gels. Then samples were electrotransferred onto a nitrocellulose membrane. Membranes were blocked for 1.5 h in 5% skimmed milk in PBS buffer at r.t. Next, membranes were incubated for 18 h at 4°C with 1:200 dilution of pooled mink sera or 1:500 dilution of pooled mouse sera. After washing, blots were incubated (1 h, at r.t.) with rabbit anti-mink IgG HRP conjugated antibodies (1:1000) (Biorbyt Ltd., U.K.) or rabbit anti-mouse IgG HRP conjugated antibodies (1:4,000) (Sigma-Aldrich, Poland). Membranes were developed with SuperSignal West Pico Luminol/Enhancer Solution (Pierce) on Kodak films.

ELISA. Plates were coated with *T. canis* or *T. leonina* ES antigens (2.5 µg/mL) in carbonate buffer, pH 9.6, for 12 h. Then plates were washed with PBS/0.05%Tween (PBS-T) buffer and blocked with 5% skimmed milk in PBS-T. After washing, plates were incubated with mink sera (1:400) for 1 h at r.t. Plates were again washed and incubated with rabbit anti-mink IgG HRP conjugated antibodies (1:5,000) (Biorbyt Ltd.) for 1 h at RT. Following washing, plates were developed with TMB Substrate (Sigma-Aldrich, Poland) and read in an MRX plate reader (Dynatech Laboratories, U.K.) at 450 nm. Cut-off values were calculated as the mean OD from the control group +3×SD. The statistical significance of differences between groups was determined with a non-parametric Mann-Whitney test, and a value of $P < 0.05$ was considered to be significant. The analysis was carried out using Statgraphics Plus 4.1 software (Statgraphics Technologies, USA).

Results

Faecal examinations. There were no parasitic worm eggs found in examined faeces. Subsequently, the autopsies revealed no pre-adult or adult nematodes nor other parasitic worms present within the lumen of mink gastrointestinal tracts. However it was noticed that some mink had a mild intestinal coccidia infection at the beginning of and during the study. The infection in particular individuals ranged from 100 to 26,800 oocysts per 1 g of faeces.

Post mortem examination. The autopsies of mink from all the experimental groups did not show any gross lesions which could be considered directly related to the roundworm infections. Autopsies were also performed on the bodies of mink which died before the end of the study (two mink from group G2 died in the 10th and 14th weeks p.i., and one mink from group G4, died in the 10th week p.i.). Necropsy revealed cardio-pulmonary insufficiency.

Tissue digestion. Only single *T. leonina* larvae were found during tissue digestion (Table 2 and Figs 1 and 2). No *T. canis* larvae were noted in examined tissue samples originating from animals of groups G1 and G2.

Western blotting. The test confirmed the presence of *T. canis*- and *T. leonina*-specific IgG antibodies in sera from the infected mice which served as a source for tissue L3 larvae for mink infection (Fig. 3). Roundworm-specific IgG were also noted in sera of mink from all infected groups (Fig. 3). There were no bands on blots incubated with mouse- and mink-negative sera (data not shown).

ELISA. All infected mink developed IgG antibodies specific to larval ES products (Fig. 4), with

the highest antibody levels observed in mink infected with *T. canis* eggs. The infection by paratenic host tissue ingestion resulted in a lower antibody level. OD values differed significantly between the egg-infected and paratenic–host-infected groups of mink.

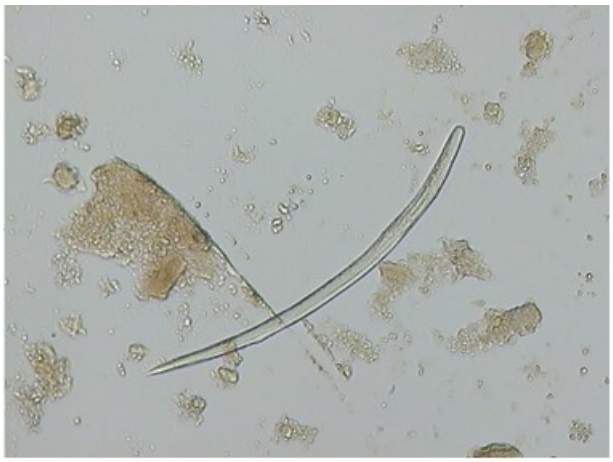

Fig. 1. *T. leonina* larva isolated from digested tissues of mink infected with eggs (G3)

