IJP: Parasites and Wildlife 12 (2020) 176–180

Contents lists available at ScienceDirect

IJP: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Trichinella britovi infection and muscle distribution in free-living martens (*Martes* spp.) from the Głęboki Bród Forest District, Poland

Aleksandra Cybulska*, Aleksandra Kornacka, Rafał Skopek, Bożena Moskwa

Witold Stefański Institute of Parasitology, Polish Academy of Sciences, 00-818, Warsaw, Twarda 51/55, Poland

ARTICLE INFO

Keywords:
Trichinella britovi
Wildlife
Martens
Martes spp.
Predilection sites
Maintenance

ABSTRACT

Trichinella nematodes occur in many carnivorous and omnivorous animal species in the sylvatic cycle. Due to their widespread occurrence throughout Poland and diet, free-living Mustelids can act as a potential reservoir for nematodes of the genus *Trichinella* and play a role in their circulation. The study was designed to determine the presence and predilection sites for *Trichinella* nematodes in martens (*Martes* spp.) from the Głęboki Bród Forest District, Poland.

Trichinella britovi larvae were detected by molecular methods in 17.54% examined martens (prevalence: 41.67% among pine martens and 13.88% among *Martes* spp.). The intensity of infection varied from 0.17 to 37.29 larvae per gram (LPG) (mean 5.43; median 3.4). The highest larval burdens were detected in the tongue in pine martens (*Martes martes*) and the diaphragm in *Martes* spp., respectively; the lowest levels were found in the masseter in pine martens and the tongue in *Martes* spp. No statistically significant difference in the intensity of infection was observed between males and females in either group.

Our findings indicate that *T. britovi* is present in martens from the Głęboki Bród Forest District, and the predilection sites for the nematode may differ between males and females. However, due to the low number of examined animals, further studies are necessary to confirm whether they are an important element in the maintenance of *Trichinella* nematodes in the examined area.

1. Introduction

Nematodes of the genus *Trichinella* are widespread food-borne agents which circulate in domestic and wild animals (Pozio and Zarlenga, 2013). Many free-living carnivores, such as red foxes (*Vulpes vulpes*), raccoon dogs (*Nyctereutes procyonoides*), raccoons (*Procyon lotor*), lynxes (*Lynx lynx*) and wolves (*Canis lupus*) are involved in the circulation of *Trichinella* nematodes in the European natural environment (Gottstein et al., 2009; Bień et al., 2016; Deksne et al., 2016; Kärssin et al., 2017; Kołodziej-Sobocińska et al., 2018; Cybulska et al., 2018, 2019). In Central Europe, the two most common species circulating in wildlife animals are believed to be *T. spiralis* and *T. britovi* (Gottstein et al., 2009; Pozio and Zarlenga, 2013; Feidas et al., 2014).

One group of carnivores occurring in parts of Europe and Asia are the martens (*Martes* spp.) (Proulx et al., 2005). Two marten species are found in Poland, and Europe in general: the pine marten (*Martes martes*) and the stone marten (*Martes foina*) (Proulx et al., 2005; Goszczyński et al., 2007). The pine marten reaches higher population densities in mature or old coniferous, deciduous or mixed forests, while the stone marten is better adapted to suburban and urban areas, and is presented in its highest densities in agricultural, industrial and urban areas (Proulx et al., 2005). Either way, both species of marten prey on small mammals, birds, insects and fruits as staples of their diet, in proportions dependent on food supply and prey density (Posłuszny et al., 2007).

Unfortunately, few studies have compared the prevalence of *Trichinella* larvae between Mustelids and red foxes, and little is known of their muscle distribution in Mustelids. Therefore, the present study was performed to determine the occurrence of *Trichinella* spp. nematodes in martens, and to evaluate the distribution of larvae in muscle samples taken from them.

2. Materials and methods

2.1. Ethical approval

The animals examined in this study were obtained within Project Life + "Active protection of lowland populations of Capercaillie in the Bory Dolnośląskie Forest and Augustowska Primeval Forest" (Life 11, NAT/PL/428). The frozen carcasses were delivered to the Laboratory of the Witold Stefański Institute of Parasitology, Polish Academy of Sciences.

