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The occurrence and muscle distribution of *Trichinella britovi* in raccoon dogs (*Nyctereutes procyonoides*) in wildlife in the Głęboki Bród Forest District, Poland

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ABSTRACT

The raccoon dog (*Nyctereutes procyonoides*) is an introduced, invasive species in Europe. Literature data show that raccoon dogs act as a reservoir of many dangerous parasites, including nematodes of the genus *Trichinella*. The aims of the study were to determine the prevalence of *Trichinella* spp. infection in raccoon dogs collected from the Głęboki Bród Forest District between 2013 and 2016, and to evaluate their distribution in the muscle tissue of the host. The larvae of *Trichinella* spp. were detected in 45 raccoon dogs (39.82%), and all of them were identified as *T. britovi.* No mixed infection was observed. The intensity of infection ranged from 0.02 to 622.92 larvae per gram (LPG), and the highest mean was observed in the tongue and lower forelimb in both examined sexes. The raccoon dog may play a significant role as a reservoir of *T. britovi* in the wildlife in the examined area.

1. Introduction

The raccoon dog (*Nyctereutes procyonoides*) is an omnivore which was originally indigenous to East Asia, but was introduced into the Western part of the former Soviet Union in 1920s due to human activity (Lavrov, 1971; Nowak, 1984; Pitra et al., 2010), from where it later migrated to numerous European countries (Al-Sabi et al., 2013). In Poland, the first raccoon dogs were noticed among wildlife in 1955 in the eastern and north-eastern parts of the country (Dehnel, 1956). Due to their high adaptability to various environmental and climatic conditions, raccoon dogs readily adapted to the environment of Eastern Europe (Święcicka et al., 2011), and raccoon dogs are currently observed almost throughout the territory of Poland (Skorupski, 2016).

In Europe, the raccoon dog is known to be an important vector of various parasites which can infect domestic and wild animals (Al-Sabi et al., 2013; Laurimaa et al., 2016); it is well documented that some of these parasites can be highly hazardous to human health, such as *Echinococcus multilocularis* or *Trichinella* spp. (Mayer-Scholl et al., 2016; Kärssin et al., 2017).

Parasitic nematodes of the genus *Trichinella* are found in all continents except Antarctica (Pozio and Murrell, 2006). Literature data suggests that all *Trichinella* species predominantly parasitize wild carnivores and omnivores (Pozio, 2001) such as wild boars, red foxes, wolves, raccoon dogs and raccoons (Gottstein et al., 2009; Pozio and Zarlenga, 2013; Bień et al., 2016; Cybulska et al., 2016, 2018). Up to date, four *Trichinella* species, *viz. T. spiralis*, *T. britovi*, *T. nativa* and *T. pseudospiralis*, are present in Europe (Pozio, 2001), including Poland (Chmurzyńska et al., 2013; Moskwa et al., 2013).

The raccoon dog is suspected of causing damage to native fauna through its feeding habits (Süld et al., 2014). Although its diet primarily consist of insects, plants and small mammals, it can also include carrion or birds (Sutor et al., 2010; Święcicka et al., 2011). Due to its feeding habits, raccoon dog can be a host of various species of nematodes of the genus *Trichinella*.

Therefore, the aims of the study were 1) to determine the prevalence of *Trichinella* spp. infection in examined raccoon dogs, 2) to determine the species of these isolated larvae, 3) to evaluate the distribution of nematode larvae identified in muscle samples of examined animals.

2. Materials and methods

2.1. Ethical approval

All animals examined in this study were hunted within Project Life + "Active protection of lowland populations of capercaillie in the Bory Dolnoslaskie Forest and Augustowska Primeval Forest" (Life 11, NAT/PL/428); therefore, no separate agreement with Second Warsaw Local Ethics Committee for Animal Experimentation for Witold Stefański Institute of Parasitology was needed to conduct research on

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Fig. 1. The location of the sampling area (Głęboki Bród Forest District, Poland).

animal tissue (due to the Resolution No. 22/2006 of the National Ethics Committee for Animal Experiments, 07 November 2006).