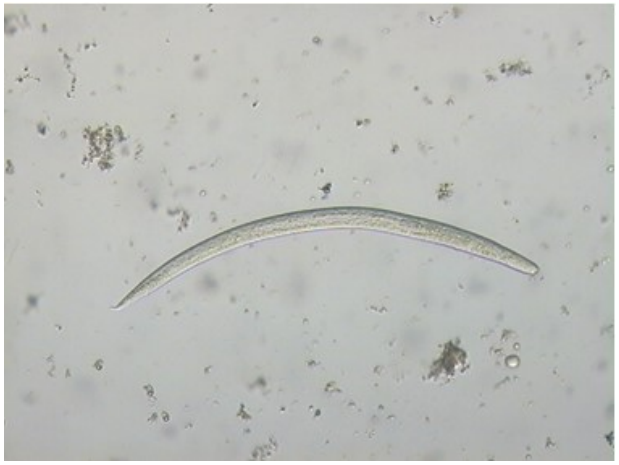

Fig. 2. *T. leonina* larva isolated from digested tissues of mink infected with tissue larvae (G4)

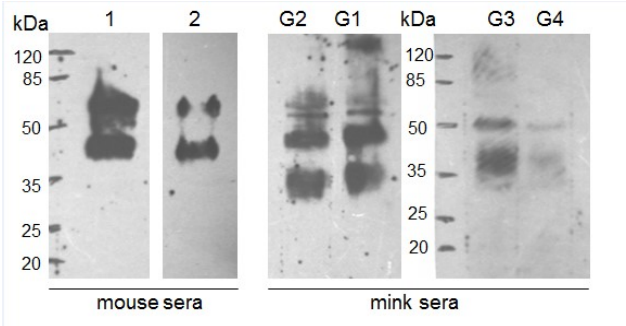

Fig. 3. Western blotting analysis of *T. canis*- and *T. leonina*-specific IgG antibodies in pooled sera from: 1 – mice infected with *T. canis*; 2 – mice infected with *T. leonina*; G1 – mink infected with *T. canis* eggs; G2 – mink infected with *T. canis* larvae in mouse tissue; G3 – mink infected with *T. leonina* eggs; G4 – mink infected with *T. leonina* larvae in mouse tissue

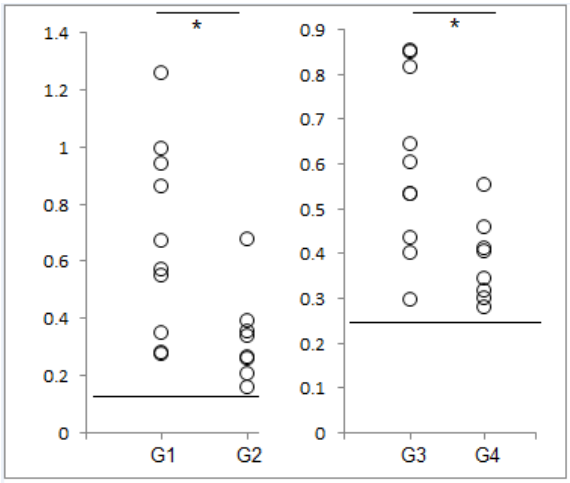

Fig. 4. IgG antibody response of mink to *T. canis* and *T. leonina* ES antigens determined by ELISA. Circles represent OD values from individual animals. Cut-off values were calculated as mean OD value from the control group +3×SD (marked as horizontal line). Statistically significant differences are marked with an asterisk ($P < 0.05$)

Table 2. Summary results of investigations of canine roundworm infections in mink

Group of mink	Eggs in faeces	Worms in the gastro-intestinal tract	Larvae in tissues (number of individuals)	Parasite-specific IgG in sera
G1	0	0	0/10	positive
G2	0	0	0/10	positive
G3	0	0	4/10	positive
G4	0	0	1/9	positive
Control	0	0	0/10	negative

