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Sex-biased polyparasitism in moose (*Alces alces*) based on molecular analysis of faecal samples

Magdalena Świsłocka [a,*], Anetta Borkowska [a], Maciej Matosiuk [a], Magdalena Czajkowska [a], Norbert Duda [b], Rafał Kowalczyk [c], Mirosław Ratkiewicz [a]

[a] Department of Zoology and Genetics, Faculty of Biology, University of Bialystok, Ciołkowskiego 1J st, 15-245, Białystok, Poland
[b] Zespół Szkół Ogólnokształcących No 2 W Białymstoku, Narewska 11, 15-840, Białystok, Poland
[c] Mammal Research Institute, Polish Academy of Sciences, Stoczek 1, 17-230, Białowieża, Poland

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ABSTRACT

Simultaneous infection with multiple parasite species in an individual host is often observed in wild populations. The understanding of parasite species distribution across populations of wild animals is of basic and applied importance, because parasites can have pronounced effects on the dynamics of host population. Here, we quantified prevalence and endoparasite species richness in moose and explored sex-biased polyparasitism using diagnostic PCR method coupled with DNA sequencing of moose faecal samples from the Biebrza River valley, North-Eastern Poland. This is the largest moose population in Central Europe that has not been harvested for almost 20 years. We also evaluated the appropriate quantity of faeces for detecting DNA of parasite species. Faecal samples were screened for molecular markers of 10 different species of endoparasites. Endoparasite prevalence was high in the studied population. Almost all of the samples (98%) tested positive for at least one parasite species, and we found polyparasitism in the majority of the tested individuals. The number of different parasite species found in a single individual ranged from 0 to 9. The parasite species richness was significantly higher in male than in female individuals. The most prevalent were liver fluke *Parafasciolopsis fasciolaemorpha* and gastrointestinal nematodes *Ostertargia* sp. Of the ten endoparasite species detected, only the prevalence of the tapeworm *Moniezia benedeni* was significantly higher in males than in females. Additionally, we identified co-occurrence associations of parasite species, which tended to be random, but we noted some evidence of both positive and negative associations. Our findings promote applications of molecular methods for parasite species identification from non-invasively collected faecal samples in management and scientific study of moose population, which should include investigation of parasite status, and in health monitoring programs for other wild cervids.

1. Introduction

High population densities combined with declines in natural forage availability (Mathisen et al., 2014), cohort effects (Wam et al., 2010), and climate warming (van Beest and Milner, 2013) may be important factors in the transmission of parasites between individuals. The understanding of the distribution of parasite species across wild animal populations is of basic and applied importance, because parasites can have pronounced effects on body mass and the condition of their hosts, thereby influencing host population dynamics (Davidson et al., 2015). Polyparasitism, i.e. simultaneous infections with multiple species in an individual host is to be expected in natural populations (review Bordes and Morand, 2011). However, widespread heterogeneities in parasite loads suggest that not all animals carry the same number or types of parasites.

Within many vertebrate species, prevalence and intensity of parasitism is often higher in males than females (male-biased parasitism, e.g. Nunn et al., 2009; Klein and Flanagan, 2016; Cozzarolo et al., 2019). A sex-biased infection could be related to differences between sexes in susceptibility, mainly caused by the effect of sex hormones on immunity, sexual size dimorphism and development, physiology, behaviour, and different exposure to parasites (Duneau and Ebert, 2012; Habig et al., 2018). Sex-biased parasitism may be linked to sex-biased transmission and can lead to the sex-specific selective pressures on hosts and parasite

* Corresponding author.
E-mail address: magdaswi@uwb.edu.pl (M. Świsłocka).

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adaptations to the host sex suffering greater parasitism (Cozzarolo et al., 2019).