2.2. Sample collection

Muscle samples (the diaphragm pillars, lumbar and costal parts of the diaphragm; the tip and body of the tongue; the superficial, middle and the deep portion of the masseter; upper and lower forelimb; upper and lower hindlimb) were collected from the carcasses of 113 raccoon dogs (49 females and 64 males). The collection took place in the years from 2013 to 2016 in Głęboki Bród Forest District (Fig. 1). Where possible, the samples were collected in the Laboratory of the Institute of Parasitology, PAS. However, some samples were collected by hunters in the field. All staff were instructed to collect the same muscle portions. The tissues were kept at −20 °C for further analysis. The day before the digestion, the muscle samples were thawed at room temperature and then weighed on a laboratory balance. The mass of examined muscles varied according to amount acquired from the source; ranging from 3.84 to 26.3 g (mean 12.63; median 12.70) for muscle tissue obtained from the diaphragm; from 6.30 to 21.72 g (mean 13.21; median 13.15) for tissue from the tongue; from 1.04 to 10.79 g (mean 4.79; median 5.01) from the masseter; from 10.71 to 41.45 g (mean 24.74; median 25.19) from the upper forelimb; from 3.88 to 39.13 g (mean 13.00; median 12.06) from the lower forelimb; from 7.58 to 41.62 g (mean 23.02; median 22.32) from the upper hindlimb; from 6.45 to 26.09 g (mean 14.62; median 14.32) from the lower hindlimb. The muscle samples were tested individually for the presence of *Trichinella* spp. using HCl–pepsin digestion according to EC Regulation No. 2015/1375 (European Commission, 2015). The larvae were identified as *Trichinella* based on their morphometrical characteristics (Anderson et al., 2009). The larvae were counted; the intensity of infection was calculated as the number of larvae per gram (LPG) of muscle tissue. The larvae were stored in ethanol at −20 °C for molecular identification at the species level.

2.3. DNA extraction

Total nucleic acid was isolated from a minimum of 10 single larvae (if available) from each animal to account for the possibility of mixed infections (Zarlenga et al., 1999).

2.4. PCR amplification

The larvae were identified at species level by multiplex polymerase chain reaction (PCR) as described by Zarlenga et al. (1999), with some modifications. All reactions were performed using a 25 μl volume of reaction mixture containing 2 μl of DNA, 1U of Taq polymerase (Thermo Fisher Scientific), 2.5 mM of $MgCl_2$, 10 mM of each dNTPs, and 20 mM specific primers. PCR was carried out using a BioRad T100™ Thermal Cycler. Reference strains of *Trichinella* species were used as positive controls (*T. spiralis*–ISS003, *T. nativa*–ISS042, *T. britovi*–ISS002, and *T. pseudospiralis*–ISS013). The PCR products were analyzed on 2% agarose gels stained with GelRed (Biotium) in TAE buffer at 70 V, and analyzed under UV light using the ChemiDoc™ MP Imaging System (BioRad).

2.5. Statistical analysis

The Chi-Square test was used for statistical analysis (http://www.socscistatistics.com). Pearson's correlation matrices (STATISTICA 6.0, StatSoft, Poland) were performed to study dependences between the numbers of larvae per gram (LPG) of every examined muscle (Table 3). The samples were divided into four groups according to the intensity of infection, and the one-sample *t*-test (STATISTICA 6.0, StatSoft, Poland) was used to calculate significant dependences for average LPG in examined muscle according to these groups (Table 2, part B). A Binomial Confidence Intervals program was used for calculating Confidence Interval (CI) at a 95% confidence level (http://statpages.info). A p-value < 0.05 was considered significant.