Discussion

The problems associated with toxocarosis caused by *T. canis* and *T. leonina* are widely studied. At their centre is recognition of the crucial points of the epidemiological aspects of the infection as reviewed by Overgaauw (29). More recently the same author described veterinary and public health issues of pet toxocarosis (30). It is generally accepted that ingestion of infective eggs, unwashed hands, and raw vegetables are the main sources of the infection in humans. Studies reviewed by Kłapeć and Stroczyńska-Sikorska (17) revealed that soil contamination with *Toxocara* eggs in rural areas in different regions of Poland varied from 5% to 38.2% of samples. Gawor *et al.* (7), summarizing their investigations, suggested that a high risk of human toxocarosis exists due to soil contamination with *Toxocara* eggs in both urban public sites and in rural settlements (especially in the Mazowieckie Province). In addition, Gawor and Marczyńska (8) reported the results of a study on the level of awareness of the risk of zoonotic parasite infections in rural communities. They showed a general lack of knowledge, particularly of preventive measures against pet–carnivore-originated toxocarosis. Finally, Mizgajska-Wiktor *et al.*

(23) summarized 20 years of soil contamination studies and showed that an average of 14.9% of samples contained roundworm eggs. The authors indicated that egg distribution in rural areas was generally lower than in urban ones.

Thus, many authors recognised direct excretion of *Toxocara* spp. eggs by infected pets into the human environment as a main risk factor of human toxocarosis (29). In consequence, these facts allayed some doubts concerning the presumed risk of toxocarosis transmission from pets *via* the paratenic host (a mouse) to farm mink and finally to humans consuming vegetables cultivated on fields fertilised with mink manure. The direct involvement of small rodents in the environmental transmission of *Toxocara* spp. and *T. leonina* was elaborately reviewed by Okulewicz *et al.* (28). Similarly, such a route of nematode transmission to farm mink *via* a rodent host had supposedly been involved in a case of trichinosis in farm mink in Estonia. *Trichinella* infections were also detected in blue foxes and brown rats living near a fur farm (22). The American mink (*Neovison vison*) as an invasive species living free in the natural environment was also recognised as a sylvatic reservoir for *Trichinella* spp. in Poland. Those authors proved that American mink had become involved in the circulation of trichinellosis (13). The role of small rodents in transmission of toxocarosis in enclosed spaces was also underlined by Okulewicz (27), who reported that small rodents as paratenic hosts play the major role in the transmission of toxocarosis in zoological gardens. That partially reflects conditions found on the mink farm.

Regarding nematodes potentially dangerous to humans in Poland, these were found in wild carnivores closely related to mink living in the Białowieża Forest (9). A cat roundworm (*Toxocara cati* (*T. mystax*)) was found in the gut of a marten (*Martes martes*). Authors also reported a hookworm (*Uncinaria stenocephala*) infection in European polecats (*Mustela putorius*). Other researchers (31) did not report mink as a host for roundworms in Poland. No roundworms were found in wild mink in studies performed in the USA (6) or France (35). Conversely, in Poland such canine roundworms as *T. canis* and *T. leonina* were regularly found in other farmed fur animals such as red foxes (1, 12, 21), and generally in other wild canids living in the country (31). The infections caused by these parasites were found in raccoons and red foxes in north-eastern Poland with prevalences of 15.1% and 33.3%, respectively (15). Hookworm infections of both host species were also reported in this study. Additionally, the American mink was identified as a competent host for some native European parasites. Amongst other parasites six nematode species were found in wild-living mink which were known to be native to European mustelids (20). These data suggest that in some conditions, exposed mink might become infected with a range of local parasite species.

The presented study was performed to investigate *T. canis* and *T. leonina* infections in farm mink and the possibility of mink becoming the definitive host for these parasites. For this reason the fourth week post infection was chosen to start coprological tests. Presumably, it would have been the shortest possible prepatent time of infection, because it is known to be in canine roundworm life cycles. Faecal examinations performed during the experiment did not reveal any roundworm eggs. Furthermore, post mortem examination of the gastro-intestinal tracts of mink showed that there was neither any pre-adult or adult *T. canis* nor *T. leonina*. This proves that these nematodes were not able to complete their life cycles in the mink host.

