MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2015) **15**, 1112–1119 doi: 10.1111/1755-0998.12382

A combined parasitological molecular approach for noninvasive characterization of parasitic nematode communities in wild hosts

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Abstract

Most hosts are concurrently or sequentially infected with multiple parasites; thus, fully understanding interactions between individual parasite species and their hosts depends on accurate characterization of the parasite community. For parasitic nematodes, noninvasive methods for obtaining quantitative, species-specific infection data in wildlife are often unreliable. Consequently, characterization of gastrointestinal nematode communities of wild hosts has largely relied on lethal sampling to isolate and enumerate adult worms directly from the tissues of dead hosts. The necessity of lethal sampling severely restricts the host species that can be studied, the adequacy of sample sizes to assess diversity, the geographic scope of collections and the research questions that can be addressed. Focusing on gastrointestinal nematodes of wild African buffalo, we evaluated whether accurate characterization of nematode communities could be made using a noninvasive technique that combined conventional parasitological approaches with molecular barcoding. To establish the reliability of this new method, we compared estimates of gastrointestinal nematode abundance, prevalence, richness and community composition derived from lethal sampling with estimates derived from our noninvasive approach. Our noninvasive technique accurately estimated total and species-specific worm abundances, as well as worm prevalence and community composition when compared to the lethal sampling method. Importantly, the rate of parasite species discovery was similar for both methods, and only a modest number of barcoded larvae (n = 10) were needed to capture key aspects of parasite community composition. Overall, this new noninvasive strategy offers numerous advantages over lethal sampling methods for studying nematode–host interactions in wildlife and can readily be applied to a range of study systems.

Keywords: DNA barcoding, faecal egg count, helminth identification, host–parasite interactions, ITS-2, molecular diagnostics

Received 11 November 2014; revision received 23 January 2015; accepted 26 January 2015

Introduction

Parasitic nematodes have significant impacts on their human, livestock and wildlife hosts (Hudson 1986; Albon *et al.* 2002; Charlier *et al.* 2009; Lustigman *et al.* 2012). However, methods to detect, identify and quantify nematode infection in wildlife lag far behind approaches used in humans and livestock (Roeber *et al.* 2013). In livestock and humans, conventional parasitological diagnostic methods have been supplemented by genetic identification techniques, but both conventional and molecular methods have important shortcomings when applied to assessing nematode infection in wild hosts.

Conventional parasitological methods for diagnosing nematode infections include morphological identification of adult worms from necropsied hosts, and noninvasive detection of immature stages (eggs or larvae) in host faeces. Quantification of adult nematodes during necropsy is accurate, species-specific and has long been considered the gold standard for assessing nematode infection in livestock. However, in wildlife, lethal sampling often raises logistical and ethical challenges and morphological identification of specimens is more difficult, typically

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requiring highly trained professionals. As a noninvasive alternative, nematode larvae can be hatched from faecal cultures and identified based on morphological characteristics (White 1927). Although larval identification keys are available for some common nematode species of livestock (Keith 1953; van Wyk & Mayhew 2013), the utility of this approach in wildlife is limited by issues such as variation in larval morphology by host species, overlap in defining traits among parasite species and the presence of novel species not found in domestic ungulates or in livestock keys (McMurtry *et al.* 2000). Faecal egg counting techniques (e.g. Ministry of Agriculture, Fisheries & Food 1980) are another noninvasive alternative; they are easy to perform, but the degree to which egg counts accurately reflect adult worm burdens can vary (McKenna 1981; Gasbarre *et al.* 1996; Cabaret *et al.* 1998; Seivwright *et al.* 2004; Liccioli *et al.* 2012; Jorge *et al.* 2013; Roeber *et al.* 2013). Furthermore, many nematode eggs of both livestock and wildlife can only be morphologically differentiated to order- or family level, preventing fine-scale partitioning of parasite species composition with this technique.

