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## An evaluation of two potential risk factors, MHC diversity and host density, for infection by an invasive nematode *Ashworthius sidemi* in endangered European bison (*Bison bonasus*)

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### ABSTRACT

Parasites are thought to increase the risk of host extinction, but their dynamics in endangered species have not been well investigated. The free-living European bison population in Białowieża Forest has recently been massively invaded by a blood-sucking nematode, *Ashworthius sidemi*. This nematode originated in Asia and was probably transmitted to Europe with the introduction of the sika deer. Here, we investigate the impacts of genetic factors (the Major Histocompatibility Complex class II genes responsible for extracellular parasite recognition) and management practices (supplementary feeding affecting winter population density) on the intensity of *A. sidemi* infections in bison. All but two out of 110 animals investigated between 2005 and 2009 were infected with *A. sidemi*, and the intensity of infection increased significantly with time. Due to a severe population bottleneck experienced by the bison, only four class II DRB alleles are retained in the Białowieża population. We found that despite high sequence divergence, neither any of the alleles nor DRB heterozygosity was significantly associated with infection intensity. We did find, however, that winter density of bison herds was positively associated with infection intensity. Winter bison population densities were in turn predicted by the intensity of supplementary feeding.

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### 1. Introduction

The roles of parasites (*sensu lato*, including microbial pathogens) as causal factors in the extinction of species are poorly understood due to limitations in existing evidence (Smith et al., 2006). Nonetheless, in currently threatened species, parasites are likely to increase extinction risk by interacting with other factors, such as habitat loss or climate change (Smith et al., 2009). Emerging diseases and invasive parasites may be of particular importance in this respect (Daszak et al., 2000; Jones et al., 2008).

The spread of parasites is likely to be contingent both on population parameters such as population density and on genetic factors that determine susceptibility to infection. A prominent gene class affecting resistance to parasites are Major Histocompatibility Complex (MHC) genes. These genes code for MHC proteins, which bind parasite-derived antigens and present them to lymphocytes, thus initiating the adaptive immune response (Janeway et al., 2004). The high polymorphism of MHC genes, unrivalled by any

other vertebrate genes, is thought to result from an evolutionary arms-race with rapidly evolving parasites (Bernatchez and Landry, 2003; Sommer, 2005; Piertney and Oliver, 2006).

Genetic variation is much reduced in endangered species, and this is also the case with MHC genes (reviewed in Radwan et al., 2010). It has been suggested that reduction in MHC variation may make endangered species particularly susceptible to infection, especially with emerging pathogens (O'Brien and Evermann, 1988; Hughes, 1991). It is possible that MHC variants capable of presenting antigens of a given pathogen may no longer be present in the threatened population, making endangered species more susceptible to extinction. Furthermore, evolution of parasite adaptations allowing them to escape presentation by hosts should be easier if the number of MHC variants existing in a population is low.

On the other hand, for many parasites, transmission depends on host population density. As the population densities of threatened species are often low, such species may escape pressure from parasites, which can then be expected to suffer extinction as a consequence of low transmission rates (McCallum et al., 2001). However, while population density has been shown to correlate with parasite abundance across species (Arneberg et al., 1998; Nunn et al.,

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2003), the evidence for density-dependent parasite transmission is still limited (Greer et al., 2008; Smith et al., 2009 and references therein).

Here, we investigate the effects of the MHC II DRB genotype and the population density of European bison *Bison bonasus* during winter on the intensity of infection with an invasive nematode *Ashworthius sidemi* (Trichostrongylidae). European bison became extinct from its natural habitats in the 20th century, but a population was successfully re-established in Białowieża Forest (in Poland) from animals that had survived in captivity (Pucek, 1991). This lowland subspecies of bison was restored via intensive captive breeding and a series of re-introductions, and species numbers now consist of about 1600 free-ranging individuals distributed between 12 populations over the territories of Poland, Belarus, and Lithuania (Pucek et al., 2004); European bison Pedigree Book, 2008). The IUCN Redlist considers European bison to be a vulnerable species (IUCN, 2009). Infections have been listed in the IUCN Conservation Action Plan as one of the most important actual and potential threats to the European bison population (Pucek et al., 2004).

