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The proof is in the poo: Non-invasive method to detect endoparasitic infection

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Abstract

Almost every animal trait is strongly associated with parasitic infection or the potential exposure to parasites. Despite this importance, one of the greatest challenges that researchers still face is to accurately determine the status and severity of the endoparasitic infection without killing and dissecting the host. Thus, the precise detection of infection with minimal handling of the individual will improve experimental designs in live animal research. Here, we quantified extracellular DNA from two species of endoparasitic worm that grow within the host body cavity, hairworms (phylum Nematomorpha) and mermithids (phylum Nematoda), from the frass of their insect host, a cave wētā (Orthoptera: Rhaphidophoridae) and an earwig (Dermaptera: Forficulidae), respectively. Frass collection was done at two successive time periods, to test if parasitic growth correlated with relative DNA quantity in the frass. We developed and optimized two highly specific TaqMan assays, one for each parasite-specific DNA amplification. We were able to detect infection prevalence with 100% accuracy in individuals identified as infected through post-study dissections. An additional infection in earwigs was detected with the TaqMan assay alone, probably because some worms were either too small or degraded to observe during dissection. No difference in DNA quantity was detected between sampling periods, although future protocols could be refined to support such a trend. This study demonstrates that a noninvasive and minimally stressful method can be used to detect endoparasitic infection with greater accuracy than dissection alone, helping improve protocols for live animal studies.

KEYWORDS
diagnostic tool, environmental DNA, Mermithidae, Nematomorpha, parasite detection, quantitative PCR

Jean-François Doherty and Upendra R. Bhattarai contributed equally to this article.

1 | INTRODUCTION

Researchers face numerous methodological challenges when studying live animals, especially individuals collected from natural populations. Not only do these animals have a range of uncontrolled variables that impact study design, their individual fitness and behaviours can be strongly associated with the parasites they naturally carry or are exposed to (Ezenwa et al., 2016). In particular, the impacts of internal parasites (endoparasites) are inherently difficult to quantify, due to their cryptic nature. Endoparasites must evade host internal defence reactions to develop (Schmid-Hempel, 2009) and they often require multiple hosts to complete their life cycle (Auld & Tinsley, 2015; Poulin, 2007). The hidden interactions that occur between hosts and their endoparasites can also vary greatly with the developmental stage of the latter. For instance, parasites that require trophic transmission between hosts can alter the behaviour or appearance of the prey host when they are ready for transmission (Bhattarai et al., 2021; Poulin, 2010). Given the inevitable impacts that endoparasites have on natural populations, researchers need to include parasitism as an integral part of their study designs with live animals, which ideally requires the ability to identify infected individuals without killing and dissecting every subject. Invisible endoparasites can also be hugely important to other research areas, ranging from studies on host–parasite compatibility and immune responses (Schmid-Hempel, 2009), to applications in conservation biology (Milotic et al., 2020), where destructive sampling is obviously counterproductive.

The adoption of advanced molecular tools like next-generation sequencing has been occasionally slow in parasitology, even though these technologies hold great potential for uncovering the molecular underpinnings of host–parasite interactions (Selbach et al., 2019). A promising avenue of research in molecular parasitology is the use of environmental DNA (eDNA) to explore and quantify the hidden diversity of parasites in natural ecosystems (Bass et al., 2015). This includes extracting intracellular DNA from intact parasites (eggs or other life stages) collected in environmental samples such as soil, water, and faeces (e.g., Huggins et al., 2017; Jiang et al., 2005; Yamanouchi et al., 2019) and amplifying extracellular DNA collected directly from the environment (e.g., Sengupta et al., 2019; Thomas et al., 2022). Screening hosts for endoparasites in a wild population using eDNA is not straightforward, since tracking and monitoring individual hosts are not often feasible (Bass et al., 2015). In the laboratory, however, eDNA methods can be used to accurately detect the infection status of individual hosts, especially if infected hosts cannot be morphologically distinguished from uninfected conspecifics. For example, three-spined stickleback infected with the tapeworm *Schistocephalus solidus* (a parasite known to cause behavioural change in this host) can be detected by measuring abdominal distension (Dingemanse et al., 2009), although this method is unreliable when the parasite is either too small or if distension is caused by something else (Barber, 1997). Recently, identification of infected sticklebacks through eDNA (by extracting serous fluid with a needle from the internal cavity in sticklebacks) was found to be highly reliable; quantitative PCR also revealed that *S. solidus* mass correlated with the amount of eDNA collected from each host (Berger & Aubin-Horth, 2018). Nematode DNA has also been detected in the faeces of eel hosts (Jousseaume et al., 2021), although this required massaging eels to collect faeces. These examples required invasive methods to obtain parasite DNA, which could lead to manipulation-induced biases impacting subsequent live animal research.

Animal handling and housing in the laboratory inevitably impacts the data acquired through experimentation, which could result in stress-induced differences in behaviour and other measurable traits (Bailey, 2018; Ferdowsian & Beck, 2011; Sensini et al., 2020). If researchers aim to study naturally occurring behaviours (whether impacted by parasites or not) in the laboratory, reducing artificial stress must be of prime importance. The use of eDNA, collected in a non-invasive manner from the environment, may be a better strategy in determining the infection status of individuals collected from natural populations (Bass et al., 2015), all while minimizing physical handling and the stress that accompanies it. For studies on host–parasite interactions, reducing the stresses incurred from unnatural handling and artificial laboratory conditions should therefore produce data that approach what is observable in nature, allowing us to better quantify the real impacts that parasites have on host phenotype.