* Corresponding author. Witold Stefański Institute of Parasitology, Polish Academy of Sciences, Twarda 51/55, 00-818, Warsaw, Poland.
E-mail address: cybulska.aleksandra@twarda.pan.pl (A. Cybulska).

https://doi.org/10.1016/j.ijppaw.2020.06.003
Received 10 March 2020; Received in revised form 9 June 2020; Accepted 9 June 2020
Available online 17 June 2020

Fig. 1. The location of the sampling area (the Głęboki Bród Forest District, Poland) is marked by a red square.

2.2. Collection of material

Muscle samples were collected from the carcasses of 57 martens (33 males and 24 females) from 2013 to 2016 in the Głęboki Bród Forest District (Fig. 1). From each animal, a set of different muscles (diaphragm, tongue, masseter) and muscle groups (upper and lower forelimb, upper and lower hindlimb) was collected during necropsy in the Laboratory of the Institute of Parasitology, PAS. Muscle samples were collected from nine stone martens (*Martes foina*) and 12 pine martens (*Martes martes*). However, as the carcasses were skinned in the field by the hunters who acquired them, in 36 cases it was not possible to define the marten to a species; in these cases, the animals were determined by genus.

The tissues were kept at −20 °C for further analysis. The day before digestion, the samples were thawed at room temperature and then weighed on a laboratory scale. The mass of examined muscle tissue ranged from 0.67 to 15.56 g (mean 3.97; median 3.78) for the diaphragm; from 1.86 to 5.31 g (mean 3.46; median 3.47) for the tongue; from 0.53 to 3.16 g (mean 1.54; median 1.42) for the masseter; from 3.39 to 14.06 g (mean 8.39; median 7.63) for the upper forelimb; from 1.77 to 10.54 g (mean 4.93; median 4.46) for the lower forelimb; from 4.43 to 19.18 g (mean 9.97; median 9.53) for the upper hindlimb; from 2.97 to 13.30 g (mean 5.90; median 4.31) for the lower hindlimb. The muscle samples were tested individually using HCl–pepsin digestion according to EC Regulation No. 2015/1375 (European Commission, 2015). The obtained larvae were counted; the intensity of infection was calculated as the number of larvae per gram (LPG) of muscle tissue. The larvae were collected individually and then were stored in 70% ethanol at −20 °C for further molecular identification at the species level.

2.3. DNA extraction and PCR amplification

According to the protocol described by Zarlenga et al. (1999), total nucleic acid was isolated from a minimum of 10 single larvae (if available) from each animal to avoid the possibility of mixed infections, in the event that mixed infection occurred. Multiplex polymerase chain reaction (PCR) was used to identify the larvae at the species level. All reactions were carried out using the BioRad T100™ Thermal Cycler, using specific primers described by Zarlenga et al. (1999). Reference strains of nematodes (*T. spiralis*–ISS003, *T. nativa*–ISS042, *T. britovi*–ISS002, and *T. pseudospiralis*–ISS013) and nuclease-free water were used as positive and negative controls, respectively. The PCR products were electrophoresed in 2% agarose gels stained with GelRed® Nucleic Acid Gel Stain (Biotium), in TAE buffer at 70 V. Following this, gels were analyzed under UV light using the ChemiDoc™ MP Imaging System (BioRad).

2.4. Statistical analysis

The Chi-Square test (http://www.socscistatistics.com) and Pearson's correlation matrices (STATISTICA 6.0, StatSoft, Poland) were used for statistical analysis. A Binomial Confidence Intervals program (http://statpages.info) was used for calculating the Confidence Interval (CI) at a 95% confidence level. A p-value < 0.05 was considered significant.

3. Results

Trichinella muscle larvae were detected in 10 of the 57 examined animals, including five of the 12 animals identified as pine martens, and another five of the 36 martens not identified to genus level (*Martes* spp.). All larvae were classified by molecular method as *T. britovi*. None of the examined stone martens were infected. The prevalence was found to be 41.67% among pine martens and 13.88% among *Martes* spp., with no statistically significant difference being observed between males and females in either case ($p < 0.05$, Chi-Square test).

The intensity of infection varied from 0.53 to 37.29 LPG (mean 6.97; median 5.56) among pine martens; and from 0.17 to 12.48 LPG (mean 4.12; median 3.14) among *Martes* spp. (Table 1, part A). The LPG per infected animal amounted to 2.29 for the female pine martens, and 1.50, 5.56, 8.68 and 16.69 for the four males (mean 8.13; median 7.12). Similarly, the value for *Martes* spp. amounted to 3.10, 3.14 and 5.90 for the females (mean 4.05; median 3.14), and 1.08 and 7.38 for the males (mean 4.23; median 4.23).

