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Contrasting patterns of structural host specificity of two species of *Heligmosomoides* nematodes in sympatric rodents

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Abstract Host specificity is a fundamental property of parasites. Whereas most studies focus on measures of specificity on host range, only few studies have considered quantitative aspects such as infection intensity or prevalence. The relative importance of these quantitative aspects is still unclear, mainly because of methodological constraints, yet central to a precise assessment of host specificity. Here, we assessed simultaneously two quantitative measures of host specificity of *Heligmosomoides glareoli* and *Heligmosomoides polygyrus polygyrus* infections in sympatric rodent hosts. We used standard morphological techniques as well as real-time quantitative PCR and sequencing of the rDNA ITS2 fragment to analyse parasite infection via faecal sample remains. Although both parasite species are thought to be strictly species-specific, we found morphologically and molecularly validated co- and cross-infections. We also detected contrasting patterns within and between host species with regard to specificity for prevalence and intensity of infection. *H. glareoli* intensities were twofold higher in bank voles than in yellow-necked mice, but prevalence did not differ significantly between species (33 vs. 18 %). We found the opposite pattern in *H. polygyrus* infections with similar intensity levels between host species but significantly higher prevalence in mouse hosts (56 vs. 10 %). Detection rates were higher with molecular tools than morphological methods. Our results emphasize the necessity to consider quantitative aspects of specificity for a full view of a parasites' capacity to replicate and transmit in hosts and present a worked example of how

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modern molecular tools help to advance our understanding of selective forces in host-parasite ecology and evolution.

Introduction

Host specificity is one of the most important properties of parasite taxa with consequences for epidemiology, virulence evolution and host-parasite co-evolution (Poulin 2011; Woolhouse et al. 2001). Traditionally, host specificity is assessed as the number of host species used by a certain parasite species (Poulin et al. 2011). Studies using this approach show that host ranges can vary drastically between parasite species: some parasites are able to infect a wide range of different hosts, whilst others are specialists that only make use of a limited number of host species (Malenke et al. 2009; Pedersen et al. 2005; Rausch 1957).

Measurements of host specificity go well beyond the mere count of the number of host species used by a parasite, and it is useful to distinguish host range and host specificity (Rohde and Rohde 2008). In addition to information on the number of different hosts used, host specificity uses information on the relatedness of host species (Poulin and Mouillot 2003), the consistency in host use across a parasite's geographic range (Krasnov et al. 2011) and most importantly the quantitative distributions of a parasite across host species (*structural specificity*) (Poulin et al. 2011). Structural specificity itself combines two quantitative measures: the likelihood of infection in different host species (i.e. prevalence) and the ability to replicate in and spread from different hosts (i.e. infection intensity) (Lymbery 1989). We will refer to these particular two aspects as specificity for prevalence and for infection intensity, respectively. As a particular parasite may have equal

prevalence in two host species but differ in growth rate or fecundity so that one of the host species is much more important for transmission than another (high specificity for infection intensity), it is important to consider both aspects to obtain a correct measure of structural host specificity (Poulin et al. 2011).

Helminth infections in vertebrate hosts are commonly used models in host-parasite specificity studies. Whilst the majority of previous papers have focused on prevalence of helminth infection in hosts (Poulin 2007; Poulin et al. 2011; Schmid-Hempel 2011) both aspects of structural specificity have rarely been considered simultaneously (but see Marques et al. 2011; Muñoz et al. 2006). In addition, molecular advances suggest that helminth host specificity is underestimated when using morphological methods only, as they are characterized by low levels of resolution and detection (Bickford et al. 2007; Hung et al. 1999; Poulin and Leung 2010). This is particularly true for non-invasive studies of helminth infections where the identification of a parasite morpho-species is based on the identification of parasite eggs and larvae retrieved from faeces only (Zajac and Conboy 2006). Best results in taxonomic classification are achieved when adult worms are available for species identification, which is usually not the case in non-invasive studies.