The moose (*Alces alces*), as the largest cervid species in European temperate zone, is an important game animal, which can also considerably alter the ecosystem structure and functioning (Graham et al., 2010). In Poland, moose population recovered since introducing the ban on hunting imposed in 2001 (Raczyński and Ratkiewicz, 2011), and the largest autochthonous population occupies the Biebrza River valley, the most expansive natural complex of marshland in Central Europe. The hunting ban is still in force, thus creating a unique situation for the moose population on the European scale. It gives the opportunity to study the natural performance of a non-harvested moose population, which reaches high densities. Although endoparasites can considerably affect wildlife population dynamics by influencing host fecundity and survival, little is known about this group of parasites in the Polish moose populations. The only two previous population studies, based on microscopic observation of eggs, oocysts, and larvae shedding, allowed Kuligowska et al. (2014) and Filip-Hutsch et al. (2020) to detect several species or groups of endoparasites from moose faeces collected in the Polesie National Park and the Biebrza valley (E and NE Poland).

Distinct endoparasite species have different properties regarding localization in a host, pathogenicity, prepatent period, and fecundity, but their eggs, oocysts, larvae or tissues usually are excreted in host faeces. Conventional identification based on macroscopic methods is labour-intensive and time-consuming, and requires specialized expertise due to the overlapping morphological characteristics among different species, and hence may be error-prone (Davidson et al., 2015; Filip-Hutsch et al., 2020). The prevalence and intensity of parasitic infections differ depending on the season and climate conditions. The seasonality of parasite eggs/oocysts/larvae shedding into the environment may strongly affect the results of parasite study, which are based only on the presence of different developmental stages in the faeces. Therefore the number of eggs/oocysts/larvae per 1 g of faeces may not reflect the infection intensity.

DNA-based molecular techniques are more sensitive and less subjective, and allow accurate identification of parasite species from non-invasive faecal sampling of their hosts, which makes them the ideal methods to assess parasite species richness in a natural host population. However, some studies suggest that successful parasite gene amplification with field samples needs an optimized DNA extraction method to lyse oocyst/egg wall and to prevent PCR inhibition of faecal material (e.g. Tang et al., 2018). The objectives of this study were (1) to test whether parasite assessment from faecal samples require a special DNA extraction method or if it can be carried out using DNA obtained from moose faeces during a survey of its population genetic structure, (2) to quantify endoparasite richness and explore sex-biased parasitism using molecular methods for identification of both parasite species and moose individual genotypes from non-invasively collected faecal samples, and (3) to examine parasite association between coinfecting parasite species in the studied moose individuals.

2. Material and methods

2.1. Faeces sampling and molecular analyses

Seventy two fresh samples of moose faeces were collected in winter (from November 2012 to March 2013) in the Biebrza River valley (22°35′E, 53°26′N), North-Eastern Poland. The majority of samples were collected from moose individuals localized by GPS-tracking (25 collared individuals; for details see Borowik et al., 2020). Some samples were also collected from occasionally encountered individuals during field visits. Three droppings were taken from a single heap and frozen at −20 °C to preserve the parasites until molecular identification could take place. DNA was extracted and purified using the DNeasy Blood and Tissue Kit (Qiagen, Germany) from ~0.025 g of faecal samples, according to the manufacturer's instructions (Protocol 1). To avoid pseudo-replication, each faecal sample was examined for 11 microsatellite loci and ~333-bp fragment of the SRY gene in order to genetically identify individuals and their sex (Świsłocka et al., 2013; Świsłocka et al., 2015). The 72 multi-locus microsatellite profiles were assigned to 53 different individuals, 26 males and 27 females using CERVUS 3.0.3 (Kalinowski et al., 2007).

DNA samples were screened for parasite infection using 10 newly designed primer pairs (Primer3 v0.4.0 software; Rozen and Skaletsky, 2000) for molecular identification of tapeworms *Moniezia benedeni* (Cestoda, Anoplocephalidae) and *Echinococcus granulosus* (Taeniidae), the liver fluke *Parafasciolopsis fasciolaemorpha* (Trematoda, Fasciolidae), cerebrospinal nematodes: *Elaphostrongylus alces*, *E. cervi*, and *Parelaphostrongylus tenuis* (Nematoda, Protostrongylidae), lungworms *Dictiocaulus capreolus* and *D. cervi* (Dictiocaulidae), and gastrointestinal nematodes *Ostertagia antipini* and *O. leptospicularis* (Trichostrongylidae; Table S1). The ten parasite species mentioned above were selected based on the criteria of prevalence/uniqueness in the moose and the availability of their gene sequences in the NCBI database, which made it possible to design species-specific primers of molecular identification.