3. Results

Based on their morphology, *Trichinella* larvae were identified in 45 of the 113 examined raccoon dogs. The overall prevalence of *Trichinella* larvae was 39.82%, i.e. 44.90% among females and 35.94% among males, and all of the isolated larvae were classified as *T. britovi*. The intensity of infection ranged from 0.02 to 622.92 LPG (Table 1). A statistically significant difference in LPG was observed between males and females ($p < 0.05$, Chi-Square test).

With regard to the muscle distribution of *T. britovi* in examined raccoon dogs with respect to the sex of the animal, the highest mean LPG was recorded in the tongue, lower forelimb and masseter among examined females, and in the tongue, lower forelimb and lower hindlimb among males. Additionally, to examine the intensity of infection, expressed as average LPG per animal, the results were divided into four groups as follows: < 1 LPG, 1–10 LPG, 10–100 LPG and > 100 LPG (Table 2).

Table 1
Prevalence and larval burden of *Trichinella britovi* in raccoon dogs of the Głęboki Bród Forest District, Poland.

Gender	No. of infected/examined animals	Prevalence %/CI_{95}	Intensity of infection	
			range (LPG)/SD	mean/median (LPG)
Female	22/49	44.90/30.67–59.77	0.03–622.92/110.04	78.46/30.51
Male	23/64	35.94/24.32–48.90	0.02–390.16/54.88	37.06/10.94
Total	45/113	39.82/30.73–49.46	0.02–622.92/87.08	56.06/21.44

Table 2
Larval burden of *Trichinella britovi* in muscles and muscle groups of the raccoon dogs from the Głęboki Bród Forest District, Poland. Part A – reflect to general data obtained for four groups describing the different intensity of infection; Part B – average LPG in examined muscle with respect to four groups describing the different intensity of infection. Significant dependences ($p < 0.05$) are marked by asterisks (*).

Part A					Part B						
Intensity of infection	N	Prevalence (%)/CI$_{95}$	Range of average LPG per animal	Mean/Median	Diaphragm	Tongue	Masseter	Upper forelimb	Lower forelimb	Upper hindlimb	Lower hindlimb
Female											
< 1	7	14.29/5.94–27.24	0.01–0.36	0.07/0.02	0.06*	0.25	0.06	0.01	0.08	0.03	0.02
1–10	3	6.12/1.28–16.87	3.08–7.29	5.07/4.85	5.85	4.54	4.38	3.52	8.99	3.57*	4.66
10–100	6	12.25/4.63–24.77	19.04–74.91	40.14/32.49	31.62*	46.19*	25.35*	25.57*	82.16*	30.99*	39.10*
> 100	6	12.25/4.63–24.77	111.22–331.26	176.32/156.77	138.70*	240.69*	251.82*	118.70*	200.31*	140.51*	143.49*
Total	22	44.90/30.67–59.77	0.02–331.26	59.75/20.94	47.27*	78.94*	76.21*	39.83*	78.29*	47.27*	50.44*
Male											
< 1	5	7.81/2.58–17.30	0.01–0.87	0.21/0.04	0.31	0.13*	0.05	0.15	0.46	0.17	0.20
1–10	8	12.5/5.55–23.15	1.94–7.03	3.68/3.40	2.82*	3.34*	3.86*	2.56*	6.66*	2.39*	4.10*
10–100	7	10.94/4.51–21.25	14.21–87.28	42.39/40.51	47.40	54.39*	37.73*	25.48*	64.59*	29.67*	37.49*
> 100	3	4.69/0.98–13.09	100.69–157.88	124.85/115.99	114.03*	190.73	110.40	103.93	102.98	109.30*	142.61*
Total	23	35.94/24.32–48.90	0.03–157.88	30.51/4.25	30.35*	42.62*	27.24*	22.24*	35.50*	24.16*	31.48*

N – respect to positive samples.

A Pearson's correlation matrix (R) greater than 0.5 indicates the presence of a strong positive correlation between individual muscles or muscle groups with regard to their LPG (Table 3).