Molecular barcoding techniques have recently become a viable solution to the issue of noninvasive, species-specific nematode identification in livestock (e.g. Roeber *et al.* 2013; Bisset *et al.* 2014). A few studies have also begun applying these methods to wildlife (Dallas *et al.* 2000; Wimmer *et al.* 2004; Archie & Ezenwa 2011; Carlsson *et al.* 2012) to detect the presence of various nematode species. In livestock, molecular methods for obtaining quantitative data on nematode infection rely on using species-specific primers (Bott *et al.* 2009; Bisset *et al.* 2014). In wild hosts, because the list of expected nematode species often includes undescribed species and species not shared with domesticated hosts (e.g. Hoberg *et al.* 2008; Moravec & Jirku 2014; Rahimian *et al.* 2014), quantitative molecular techniques may underestimate parasite diversity and inaccurately represent community composition. Here, we tested whether accurate inferences about gastrointestinal nematode communities in wildlife hosts can be drawn using a novel combination of parasitological and genetic techniques. Specifically, we compared data on nematode abundance and species composition derived from lethal sampling to a noninvasive strategy that combined faecal egg counts (FECs) and molecular barcoding of larvae. To examine the congruence between the two methods, we evaluated whether (i) abundance estimates derived from a combination of molecular identification of larvae and FECs reflected species-specific abundance of adult worms and adult community composition, and (ii) sample size influenced the accuracy of abundance and community composition estimates derived from noninvasive data.

Materials and methods

Sample collection

African buffalo (*Syncerus caffer*) were sampled for nematode infection from June 2008 to August 2012 in Kruger National Park, South Africa, as part of a longitudinal study of parasite interactions. Approximately every 6 months, individuals were captured by helicopter or vehicle and then immobilized for noninvasive faecal sampling, 127 of which were the focus of this study (Ezenwa & Jolles 2015). We used a modified McMaster protocol to quantify strongyle (Nematoda: Trichostrongylidae) FECs (Ezenwa 2003). Next, we cultured between 1 and 50 g of each faecal sample for approximately 10 days to isolate third-stage larvae. Larvae were isolated using a modified Baermann technique, ex-sheathed with a 0.15% sodium hypochlorite solution and stored in 95% ethanol until genetic analysis (Archie & Ezenwa 2011). To identify larval specimens to species level, we extracted DNA from individual larvae (Archie & Ezenwa 2011), and then amplified and sequenced the ITS-2 region (Sim *et al.* 2010). Species-specific larval abundance estimates were calculated by multiplying the proportional abundance of each ITS-2 sequence type by the total FEC (Oliveira *et al.* 2009; Budischak *et al.* 2012).

In June–August 2012, a subset of 105 study animals were euthanized following the South African National Parks Standard Operating Procedure for Lethal Population Control. Thirty-three individuals testing positive for the presence of worms via noninvasive faecal sampling were randomly selected for adult parasite isolation. For this subset, faecal samples were collected from the rectum immediately after death for FEC and larvae collection as described above; however, because animals tended to defecate heavily during the animal capture procedure used for this portion of the study, depletion of faeces from the rectum limited the availability of faeces for larval culture to 8 of 33 animals. To quantify adult nematodes, the abomasum and small intestine were collected from each animal and rinsed using 6 L or 12 L of water, respectively, following standard procedures (Wood *et al.* 1995). Contents were mixed, and a 2.5% aliquot was removed and preserved in 5% phosphate buffered formalin (PBF). Formalin-fixed adult nematode specimens were isolated from GI contents by rinsing samples through 250 and 44 μm sieves. Specimens were counted and morphologically identified at the USDA Agricultural Research Service, US National Parasite Collection (USNPC) and Animal Parasitic Diseases Laboratory. Representative voucher specimens are archived in the USNPC, and the national collection is now held by the National Museum of Natural History, Smithsonian

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Institution. Counts for each 2.5% sample were multiplied by 40 to estimate total abundance.