PCRs for each primer pair were carried out in 10 μL volumes, and the reaction mixtures consisted of 2 μL of extracted DNA as a template (~20 ng), 4.5 μL of Qiagen Multiplex PCR Master Mix (1x), 0.9 μL mix of primers and 2.6 μL of Qiagen nuclease-free water. The thermocycling parameters were as follows: initial denaturation step at 95 °C for 15 min and 38 cycles with denaturation at 94 °C for 30 s, annealing at 57 °C for 90 s, extension at 72 °C for 60 s, and final elongation for 30 min at 60 °C. PCRs were performed in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems). PCR products were purified with the Clean-Up kit (A&A Biotechnology) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Unincorporated dideoxynucleotides were eliminated from the sequencing reaction using the ExTerminator Kit (A&A Biotechnology). We carried out detection of sequencing reaction products on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequencing results were edited and aligned using BioEdit (Hall, 1999). To determine parasite species, the DNA sequences were compared to the GenBank references (Table S1) by BLAST (http://www.ncbi,nlm.nih.gov/).

Molecular assays for parasite infection diagnosis using faecal samples are greatly improved through more effective techniques of DNA extraction (Tang et al., 2018). Thus, to evaluate the appropriate quantity of faeces sufficient for the detection of the DNA of parasite species, the second dropping from 53 faecal samples came from 53 different individuals of moose were destined to DNA extraction using Protocol 2 (Supplementary material 1). DNA was extracted using two commercial kits simultaneously: DNeasy Blood and Tissue Kit and QIAamp DNA Stool Mini Kit (Qiagen, Germany) adapted for ~6 g of faecal sample, a single faecal dropping. DNA extracted by Protocol 2 was submitted to the PCR under the conditions described above, using primer pairs designed for identification of the liver fluke *P. fasciolaemorpha* and nematode worms *E. alces* and *E. cervi*. We used two different faecal droppings from each individual to extract DNA according to different protocols. The sample was considered positive if it was tested positive by at least one of the extraction methods mentioned above.

2.2. Statistical analysis

The proportion of infected individuals within the population or sex group (prevalence) and their 95% confidence intervals (CI) were calculated using OpenEpi (http://www.openepi.com/Proportion/Proportion.htm) based on Wilson score interval. The number of parasite species per host (parasite species richness) was also calculated. Relation between prevalence of detected parasite species and an extraction method or host sex was evaluated by Chi-squared test using Quantitative Parasitology 3.0 (Rózsa et al., 2000). Mann-Whitney *U* test and Chi-squared test for trend were conducted to compare parasite species richness between sexes (https://www.medcalc.org). P values smaller

than 0.05 were considered significantly different. Probabilistic model of species co-occurrence (Veech, 2013), implemented in the "cooccur" package 1.3 version (Griffith et al., 2016) of the statistical computing language R (R Core Team, 2015), was applied to detect pairs of parasite species that share hosts more or less frequently than expected in all analyzed host individuals, as well as for males and females independently. Pairs of species with the expected co-occurrence lower than 1 were removed from the analysis.

3. Results

Both extraction protocols have resulted in satisfactory quantities and qualities of genomic DNA for molecular detection of parasites. PCR results indicated that the yield of DNA of the more abundant liver fluke *P. fasciolaemorpha* and rarer worms, e.g. *E. alces* and *E. cervi*, was sufficient regardless of extraction protocols. Prevalence differences between the extraction methods were low and not statistically significant, and ranged from 7.8% for *P. fasciolaemorpha* to 0% for *E. alces* (Table 1).