The population in Białowieża Forest (which is located in both in Poland and Belarus) consists of about 820 individuals that have been separated since 1981 by a border fence; about 460 bison now live on the Polish side of this fence. However, this population is derived from just seven ancestors and consequently harbours little genetic variation (Pertoldi et al., 2009; Tokarska et al., 2009a,b; Wójcik et al., 2009). Depletion of genetic variation has also affected the MHC DRB locus: only four alleles are now found in the Białowieża population (Radwan et al., 2007) compared to over 105 alleles found in domesticated cattle (<http://www.ebi.ac.uk/ipd/mhc/bola/index.html>) and 15 alleles found in American bison (*Bison bison*), which has also experienced a bottleneck, although a less extreme one than the European bison (Traul et al., 2005).

The European Bison has recently been reported to suffer from an increasing rate of infection with the blood-sucking helminth *A. sidemi*. The parasite is typically found in Asiatic deer (especially *Cervus nippon*) and was spread to Poland by infected red deer migrating from neighbouring countries (Drózdź et al., 1998). The first infected bison were noted in 1999 in the Belarussian part of the Białowieża Forest (Kochko, 2003), then, in 2000 in the Polish part of the Forest (Drózdź et al., 2003). Since, the nematode has spread rapidly, significantly reducing the occurrence of other gut nematodes previously found in the bison (Demiaszkiewicz et al., 2008). The infection causes pathological changes such as an oedema, hyperaemia and effusion in the abomasum and duodenum mucosa. These pathologies are most clearly seen in highly infected calves, and they can lead to chronic diarrhoea, deterioration and death of the young animals (Demiaszkiewicz et al., 2009).

MHC class II genes, such as the highly polymorphic DRB gene, are responsible for recognition of antigens from extracellular parasites, including macroparasites. Associations between DRB genotypes and susceptibility to helminth infections have been reported (e.g. Paterson et al., 1998; Froeschke and Sommer, 2005; Harf and Sommer, 2005; Meyer-Lucht and Sommer, 2005; Kloch et al., 2010). While there are only four alleles of MHC DRB3 in the Białowieża population, the alleles are highly divergent. According to Hedrick (2003), high divergence may be maintained in endangered species by heterozygote advantage, where possessing two highly dissimilar alleles increases the probability that at least one of them will bind a parasite-derived antigen. We tested two predictions following from this reasoning: that heterozygotes will be less infected than homozygotes, and that infection intensity will be associated with possessing particular MHC DRB3 alleles. DRB3 locus was studied because it is the only widely expressed DRB locus in cattle, and is the most polymorphic class II gene in cattle and other ruminants (Mikko et al., 1999). We examined variation at exon 2 of the DRB3 gene, which

is the most polymorphic exon coding the structure of the antigen binding groove of the MHC molecule.

Since its re-introduction to Białowieża Forest, the bison herd was provided with supplementary food (mainly hay) during the winter. Apart from improving survival of the bison, this practice is aimed at reducing potential damage to the stands of tree and crops and at mitigating the dispersion of the population out of the Białowieża Forest. The hay is amassed in five main, and several smaller, feeding stations through the summer, and supplied until late March. This supplementary feeding practice effectively aggregates bison herds near the feeding stations from late October to March (Krasińska and Krasiński, 2007). Intensively fed herds (food is delivered to the feeding sites 3–5 times a week) create large aggregations of 56–104 individuals that winter in a relatively small area of 1.1–6.7 km<sup>2</sup> around the feeding sites. Less intensively fed or unfed bison, in contrast, cover larger home ranges (1.6–15.5 km<sup>2</sup> and 7.9–52.3, respectively) and stay in smaller herds (29–59 individuals). Because nematode eggs are found in bison faeces year round (A. Pyziel, R. Kowalczyk and A. Demiaszkiewicz, unpublished), increased herd density during winter aggregations may facilitate transmission of *A. sidemi*, which occurs by ingesting an invasive larval stage. Therefore, we investigated the following two relationships: that between feeding intensity and winter aggregation density, and that between aggregation density and the intensity of infection with *A. sidemi*.

## 2. Methods

We analysed 110 individuals culled by the National Park staff in Białowieża Primeval Forest, north-eastern Poland, between 2005 and 2009. The bison carcasses were necropsied within 1–2 h after death. All of the abomasa and duodena were completely examined for worms using the sedimentation method, leaving the sediment containing worms after remaining stomach content have been washed away. The sediment was preserved in 2–3% formalin. Collected nematodes were conserved in 75% ethyl alcohol with the addition of 5% glycerol. After evaporation of alcohol, non-permanent preparations were made in glycerol of all the nematodes for species identification (Drózdź et al., 1998). Intensity of infection of each individual with *A. sidemi* was determined by counting all of the extracted nematodes under a microscope.