4. Discussion

According to data published by DAISIE European Invasive Alien Species Gateway (2006) the present range area of the raccoon dog in Eastern and Central Europe, includes Austria, Belarus, Bulgaria, Denmark, Estonia, Finland, Germany, Italy, Hungary, Latvia, Lithuania, Moldova, the Netherlands, Norway, Poland, Western Russia, Romania, Slovakia, Sweden, Switzerland and Ukraine. According to the Commission Implementing Regulation ((EU 2017/1263 2017)), the raccoon dog has been included to the List of Invasive Alien Species of Union concern, which poses a significant threat to biodiversity in Europe. It is documented that locally the raccoon dog may be threat to populations of waterfowl and amphibians (Kauhala and Kowalczyk, 2011), and literature data suggests that it may compete with native medium-sized carnivores, such as the Eurasian badger (*Meles meles*) and the red fox (*Vulpes vulpes*) (Jędrzejewska and Jędrzejewski, 1998; Kowalczyk et al., 2008).

In Poland, first individual raccoon dogs were noticed in the eastern part of the country, particularly the Białowieża Forest, in 1955 through expansion from Lithuania, Belarus and Ukraine (Dehnel, 1956). From here, it migrated from north-eastern Poland to other regions, and is currently found throughout the whole country, with the exception of higher parts of the mountains in the south (Kauhala and Kowalczyk, 2011). According to available data from the Polish Hunter Association, in 2011, this carnivore was identified in 91% of hunting grounds. The raccoon dog population was estimated as 54 500 individuals in 2011 (data available at https://pzlow.pl/); and its annual hunting acquisition is steadily increasing, from over 11 000 animals in 2011 to nearly 16 000 individuals in 2016 (Skorupski, 2016).

It has been reported that in other European countries, raccoon dogs act as a reservoir for four *Trichinella* species, *viz. T. spiralis*, *T. britovi*, *T. nativa* and *T. pseudospiralis*, as single or mixed infection. However, the prevalence varies between different European regions, from 0% in Denmark and Austria, 1.9% and 4% in Germany, 28% and 33.2% in Finland, 29.3% and 32.5% in Lithuania, and 35.5% in Latvia, to 42% and 57.5% in Estonia (Malakauskas et al., 2007; Airas et al., 2010; Pannwitz et al., 2010; Bružinskaitė-Schmidhalter et al., 2011; Al-Sabi et al., 2013; Mayer-Scholl et al., 2016; Duscher et al., 2017; Kärssin et al., 2017; Antti et al., 2018).

In Poland, the raccoon dog is not routinely examined for the presence of nematodes of the genus *Trichinella*. Therefore data on its occurrence is mainly based on scientific studies, which show that it is typically infected with *T. spiralis* and *T. britovi* (Cabaj et al., 2005; Osten-Sacken and Solarczyk, 2016), with one case of mixed infection with *T. spiralis* and *T. britovi* being reported (Gołąb et al., 2009). However, raccoon dogs from the Głęboki Bród Forest District tested in the present study were infected with *T. britovi* alone; this is not surprising as it is well documented that carnivorous mammals are preferential hosts for *T. britovi* (Pozio and Zarlenga, 2013). *T. britovi* infection has been described in 74% of raccoon dogs examined in Lithuania, and in 40.5% and 24.8% of tested individuals in Estonia (Malakauskas et al., 2007; Kärssin et al., 2017). In contrast, the most commonly-identified *Trichinella* species identified in raccoon dogs in Finland was *T. nativa*, followed by *T. spiralis* and *T. britovi* (Airas et al., 2010); this contrasts with Antti et al. (2018) who found *T. nativa* to be prevalent among raccoon dogs in Finland, followed by *T. britovi*. Additionally, Mayer-Scholl et al. (2016) found *T. spiralis* to be the most widely-infecting species in raccoon dogs in Germany. These findings indicate that the frequency of the occurrence of *Trichinella* species varies across Europe, and suggests that the distribution of *Trichinella* nematodes in wildlife may depend on the local environment and the climate.