Overall, 98.1% (52 out of 53, CI: 90.1–99.7) of the samples were positive for at least one parasite species (up to 9 in a single host), and a substantial fraction of moose were infested with 6 or 5 endoparasite species (22.6% and 18.9%, respectively; Fig. 1). The most prevalent were the liver fluke *P. fasciolaemorpha* and gastrointestinal nematodes *Ostertagia* sp. which were identified in over 75% of the tested samples (Fig. 1A). They were the most frequently found parasites both in males and females, and their prevalence did not differ significantly between the sexes. Of the ten endoparasite species, the tapeworm *M. benedeni* was found in 54.7% of the samples (29 out of 53, with CI: 41.4–67.3) and the proportion of infected animals was significantly higher in males than in females (Table 2).

We identified six different haplotypes representing the liver fluke (GenBank accession no. MT889713 – MT889718). The obtained DNA sequences of the other nine endoparasite species did not show any polymorphism and were identical to those deposited in the GenBank database (Table S1).

Of the 27 moose females, 26 (96.3%, CI: 81.7–99.3) were positive for at least one endoparasite species, with the median of 4. All males were positive for at least three endoparasite species (median = 6), with higher parasite richness compared to females ($U = 214$, $P < 0.05$, Fig. 1B). There were differences between sexes in the proportion of individuals infected by different numbers of parasite species (from 1 to 9 species; Chi-squared test for trend, $\chi^2 = 5.65$, $P < 0.05$). The proportion of infected females decreased significantly as the parasite species richness increased (Chi-squared test for trend, $\chi^2 = 11.91$, $P < 0.001$), while this relation was not observed in males (Chi-squared test for trend, $\chi^2 = 1.77$, $P > 0.05$). There were single females, in which 7, 8 and 9 parasite species were found (Fig. 1B).

The analysis of co-occurrence revealed that most of the classifiable parasite species pairs showed random associations, 80.0% (36/45) in all hosts, 88.9% (40/45) in males, and 86.8% (33/38) in females. Nine positive associations between parasite species pairs were found in all hosts, and five in females only. Most of the positive associations were detected for *O. leptospicularis*, four in all hosts (*M. benedeni*, *E. alces*, *D. cervi*, *O. antipini*) and three in females (*P. fasciolaemorpha*, *E. alces*, *O. antipini*), whereas, analysis performed on parasite pairs occurring in moose males indicated three positive and two negative associations. *M. benedeni* – *P. fasciolaemorpha* and *D. cervi* – *E. alces* pairs shared a male host less frequently than expected. A heatmap showing associations between parasite species pairs determined using the probabilistic co-occurrence model is shown in Fig. 2.

Table 1
Prevalence (%) and the 95% confidence intervals (CI) of *Parafaciolopsis fasciolaemorpha* (*Pf*), *Elaphostrongylus alces* (*Ea*) and *E. cervi* (*Ec*) obtained using different extraction methods (Protocol 1 and 2). *P*, the probability value in Chi-squared test. No. of samples = 53.

Species	Protocol 1		Protocol 2		*P*
	No. positive	% (CI)	No. positive	% (CI)	
Pf	27	50.9 (37.9–63.9)	31	58.5 (44.2–71.6)	0.4351
Ea	15	28.3 (18.0–41.6)	15	28.3 (18.0–41.6)	0.9999
Ec	3	5.7 (1.9–15.4)	2	3.8 (1.0–12.8)	0.6468

4. Discussion

Parasitic infections are one of the factors influencing the health status and condition of wild ruminants which often serve as the first link in the spread of parasites in the natural environment (Filip et al., 2017). In many cases, helminth parasites are characterized by subclinical effects, but particularly in overpopulation they are key to bottom-up population control and can cause a decline in population size (Davidson et al., 2015; Filip and Demiaszkiewicz, 2016). Although moose are characterized by relatively high susceptibility to parasitic diseases, studies on parasite species richness in moose population in Poland have been scarce (Filip-Hutsch et al., 2020) when compared with other ungulate species such as the European bison (*Bison bonasus*; Karbowiak et al., 2014a, 2014b; Kołodziej-Sobocińska et al., 2018; Krzysiak et al., 2020) or red deer (*Cervus elaphus*; Pilarczyk et al., 2005; Kowal et al., 2015; Pyziel et al., 2017; Demiaszkiewicz et al., 2018). This study is the first attempt at molecular identification of endoparasite species in one of the densest, non-harvested moose populations in Central Europe (the Biebrza valley, NE Poland).