The highest mean LPG was recorded in the diaphragm (10.16 LPG) of the one infected female pine marten, and in the tongue (16.75 LPG) and diaphragm (10.20 LPG) for males. Among the *Martes* spp., the highest mean LPG was observed in the diaphragm (4.78 LPG) and tongue (4.42 LPG) among the females, and in the upper forelimb (6.58 LPG) and the lower hindlimb (6.02 LPG) among the males (Table 1, part B).

A Pearson's correlation matrix (R) greater than 0.0 indicates the presence of a positive correlation between individual muscles or muscle groups with regard to their LPG, while a value of 0.5 indicates a strong positive correlation. However, an R value less than −0.5 was observed between the LPG values in the diaphragm and in the other examined muscles among females, indicating a strong negative correlation (Table 2).

4. Discussion

The natural ecosystem plays an important role in the maintenance of *Trichinella* nematodes in the sylvatic cycle (Moskwa et al., 2012). In the presented study, only *T. britovi* was identified as an epidemiological agent in the examined martens. It is well known that *T. britovi* is the most prevalent species of *Trichinella* among wild carnivores, and it possesses a wide distribution, covering most of Europe, North and West Africa, and Southwest Asia (Gottstein et al., 2009).

In Poland, data regarding the occurrence of *Trichinella* infection in wild animals, such as martens, is based only on individual scientific studies. Cabaj (2006) report the presence of a *Trichinella* spp. nematode in one examined marten from the Warmińsko-Mazurskie District, while Moskwa et al. (2012) confirmed the occurrence of *T. britovi* in two of three examined martens from the same research area. Our research shows that animals from the Głęboki Bród Forest District are hosts for *T.*

Table 1
Larval burden of *Trichinella britovi* in muscles and muscle groups of the martens from the Głęboki Bród Forest District, Poland. Part A – general prevalence with respect to gender; Part B – mean LPG in the examined muscles and muscle groups with respect to gender.

Part A						Part B						
Species	Gender	Infected/examined animals	Prevalence (%)/$CI_{95\%}$	LPG range/SD	Mean/median (LPG per animal)	Diaphragm	Tongue	Masseter	Upper forelimb	Down forelimb	Upper hindlimb	Down hindlimb
Martes martes	Male	4/11	36.36/10.93–69.21	0.53–37.29/8.01	8.13/7.12	10.20	16.75	3.69	6.08	8.74	6.56	4.91
	Female	1/1	100/2.50–100.00	2.71–10.16/3.74	2.29/2.29	10.16	3.18	–	–	2.71	–	–
Total		5/12	41.67/15.17–72.33	0.53–37.29/7.68	6.97/5.56	10.19	14.03	2.95	4.86	7.54	5.25	3.93
Martes spp.	Male	2/17	11.76/1.46–36.44	0.17–12.48/4.29	4.23/4.23	5.38	1.24	1.97	6.58	3.61	4.84	6.02
	Female	3/19	15.79/3.38–39.58	0.81–8.70/2.14	4.05/3.14	4.78	4.42	3.95	3.26	4.15	4.16	3.62
Total		5/36	13.88/4.67–29.50	0.17–12.48/3.12	4.12/3.14	5.02	3.15	3.16	4.59	3.93	4.43	4.58

britovi, and that the parasite can be detected throughout the examined area in the marten population.

It has been reported previously that martens can be infected by *T. britovi* and *T. spiralis*, and mixtures of *T. britovi/T. nativa/T. spiralis*, *T. nativa/T. britovi* and *T. spiralis/T. britovi* (Malakauskas et al., 2007; Hurníková et al., 2009; Moskwa et al., 2012; Oltean et al., 2014; Deksne et al., 2016; Kirjušina et al., 2016; Klun et al., 2019), however, the prevalence depends on the examined region. Our present findings reveal that 41.67% of pine martens and 13.88% of *Martens* spp. were infected by *T. britovi*, and none of examined stone martens were positive for *Trichinella*. Nevertheless, in other European countries, *T. britovi* was identified in 4.8% of stone martens from Serbia (Klun et al., 2019), 45.8% of stone martens in Latvia (Deksne et al., 2016), 7.9% of stone martens in Italy (Remonti et al., 2005), two of four examined stone martens from Romania (Oltean et al., 2014), and in one of three stone martens and one of two pine martens from Slovakia (Hurníková et al., 2009). Furthermore, *Trichinella* spp. was described in 4.44% of pine martens from Spain (Segovia et al., 2007) and 28.6% of stone martens from Bulgaria (Kirkova et al., 2011). According to Bandino et al. (2015), none of 18 martens from Sardinia, Italy tested for *Trichinella* spp. was positive.

