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

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**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 Zarlenza, 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 Zarlenza, 2013; Feidais 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 (Połówczyński 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 Capercaille 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 Stefan ski Institute of Parasitology, Polish Academy of Sciences.
reactions were carried out using the BioRad T100™ Thermal Cycler, in the event that mixed infection occurred. Multiplex polymerase chain nucleic acid was isolated from a minimum of 10 single larvae (if necessary), collected 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 Zarlenka 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 Zarlenka 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 the three examined martens from the same research area. Our research shows that animals from the Głęboki Bród Forest District are hosts for T. britovi.
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.

| Species             | Part A | Part B |
|---------------------|--------|--------|
|                     | Gender | Infected/examined animals | Prevalence (%)/CI 95% | LPGrange/SD | Mean/median (LPG per animal) |
|                     |        |                    |                        |            |                          |
|                     | Male   | 4/11               | 36.36/10.93–69.21      | 8.74       | 8.13/7.12               |
|                     | Female | 1/1                | 100/2.50–100.00        | 2.71       | 2.29/2.29               |
| Martes martes       |        | 5/12               | 41.67/15.17–72.33      | 10.20      | 14.03                   |
|                     | Total  | 5/17               | 43.2%                  | 5.38       | 4.59                    |
|                     | Female | 1/3                | 100/2.50–100.00        | 2.71       | 2.29/2.29               |
|                     |        | 2/3                | 66.67/10.0–100.0       | 6.79       | 5.62                    |
|                     |        | 4/3                | 100/2.50–100.00        | 2.71       | 2.29/2.29               |
|                     |        | 1/3                | 33.33/10.0–100.0       | 2.71       | 2.29/2.29               |
|                     |        | 6/3                | 100/2.50–100.00        | 2.71       | 2.29/2.29               |

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*. 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), 28.6% of stone martens from Bulgaria (Kirkova 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 Poland (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 (Senutaite and Grikenienė, 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% of females of *Martes* spp. was positive.

*Trichinella* spp. infection in wildlife may depend on the local environment, for example food supplies, population in forest areas (Malakauskas et al., 2007), and natural causes in the nearby Augustowska Forest, also located within the Głęboki Bród Forest District (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.
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; Poslusny 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 (Poslusny 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; Poslusny 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 (Dvorozniaková 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 Zarlinga, 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., 2011). Considering all infected martens, in most cases, the Pearson’s correlation matrix showed significant dependence between the LPG values in the diaphragm and in the other examined muscles. 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.

### Table 2

| Male          | Female          | Diaphragm | Tongue | Maseter | Upper forelimb | Lower forelimb | Upper hindlimb | Lower hindlimb |
|---------------|-----------------|-----------|--------|---------|----------------|----------------|----------------|----------------|
| Diaphragm     |                 | 0.91*     | −0.79  | −0.67   | −0.83          | −0.97*         | −0.76          | −0.90          |
| Tongue        |                 | 0.97*     | 0.95   | 0.90    | 0.66           | 0.92           | 0.98*          | 0.89           |
| Maseter       |                 | 0.94      | 0.94   | 0.91    | 0.84*          | 0.91*          | 0.59           | 0.91           |
| Upper forelimb|                 | 0.68      | 0.68   | 0.74    | 0.43           | 0.71           |                |                |
| Lower forelimb|                 | 0.90*     | 0.90*  | 0.89*   | 0.43           | 0.71           |                |                |
| Upper hindlimb|                 | 0.46      | 0.46   | 0.51    | 0.80           |                |                |                |
| Lower hindlimb|                 | 0.20      | 0.20   | 0.51    | 0.90           |                |                |                |

### 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 Cybulskas: 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.

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