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Molecular identification of potential intermediate hosts of *Aulonocephalus pennula* from the order Orthoptera

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Research Paper

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Abstract

Aulonocephalus pennula is a heteroxenous nematode that commonly infects a declining game bird, the northern bobwhite quail (*Colinus virginianus*). There is a lack of information on the life cycle of *A. pennula* and the potential effects of infection on bobwhites. In order to better understand the life cycle of this parasite, various species from the order Orthoptera were collected from a field site in Mitchell County, Texas. Using polymerase chain reaction (PCR), nine potential intermediate hosts were identified from the 35 orthopteran species collected. Later, ten live specimens were collected to identify larvae within the potential intermediate hosts. Larvae were present in three of these and were sent for sequencing. Similarly, the presence of larvae was confirmed from extra tissues of samples identified as positive with PCR. This was the first study to document potential intermediate hosts, but future studies are needed to confirm that these species are capable of transmitting infection to bobwhite. However, this study demonstrates that PCR has increased sensitivity and may be a valuable tool when determining intermediate hosts.

Introduction

Nematodes of the family Subuluridae are heteroxeneous parasites present in the intestines or caeca of several orders of birds (Anderson, 2000). However, research on the life history of subulurids is limited (Quentin & Poinar, 1973). Of the ten genera of Subuluridae found in vertebrates, only two of the genera have been investigated further than the definitive host (Anderson, 2000; Anderson *et al.*, 2009). The most studied of these is *Subulura brumpti*, which is commonly found in the caeca of Galliformes (Anderson, 2000; Pinto *et al.,* 2004; Peterson, 2007). *Subulura brumpti* utilizes a variety of arthropod intermediate hosts (Alicata, 1939), has a prepatent period of 45 days (Abdou & Selim, 1963) and has pathological consequences (Nagarajan *et al.*, 2012). Although another subulurid, *Aulonocephalus pennula*, has a higher prevalence and abundance in some Galliformes than *S. brumpti*, surprisingly little is known about its life history (Peterson, 2007).

Aulonocephalus pennula was first described by Chandler (1935) as an intestinal parasite residing predominantly in the caeca, and a study of subulurid head anatomy suggested that *A. pennula* was capable of attaching (Inglis, 1958). Although additional studies are needed to determine the pathogenicity of *A. pennula*, it has been associated with gross pathology (Rollins, 1980), distension of the caeca (Olsen & Fedynich, 2016) and lack of digesta within the caeca (Dunham *et al.*, 2017a). It has also been hypothesized that *A. pennula* impairs caecal function, which may lead to malnutrition and negatively affect reproduction, especially during drought years (Rollins, 1980; Lehmann, 1984; Dunham *et al.,* 2017a).

This is concerning as the caecum has a variety of functions thought to be important during times of stress, such as absorption of water, breakdown of cellulose, nitrogen absorption and immune response (Fenna & Boag, 1974; Clench & Mathias, 1995). Other parasites have been known to increase nitrogen output, reduce fecundity and survival, and cause gross pathology within the caecum (Lehmann, 1984; Hudson *et al*., 1992; Greiner & Ritchie, 1994; Petkevičius, 2007). For example, the helminth *Obeliscoides cuniculi* reduces survival in the snowshoe hare (*Lepus americanus*) (Murray *et al*., 1997) and *Trichostronylus tenuis* can regulate red grouse (*Lagopus lagopus scoticus*) populations by reducing fecundity (Hudson *et al.,* 1998). Furthermore, the caecal worm, *A. pennula*, has recently been associated with a die-off of northern bobwhite quail (*Colinus virginianus*) in Texas (Henry *et al.,* 2017).

Bobwhites are economically important game birds that have been experiencing a decline throughout their range (Johnson *et al.*, 2012; Sauer *et al.*, 2013). Habitat loss, fragmentation and land-use changes are typically attributed to this decline (Rollins, 2007; Hernández *et al*., 2013). However, bobwhites are declining at a similar rate in regions considered to have a suitable habitat, such as the Rolling Plains ecoregion of Texas (Rollins, 2007; Xiang *et al*., 2013), suggesting that other factors may be influencing bobwhite populations. In

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spite of urging by Robel (1993) and Brennan (2002) for further research regarding factors such as parasitism, there has been a lack of studies on the impacts of parasites (Peterson, 2007). When the 2010 quail populations of the Rolling Plains failed to irrupt under favourable conditions, the initiative Operation Idiopathic Decline (OID) investigated the potential role parasites may be playing in the decline. One finding from OID was the abundance of the caecal worm, *A. pennula*, which infected 73% of bobwhites (Bruno, 2014), and Dunham *et al.* (2017b) later reported that infection rate was as high as 99% within some areas of the Rolling Plains.