Notwithstanding, non-invasive methods are indispensible for the majority of evolutionary and ecological studies, and morphological methods are continuously used as standard method to identify intensity levels of helminth infection. Real-time quantitative PCR (qPCR) is more and more replacing microscopic identification of infections and ameliorating the specificity of quantification (Gordon et al. 2011). In recent publications, copro-qPCR, the molecular detection of gastrointestinal parasites via parasite eggs shed in host faeces, proved to be a sensitive and specific method. Its usefulness has been demonstrated for example in the assessment of *Strongyloides stercoralis* infections using human stool samples (Verweij et al. 2009) or the detection of *Schistosoma japonicum* infections in water buffaloes (Wu et al. 2010). In both examples, authors found a fold detection rate of faecal eggs as compared to results from morphological studies.

Heligmosomoides polygyrus is a trichostrongyle nematode parasite, which is common in small mammals (Baylis 1926; Behnke et al. 1991; Lewis 1987). Due to its easy maintenance as well as due to the fact that it establishes chronic infection in different strains of mice, the laboratory subspecies strain *H. polygyrus bakeri* has also become an important model in studies on helminth infection under laboratory conditions (see Behnke and Harris 2010; Maizels et al. 2011 for details on nomenclature). In natural populations, the sub-species *H. polygyrus polygyrus* is a dominant helminth in European mice of the genus *Apodemus* sp. (Baylis 1926; Klimpel et al. 2007; Lewis 1987). The closely related *Heligmosomoides glareoli* has its main host in bank voles *Myodes glareolus*

(Baylis 1928; Biserkov 1998; Haukisalmi and Henttonen 2000). A few reports suggest that infections are not exclusively restricted to these hosts and that cross-infections between host species may occur occasionally. Meszaros (1978) and Murai et al. (1992), for example, reported independent records of adult *H. p. polygyrus* in bank voles, suggesting that this nematode can actually also infect and develop to maturity in microtine hosts. Nonetheless, these two closely related nematode species are generally considered host specific (Quinnell et al. 1991).

Molecular identification and discrimination of *Heligmosomoides* species are possible with primers targeting the internal transcribed spacers (ITS1 and ITS2) of the ribosomal DNA or the mitochondrial cytochrome c oxidase I (COI) gene using adult worms as DNA source (Cable et al. 2006). With reference sequences from *Heligmosomoides* species and subspecies as well as the methodology to extract DNA from faecal parasite eggs being available (Gasser et al. 1993; Verweij et al. 2009), all essential information is on hand, yet we are not aware of a previous study on molecular identification of *Heligmosomoides* infections based on faecal samples.

The aim of our study was to assess specificity for prevalence and intensity of nematode infections in two sympatric rodent species. Due to the ecological and experimental importance of the parasite, we focussed our study on *Heligmosomoides* infections. By using a combination of live-trapping and both non-invasive morphological and molecular parasite identification, we provide evidence for the occurrence of both parasite species in sympatrically occurring yellow-necked mouse and bank vole populations. We compare morphological and molecular prevalence data and present a species-specific molecular protocol to easily identify and quantify infections. Based on this dataset, we tested the hypotheses that the two aspects of structural specificity, i.e. specificity for prevalence and infection intensity, differ between the two closely related nematode species with regard to their preferred host species.

Material and methods

Rodent sampling and parasitological screening

Rodent hosts were caught with live traps (Ugglan special, Grahn AB, Sweden) at three sites at Revingehed, Southern Sweden, in April–May 2012 (55° 41′ 42.2″ N 13° 26′ 50.1″ E). The habitat of the three sites is characterized by moist deciduous woodland (Kalvs mosse), open moist meadows (Silvåkrakärret) and dry deciduous forest (Ekskogen). The most common rodent species in the area are yellow-necked mice (*Apodemus flavicollis*; YNM) and bank voles (*M. glareolus*; BV) (Hellgren et al. 2011). We focused the