The main objective of this study was to test whether it is possible to reliably detect endoparasites in individual animals collected from natural populations, using a noninvasive method requiring extracellular eDNA. We tested this for two distinct lineages of endoparasitic worms that display convergence in their life cycles: hairworms (phylum Nematomorpha) and mermithid roundworms (phylum Nematoda). Both grow several orders of magnitude from microscopic larvae within the body cavity of terrestrial arthropods (mainly insects) that consume either dormant cysts (hairworms) or eggs (mermithids) (Bolek et al., 2015; Poinar, 2010). When their growth is complete, both parasites exit through the host cuticle either directly into water (hairworms) or a water-saturated substrate (mermithids) to complete their life cycle. This coincides with remarkable changes in host behaviour that bring the parasites to these environments, which is thought to result from adaptive host manipulation (Herbison, Evans, Doherty, Algie et al., 2019; Herbison, Evans, Doherty, & Poulin, 2019; Thomas et al., 2002). For this study, we used the two following systems: the "hairworm" system, which consists of the hairworm *Gordius paranensis* infecting the cave wētā *Pleioplectron simplex* (Orthoptera: Rhaphidophoridae) (Schmidt-Rhaesa et al., 2000) and the "mermithid" system, which consists of the nematode *Mermis nigrescens* infecting the European earwig *Forficula auricularia* (Dermaptera: Forficulidae) (Presswell et al., 2015). We hypothesise that extracellular parasite DNA, despite the worms' position outside the gut, ends up in the digestive tract of the insect and, ultimately, in the frass. To test this, we collected frass from individual insects sampled from natural populations where these parasites are known to occur, and extracted and quantified parasite DNA from the frass using real-time quantitative PCR (qPCR). Additionally, to test whether parasite growth correlates with the quantity of DNA detected in the frass, we performed a second frass collection from the

same individuals at a later time point. The novelty of this methodological approach is the use of a noninvasive and minimally stressful technique to quantify extracellular eDNA from distinct lineages of endoparasites to identify naturally infected individuals.

2 | MATERIALS AND METHODS

2.1 | Insect collection

Insects were collected from populations known to naturally harbour hairworms or mermithids. Cave wētā (harbouring hairworms) were collected at night from the stream banks at Cass Field Station, Canterbury, New Zealand (43°02′ 09″ S, 171°45′ 37″ E) and earwigs (harbouring mermithids) were collected from flower heads at Dunedin Botanic Garden, Otago, New Zealand (45°51′ 25″ S, 170°31′ 08″ E). These were kept separately throughout the experiment in a temperature-controlled room at the Animal Containment Facility (Department of Zoology, University of Otago) under the following conditions: daily temperature cycling between 12 and 15°C (night and day) with a photoperiod set to L14:D10 and a relative humidity of 65%. To assure accuracy, conditions in the room were continually monitored with HOBO U12-012 data loggers (Onset, Massachusetts, USA). Cave wētā and earwigs were housed separately in clear 20-L containers with dirt beds, each fitted with opaque plastic tubes (cave wētā) or a combination of flower heads and pieces of cardboard (earwigs) for shelter. Both species were provided with water and fed a 50/50 mixture of ground oats and commercial cat food ad libitum; insects were housed under these conditions for a week to acclimate before they were isolated for collection of frass.

2.2 | Insect frass sampling

Individuals that survived the acclimation period (48/50 cave wētā and 47/50 earwigs) were then isolated to collect their frass. Each cave wētā (n = 48) was placed into individual 1-L clean transparent plastic containers fitted with egg cartons for shelter (Figure S1). Because of their smaller size, earwigs (n = 47) were placed into 100-mL clean transparent plastic containers with hinged lids to facilitate handling (lids were perforated eight times with a size 0 insect pin to circulate air). Earwig containers each had a short piece of opaque plastic pipe for shelter (Figure S1). Every insect was provided with food and water ad libitum (see above). Cave wētā frass was collected after 72 h and earwig frass was collected after 7 days, since earwigs produced much smaller quantities of frass over time and we had to accumulate enough starting material for DNA extractions. Two weeks after the end of both respective frass collection periods, the containers were cleaned with 70% ethanol, wiped dry, and fresh food and water were provided (insects, hidden within their shelters, were removed temporarily during cleaning), and new frass was collected again after the same amount of time as before for each species. Samples from both collection periods were placed in individual microtubes and stored frozen at −80°C until further processing. To minimize stressing the animals, the frass was collected, and the containers were cleaned as quickly as possible during the day, when insects were typically hidden or less active.

2.3 | Host dissection for visual confirmation of infection

After the second frass sampling, individuals that survived both collection periods were placed in separate microtubes and snap frozen in liquid nitrogen and stored at −80°C. Later, they were carefully opened under a dissecting microscope to look for worms. The presence and number of worms were recorded.