Although *A. pennula* has been reported in bobwhites from Texas since 1941 (Webster & Addis, 1945), no studies have determined the intermediate host (Lehmann, 1953, 1984; Demarais *et al.*, 1987; Dunham *et al.*, 2017a, b). This is likely due to traditional techniques, such as dissection, being labour intensive and lacking sensitivity (Watts *et al.*, 1999; Kozak & Wędrychowicz, 2010). However, advances in molecular tools can increase sensitivity and are more time efficient and cost effective, particularly in species that lack host specificity (Gasser, 2006; Thompson *et al.*, 2007). Because subulurids lack intermediate host specificity (Anderson, 2000), molecular tools such as polymerase chain reaction (PCR) could be valuable when investigating the life cycle of *A. pennula*. The aim of this study was to identify potential intermediate hosts of *A. pennula* using PCR.

Materials and methods

Field sampling

Sampling was focused on orthopterans (grasshoppers, crickets and katydids) because *A. pennula* is thought to have an insect intermediate host (Anderson, 2000; Peterson, 2007) and orthopterans are important in the bobwhite diet (Hernández & Peterson, 2007). Orthopterans were collected in Mitchell County, Texas, as previous research has shown a high prevalence of *A. pennula* in bobwhites (Dunham *et al.*, 2017b; Henry *et al.*, 2017). Orthopterans were collected by sweep nets and by hand on two transects from May 2016 to July 2016. After collection, orthopterans were placed in individual bags and identified within 24 h, using an online key (Brust *et al.*, 2014) and a field guide (Capinera *et al.*, 2004). Following identification, they were stored frozen until DNA extraction could be conducted.

Genomic DNA extractions from insects/intermediate host

Prior to DNA extraction, samples were pooled (based on identification and month of collection) because this has been shown to be an effective method for screening large samples in other species (Goodman *et al.*, 2003; Guevara *et al.*, 2003). The number in each pooled group was based on size and amount collected. Each pooled group was weighed and then homogenized by finely chopping with a scalpel blade or a blender, depending on size. The head, wings and legs of adult grasshoppers were cut off prior to homogenizing. This was done because subulurids are found within the body cavity of the insect (Anderson, 2000), and it would allow for better lysing during the extraction process. DNA was extracted from samples using Qiagen DNeasy blood and tissue kits (Qiagen, Germantown, Maryland, USA), and a negative extraction sample was used every 20th sample. All eluted genomic DNA and extra tissues were stored at −20°C for further use.

Molecular testing for A. pennula larvae

PCR reactions were done in 10 µl volumes with previously described primers, Apen F (10 µM) and Apen R1 (10 µM) (Kalyanasundaram *et al.*, 2017), and primers ND2_70F (50 µM) and ND2_149R (50 µM) (Kistler *et al.*, 2016b) for the housekeeping gene. For each reaction 5 µl of 2× Red dye master mix, 0.25 µl of each primer, 1 µl of nuclease-free water, 2 µl of extracted DNA template and 1 µl of housekeeping template were used. The following PCR program was used: 95°C for 3 min; 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s; and finally 72°C for 5 min. A 2% agarose gel stained with ethidium bromide (10 g/ml) was used to visualize the PCR products.

Larval identification in intermediate host

Following molecular testing, in August of 2017 live specimens of species that had tested positive were collected to confirm the presence of larvae. If larvae were found, they were sent for sequencing to confirm that they were indeed *A. pennula*. For cross-confirmation, the same PCR program was run but with newly designed *Oxyspirura petrowi* ITS1 primers (oxyITS1F 5′- GCAACGCTACTTATTACCACA CC-3′ and oxyITS1R 5′-TGGCAGCATACTTGCATCAATGC-3′). Samples that had tested positive for *A. pennula*, and for which additional tissue was available, were also examined for larvae. The extra tissue was placed on a slide with 0.1× phosphate-buffered saline (PBS). The slide was then placed under the microscope and examined for larvae. All larvae were collected and then extracted as described above. PCR was also run as described above on DNA extracted from larvae.

Results

Thirty-five species from the order Orthoptera were collected, of which nine species showed amplification for *A. pennula* DNA. Of the nine species of grasshoppers collected from the subfamily Gomphocerinae, five tested positive for *A. pennula* larvae; while only two of the 11 species collected for Melanoplinae were positive (table 1). For Oedipodinae, two of the three species tested positive. For both Gomphocerinae and Oedipodinae, the number of positive samples increased from May to July, and Gomphocerinae contained the species *Opeia obscura,* which was positive most frequently (92%). However, neither of the species collected for the subfamily Cyrtacanthacridinae and family Romaleidae were positive for *A. pennula*. There were also no positives from the order Ensifera (table 2).