study on these two host species. Traps were baited with grains and carrots. Trapped rodents were sexed, weighed and transferred to individual cages at Stensoffa research station (Lund University), in order to obtain fresh, non-contaminated faecal samples. Animals were provided with food and water ad libitum and released after one night at their original point of capture. Faecal samples were collected from cages and split into two aliquots which were then stored in 10 % neutral buffered formalin for morphological analyses or frozen at −20 °C for molecular analyses, respectively. To obtain control DNA from adult worms, seven bank voles and five yellow-necked mice were dissected. Small intestines were screened for the presence of *Heligmosomoides* sp., microscopically identified and stored in 70 % EtOH for later DNA extraction.

Morphological identification and quantification of parasite infection

For morphological analyses, faecal samples from 46 bank voles and 29 yellow-necked mice were processed with a modified form of the formalin-ethyl-acetate sedimentation as previously described by Clough (2010). Wet mounts were prepared with 20-mg faecal sediment, analysing individual samples for *Heligmosomoides sp.* prevalence (presence/absence) and infection intensity (egg output per gram faeces) (following definitions from Bush et al. 1997). Earlier experimental studies suggested that *Heligmosomoides* infections estimates of the number of eggs per gram faeces are directly proportionally correlated to the number of parasites present in the intestines (Keymer and Hiorns 1986). In addition, adult worms retrieved from both voles and mice were identified morphologically based on original species descriptions from Baylis (1926, 1927, 1928) and Biserkov (1998) to validate molecular species identification (see below).

DNA extraction from individual worms and species identification

DNA was extracted from 36 *Heligmosomoides* adult worms retrieved from dissections following the protocol of Laird et al. (1991). Worms of each species were pooled in groups of five to ten in order to increase the amount of DNA per sample. Extracted DNA was dissolved in 50 μl ddH$_2$O, the content was quantified with a Thermo Scientific NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and diluted to a concentration of 25 ng μl^{-1} for PCR assays. We used general ITS primers NC5 and NC2R from Hung et al. (1999) in 25-μl PCR runs containing 25-ng DNA template, 1.5 mM MgCl$_2$, 1× PCR buffer (Applied Biosystems, Foster City, CA, USA), 0.5 mM of each dNTP and 1 mM of each primer and 2.5 U AmpliTaq DNA- polymerase (Applied Biosystems). The standard thermal profile used was 2 min at 94 °C, followed by 94 °C for

30 s, 57 °C for 30 s, 72 °C for 45 s (30 cycles) and 72 °C for 10 min on a GeneAmp PCR systems 9700 thermal cycler (Applied Biosystems). Sequencing was performed with a BigDye™ terminator cycle sequencing kit (Applied Biosystems) in an ABI 3100 capillary sequencer (Applied Biosystems).

DNA extraction from faecal samples

The dataset for molecular analyses comprised faecal samples from 102 individual samples (63 bank voles and 39 yellow-necked mice). We used protocols from Gasser et al. (1993) and Verweij et al. (2009) for DNA extraction from faecal eggs with some modifications. Approximately 50-mg unpreserved and thawed faeces (~5 faecal droppings) were suspended in 200 μl of 1× PBS. After heating for 10 min at 100 °C, suspensions were treated with 200 μl of 1 % sodium dodecyl sulphate (SDS) containing 500 μg/ml proteinase K and incubated for 2 h at 55 °C. DNA was then isolated using QIAamp DNA Stool Mini Kit spin columns (QIAgen, Hilden, Germany) and eluted with 200 μl AE buffer provided with the kit. DNA concentrations were quantified using a NanoDrop and concentrations were adjusted to 25 ng μl^{-1} for PCR.