Genetic analysis of additional adult specimens was used to obtain reference sequence information for species-specific identification of larvae. Specimens used for these analyses were obtained by pooling aliquots of abomasum and small intestine contents and then isolating worms following an agar-gel procedure (Slotved *et al.* 1996). Briefly, pooled samples were mixed with an equal volume of 2% agarose and then poured in a thin layer over a cloth sheet. Sheets were hung in warm phosphate buffered saline (PBS) overnight to facilitate nematode migration out of the agarose. Specimens were collected from the agarose 8–12 h later and preserved in 70% ethanol. Morphological identification occurred at the USDA, followed by genetic analysis at the University of Georgia. For genetic analysis, DNA was extracted from the midbody region of morphologically identified adult specimens as described for larvae above (Archie & Ezenwa 2011) and amplified using nested primers developed by Sim *et al.* (2010); thus, sequence data and morphology could be assessed concurrently from single identified adult parasites linked to archived vouchers.

Data analysis

We used matched lethal (adult) and noninvasive (egg and larvae) data to examine the effects of sampling technique on estimates of total and species-specific worm abundance. For total abundance, we examined the relationship between the number of adult worms isolated from each host and the corresponding FEC using a Spearman rank correlation test. Both lethal and noninvasive methods had their limitations for determining species-specific abundances; adult females of one genus (*Haemonchus*) could not be differentiated morphologically and the larval culture technique used precluded collection of larvae of one species (*Parabronema* sp.). Accordingly, the two *Haemonchus* species were combined and *Parabronema* was excluded from species-specific abundance analyses. We tested whether adult worm counts were correlated with larval abundance estimates using the two most common nematode species and hosts with sufficient numbers of identified larvae ($n = 8$ hosts). Adult-larvae comparisons were done using linear regression.

To test whether noninvasive nematode sampling techniques could accurately capture worm community composition, we compared species-specific prevalence, relative abundance and community similarity estimates between lethal and noninvasive methods. For prevalence, we determined whether the overall prevalence of each species differed between adult and larval sampling methods using *Z*-tests. For relative abundance, we used paired *t*-tests to examine whether the fraction of identified larvae of each worm species in a host differed from the fraction of adults observed. Finally, to ascertain whether noninvasive techniques could capture parasite community differences, we estimated pairwise similarity in worm species community composition between hosts using the Bray–Curtis dissimilarity index and then examined the degree to which these community estimates were correlated between methods using Mantel tests (Oksanen *et al.* 2013).

To test the sensitivity of the noninvasive method to sample size, we examined the effects of the number of hosts sampled and number of larvae per host sampled (subsample size) on worm richness estimates and relative abundance. First, we tested the effect of host sample size on worm species richness for lethal vs. noninvasive parasite data by calculating species accumulation curves using a Jackknife 1 approach (Colwell 2013). Second, we examined the number of barcoded larvae necessary to accurately capture species abundances by comparing the difference in relative abundance between methods (adult % − larvae %) to the number of identified larvae from each host. Two worm species dominated the parasite community and their proportional abundances sum to 1; thus, the absolute value of difference between methods reflects dissimilarities for both species. Next, for a broader picture of the number of barcoded larvae needed for reliable community estimates, we used a total of 127 study animals with larval data to examine how species richness varied with the number of larvae identified. Specifically, we used bootstrapping to calculate nematode richness and 95% confidence intervals for each host at a range of larval sample sizes (5–45 larvae) and then examined how the mean difference between observed and bootstrapped richness varied with the number of larvae sampled. This bootstrap analysis included *Haemonchus placei* (Hp), *Haemonchus bedfordi* (Hb), *Cooperia fuelleborni* (Cf) and 'Other', which pooled *Trichostrongylus* sp., and seven other rare parasite types for which no corresponding adults or sequences in GenBank were found. Lastly, we compared the mean differences between observed and bootstrapped values of species-specific relative abundance ($n = 1000$ replicates) across a range of larval sample sizes. From these approaches, we identified a cut-off sample size below which relative abundance estimates were inaccurate.