Many endoparasite species, such as nematodes, cannot be identified with certainty using traditional morphological or morphometric techniques of faecal samples analysis. Adult forms of nematodes or tapeworms collected during animal dissections can be in general identified using morphological features, but the more easily obtainable developmental stages, such as eggs and larvae, are less distinguishable especially to the species level (Tang et al., 2018). DNA-based methods detect past infection events and are not dependent on the parasite stage. Said et al. (2018) showed that molecular tools enabled the detection, for the first time, of five gastrointestinal nematode species in North African wild ruminants, while a microscopic analysis of the faecal samples allowed for the identification of only one genus. Successful amplification of the DNA of three different parasite species (*P. fasciolaemorpha*, *E. alces*, and *E. cervi*) from DNA samples extracted from moose faeces according to different protocols carried out in this study showed that standard DNA extraction and purification method using even a small fragment of a faecal dropping has potential applicability for diagnosis of parasitosis in wild ungulates. The study showed that molecular methods are more effective than microscopic ones, as they determine the occurrence of rare endoparasite species such as *D. capreolus*, which was not recognized in the faecal samples by Filip-Hutsch et al. (2020) by microscopic methods in the analyzed moose population from the Biebrza valley. We were able to identify different species of *Moniezia* sp., *Elaphostrongylus* sp. and *Dictyocaulus* sp., which were usually identified at genus level based on morphological characteristics of their eggs and larvae (Davidson et al., 2015; Filip-Hutsch et al., 2020).

Despite many advantages, the molecular approach to parasitological study for the detection and confirmation of the presence of DNA of specific parasite species in faeces is only a qualitative method, but not quantitative. The presence of a parasite does not necessarily mean a high intensity of the infection. In some cases, the intensity of the infection has a much greater influence on the infected host than the endoparasite species richness (Shemshadi et al., 2017; Allan et al., 2020). On the other hand, greater parasite species richness may have deleterious impact on hosts because of a higher parasitic diversion of resources or cumulative damage to the host tissues due to a greater overall parasite load (Bordes and Morand, 2011). Only full knowledge of the endoparasite species