2.4 | Parasite and host DNA extraction

The frass from 39 cave wētā and 44 earwigs were collected at both sampling periods and were used for DNA extraction. Some insects died between the sampling periods (six cave wētā and three earwigs) and three cave wētā frass samples were heavily degraded after thawing and could not be recuperated. Between samples, forceps and other equipment used to extract the frass were washed in a 1% bleach solution, rinsed with 75% ethanol, then rinsed with distilled water. DNA from each individual frass sample was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) (Tsuji et al., 2019) and control genomic DNA (gDNA) from both parasite (around 1 cm of hairworm and a full mermithid) and host tissues (cave wētā leg and earwig head and thorax) was extracted using DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. DNA from cave wētā frass was re-extracted to verify potential contamination in the melt curve analysis (see below). Parasite DNA extractions (positive control) and frass DNA extractions were performed on separate days to avoid any cross-contamination. These gDNA served as positive and negative control templates, respectively, during PCR optimisation and subsequent reactions (see below). Extracted DNA was quantified using Qubit 2.0 (Invitrogen) and stored at −20°C when not in use.

2.5 | Designing TaqMan assays and confirming specificity

We aimed to amplify between 100–300 bp of parasite DNA for the qPCR specificity, since shed DNA has to pass through the digestive tract of the host and is likely to be degraded. For hairworms, we amplified two sets of primers that were designed for New Zealand hairworms, following the conditions from Tobias et al. (2017): NZHW_CO1_F and NZHW_CO1_R targeting the mitochondrial COI gene; HW_Grp5_ITS_F and HW_Grp5_ITS_R targeting ITS. Host DNA was amplified with the "universal" primers

LCO1490 and HCO2198 (Folmer et al., 1994) following the conditions from Tobias et al. (2017). These were sequenced with Sanger sequencing by the Genetic Analysis Services at University of Otago. Based on these sequences, we designed multiple sets of primers using Geneious Prime (version 2019.1.3) that amplified between 100–300 bp within these two regions (Table 1) and selected COI_241_F: 5″–CAGGTTGTCCTACAGGTTGGA–3″ and COI_476_R: 5″–CCCCAGCCAAAACAGGAAGT–3″ (product size of 235 bp) after several rounds of conventional PCRs testing for specificity. We selected this primer pair because it performed best under stringent conditions, amplifying a single hairworm DNA band at a high annealing temperature of 65.5°C. The sequenced amplicon was aligned against both host and parasite DNA in Geneious Prime (version 2019.1.3) to verify specificity. A TaqMan probe 79P (5″/56-FAM/AGCTAACACCTGCAATGTGT AACGA/36-TAMSp/3″) targeting the amplicon was designed using the Geneious Prime probe designer tool.

For mermithids, we used Primer BLAST, a web-based tool from NCBI, to design primers. The primer pair 7u.28sF and 7u.28sR (product size of 151 bp), which showed a persistent targeted amplification at a high annealing temperature of 63.5°C, was selected for further processing through several rounds of specificity tests with conventional PCRs (Table 1). A TaqMan probe 7u.28sP (5″/56-FAM/TTAGAGCGCGTAATGAATGGGCGA/36-TAMSp/3″) was designed to complement the target amplicon using Integrated DNA Technologies (IDT) software. The specificity of the selected primers and the probe to the mermithid and against the host DNA was validated by aligning them against the respective genome assembly (Bhattarai et al., 2022) using Geneious Prime (version 2022.0.2).

2.6 | qPCR optimisation and sequence confirmation

All qPCRs were performed on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific). The qPCRs were further optimized with SYBR Green Real-Time PCR (Thermo Fisher Scientific) assays using parasite gDNA under the following conditions: initial denaturing step at 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s, and a standard single cycle for the melt curve of 95°C for 15 s, 60°C for 1 min, and 95°C for 1 s. The melt curve analysis was performed to ensure no undesired amplification occurred. For hairworms, we did a PCR with a seven-step serial dilution of hairworm gDNA and sterile Milli-Q water as a negative control, starting at a concentration of 24.2 ng/μl. We quantified DNA from these dilutions with the Qubit 1× dsDNA High Sensitivity Assay Kit (Invitrogen) and regressed them against their respective amplification cycle value (Cq value) obtained from the qPCR. The resulting linear regression was used to calculate the Cq value at the *x*-intercept, allowing us to estimate the lowest detection threshold. This was also done for each plate of the TaqMan assays (see below). For mermithids, a qPCR with six serial ten-fold dilutions of mermithid gDNA at a starting concentration of 36.0 ng/μl was carried out with the optimized PCR conditions to verify the lowest detection threshold. All the qPCRs were performed in duplicates (hairworms) or triplicates (mermithids), used blank reactions, and positive (parasite gDNA) and negative (host gDNA) controls unless otherwise specified.