Results

Lethal sampling of 33 host individuals identified seven species of strongyle nematodes: Hp, Hb, Cf, an undescribed *Parabronema* species (P), an undescribed *Trichostrongylus* species (T), *Africanastrongylus giganticus* (Ag) and *Africanastrongylus buceros* (Ab). Cf was the most

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common worm detected based on prevalence (94% of hosts) and total abundance (96% of specimens), followed by H (88% prevalence, 2% of specimens) and then by P (79% prevalence, 1.5% of specimens). T, Ab and Ag were rare; each was found in two or fewer hosts (<6% prevalence) and comprised <0.05% of the total worm specimens. For the eight hosts with sufficient matched adult and larval samples, the two most rare worm species (Ab and Ag) were not detected by either method, so are not included in the comparisons below. Two adult T specimens each were found, two of the eight hosts with matching adult and larval samples, but these rare T infections were not detected by the noninvasive method.

Estimates of total worm abundance from lethal adult worm counts and noninvasive FECs were highly correlated (Spearman correlation: $r_s = 0.76$, S = 1066, $P < 0.0001$, $n = 33$; Fig. 1a). Excluding a single individual with a high level of infection (60 000 worms) from the analysis did not change the FEC–adult abundance relationship ($r_s = 0.74$, S = 1068, $P < 0.0001$). Notably, the noninvasive, combined parasitological molecular approach was able to accurately capture species-specific worm abundances for the subset of hosts with a sufficient larval sample size ($n = 8$ hosts). Adult worm counts were significantly correlated with larvae-based abundance estimates for Cf ($r = 0.75$, $t_6 = 2.74$, $P = 0.034$; Fig. 1b), and the relationship between these estimates was strong, although marginally significant for H ($r = 0.69$, $t_6 = 2.36$, $P = 0.056$; Fig. 1c).

Noninvasive methods were also able to capture several key aspects of nematode community composition among the eight hosts with both adult and larval data. For both methods, Cf was numerically dominant, accounting for over 98.6% of larvae identified and 98.8% of corresponding adult specimens, and there was no difference across hosts in the relative abundance of Cf by method ($t = -0.24$, $P = 0.82$). Relative abundances of H and T also did not differ by sampling method (H: $t = 0.34$, $P = 0.74$; T: $t = -1.23$, $P = 0.26$). Prevalence of Cf (A: 100%, L: 100%) and T (A: 25%, L: 0%, $Z = 1.51$, $P = 0.13$) did not differ between the adult and larval methods, but H prevalence was higher in adult samples compared to larval samples (A: 100%, L: 38%; $Z = 2.7$, $P = 0.007$). Overall, the noninvasive method detected comparable patterns of community composition as did the lethal method as indicated by a significant positive correlation between pairwise Bray–Curtis dissimilarity indices estimated from adult vs. larval sampling (Mantel test: $r = 0.32$, $P = 0.014$).

Lastly, we sought to determine the sample sizes necessary for accurately estimating nematode community composition using noninvasive methods. Species accumulation curves for lethal and nonlethal methods showed that the number of worm species detected increased with the number of hosts sampled at similar rates, although the rate was slightly slower for the noninvasive technique (Fig. 2). The species accumulation curves also illustrate that both sampling methods probably missed some rare species (Fig. 2). Furthermore, a comparison of proportional species abundances derived from the lethal and noninvasive methods showed that a sample size threshold of 10 larvae per host was sufficient for accurately determining the proportional abundance of each parasite species (Fig. 3a). At a cut-off sample size of 10 larvae, within-host worm richness was underestimated by only 0.61 species (95% CI: 0.54–0.68 species). Unsurprisingly, as the number of larvae identified per