DNA was extracted from blood and tissue samples using the Qiagen extraction kit according to the manufacturer's protocol. Genotyping methods followed those described in Radwan et al. (2007). Briefly, a 236 bp (excluding primers) fragment of the 2nd exon (covering >90% of the total exon length) of the DRB3 MHC gene was amplified using HLO30 and HLO32 primers labelled with the fluorochromes FAM and HEX. Genotyping was performed using the SSCP (Single Strand Conformation Polymorphisms) method on an ABI 3130xl genetic analyser.

Winter density for herds forming separate winter aggregations was calculated on the basis of herd size and winter home range. Herd size was calculated as the average number of bison aggregating in winter at feeding sites or, in the case of non-fed bison, the largest observed number of bison roaming together (Mysterud et al., 2007). Winter home range size was the average area (for winters 2005–2009) covered by radio-collared bison (from 1 to 3 individuals per herd) between December 1st and February 28th. Winter ranges were calculated as the Minimum Convex Polygon with 100% of localizations.

Data on the intensity of winter feeding were obtained from the managers of the national park. Three levels of feeding intensity were used in the analyses: high intensity (3–5 times a week), low intensity (1–2 times a week) and no feeding.

Conformance of the allele frequencies with Hardy–Weinberg expectations was tested using the complete enumeration

algorithm of Louis & Dempster (1987) as implemented in Genepop 4 (Rousset 2008).

We expected that the intensity of infection should be affected by population density, and not directly by supplementary feeding. Supplementary feeding, in turn, was predicted to have a significant effect on density. Therefore, we first analysed the effect of supplementary feeding on density, and then, in a separate model, we investigated the effect of density (along with other factors, see below) on infection intensity. As we predicted a positive effect of the intensity of feeding on winter density, the association between these variables was tested as a linear contrast within one-way ANOVA. The number of *A. sidemi* was Box-Cox transformed  $((y^{0.23} - 1)/0.23)$  for statistical analyses to achieve normality of distribution (untransformed means and standard deviations were presented in figures for ease of interpretation). The impact of MHC genotype and winter density on the intensity of infection was assessed using a general linear model, with alleles and sex as factors, and age, year and winter density as covariates. As winter density or year could conceivably impact the way genotype, sex or age respond to infection with *A. sidemi*, we also initially included interactions with these variables into our models. Non-significant interactions ( $P > 0.2$ ) were sequentially removed, and none was retained in the final model. To estimate effect sizes we computed partial eta squared ( $\eta_p^2$ ) (Cohen 1973).

In another model, the effect of MHC genotype, instead of effects of MHC alleles, was tested (with remaining variables as above). In a third model, we also tested the general impact of MHC heterozygosity, i.e. all heterozygotes were pooled into one level, and homozygotes into another level.

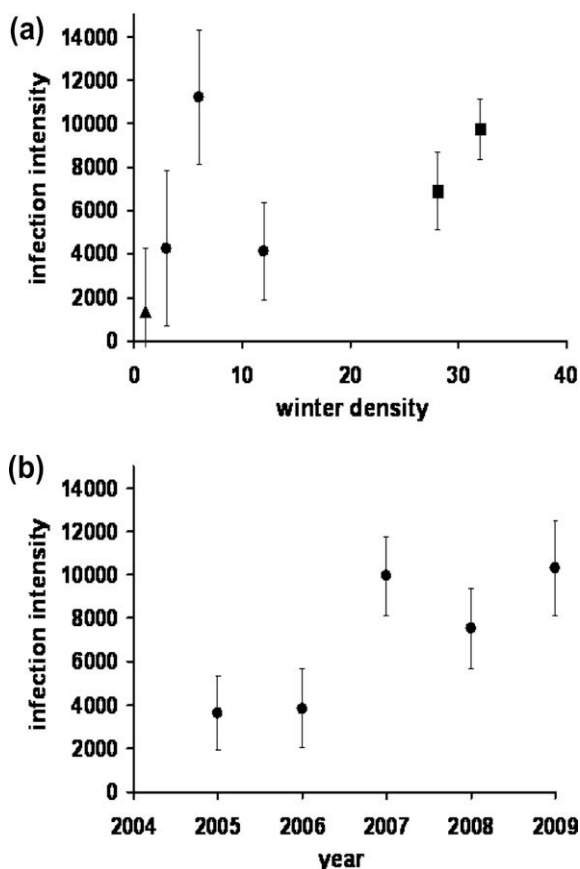


Fig. 1. The impact of winter aggregation density of bison (a) and time (b) on the intensity of *A. sidemi* infection (mean  $\pm$  SE number of parasites) in European bison in Białowieża Forest. Symbols in (a): squares, high feeding intensity; circles, medium feeding intensity; triangle, no feeding.