Conventional PCRs were performed with two hairworm and mermithid gDNA samples as templates to confirm the amplification size and product composition. In both systems, we used parasite gDNA as positive controls and host gDNA and blank reactions as negative controls. The PCR reaction for hairworms was optimized under the following conditions: initial denaturing step at 94°C for 2 min, followed by 45 cycles of 94°C for 30 s, 65.5°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. For mermithids, we optimized the PCR reaction under the following conditions: initial denaturing step at 95°C for 5 min, followed by 50 cycles of 95°C for 15 s, 63.5°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were subjected to gel electrophoresis in 1% agarose gel for visual size confirmation. The products were purified using Sephadex g50 spin columns (Sigma-Aldrich) and Sanger sequenced to validate the target DNA template. Furthermore, the specificity of the probes was also confirmed with a TaqMan assay using TaqMan Fast Advanced Master Mix (Applied Biosystems) on positive and negative controls with the thermal condition of initial denaturation at 95°C for 5 min, followed by 50 cycles of 95°C for 30 s and 65.5°C (hairworms) or 63.5°C (mermithids) for 30 s. Fluorescence thresholds were left to automatic detection.

2.7 | TaqMan analyses of frass DNA

All the cave wētā frass samples from both time points ($n = 78$) were used and all the infected and six randomly chosen uninfected earwigs were selected and the frass from both time points ($n = 18$) were used. For hairworms, the seven standard dilutions (with negative control), frass DNA samples, two negative gDNA controls (cave wētā), and two no template controls were used for each qPCR plate. For earwigs, selected frass DNA samples, five negative gDNA controls (earwig), a positive gDNA control (mermithid), and a no template control were used for the qPCR analysis with the optimized assay. Each qPCR reaction was run with either two (hairworm) or three (mermithid) technical replicates. Each reaction for hairworms (total of 10 μL) consisted of 5 μL of master mix (TaqMan Fast Advanced Master Mix, Applied Biosystems), 1 μL of template DNA, 0.5 μL of both primers and probe, and the remaining volume with sterile Milli-Q water. Each reaction for mermithids (total of 20 μL) consisted of 10 μL of master mix, 2 μL of template DNA, 0.5 μL of both primers and probe, and the remaining volume with sterile Milli-Q water. The qPCRs were conducted in the QuantStudio 3D Digital Real-Time PCR System (Applied Biosystems). The results were interpreted with the Design and Analysis Software (version v2.6.2) in the Thermo Fisher Connect Platform.

TABLE 1 Primers and probes tested in this study.

Host–parasite system	Primers and probes	Template	Target	Source
Cave wētā and hairworm	LCO1490	Host DNA	COI	Folmer et al. (1994)
	HCO2198	Host DNA	COI	Folmer et al. (1994)
	NZHW_CO1_F	Parasite DNA	COI	Tobias et al. (2017)
	NZHW_CO1_R	Parasite DNA	COI	Tobias et al. (2017)
	HW_Grp5_ITS_F	Parasite DNA	ITS	Tobias et al. (2017)
	HW_Grp5_ITS_R	Parasite DNA	ITS	Tobias et al. (2017)
	COI_150_F	Parasite DNA	COI	Designed for this study
	COI_150_R	Parasite DNA	COI	Designed for this study
	COI_241_F	**Parasite DNA**	**COI**	**Designed for this study**
	COI_241_R	Parasite DNA	COI	Designed for this study
	COI_476_F	Parasite DNA	COI	Designed for this study
	COI_476_R	**Parasite DNA**	**COI**	**Designed for this study**
	COI_488_F	Parasite DNA	COI	Designed for this study
	COI_488_R	Parasite DNA	COI	Designed for this study
	79P	**Parasite DNA**	**COI_241_F and COI_476_R amplicon**	**Designed for this study**
Earwig and mermithid	1u.18sF	Parasite DNA	18S rRNA	Designed for this study
	1u.18sR	Parasite DNA	18S rRNA	Designed for this study
	1.2 F	Parasite DNA	18S rRNA	Presswell et al. (2015)
	9 R	Parasite DNA	18S rRNA	Presswell et al. (2015)
	Nem_18S_F	Parasite DNA	18S rRNA	Huggins et al. (2017)
	Nem_18S_R	Parasite DNA	18S rRNA	Huggins et al. (2017)
	4u.glb-bF	Parasite DNA	Glb-b	Designed for this study
	4u.glb-bR	Parasite DNA	Glb-b	Designed for this study
	5u.glb-eF	Parasite DNA	Glb-e	Designed for this study
	5u.glb-eR	Parasite DNA	Glb-e	Designed for this study
	6u.18s.itsF	Parasite DNA	18S rRNA	Designed for this study
	6u.18s.itsR	Parasite DNA	18S rRNA	Designed for this study
	8u.18sF	Parasite DNA	18S rRNA	Designed for this study
	8u.18sR	Parasite DNA	18S rRNA	Designed for this study
	9u.18sF	Parasite DNA	18S rRNA	Designed for this study
	9u.18sR	Parasite DNA	18S rRNA	Designed for this study
	10u.18sF	Parasite DNA	18S rRNA	Designed for this study
	10u.18sR	Parasite DNA	18S rRNA	Designed for this study
	7u.28sF	**Parasite DNA**	**28S rRNA**	**Designed for this study**
	7u.28sR	**Parasite DNA**	**28S rRNA**	**Designed for this study**
	7u.28sP	**Parasite DNA**	**7u.28sF and 7u.28sR amplicon**	**Designed for this study**

Note: Rows in bold indicate the primers that were selected for the qPCR analyses.