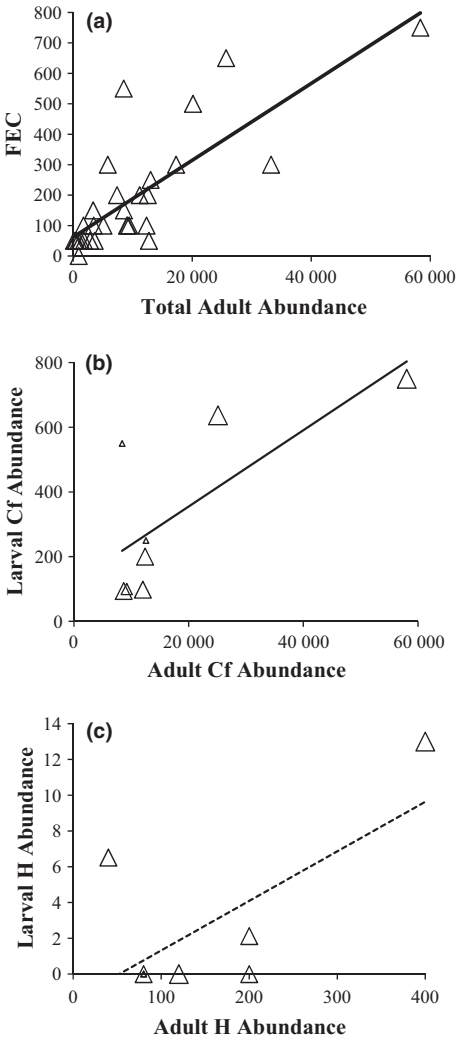

Fig. 1 Estimates of abundance from lethal adult worm sampling and noninvasive faecal egg counts (FEC) were significantly correlated (a). Species-specific abundance estimates from adult sampling were correlated with noninvasive larval abundance estimates for (b) *Cooperia fuelleborni* (Cf) and (c) H. The size of points reflects the number of larvae sampled in (b) and (c).

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host increased, the number of species missed (observed − bootstrap estimate) decreased (Fig. 3b). From a species-specific perspective, the error in estimating proportional abundance was small across all sample sizes, and variability decreased as the number of larvae identified per host increased (Fig. 3c).

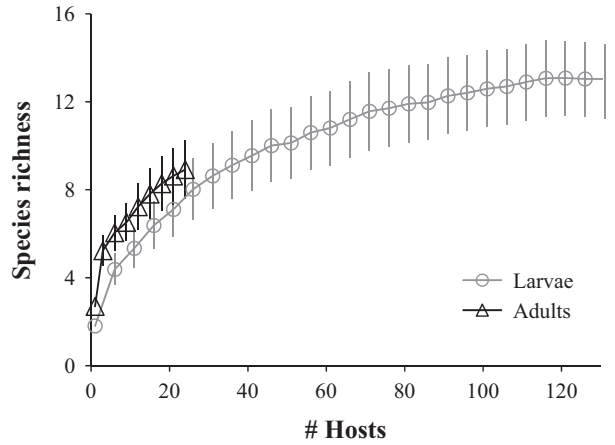

Fig. 2 Jackknife species accumulation curves (±1 SD) for lethally sampled adult worms and noninvasively sampled larvae.

Discussion

Using a novel combination of molecular barcoding techniques in conjunction with parasitological diagnostic methods (FECs and larval culture), we were able to accurately assess species-specific abundance and community composition of nematodes in a wild host. Our method represents a reliable, noninvasive approach for obtaining quantitative data about nematode communities from wildlife. First, we were able to estimate species-specific worm abundance and prevalence by integrating molecular larval identification with FEC data, and results showed a strong correlation to abundance estimates derived from lethal adult worm sampling. Next, community composition estimates based on the noninvasive approach closely mimicked estimates based on lethal sampling, but additional studies with larger paired-sample sizes would be helpful for establishing the reliability of the noninvasive approach. Finally, in terms of sampling effort, we showed that species accumulated at a similar rate with number of hosts sampled for the noninvasive method compared to the lethal method, and a sample size as low as 10 larvae per host produced good estimates of the proportional abundance of worm