Table 3
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		0.78*	0.51*	0.98*	0.96*	0.96*	0.98*
Tongue	0.81*		0.41	0.84*	0.79*	0.87*	0.84*
Masseter	0.74*	0.52*		0.49*	0.45*	0.44*	0.46*
Upper forelimb	0.85*	0.96*	0.66*		0.96*	0.99*	0.99*
Lower forelimb	0.67*	0.53*	0.84*	0.62*		0.95*	0.98*
Upper hindlimb	0.88*	0.87*	0.79*	0.97*	0.72*		0.98*
Lower hindlimb	0.64*	0.58*	0.93*	0.72*	0.88*	0.81*	

It is worth adding that the Głęboki Bród Forest District is geographically located near Białowieża Forest, where the first raccoon dogs were observed. A high percentage (almost 40%) of examined animals infected with *Trichinella* indicate that raccoon dogs may play an important role in the maintenance of *T. britovi* among wildlife in the tested area.

Little is known about predilection among muscles in wild raccoon dogs. Available data are limited or are derived from experimentally-infected animals. Maas et al. (2016) found *T. spiralis* in one of nine examined raccoon dogs, and identified 89.3 LPG in the forelimb muscle and 32.4 LPG in the tongue of the infected animal. The highest larval burdens were found in the front leg muscle, eye muscles and tongue in raccoon dogs experimentally infected with *T. spiralis* and *T. nativa* (Mikkonen et al., 2001). In general, in the present study, the highest larval burdens were detected in the tongue, lower forelimb muscles and masseter in examined females, and in the tongue, lower hindlimb and upper hindlimb muscles in examined males. However, as varying intensities of *T. britovi* infection were observed, from very low intensities to massive infections, the tested animals were divided into four groups based on the following intensities of infection: < 1 LPG, 1–10 LPG, 10–100 LPG and > 100 LPG (Table 2).

Mikkonen et al. (2001) suggest that differences between predilection sites are more obvious when the animal is heavily infected, which has also been noted in studies conducted in another invasive species in Poland, the raccoon (*Procyon lotor*) (Cybulska et al., 2018). Similarly, our findings note a low intensity of infection in the < 1 LPG group of examined raccoon dogs, and it is hard to estimate which muscle is most likely to be predictive of the occurrence of *T. britovi* in raccoon dogs. Therefore, we conclude that the muscle distribution of *Trichinella* is random in animals with low intensity of infection.

The highest larval burdens were observed in lower forelimb muscles in the 1–10 LPG and 10–100 LPG groups, in both sexes. However, in the > 100 LPG group, the predilection muscles for *T. britovi* were the masseter in females and the tongue in males. Mikkonen et al. (2001) recommend using the tongue and *flexor carpi ulnaris* muscle as practical indicator muscles for *Trichinella* during routine examination of raccoon dogs in research studies. Finally, although it has been noted that the location of predilection sites seems to be more dependent on the host species than the parasite species (Kapel et al., 1995, 1998), the predeliction site in the present study was also found to vary between sexes, as did the intensity of *Trichinella* infection.

5. Conclusion

Raccoon dogs can play an important role in spreading *Trichinella* nematodes through the sylvatic cycle in Europe, because it is a fast-spreading, invasive species itself. The results of our study confirm that raccoon dogs from the Głęboki Bród Forest District act as a host of *T. britovi*, and its high prevalence level suggest that they may be an important element in the sylvatic cycle of this nematode species in the examined area; however, the nationwide epidemiological situation remains unclear. Therefore, further epidemiological surveillance is needed in these animals to assess the risk of spread of *Trichinella* and other dangerous parasites.

Authors' contributions

AC, BM – conceived and designed the study area; AC, AK – performed experiments; AC, AK, BM – collected muscle samples; AC – analyzed the data and wrote the manuscript.

All authors read and approved the final manuscript.

Declaration of interests

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.

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.05.003.

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