3 | RESULTS

3.1 | PCR optimisation and verification

During the qPCR optimization steps for both the SYBR Green and TaqMan assays, we observed some unwanted amplification in the hairworm system, and none in the mermithid system. For hairworms, agarose gel electrophoresis and gel imaging confirmed the amplified product had the desired 235 bp size (Figure 1a). However, the melt curve analysis from the SYBR Green assays showed a strong single peak and a distinct smaller peak (Figure 1b). Sanger sequencing of the amplified product and subsequent alignment to hairworm sequences showed 100% sequence identity with *G. paranensis* COI. For mermithids, agarose gel electrophoresis and gel imaging confirmed the amplified product had the desired 151 bp size (Figure 1c). Melt curve analysis from the SYBR Green assays confirmed a single PCR product (Figure 1d). Sanger sequencing of the amplified product followed by a BLAST database search showed 100% identical and complete sequence similarity with the targeted 28 s rRNA genomic region of the *M. nigrescens* genome.

3.2 | Test of qPCR detection threshold

The detection threshold is the lowest concentration of parasite DNA at which detection is possible in the optimized qPCR TaqMan assay. For hairworms, three qPCR plates were required to process all the samples, and for each plate we obtained a linear regression from the Cq values of the seven standard serial dilutions of parasite gDNA (and negative control) against the DNA quantity measured in each dilution, allowing us to estimate a Cq detection threshold of 33.15 for the first plate, 32.40 for the second plate, and 32.96 for the third plate (Table S1). For all three plates, the first standard was not included to increase linear fitting. Based on these regressions, any Cq value above these thresholds, respective of the plate, would estimate a negative DNA quantity, which can be interpreted as a null quantity. For mermithids, we tested with a six-serial dilution of parasite gDNA, diluting 10 times in succession. We were able to achieve a robust detection with a Cq value of 38.37 ± 0.89 (mean ± standard deviation) in parasite DNA at a theoretical concentration as low as 0.00036 ng/μL (Table S1).

3.3 | Infection prevalence through dissection

Three cave wētā were infected with hairworms, for a prevalence of 7.7% (3/39), including two single infections and one double infection. Three earwigs were infected with mermithids, resulting in a prevalence of 6.8% (3/44). One earwig was infected with a single mermithid, another was infected with five, and the third was infected with six.

3.4 | Infection prevalence through qPCR of frass DNA

For hairworms, the TaqMan assay detected parasite DNA from the second collection period in two of the cave wētā identified as infected through dissections (Table 2). For the third cave wētā identified as infected, parasite DNA was detected from the first collection period. No parasite DNA was amplified from the frass of cave wētā identified as uninfected through dissection. For mermithids, the TaqMan assay detected parasite DNA at both time points from the frass of all three earwigs identified as infected from the dissections. However, the signal did not increase with time, as seen with the Cq values (Table 3). For the earwigs identified as uninfected from dissections, we randomly selected six individuals with their frass DNA from both time points, providing 12 frass DNA samples for the TaqMan assay. All these qPCRs, apart from two samples (EP17.1 and EP17.2), showed negative results, confirming no parasite DNA was present in those samples and subsequently no mermithid infection in the host. The two frass samples with positive mermithid qPCR results were collected from the same individual (EP17).

4 | DISCUSSION

Identifying endoparasitism in individuals from natural populations represents one of the biggest methodological challenges for live animal research, which is important since any given trait is strongly associated with overall health and can thus be impacted by internal parasites. Most screening processes rely either on post-study

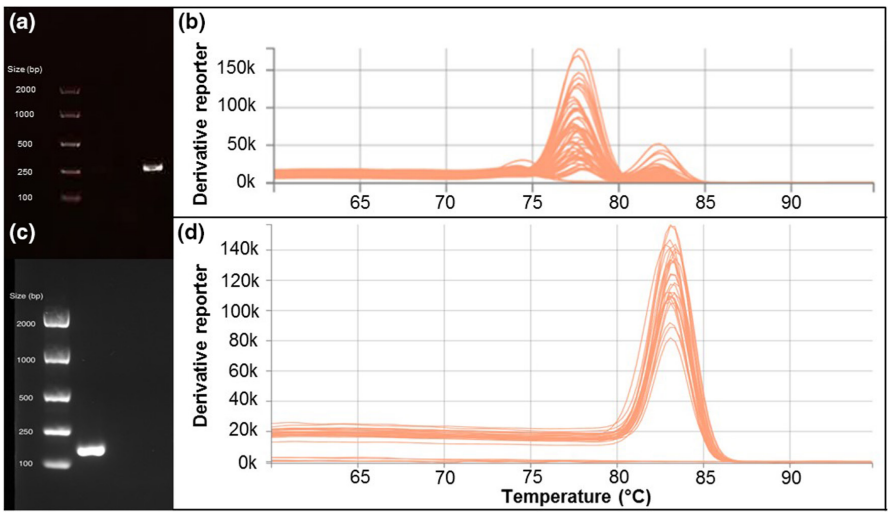

FIGURE 1 Primer specificity test. (a) Agarose gel of hairworm (*Gordius paranensis*) DNA amplified with COI_241_F and COI_476_R and an amplified product (fourth lane) at the anticipated size of 235 bp. (b) Melt curve analysis from the optimized SYBR Green qPCR assay with a hairworm gDNA template. (c) Agarose gel image of mermithid (*Mermis nigrescens*) DNA amplified with the 7u.28sF and 7u.28sR primer pair and an amplified product (second lane) at the anticipated size of 151 bp. (d) Melt curve analysis from the optimized SYBR Green qPCR assay with a mermithid gDNA template. For both gels, EasyLadder I (Meridian Bioscience) was used as a reference (first lane). The numbers represent the size of corresponding bands in base pairs. Melt curve analyses were obtained by reducing the temperature by 1.6°C/s from 95 to 60°C.