Our findings proved that both parasite species were able to infect and develop in farm mink as the paratenic host. First, we found single *T. leonina* migrating larvae in digested tissue of parenchymal organs and skeletal muscle of infected mink. Secondly, all infected animals developed a specific IgG response to parasite ES products.

Serological diagnostic tests such as ELISA and western blotting are considered adequate methods to confirm toxocarosis in a paratenic host, including humans. ELISA detecting IgG antibodies against *Toxocara* ES antigens has become the reference test for toxocarosis immunodiagnosis. These tests show high sensitivity and specificity (5). Several antigens of about 24, 28, 30, 35, 56, 67, 117, 136, and 152 kDa are recognised by antibodies present in infected human sera (32). Similarly, an immunoblot profile of *T. canis* ES antigens with hyperimmune serum raised in rabbits, which are also paratenic hosts, shows a similar pattern: 21, 25, 30, 37, 45, 50, 57, 69, 77, and 105 kDa (34).

The antigens recognised during our study are in line with these results. IgG antibodies in sera from infected mice showed molecular masses of about 45 and 70 kDa. Sera from mink infected with *T. canis* or *T. leonina* recognised antigens of about 33, 45, 50, and 100 kDa, and sera from mink infected with *T. canis* additionally recognised an antigen with a molecular mass over 120 kDa. Such high molecular mass antigens were also recognised by antibodies from *T. canis* infected mice, rabbits, and humans (25).

Levels of serum IgG-specific to *T. canis* and *T. leonina* ES antigens were significantly higher in all experimental mink than in control uninfected animals. A similar level of roundworm-specific antibodies in mink infected with *T. canis* and *T. leonina* eggs was observed. Moreover, it was also noted that IgG response differed significantly amongst groups infected with larvated eggs and tissue larvae. It is very likely that a more enhanced antibody response reflects a stronger host reaction to a more massive invasion caused by the ingestion of parasite eggs. One could assume that the infection elicited by tissue larvae was less intensive as some roundworm larvae could have been terminated by the immune system of the paratenic

host (mouse), limiting the final infective dose for the mink.

In our study only single *T. leonina* larvae were found in digested tissues of parenchymal organs and striated muscles of mink. As a mixture of organ tissues of individual mink was examined, it was not possible to determine from which of these organs the larvae actually originated. Kang *et al.* (14) noticed that *T. leonina* larvae were able to spread all over the body of mice used as an experimental paratenic host model. In that study larvae were found in various organs such as brain, heart, intestine, liver, lungs, muscle, and spleen. It is known however that *Toxocara* larvae show different migration patterns depending on the particular paratenic host species (33).

Unfortunately, in our experiment no *T. canis* larvae were found in digested tissues. This could possibly be explained by the limited size of the mixed tissue samples - larvae could have been present in other parts of examined parenchymal organs or skeletal muscle.

Our results confirm that farm mink can become paratenic hosts of canine roundworm parasites after ingesting not only larvated eggs, but also infected mouse tissue. If such a situation occurred on farm (for example during feed preparation), mink could potentially develop an infection resulting in a set of quite complex clinical symptoms of toxocarosis. That would definitely have some impact on the welfare and health status of farm mink. The establishment of *T. canis* larvae in particular organs of a paratenic host may provoke local inflammation processes widely known in human medicine as ocular larva migrans and visceral larva migrans syndromes (16). Therefore our results support the necessity of controlling small rodents on mink farms and auditing and reporting this according to the Veterinary Inspection regulations.

The results of our study conclusively confirmed infection of canine roundworms in farm mink, together with a specific IgG antibody response to both larval (*T. canis* and *T. leonina*) ES products in all experimental mink. It was also noted that both parasite species were not able to complete their life cycles within the mink as the definitive host. Thus, farm mink may only be a paratenic host and not the definitive host for these parasites of carnivores.

Therefore, it can be generally concluded that farm mink are not involved in the epidemiology of these zoonotic infections and their manure and tissue do not play any role in transmission of the toxocarosis to humans. Consequently, results of the study also imply that farm mink faeces need not be considered a source of canine roundworms eggs in soil fertilised with mink manure.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The project was approved by the 3rd Local Ethical Committee for the Experiments on Animals at Warsaw University of Life Sciences.

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