Measures of infection prevalence and intensity using real-time PCR (qPCR)

Both infection prevalence and intensity were assessed with real-time quantitative PCR using an MX3000 qPCR system (Stratagene) with SYBR green-based detection (Platinum SYBR Green qPCR SuperMIX-UDG, Invitrogen, Carlsbad, USA). We measured infection intensity in terms of faecal egg output (Keymer and Hiorns 1986) by quantifying the number of *H. glareoli* and *H. p. polygyrus* DNA copies in faecal DNA extracts. Species-specific primers within ITS2 were designed separately for *H. glareoli* and *H. p. polygyrus* infections using Primer 3 (Rozen and Skaletsky 2000). We based the primer design on sequences obtained from own sequences of isolated worms and from reference sequences for *H. glareoli*, *H. p. polygyrus*, *H. bakeri* and *Heligmosomum mixtum* from GenBank (Cable et al. 2006). Primers were GlaF3 5′-TGAT TCGCGTATCGATGAAA-3′ and GlaR2 5′-TGGGAATCAT CAACGACGTA-3′ for *H. glareoli* infections and PolyF5 5′-GATCACATGTGTCGTGTTGTATCT-3′ and PolyR5 5′-AACGCATGTACACTGTGTCGTA-3′ for *H. p. polygyrus* infections.

Each qPCR reaction contained 12.5 μl SuperMIX, 0.1 μM ROX, 0.4 μM of each primer and 4 μl template DNA resulting in a total volume of 25 μl. The temperature profile consisted of initial incubations at 50 and 95 °C for 2 min each, 43 cycles of 95 °C for 15 s, 57 °C for 30 s and 72 °C for 30 s. A final melting temperature analysis was performed between 55 and

95 °C to ensure accurate identification of target amplicons. Length of amplicons were 228 bp (*H. glareoli*) and 240 bp (*H. p. polygyrus*) with melting temperatures of 83.45–84.45 °C (*H. glareoli*) and 82.95–83.95 (*H. p. polygyrus*), respectively.

Three negative samples and a standard curve were included in each qPCR run. Standards were prepared by purifying long ITS sequences (*H. glareoli* 1,012 bp, *H. p. polygyrus* 1,063 bp, amplified with primer NC5 and NC2) with a MinElute PCR Purification Kit (Qiagen) and ran as serially diluted standard (steps of 1:5) on each qPCR plate. The most concentrated standard well contained 9.14×10^3 DNA copies with Ct-value of 21.9 (for *H. g.*) and 5.26×10^2 DNA copies with Ct-value of 22.1 (*H. p. polygyrus*). The standardized number of DNA copies per parasite species per sample was corrected for the amount of original faecal DNA applied as measured by NanoDrop. Each sample was run in duplicates on different plates.

To estimate repeatability of faecal parasite, DNA extraction and qPCR DNA quantification, we split 11 positive samples into two and used them as technical replicates. We performed two variance component analyses with individual as random factors, first focussing on repeatability between different extractions and, second, analysing repeatability between different analyses of the same sample. Repeatability of infection intensity between different extractions of sample aliquots of one individual was 0.79, and repeatability between different analyses of the same sample was 0.97.

Statistical analyses

Infection prevalence (0 or 1) was analysed by means of generalized linear models, using proc genmod in SAS 9.3, with binary error distribution. Infection intensity (parasite DNA copies per faecal sample with intensities >0) was analysed by means of general linear models, using proc glm. In both types of analyses, we included the factors host species, site and sex. To control for variation in age, we also included weight (Z-transformed within each species) as a covariate. We eliminated non-significant (at $p > 0.1$) interactions and main effects in a step-wise manner. Observed frequencies of co-infections were compared to expected frequencies from random co-occurrence in all host species by means of chi-square tests (Fisher exact test for small sample sizes) conducted in SPSS.

Results

Heligmosomoides sp. prevalence based on morphological analyses was 25 % (species-specific prevalence, BV 15 %, YNM 48 %; total $n = 75$). Molecular analyses confirmed that *Heligmosomoides* morpho-species actually consisted of the two closely related species *H. glareoli* and *H. p. polygyrus* (GenBank accession nos. KM215273 and KM215274). Molecular identification of the two species was confirmed by morphological identification of adult worms (Fig. 1). We detected no specimen of *H. mixtum* infection in our samples. Of all 102 samples analysed with qPCR, 49 % were infected with at least one of each *Heligmosomoides* infection (species-specific prevalence, BV 43 %, YNM 64 %). Thus, molecularly assessed overall prevalence was approximately twofold when compare to morphological results.