TABLE 2 TaqMan qPCR results for hairworm (*Gordius paranensis*) DNA amplified from cave wētā (*Pleioplectron simplex*) frass collected at two time points.

Sample	Source	Infection (number of worms)	Total DNA concentration (ng/μl)	Plate	Cq value (mean ± SD)
CP1.1	Frass	No	[b]	3	35.76
CP1.2	Frass	No	0.09	3	Undetermined
CP2.1	Frass	No	[b]	3	37.65
CP2.2	Frass	No	0.81	3	37.04
CP3.1	Frass	No	0.18	3	Undetermined
CP3.2	Frass	No	[b]	3	37.47
CP4.1	Frass	No	0.08	1	34.03
CP4.2	Frass	No	0.70	1	35.34
CP5.2	Frass	No	0.15	1	35.15
CP6.1	Frass	No	0.19	1	36.10
CP6.2	Frass	No	0.38	1	35.87
CP7.1	Frass	No	0.12	1	33.93
CP7.2	Frass	No	0.38	1	Undetermined
CP8.1	Frass	No	0.06	1	33.28
CP8.2	Frass	No	0.28	1	37.64
CP9.1	Frass	No	0.05	1	36.54
CP9.2	Frass	No	0.63	1	36.24
CP10.1	Frass	No	[b]	1	36.52
CP10.2	Frass	No	0.34	1	36.42
CP11.1	Frass	No	0.06	1	Undetermined
CP11.2	Frass	No	0.06	1	36.74
CP12.1	Frass	No	[b]	1	34.94
CP12.2	Frass	No	0.13	1	36.68
CP13.1	Frass	No	[b]	1	35.31
CP13.2	Frass	No	0.53	1	Undetermined
CP14.1	Frass	No	[b]	1	35.73
CP15.1	Frass	No	[b]	1	Undetermined
CP15.2	Frass	No	0.45	1	Undetermined
CP16.1	Frass	No	0.06	1	36.87
CP16.2	Frass	No	0.26	1	36.91
CP17.1	**Frass**	**Yes (2)**	**0.21**	**3**	**31.33 ± 0.28**
CP17.2	Frass	Yes (2)	0.42	3	33.51 ± 0.77
CP18.1	Frass	No	0.11	1	37.05
CP19.1	Frass	No	[b]	1	36.74
CP20.1	Frass	No	0.06	1	Undetermined
CP20.2	Frass	No	0.18	1	35.19
CP21.1	Frass	No	[b]	1	Undetermined
CP21.1	Frass	No	0.12	1	36.66
CP22.1	Frass	No	0.06	1	36.72
CP22.2	Frass	No	2.36	1	36.96
CP23.1	Frass	No	0.41	1	36.36
CP23.2	Frass	No	1.05	1	36.07
CP24.1	Frass	No	2.00	1	36.33
CP25.1	Frass	No	0.35	1	31.35

TABLE 2 (Continued)

Sample	Source	Infection (number of worms)	Total DNA concentration (ng/μl)	Plate	Cq value (mean ± SD)
CP25.1	Frass	No	0.35	1	Undetermined
CP25.2	Frass	No	0.60	1	36.09
CP26.1	Frass	No	0.08	2	33.74
CP26.2	Frass	No	0.51	2	35.79
CP27.1	Frass	No	[b]	2	35.86
CP27.2	Frass	No	0.15	2	36.03
CP28.1	Frass	No	[b]	2	36.72
CP28.2	Frass	No	0.25	2	36.17
CP29.1	Frass	No	0.06	2	34.58
CP30.1	Frass	No	0.26	2	34.95
CP30.2	Frass	No	0.44	2	36.15
CP31.1	Frass	Yes (1)	0.38	3	36.13[a]
CP31.2	**Frass**	**Yes (1)**	**2.93**	**3**	**32.44 ± 0.78**
CP32.1	Frass	No	0.59	3	36.01
CP32.2	Frass	No	0.29	3	36.21
CP33.1	Frass	No	0.14	3	36.14
CP33.2	Frass	No	0.74	3	33.14
CP34.1	Frass	Yes (1)	0.22	3	36.39 ± 0.01
CP34.2	**Frass**	**Yes (1)**	**0.25**	**3**	**31.03 ± 0.03**
CP35.2	Frass	No	0.35	2	36.55
CP36.1	Frass	No	0.07	2	33.79
CP36.2	Frass	No	0.37	2	36.10
CP37.1	Frass	No	0.28	2	35.80
CP37.2	Frass	No	0.80	2	36.56
CP38.1	Frass	No	[b]	2	35.27
CP38.2	Frass	No	0.53	2	34.94
CP39.1	Frass	No	2.76	2	35.78
CP39.2	Frass	No	0.65	2	34.67
CP40.1	Frass	No	0.28	2	36.14
CP40.2	Frass	No	2.47	2	35.66
CP41.1	Frass	No	[b]	2	34.56
CP42.1	Frass	No	0.63	2	35.42
CP42.2	Frass	No	3.13	2	34.38
CP43.2	Frass	No	4.65	2	35.75
CP44.1	Frass	No	1.10	2	34.78
CP45.1	Frass	No	0.13	2	36.66
CP45.2	Frass	No	2.51	2	35.60
CP46.1	Frass	No	1.62	2	33.28
CP46.2	Frass	No	2.54	2	34.53
CP47.1	Frass	No	0.32	2	35.60
CP47.2	Frass	No	0.85	2	35.40
CP48.1	Frass	No	0.46	2	35.08
CP48.2	Frass	No	1.63	2	36.23
CW1	Cave wētā	No	NA	1	36.21
CW2	Cave wētā	No	NA	1	Undetermined