Trichinella spp. infection is well documented in the Baltic States, where nematodes were detected in 30.4% of tested martens from Estonia, and in between 40% and 62.5% of tested martens from Lithuania (Senutaitė and Grikienienė, 2001; Malakauskas et al., 2007). In Latvia, the prevalence of *Trichinella* nematodes varied from 28.6% to 56.2% of examined martens depending on the study (Malakauskas et al., 2007; Deksne et al., 2016). In addition, another Latvian study found 43.2% of examined male and 48.4% of female pine martens to be infected by *Trichinella* spp., as well as three of six tested male and one of two female stone martens. Moreover, *Trichinella* nematodes were discovered in one of six examined male pine martens and in two of three male stone martens from Lithuania (Bērziņa et al., 2013). In contrast, in the present study, the occurrence of *T. britovi* was noted in 36.36% of the male pine martens and in one female; and in 11.76% of males and 15.79% females of *Martes* spp. It has been suggested that the similarities and differences in *Trichinella* prevalence between the Baltic States and other neighboring countries, such as Poland, may be caused by improper human behavior or the occurrence of the large carnivore population in forest areas (Malakauskas et al., 2007).

It has been pointed that the distribution of *Trichinella* spp. in wildlife may depend on the local environment, for example food supplies, animal home range, the population densities of martens and other wild animals, and the infection parameters of *Trichinella* spp. (Bērziņa et al., 2013). While the typical home range of a pine marten is 1.41 km^2 for females and 2.58 km^2 for males, and it is typically found in insular wooded areas, scrublands and coniferous forests (Zalewski et al., 2004; Zalewski and Jędrzejewski, 2006), the stone marten typically ranges up to 0.46 km^2 for females and to 1.82 km^2 for males, and is known to live in forests, woodlands and pastures, and is expanding in suburban and urban areas (Genovesi et al., 1997; Proulx et al., 2005; Wereszczuk and Zalewski, 2019). Their wide variety of habitats and life style increase the risk of *Trichinella* infection among martens and thus the chance of infecting other animals (Bērziņa et al., 2013). For example, other predators as red foxes, who can range as far as 30 km^2, occasionally feed on martens (Storch et al., 1990; Borkowski, 1994; Goszczyński, 2002). It has been documented that over 27% of red foxes examined from Podlaskie Voivodship, where the Głęboki Bród Forest District is located, is infected by *T. britovi* (Cybulska et al., 2016), as are 39.82% of raccoon dogs from the Głęboki Bród Forest District (Cybulska et al., 2019). Additionally, *T. britovi* was found in a single wolf that had died of natural causes in the nearby Augustowska Forest, also located within the Głęboki Bród Forest Distrit (Bień et al., 2016). It therefore appears that this parasite species is commonly maintained in the predators in this area, and that martens infected by *Trichinella* nematodes could be a source of infection for other animals, if they occur in the same areas.

Table 2
Pearson's correlation matrix showing the R-values of the relationships between the number of larvae per gram (LPG values) of different muscles or muscle groups. The values above the diagonal line refer to females, while those below the line refer to males. Significant dependences between tissue types ($p < 0.05$) are marked by asterisks (*).