We detected cross-infections of both *H. glareoli* in yellow-necked mice and *H. p. polygyrus* in bank voles: We found *H. glareoli* infections in seven yellow-necked mice (prevalence 18 %) and *H. p. polygyrus* in six bank voles (prevalence 10 %) (Fig. 2). Of these, two bank voles and five yellow-necked mice showed simultaneous infections of both *Heligmosomoides* species. Observed frequencies of co-infections did not differ significantly from frequencies expected from random co-occurrence (Table S1). To validate qPCR amplifications, we sequenced cross-infection sequences directly from qPCRs fragments and aligned them with own and reference sequences in GenBank. Sequence similarity of *H. glareoli* as well as *H. p. polygyrus* found in bank voles and yellow-necked mice, respectively, was 100 % within both nematode species (see Fig. S1 for alignment).

Specificity of prevalence

Prevalence of *H. glareoli* infection did not differ between host species ($\chi^2 = 2.88$, $df = 1$, $p = 0.09$; Fig. 1a), sex ($\chi^2 = 2.29$, $df = 1$, $p = 0.13$) or sites ($\chi^2 = 0.81$, $df = 2$, $p = 0.67$). In contrast, there was a significant effect of host species on the prevalence of infection with *H. p. polygyrus*, so that prevalence was lower in bank voles (10 %) as compared to yellow-necked mice (56 %) ($\chi^2 = 29.23$, $df = 1$, $p < 0.001$, Fig. 1a). There was no effect of site ($\chi^2 = 3.12$, $df = 2$, $p = 0.21$) or sex ($\chi^2 = 0.25$, $df = 1$, $p = 0.61$) on *H. p. polygyrus* prevalence levels.

Specificity of infection intensity

Host species had a significant effect on *H. glareoli* fecundity so that faecal egg output (as measured in DNA copies per faecal sample) was higher in bank voles than yellow-necked mice ($F_{1,27} = 12.24$, $p < 0.01$, Fig. 1b). Infection intensities differed between sites ($F_{2,26} = 4.73$, $p < 0.05$); highest intensities were recorded in samples coming from Kalvs mosse. Host sex had no significant effect on intensities ($F_{1,27} = 0.06$, $p = 0.80$). *H. p. polygyrus* egg output did not differ between sites ($F_{2,26} = 3.13$, $p = 0.07$) or hosts of different sex ($F_{1,27} = 2.23$, $p = 0.16$), and there was only a trend of differences between species ($F_{1,27} = 4.24$, $p = 0.05$, Fig. 1b) indicating higher fecundity of *H. p. polygyrus* in yellow-necked mice.

Fig. 1 Male *bursa copulatrix* with conspicuous long protruding spicula in *Heligmosomoides glareoli* (left) and short-contained spicula in *H. p. polygyrus* (right). Both worms have been isolated from the same yellow-necked mouse host individual


Discussion

H. glareoli and *H. p. polygyrus* are common nematode infections in voles and mice. In this paper, we provide both morphological and molecular evidence of cross- and co-infections of these parasite species in wild muroid hosts and demonstrate contrasting patterns in structural host specificity of both species.