(Continues)

TABLE 2 (Continued)

Sample	Source	Infection (number of worms)	Total DNA concentration (ng/μl)	Plate	Cq value (mean ± SD)
CW3	Cave wētā	No	NA	2	35.34
CW4	Cave wētā	No	NA	2	35.72
CW5	Cave wētā	No	NA	3	Undetermined
CW6	Cave wētā	No	NA	3	35.14
NTC	Water	NA	0.00	1	Undetermined
NTC	Water	NA	0.00	1	Undetermined
NTC	Water	NA	0.00	2	36.72
NTC	Water	NA	0.00	2	35.09
NTC	Water	NA	0.00	3	Undetermined
NTC	Water	NA	0.00	3	36.92

Note: The source of DNA and the infection status (determined by dissection) of each host are provided. Total DNA concentrations in the frass were quantified with Qubit. Mean amplification cycle values (Cq values) and standard deviations were calculated from duplicate qPCRs. Standard deviations are only provided for Cq values of samples collected from infected cave wētā. Note that Cq values below the detection thresholds respective of the qPCR plate, which indicates that parasite DNA was amplified, are identified in bold.

[a]A standard deviation could not be calculated for this sample because we obtained an undetermined Cq value for one of the replicate qPCRs.

[b]Concentration too low to quantify with Qubit.

dissections or physical handling of the host, limiting study approaches and impacting data acquisition (Bailey, 2018; Ferdowsian & Beck, 2011). In this study, we were able to amplify parasite DNA using a minimally invasive method that required optimized parasite-specific qPCRs and extracellular eDNA extracted from the frass of individual hosts, allowing us to identify infected individuals from two populations of evolutionary distinct host–parasite systems. For the cave wētā-hairworm system, we were able to confirm the infection status of all three cave wētā within which worms were found during post-study dissections. We also observed a higher relative quantity of parasite DNA in two of the frass samples collected at the second time point. However, we cannot confirm with this data that the amount of parasite DNA increased between both sampling periods, since we did not amplify parasite DNA in the frass collected from the first time point, even though these individuals were infected. This may be due to the shorter frass collection period (72 h) in this system, since less frass could have resulted in less parasite DNA extracted. For the earwig-mermithid system, parasite DNA was amplified across both frass collection periods in all individuals identified as infected through post-study dissections. However, there was no apparent increase in the relative quantity of parasite DNA amplified from the first to second time point. In addition, we were able to detect mermithid DNA in both frass samples from an earwig that was not identified as infected from the dissections. This could be explained by the presence of worm(s) that were either too small to observe under the dissecting microscope or worm(s) that had died prior to dissection and were too degraded to distinguish among host tissues. Having amplified parasite DNA at both time points from a host that appeared to be uninfected indicates that the qPCR test can be more sensitive for diagnostic purposes than dissection alone. This does not exclude the possibility that the test can also produce false positives, although it is unlikely to have occurred twice in samples taken from the same individual.

One of the most obvious limitations to this type of diagnostic test is finding parasite primers specific enough to design probes and optimize the qPCRs. For both systems, we first had to go through a series of conventional PCRs to obtain primers that amplified short fragments of parasite DNA. The insect frass collected here most probably contained DNA from species other than the parasite and the host. Therefore, our primers had to be specific enough to avoid cross-amplification with host DNA and organisms present in the frass such as bacteria and fungi (Bhadury et al., 2011; Bhadury & Austen, 2010). To overcome these potential technical issues, we combined literature searches with wet-laboratory PCR optimizations to design and validate primer pairs and TaqMan probes for the specific amplification of the COI mitochondrial region of *G. paranensis* and the 28S rRNA region of *M. nigrescens*, the two parasite species we wanted to detect using host frass. Although the gels confirmed that we obtained one PCR product in each system after optimisation, we did observe some nonspecific type amplification in the melt curve analysis for the hairworm primers. This nonspecific amplification was distinguishable through the melt curve analysis and present in nontemplate negative controls, suggesting it was probably primer dimer amplification rather than an off-target amplification (Ruiz-Villalba et al., 2017). Further optimisation with more stringent PCR conditions may have been required to avoid this unwanted amplification. The melt curve analysis for the mermithid primers showed no contamination, confirming that they were highly specific to the parasite DNA template found within the frass.