Table 1

The result of a general linear model predicting influence of different factors on the intensity of *A. sidemi* infection in the European bison in Białowieża Forest (df, degrees of freedom; MS, mean square;  $F$ ,  $F$ -value;  $p$ ,  $p$ -value;  $\eta_p^2$ , estimates effect sizes).

Factor	df	MS	$F$	$p$	$\eta_p^2$
<b>Bibo-DRB3*0101</b>	1	2.1	0.02	0.881	0.009
<b>Bibo-DRB3*0201</b>	1	20.6	0.21	0.642	0.000
<b>Bibo-DRB3*0301</b>	1	76.3	0.80	0.373	0.002
<b>Bibo-DRB3*0401</b>	1	0.6	0.01	0.934	0.001
Sex	1	155.4	1.63	0.204	0.018
Time (year)	1	2382.5	25.00	0.001	0.219
Age	1	367.1	3.85	0.052	0.041
Host density	1	723.6	7.59	0.007	0.078
Error	89	95.2			

### 3. Results

We found a positive effect of feeding intensity on the densities of winter groupings ( $F_{1,3} = 30.9$ ,  $P = 0.009$ ). Mean winter density ranged from 1.3 individuals/km<sup>2</sup> in non-fed bison to 7.5 individuals/km<sup>2</sup> for medium-intensity fed and 29.8 individuals/km<sup>2</sup> for high-intensity fed bison.

The vast majority (98.2%) of animals examined were infected with *A. sidemi* (25 of 26 sampled in 2005, 22/23 in 2006, 23/23 in 2007, 22/22 in 2008, 16/16 in 2009). Infection intensities ranged from 1 to 44310 worms per individual. The proportions of **Bibo-DRB3\*0101**, **Bibo-DRB3\*0102**, **Bibo-DRB3\*0103** and **Bibo-DRB3\*0104** loci in a population were, respectively, 0.28, 0.36, 0.31 and 0.04. Genotype frequencies were: \*0101 \*0101, 0.114; \*0101 \*0102, 0.281; \*0101 \*0103, 0.167; \*0101 \*0104, 0.035; \*0102 \*0102, 0.088; \*0102 \*0103, 0.167; \*0102 \*0104, 0.009; \*0103 \*0103, 0.105 and \*0103 \*0104, 0.035. No departures from Hardy–Weinberg expectations were detected ( $P = 0.42$ ).

A general linear model revealed that infection intensity increased with time and with the density of winter aggregations of the European bison (Fig. 1, Table 1). There was also a marginally non-significant, positive effect of age (partial  $r = 0.20$ ). However, the effects of MHC alleles and sex were not significant. An alternative model (not presented), taking into account genotypes instead of alleles, led to identical conclusions (genotype effect  $F_{1,85} = 0.48$ ,  $P = 0.86$ ,  $\eta_p^2 = 0.004$ ). Also the third model, with MHC heterozygosity used as an explanatory variable instead of particular MHC alleles, showed significant effects of time and density (not presented), but not of MHC heterozygosity on infection intensity ( $F_{1,92} = 0.002$ ,  $P = 0.963$ ,  $\eta_p^2 < 0.0001$ ).

### 4. Discussion

The dramatic increase in the prevalence and intensity of infection of the invasive nematode *A. sidemi* is a growing concern for the free-living European bison population studied here. Firstly, the intensities of infection *A. sidemi* are now an order of magnitude higher (up to 44310 worms/individual in 2009) than intensities of infections with native parasites recorded before the appearance of *A. sidemi* (maximum intensities of infection with most common gastrointestinal parasites were 377 worms/individual for *Ostertagia ostertagi*, 666 for *Ostertagia leptospicularis*, 229 for *Ostertagia kolchida*, 1334 for *Cooperia oncophora*, 1027 for *Nematodirus helvetianus* and 871 for *Aonchoteca bilobata*, Drózdź et al., 2002). Secondly, native nematodes are smaller in size and none of these species is blood-sucking. Not surprisingly, histopathological changes in abomasa and duodena observed in individuals infected with *A. sidemi* were much more drastic than effects of infestation with native parasites observed earlier (Osińska et al., 2010).