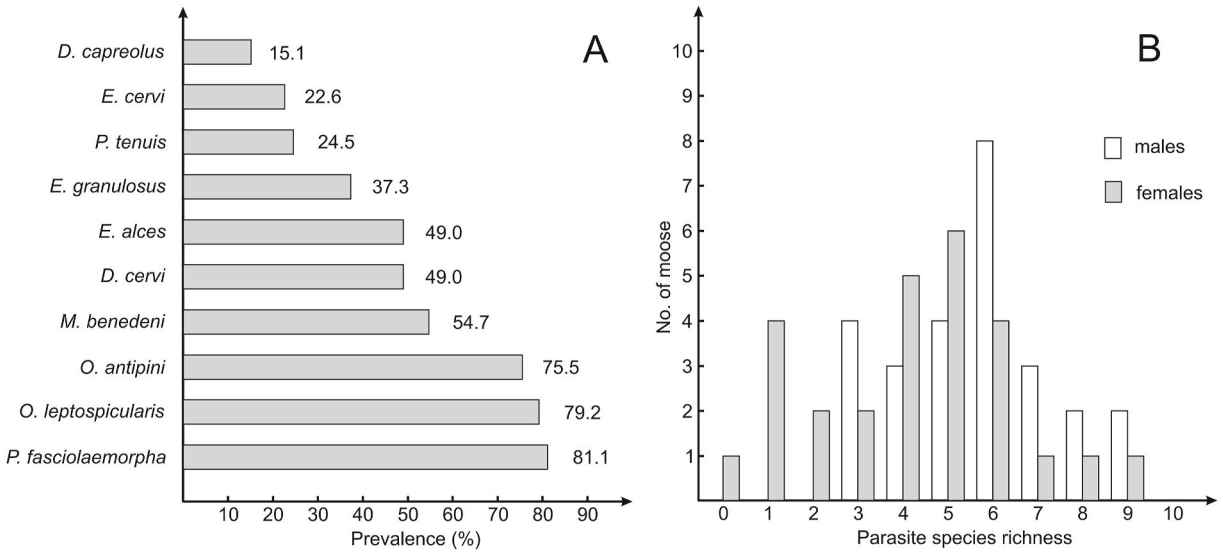

Fig. 1. Prevalence of ten endoparasite species (A) and parasite species richness in males and females (B) diagnosed using molecular methods from faecal samples of 53 moose from Biebrza valley, NE Poland (Central Eastern Europe).

Table 2
Prevalence of endoparasites obtained from molecular analysis of moose faecal samples. CI, 95% confidence interval. A significant difference between sexes in chi-square test is marked in italics.

Species	Males (N = 26)		Females (N = 27)		P value
	No. positive	% (CI)	No. positive	% (CI)	
Moniezia benedeni	18	69.2 (50.0–83.5)	11	40.7 (24.5–59.3)	*0.0372*
Echinoccocus granulosus	12	46.2 (28.8–64.5)	8	29.6 (15.8–48.5)	0.2152
Parafaciolopsis fasciolaemorpha	19	73.1 (53.9–86.3)	24	88.9 (71.9–96.2)	0.1414
Elaphostrongylus alces	16	61.5 (42.5–77.6)	10	37.0 (21.5–55.8)	0.0745
Elaphostrongylus cervi	7	26.9 (13.7–46.1)	5	18.5 (8.2–36.7)	0.5604
Parelaphostrongylus tenuis	9	34.6 (19.4–53.8)	4	14.8 (5.9–32.5)	0.9398
Dictyocaulus capreolus	6	23.1 (11.0–42.0)	2	7.4 (2.0–23.4)	0.1112
Dictyocaulus cervi	16	61.5 (42.5–77.6)	10	37.0 (21.5–55.8)	0.0745
Ostertagia antipini	21	80.8 (62.1–91.5)	19	70.4 (51.5–84.2)	0.3791
Ostertagia leptospicularis	23	88.5 (71.0–96.0)	19	70.4 (51.5–84.2)	0.1045