Both hairworms and mermithids can grow several orders of magnitude in size within the body cavity of their insect hosts (Bolek

TABLE 3 TaqMan qPCR results for mermithid (*Mermis nigrescens*) DNA amplified from earwig (*Forficula auricularia*) frass collected at two time points.

Sample	Source	Infection (number of worms)	Total DNA concentration (ng/μl)	Cq value (mean ± SD)
EP10.1	Frass	Yes (1)	0.19	32.75 ± 0.31
EP10.2	Frass	Yes (1)	0.11	34.25 ± 1.75
EP13.1	Frass	Yes (5)	0.13	33.77 ± 1.24
EP13.2	Frass	Yes (5)	0.18	36.43 ± 0.35
EP17.1	Frass	No	0.06	38.19 ± 1.33
EP17.2	Frass	No	0.83	37.92 ± 0.69
EP25.1	Frass	Yes (6)	1.57	38.01 ± 1.24
EP25.2	Frass	Yes (6)	0.32	37.88 ± 0.51
EP29.1	Frass	No	1.05	Undetermined
EP29.2	Frass	No	[a]	Undetermined
EP31.1	Frass	No	0.13	Undetermined
EP31.2	Frass	No	0.20	Undetermined
EP39.1	Frass	No	0.66	Undetermined
EP39.2	Frass	No	0.42	Undetermined
EP42.1	Frass	No	0.75	Undetermined
EP42.2	Frass	No	0.04	Undetermined
EP45.1	Frass	No	1.24	Undetermined
EP45.2	Frass	No	0.04	Undetermined
EW404	Earwig	No	3.19	Undetermined
EW406	Earwig	No	2.93	Undetermined
EW407	Earwig	No	2.78	Undetermined
EW408	Earwig	No	3.65	Undetermined
EW410	Earwig	No	2.39	Undetermined
NEM.23.1	Mermithid	NA	NA	14.54
NTC	Water	NA	0.00	Undetermined

Note: The source of DNA and the infection status (determined by dissection) of each host are provided. Total DNA concentrations in the frass were quantified with Qubit. Mean amplification cycle values (Cq values) and standard deviations were calculated from triplicate qPCRs.

[a]Concentration too low to quantify with Qubit.

et al., 2015; Poinar, 2010). We hypothesised that shed parasite DNA could somehow end up in the host gut and eventually in the frass. Our data support this, since we extracted and amplified the DNA of both parasites from our samples. However, we did not observe a positive trend in the relative quantity of DNA between the two frass collection periods. We predicted that, as these parasites grow, they would shed larger amounts of DNA into the host body cavity, which should be detected in the frass through qPCRs. Shed DNA has been positively correlated with parasite mass (Berger & Aubin-Horth, 2018); however we suspect that the allotted time between both frass collections may not have been long enough to allow substantial growth of the parasites. Depending on the species, hairworms and mermithids may take anywhere from a few weeks to several months to fully grow within their insect hosts (Bolek et al., 2015; Poinar, 2010), even under optimal conditions (Bolek et al., 2013; Hanelt & Janovy, 2004). Therefore, the difference in shed DNA quantity may have been too small for the qPCRs to detect. We suggest researchers base their sampling period length on the size of the parasite (to estimate how much DNA is likely to be excreted) and the time between samples on the growth rate of the parasite.

Amplifying intracellular or extracellular eDNA from environmental samples such as faeces has been instrumental for medical and veterinary diagnostics (e.g., Wang et al., 2020) and ecological and conservation studies (e.g., Kurose et al., 2005; Rytkönen et al., 2019). eDNA can help researchers to better explore the hidden diversity and ecology of cryptic organisms such as parasites (Bass et al., 2015). Given that many parasites impact host behaviour, from indirect pathological side effects to direct adaptive manipulation (Poulin, 1995), it is essential that researchers be able to detect infected individuals throughout studies on live animals. Without controlling for this important, yet often hidden variable, the data acquired from these studies could be far removed from what is observable in nature (Ezenwa et al., 2016). The current study shows that a noninvasive and minimally stressful extraction of extracellular eDNA from insect frass can be amplified with qPCRs to accurately detect

parasitic worms within their body cavity. This copro-diagnostic protocol can be applied to other host–parasite systems, helping researchers expand their study designs to any aspect of host–parasite ecology, in both natural and artificial settings.

AUTHOR CONTRIBUTIONS

Eddy J. Dowle, Jean-François Doherty, and Upendra R. Bhattarai conceived and designed the study; Jean-François Doherty and Upendra R. Bhattarai collected samples and ran laboratory analyses with help from Eddy J. Dowle and Sara Ferreira; Jean-François Doherty and Upendra R. Bhattarai wrote the manuscript with critical input from Eddy J. Dowle, Neil J. Gemmell, Robert Poulin, and Sara Ferreira. All authors gave their final approval for publication.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Full data sets have been made available in the Dryad Digital Repository: https://doi.org/10.5061/dryad.mcvdnck4t.

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