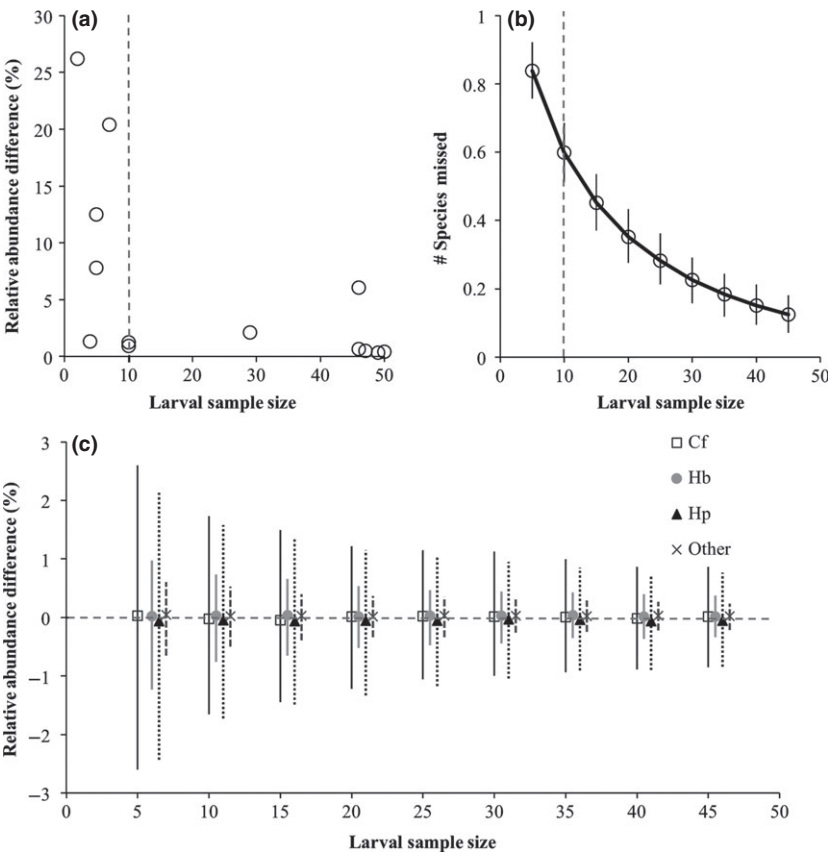

Fig. 3 (a) The difference between lethal and noninvasive estimates of the relative abundance for *Cooperia fuelleborni* (Cf) and H decreased as a function of the number of larvae identified per host. (b) Mean number of worm species missed as a function of larval sample size. (c) The mean difference between observed and estimated relative abundances (with 95% CI) for each worm species across larval sample sizes.

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species, with few species missed. However, the number larvae needed to capture nematode community composition may vary with community richness and evenness.

Parasitic helminths in general, and nematodes more specifically, can have important impacts on their hosts. Different nematode species are known to have distinct performance and fitness effects on livestock hosts and humans (Stear *et al.* 1998; Bowman 2009), yet very little is known about how individual nematode species in parasite communities contribute to the costs of nematode infections often described in wildlife species. Limited evidence from wild hosts suggests that the costs of these infections vary by species (Lello *et al.* 2004; Craig *et al.* 2006; Budischak *et al.* 2012). The noninvasive approach we outline here provides a new tool for quantitatively characterizing nematode communities infecting wild hosts and investigating species-specific consequences of infection. This approach offers numerous advantages over lethal sampling methods including the ability to sample large numbers of hosts and to sample individuals repeatedly to track longitudinal changes in parasite communities. For instance, noninvasive sampling will permit investigation of the shape of age-intensity curves, as well as the mechanisms influencing those fundamental patterns without culling numerous hosts across all age groups. Single time point studies provide only correlational data about parasite co-occurrence patterns and the relationship between infection and host immune responses; accordingly, noninvasive repeated sampling is necessary to understand the strength and consequences of interactions among co-infecting species and the efficacy and dynamics of host defences. Given that helminth co-infection is pervasive in wildlife (Petney & Andrews 1998), this approach can help extend our basic understanding of fundamental aspects of host–nematode interactions in a range of wildlife systems.