It is assumed that *H. glareoli* and *H. p. polygyrus* have (near) mutually exclusive host distributions and that they are not able to infect and develop to maturity in other hosts than their specific hosts which are voles and mice, respectively

(Quinnell et al. 1991). A study on sympatrically living bank voles and wood mice (*A. sylvaticus*) in France supports such strict host specificity of these monoxenous nematodes with *H. glareoli* being detected only in voles and *H. p. polygyrus* in mice (Pisanu et al. 2009). Wood mice can be regarded as sister species to yellow-necked mice and have close to identical parasite distributions (Klimpel et al. 2007). However, in our study, we identified adult worms of both *Heligmosomoides* species cross-infecting their specific hosts. We provided morphological evidence that both species can be detected in the same host individual (co-infection), and we validated our morphological species identification and the occurrence of

Fig. 2 **a** *Heligmosomoides glareoli* and **b** *H. p. polygyrus* prevalence and infection intensities measured as % prevalence (0 or 1) and log (DNA copies per sample) in bank voles (BV) and yellow-necked mice (YNM). *H. glareoli* was specific to its main host BV with regard to infection intensity but not prevalence, whereas *H. p. polygyrus* showed high prevalence but only low intensity specificity in YNM. Outliers are depicted with *star symbols*. In compliance with data analyses, only infections >0 are depicted in intensity plots here
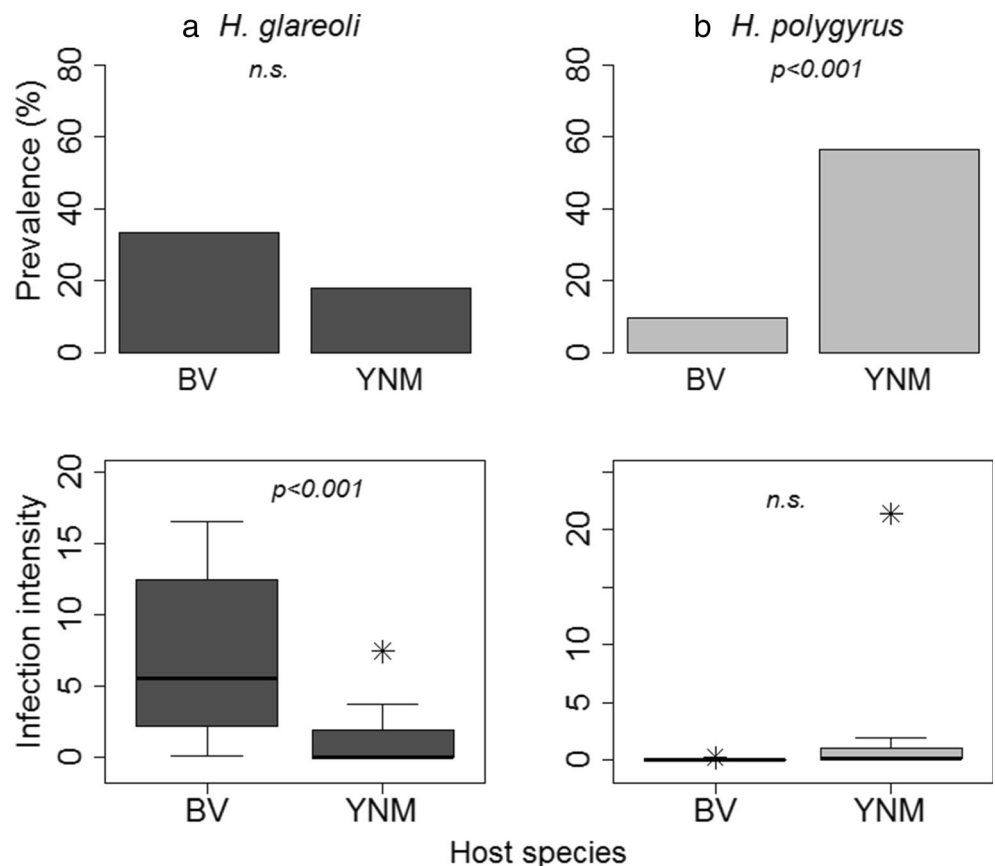
H. glareoli in yellow-necked mice and *H. p. polygyrus* in bank voles molecularly by specific real-time qPCR. Combining our evidence with two earlier reports on occasional findings of *H. polygyrus* in bank voles (Meszaros 1978; Murai et al. 1992), these results show that *H. glareoli* and *H. p. polygyrus* have, to some extent, overlapping host distributions when sharing the same habitat. However, the range of host distributions seems to be limited by the ability for *Heligmosomoides* species to develop only in phylogenetically related host species and may not be inter-transmissible by other host species of different suborders (Pisanu et al. 2009).