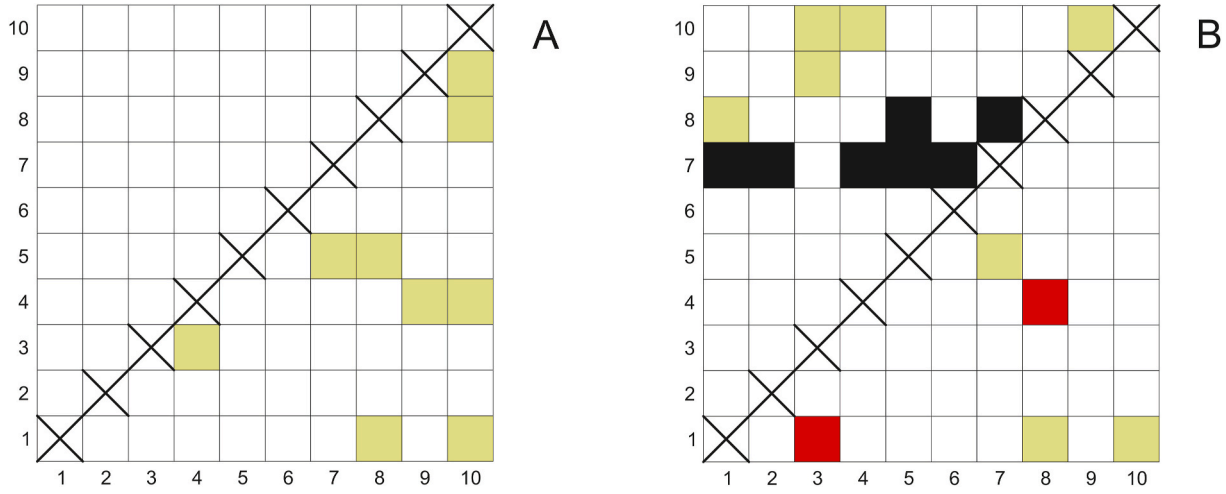

Fig. 2. Heatmap showing associations between pairs of parasite species occurring in (A) all hosts and (B) moose males (below the diagonal) and females (above the diagonal), determined using the probabilistic co-occurrence model. Positive associations in green, negative in red. Species pairs with expected co-occurrence lower than 1 and therefore removed from the analysis are marked in black. Parasite species are listed as follows: 1. *M. benedeni*, 2. *E. granulosus*, 3. *P. fasciolaemorpha*, 4. *E. alces*, 5. *E. cervi*, 6. *P. tenuis*, 7. *D. capreolus*, 8. *D. cervi*, 9. *O. antipini*, 10. *O. leptospicularis*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

richness in combination with the intensity of the infection, gender, and age of infected animals, and the specificity of the host's living environment conditions will give a reliable overview of the health status of studied populations and the risk of transmissions of pathogens to others animals and humans.

Based on molecular analysis, ten endoparasite species in total were detected in 53 moose individuals from the Biebrza valley and these results correspond to the earlier microscopic research, in which eight of them were previously identified in different moose populations (Dróżdż, 1966; Davidson et al., 2015; Filip and Demiaszkiewicz, 2016; Filip-Hutsch et al., 2020). On the other hand, *D. cervi* and *E. cervi*, which are considered to be typical red deer nematodes, were confirmed in the wild moose population for the first time. The red deer has been first reported in the Biebrza valley in the late 1970s (Gębczyńska and Raczyński, 1993). Since then, its population has increased considerably, reaching over 1600 individuals (combined official data from the Biebrza National Park, Rajgród, and Knyszyn State Forests, 2014). The sympatric occurrence of moose and red deer in NE Poland can favor endoparasite transmission between these two cervid species. The detection of *D. cervi* in almost half of the studied moose faeces suggests the invasive nature of this lungworm. On the other hand, the discovery of *E. cervi* in 22.6% of the samples confirmed that moose can become infected with this endoparasite not only under experimental conditions (Stuve and Skorping, 1987), but also in a natural environment.

The most prevalent endoparasite species were *P. fasciolaemorpha* and two gastrointestinal nematodes: *O. leptospicularis* and *O. antipini*, both in moose males and females. Moose is a typical definitive host for *P. fasciolaemorpha*. The geographical range of this species of trematodes covers a large part of central and eastern Europe (Davidson et al., 2015; Filip-Hutsch et al., 2020). This parasite commonly occurs in Polish moose populations, often with extreme invasions reaching 100% of the population (Filip et al., 2016; Filip-Hutsch et al., 2020). Over three quarters of the moose individuals from the study area were infected with nematodes *O. leptospicularis* and *O. antipini*, confirming the hypothesis that these specialist parasites occur wherever moose is present, and form the associated parasite-host system with moose (Filip and Demiaszkiewicz, 2016). The lowest prevalence was assessed for lungworm *D. capreolus* (15.1%), although the roe deer is the most abundant wild cervid species in Poland and in Europe. However, over 3000 roe deer individuals in the Biebrza valley (combined official data from the Biebrza National Park, Rajgród, and Knyszyn State Forests, 2014) could be a significant reservoir for transmission of *D. capreolus*. Further study is needed to assess prevalence and intensity of infection of this parasite species in roe deer in the study area.