While the first case of infection with this helminth was noted in the Polish part of the Białowieża Forest in 2000 in a single bison,

almost all individuals investigated since 2004 have been infected. Here, we find that the infection intensity has continually increased since 2005. One reason for the rapid spread of the nematode in the bison population may be the limited MHC diversity observed in this bison species, with alleles capable of presenting nematode-derived antigens to T-cells possibly lost during the population bottleneck. However, as suggested by Hedrick (2003), high divergence of the retained alleles may have increased the chance that at least one of these alleles will be capable of recognizing parasite-derived antigens. This suggestion was not supported by our data, as none of the four highly divergent alleles occurring in European bison (amino-acid differences among the alleles ranging from 15.2% to 21.7%) was associated with the intensity of infection with *A. sidemi*. Similarly, Radwan et al. (2007) found no association between MHC DRB alleles or DRB heterozygosity and susceptibility of bison males to *posthitis*, a disease affecting genitalia, possibly caused by bacteria (Jakob et al., 2000; Lehnen et al., 2006). Both current and earlier results thus suggest that despite their divergence, alleles retained in the Białowieża population may have a limited capacity to present antigens from some parasites to the immune system. Alternatively, susceptibility to infection may not be due to the failure of MHC to bind antigens, but to the malfunction of some other element of the immune response, resulting in bison being particularly prone to infections.

We have found, however, a highly significant effect of bison density during winter aggregations on *A. sidemi* infection intensity. Similarly, territoriality and gregariousness of hosts were reported to be associated with heavier parasite loads (strongylid nematodes and coccidia) in African bovids (Ezenwa, 2004). Habitat fragmentation, leading to increased local host densities, was associated with elevated prevalence of gastrointestinal parasites in two species of threatened forest primates (Mborah and McPeck, 2009).

Further, the aggregation density of these bison in winter was, in turn, dependent on supplementary feeding intensity. Thus, the management practice meant to help to maintain this bison population in fact mediates high levels of parasite infection. Further research is needed to identify the mechanism underlying this association. It seems likely, though, that a high population density facilitates parasite transmission due to repeated defecation of bison at fixed locations, combined with sedentary behaviour enhanced by supplementary feeding. Indeed, nematode eggs were found in faeces collected during the formation of winter aggregations: of 24 scats collected each month and examined for the presence of eggs, 3 were positive in September (12%), 1 in October (4%), 2 in November (8%), 4 in December (17%), 8 in January (33%), 9 in February (37%) and 22 in March (92%) (Pyziel, Kowalczyk, Demiaszkiewicz, unpublished data). Although we have no data on survival of *A. sidemi* eggs and larvae in field, infective larvae of other trichostrongylid nematodes may survive at low temperatures in winter (Troell et al., 2005). In the bison population studied here, hay is provided in fixed locations, directly on the forest floor. Thus, forming long-lasting winter aggregations may significantly facilitate the spread of parasites among bison. In racoon dogs, directly transmitted nematode parasites become more abundant after host density was locally increased by supplementary feeding (Wright and Gompper, 2005). Another possibility is that high population density is stressful for the bison. Social stress has been shown to cause immune suppression in some species (Bartolomucci, 2007). Additionally, as parasites are known to manipulate their host behaviour (Lefevre et al., 2009), parasitized individuals may show higher gregariousness, facilitating parasite transmission.

The argument that parasites are unlikely to cause extinction relies on the assumption that parasite transmission rates decrease with decreasing host density, often leading to parasite extinction (McCallum et al., 2001). Indeed, richness of parasites was reported to be lower in threatened primate species compared to non-threa-

tened ones (Altizer et al., 2007). However, parasites may re-emerge as a significant threat to endangered populations whose numbers and densities have increased due to successful conservation programs. Our data highlight this possibility. In particular, supplementary feeding during winter is likely to break-down the natural density-dependence of parasite transmission: regardless of how large the natural range of the herd is, all its members will aggregate in winter in relatively small areas where food is supplied, facilitating transmission. Management practices should be aimed at decreasing the intensity and prevalence of nematode infections. Reduced and highly dispersed winter feeding should result in higher herd mobility, larger winter ranges for the bison and, consequently, lower intensities of infection.

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