We found no infection of *H. mixtum*, which has been reported from other studies on bank voles as the most prevalent nematode infection in bank voles in Poland and Finland (Haukisalmi and Henttonen 1993; Kloch et al. 2010). This could be due to a geographically restricted distribution of *He. mixtum* in our sampling sites in Southern Sweden as reported from other European regions (Kloch et al. 2010).

Prevalence of cross-infections ranged from 10 to 18 %. Thus, mixed infections were not common in nature, which might explain why these cross-infections are not frequently detected in other studies. Results from our comparative data of morphological and molecular approaches showed additionally that the probability of detecting an infection via faecal parasite egg detection was twofold higher when pursued molecularly. Consequently, molecular analyses provide a more detailed reflection of parasite infections measured non-invasively via faecal samples as results from other studies indicated (Nielsen et al. 2008; Verweij et al. 2009). They are a crucial prerequisite for an accurate measurement of host specificity and need to be applied on a more frequent basis (Poulin and Keeney 2008).

The two parasite species in focus showed contrasting patterns of structural host specificity. *H. p. polygyrus* occurred with fivefold to sixfold higher frequencies in mice than in voles, while *H. glareoli* was found in near equal prevalence in both host species. In contrast, *H. glareoli* had twofold higher parasite egg outputs in faeces from voles than mice, whereas *H. p. polygyrus* infection intensities did not differ statistically significantly between host species. This suggests a diverging specificity of the two nematode species with regard to their preferred host: Specificity for prevalence of *H. p. polygyrus* infections can result in a low likelihood infecting new host species, whereas specificity for intensity of *H. glareoli* infections may translate into a higher potential of *H. glareoli* to infect different host species but with certain restrictions in its ability to replicate in and spread from different hosts (Lymbery 1989; Poulin et al. 2011). Frequencies of *H. glareoli* and *H. polygyrus* co-infections detected in our study were not significantly higher or lower than expected by chance, indicating that strong facilitation or competitive exclusion did not occur in this system (Andersson et al. 2012). This pattern complies with previous studies of *Heligmosomoides*, where interactions have been detected in some cases but generally with small effects (Behnke 2008).

Taken together, our study provides morphological and molecular evidence of cross- and co-infections of two *Heligmosomoides* species in bank voles and yellow-necked mice, which has, to our knowledge, not been reported before. This evidence provided the basis for subsequent analyses on the quantitative nature of mixed infections. We found contrasting host-parasite specialization of two closely related helminth parasites, illustrating that even if a particular parasite species shows little difference in prevalence between host species, some hosts may play a more important role for transmission than others. As both host species that we examined here occurred sympatrically at the field site, we can exclude macro-ecological separation of parasites and their hosts as explanation for the detected differences in host specificity. Instead, it may be more plausible that physiological incompatibilities between parasites and hosts are driving these host-specific patterns (cf. Combes 1991: encounter and compatibility filters). *Heligmosomoides* is but one species complex effecting rodent hosts around the world, and an extension of the study with regard to the parasite range including further parasites such as *Trichuris sp.* or *Paranoplocephala sp.* would increase the probability to identify cryptic species in the system (Hayward 2010). Applying even more comprehensive indices of host specificity (Rohde 1980) as well as including further aspects such as phylogenetic and geographical variation, as known for example from *Trichuris* infections in murid rodents (Callejón et al. 2010), will further improve the level of knowledge on host-specific patterns in wild host-parasite systems (Krasnov et al. 2011; Poulin et al. 2011). Although conducted on a small scale, our study highlights important yet neglected aspects of host specificity and presents a worked example of how modern tools can be used to advance our understanding of selective forces in host-parasite ecology and evolution.

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