Overall, our data establish a new combined parasitological and molecular strategy as a viable tool for noninvasive studies of species-specific nematode abundance and community composition in wild hosts. Our method parallels development of similar morphological/molecular protocols that have been applied across North America in evaluations of distribution and diversity among pulmonary and extrapulmonary protostrongylid nematodes in free-ranging ungulates (Jenkins *et al.* 2005; Kutz *et al.* 2007). Although the new method performed very well and could be applied to a wide range of hosts, there were some drawbacks. Noninvasive sampling was less able to detect species at lower prevalence and abundance than lethal adult sampling; however, our analyses suggest that fewer than one parasite species is likely to be missed compared to lethal sampling when adequate numbers of larvae are sampled per host. Our methods of quantifying eggs and isolating larvae could not capture one of the common species of nematode (*Parabronema* sp.) infecting our host species, but alternative parasitological approaches [e.g. faecal sedimentation (Trejomacías & Estrada 2012)] could be used to address this gap. Lethal sampling also cannot overcome all issues of species identification. For example, female specimens of *Haemonchus* could not be differentiated morphologically or genetically, as the ideal preservative for morphological identification is formalin but it inhibits PCR, whereas molecular identification could distinguish all larvae to the species level. Continued morphological characterization for species of *Haemonchus* and ostertagiines such as *Africanistrongylus*, including definition of cuticular ridge systems that are diagnostic among conspecific males and females, can provide the basis for authoritative identification on which to develop future molecular protocols (Lichtenfels *et al.* 1994; Hoberg *et al.* 2008). While all diagnostic methods have advantages and disadvantages, this new noninvasive approach offers great potential for advancing understanding of the ecology of nematode parasitism in wildlife.

Acknowledgements

We thank South Africa National Parks Veterinary Services for logistical support and assistance with the animal capture operations. For assistance in the field and laboratory, we thank R. Spaan, J. Spaan, A. Majewska, J. Alagappan, L. Austin, C. Becker, B. Beechler, E. Belinfante, E. Gorsich, C. Gondhalekar, C. Hebbale, T. Lavelle, L. Leathers, L. Megow, T. Mowla, A. Petrelli, K. Raum, N. Rogers, K. Sakamoto, P. Snyder and M. Smith. We thank S. Altizer, J. Byers, J. Maron, K. Sakamoto and the Ezenwa laboratory group for providing valuable comments on earlier drafts of the manuscript. All animal protocols were approved by the UGA Institutional Animal Care and Use Committee (AUP#: A2013 08-017-Y1-A0; AUP#: A2010 10-190-Y3-A5). This work was supported by a National Science Foundation Ecology of Infectious Diseases Grant to VOE and AEJ (DEB-1102493/EF-0723928, EF-0723918).

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S.A.B., A.E.J. and V.O.E. designed the study. All authors contributed to data collection. S.A.B. analysed data, and S.A.B. and V.O.E. wrote the manuscript.

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Data accessibility

Morphologically identified adult worm specimens are available in the US National Parasite Collection under Accession nos 106876–106895 and 107043–107127. DNA sequences of morphologically identified adult worm specimens are available in GenBank under Accession nos: *Cooperia fuelleborni*: KP688059, KP688060, KP688061; *Trichostrongylus sp*.: KP688062, KP688063; *Haemonchus placei*: KP688064; and *Haemonchus bedfordi*: KP688065. Data presented in the study are available from Dryad (doi:10.5061/dryad.7nv0h).

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