In our molecular analysis, tapeworms had intermediate levels of prevalence. The *Moniezia* genus was earlier reported in a single moose individuals in Poland, in particular in young individuals from the Kampinos Forest (Filip and Demiaszkiewicz, 2016). Prevalence of 54.7% for *M. benedeni* found in the Biebrza valley moose population seems to be very high, especially when we compare this result with data from Norway (15,6% for *Moniezia* sp.; Davidson et al., 2015) or Canada (13% for *Moniezia expansa*; Hoeve et al., 1988). Interestingly, *M. benedeni* infection at a level of 28% was found beyond the eastern border of Poland, in moose populations in Belarus (Shimalov and Shimalov, 2003). In a study by Davidson et al. (2015), all individuals infected with *Moniezia* sp. were moose calves in Scandinavia, and the prevalence of this parasite reached a level of 78% in this age class. The differences in *M. benedeni* detection in moose populations may be related to the lifecycle of this parasite species, which needs higher temperatures for further development (Narsapur and Prokopic, 1979; Filip- Hutsch et al., 2020). Due to the seasonality in *Moniezia* sp. infection, differences in prevalence might be also due to timing of the faecal sampling. The second parasite species from the family Cestoda, *E. granulosus*, is a common parasite of wild animals and very dangerous for humans. It was found in the moose from the Biebrza valley with a prevalence of 37.3%. It is worth noting that the larval forms of *E. granulosus* were previously found in 60% of studied moose from the Biebrza valley, when using microscopic methods (Dróżdż, 1966). One of the definitive hosts for this tapeworm is wolf *Canis lupus* (Filip and Demiaszkiewicz, 2016). The growing population of wolves in Poland, resulting from effective protection since 1998 (Niedziałkowski and Putkowska-Smoter, 2020), may lead to an increase of *E. granulosus* infection in moose and other wild ruminants as intermediate hosts, required for the development of eggs to the larval stages (e.g. oncosphere).

Common measures of sex-biased parasite abundance are mean intensity of infection and prevalence. Gender related trends were seen in intensity of nematode infection in Norwegian moose where females had higher mean abomasal nematode counts than males, but adult males had higher faecal egg counts than adult females (Davidson et al., 2015). Filip-Hutsch et al. (2020) noted that the prevalence of *Trichuris* spp., *Varestrongylus* sp., *Dictyocaulus* sp., and *Eimeria alces* in moose populations from the Biebrza valley was significantly higher in males than females based on microscopic methods. Molecular methods used in this study did not confirm this relationship for species from genus *Dictyocaulus* (species from the other genera mentioned above were not analyzed). However molecular analysis showed an over three times higher prevalence in comparison to traditional techniques. *M. benedeni* was the only one out of the ten parasite species, whose prevalence was significantly related to the sex of moose individuals.

Apart from the prevalence of endoparasite species and endoparasites' diversity in moose, we were able to estimate parasite associations between coinfecting parasite species in the Biebrza valley population. Parasite associations tended to be generally random for the whole studied population and for males and females separately, but we found some evidence for both positive and negative associations. Positive associations indicate that correlated exposure or unmeasured host trait variation may affect encounter and transmission of these parasite species (Poulin, 2013). Maizels et al. (2004) noted that helminth parasites may suppress the immune functions of the host, potentially promoting subsequent infections by other parasite species. Two pairs of endoparasite species coinfecting males were strongly negatively associated, which could indicate the possibility of parasite competition for space or resources, potentially modulated through the host immune response (Ulrich and Schmid-Hempel, 2012; Beechler et al., 2017; Dallas et al., 2019). Parasite associations are conditional on covariates related to geography, season, and host traits. Different timings of infections may result in an infection by one parasite species leading to an increased immune response of the host, which can protect against effective infection by another parasite species (Dallas et al., 2019).

This study shows that the prevalence of most of the endoparasite species studied was very high in moose in the Biebrza valley. Additionally, the majority of individuals were infected with several parasite species, and males had significantly higher parasite species richness than females. High population density exceeding 1.5 ind/km^2 (Raczyński and Ratkiewicz, 2011) and a significant percentage of migratory individuals within the study area (Borowik et al., 2020) may promote the spread of parasitic infections and high level of parasitism in the population from the Biebrza valley. Management of this genetically distinct (Świsłocka et al., 2008; Świsłocka et al., 2020) and non-harvested for 20 years moose population should include health monitoring programs with investigations of parasite status. Furthermore, our research proved that molecular methods can be extremely useful for parasite species identification from non-invasively collected faecal samples of moose and other cervid species.

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Declaration of competing interest

The authors declare that they have 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.2020.10.008.

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