Male	Female						
	Diaphragm	Tongue	Masseter	Upper forelimb	Lower forelimb	Upper hindlimb	Lower hindlimb
Diaphragm		−0.79	−0.67	−0.83	−0.97*	−0.76	−0.90
Tongue	0.91*		0.95	0.90	0.66	0.92	0.98*
Masseter	0.97*	0.93*		0.94	0.48	0.98*	0.89
Upper forelimb	0.75	0.42	0.68		0.68	0.99*	0.91
Lower forelimb	0.99*	0.90*	0.95*	0.74		0.59	0.80
Upper hindlimb	0.93*	0.75	0.90*	0.89*	0.91*		0.90
Lower hindlimb	0.46	0.20	0.51	0.84*	0.43	0.71	

Both martens species are generalist predators and their diet consists of small mammals, birds, insects, fruits and sometimes the carrion of other animals (Goszczyński, 1976, 1986; Storch et al., 1990; Jędrzejewski et al., 1993; Posłuszny et al., 2007; Bakaloudis et al., 2012; Balestrieri et al., 2013). However, pine martens prefer small mammals and birds, while stone martens consume more fruits (Posłuszny et al., 2007). Due to their diet, martens can easily get infected by *Trichinella* nematodes. It is also well documented that rodents are staple food for martens, and it has been shown that martens from Poland often consume bank voles (*Myodes glareolus*) (Goszczyński, 1986; Jędrzejewski et al., 1993; Posłuszny et al., 2007). Due to the limited data describing the seroprevalence of *Trichinella* spp. infection in bank voles, these animals may be suspected to play a role in the maintenance of *Trichinella* nematodes in the forest environment in Poland (Dvorožňáková et al., 2016; Grzybek et al., 2019).

Studies on zoonotic nematodes such as *Trichinella* allow the larval burden, i.e. the number of larvae per gram of muscle (LPG) in preferential muscles of wild host species to be estimated for epidemiological surveys. In our present study, overall LPG per animal was lower than 10 LPG in all examined females and in five of the six examined males, with the other male demonstrating an LPG of 16.69. Our results support the conclusion that infections of *Trichinella* nematodes in wildlife animals are mostly of low intensities (<10 larvae/g in muscle tissue) (Pozio and Zarlenga, 2013). However, our previous studies on badgers, red foxes, martens and raccoon dogs indicate higher intensities: 49.76; 69.19; 117.09 and 622.92 LPG, respectively (Moskwa et al., 2012; Cybulska et al., 2016, 2019).

So far, only one report has described the muscle distribution of *T. britovi* in pine martens, indicating the highest LPG to be present in the diaphragm and lower part of the forelimbs (Kirjušina et al., 2016). In contrast, in the present study, the highest larval burden was detected in the diaphragm of one infected female pine marten; and in the tongue and the diaphragm of the males. Among the unclassified *Martes* spp, the highest LPG was found in the diaphragm and the tongue among females, and in the upper forelimb and the lower hindlimb among males. Considering all infected martens, in most cases, the Pearson's correlation matrix (R) indicated a positive or strong positive correlation between individual muscles or muscle groups with regard to their LPG values. However, in females, a strong negative correlation was observed between the LPG values in the diaphragm and in the other examined muscles. It has been suggested that the larval density in a muscle may depend more on the muscle its motorial potential than its actual activity (Mikkonen et al., 2001). Studies conducted on experimentally-infected raccoon dogs found the limb muscles to be heavily infected despite the animals' movement being restricted (Mikkonen et al., 2001). Hence it is likely that in other wild animals, such as martens, the diaphragm and forelimb muscles may be significantly predictive of the highest larval burden. Based on our obtained results, the tongue and diaphragm for pine martens, and the diaphragm and limbs muscles for *Martes* spp. in general, may be recommended as practical indicator muscles of *T. britovi* in further studies.

5. Conclusion

Our findings confirm that martens from the Głęboki Bród Forest District serve as a host for *T. britovi* and they may also play a significant role in the maintenance of these nematodes throughout the described area. *T. britovi* is also commonly spread in different carnivorous hosts throughout the area. Therefore, further epidemiological surveillance concerning the presence of *Trichinella* species, including the larval burden in various muscles, should be continued, due to the small number of infected animals described in this research.

CRediT authorship contribution statement

Aleksandra Cybulska: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Aleksandra Kornacka:** Investigation. **Rafał Skopek:** Investigation. **Bożena Moskwa:** Conceptualization, Methodology, Writing - original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The carcasses of animals used in the study were collected within the Project Life +, no. LIFE11 NAT/PL/428.

The research was performed within the National Science Centre, Poland, project no. 2017/25/N/NZ7/02625.

Rafał Skopek MSc. conducted the experiments performed as part of this study during his internship at the Witold Stefański Institute of Parasitology, Polish Academy of Sciences.

We would like to thank the local hunters and the Staff from the Głęboki Bród Forest District for their help in collecting samples.

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