One live specimen of *Mermiria bivittata,* five of *Eritettix simplex,* one of *Ageneotettix deorum* and three of *Trachyrhachys kiowa* were also collected. Although the *T. kiowa* and *E. simplex* samples from 2016 were not positive, they were collected for live samples because we believed this was potentially due to the small sample size, as they are grass-feeding and the other species from those subfamilies were positive. Of these samples, larvae were found in one *M. bivittata, E. simplex* and *T. kiowa* (fig. 1). Sequencing results confirmed that all larvae were *A. pennula*. Also, for cross-confirmation, all the PCR results were negative for *O. petrowi* ITS1 primers. Additionally, 54 samples that tested positive and had extra tissue were examined (table 3). Larvae were only found in 11 of these samples.

Discussion

PCR is often used to confirm that the larvae found during dissections of potential intermediate hosts were indeed from the

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Table 1. Percentage of samples from the suborder Caelifera positive for *A. pennula* larvae (*N* = number of samples tested).

	May		June		July		Total	
Grasshoppers	*N*	%	*N*	%	*N*	%	*N*	%
Acrididae								
Gomphocerinae	46	17	53	40	46	52	145	37
Acrolophitus hirtipes	2	0	2	0	3	0	7	0
Ageneotettix deorum	12	17	9	67	8	88	29	52
Boopedon nubilum	7	29	8	88	5	100	20	70
Eritettix simplex	–	–	–	–	2	0	2	0
Mermiria bivittata	15	7	19	16	8	38	42	17
Opeia obscura	–	–	4	75	8	100	12	92
Paropomala wyomingensis	4	0	–	–	–	–	4	0
Psoloessa sp.	6	50	3	67	3	33	12	50
Syrbula sp.	–	–	8	0	9	0	17	0
Melanoplinae	72	0	103	1	72	1	247	0.8
Dactylotum bicolor	1	0	3	0	1	0	5	0
Hesperotettix sp.	8	0	17	0	9	0	34	0
Melanoplus angustipennis	29	0	22	0	6	0	57	0
Melanoplus bivittatus	7	0	–	–	–	–	7	0
Melanoplus differentialis	–	–	4	0	2	50	6	17
Melanoplus femurrubrum	1	0	1	0	1	0	3	0
Melanoplus flavidus	3	0	3	0	12	0	18	0
Melanoplus lakinus	3	0	13	0	6	0	22	0
Melanoplus sanguinipes	8	0	22	5	19	0	49	2
Melanoplus sp.	12	0	3	0	2	0	17	0
Phoetaliotes nebrascensis	–	–	15	0	14	0	29	0
Cyrtacanthacridinae	–	–	1	0	1	0	2	0
Schistocerca sp.	–	–	1	0	1	0	2	0
Oedipodinae	5	20	6	50	4	75	15	47
Trachyrhachys kiowa	–	–	2	0	–	–	2	0
Trimerotropis sp.	2	0	2	100	2	50	6	50
Xanthippus corallipes	3	33	2	50	2	100	7	57
Romaleidae								
Brachystola magna	2	0	2	0	–	–	4	0

parasite species of interest or to identify the parasite (Jefferies *et al.*, 2009; Lehnert *et al.*, 2010; Thiengo *et al.*, 2010); however, to our knowledge, this is the first study to use PCR as a primary means of identifying potential intermediate hosts, as opposed to dissection followed by PCR. Due to the low specificity of subulurids, which is associated with lower prevalence in an intermediate host (Wetzel & Esch, 1996), identifying the intermediate hosts of *A. pennula* with traditional methods would have proven difficult. Traditional methods are often time consuming and lack sensitivity, as demonstrated here by finding larvae in only 20% of our positive samples. However, molecular techniques have increased sensitivity and make it easier to detect lower infection levels. For example, only 2.9% of *Thelazia callipaeda* intermediate host were found to be infected following dissection, while 18% were positive with PCR (Otranto *et al.*, 2005). In this study, nine potential intermediate hosts from the suborder Caelifera were identified with the use of PCR, and two other potential intermediate hosts were identified during live specimen examination.

Interestingly, all the grasshopper species that tested positive for *A. pennula* feed primarily on grasses, while all others feed on broadleaf plants or are specialists (Capinera *et al.*, 2004). However, not all negative species can be ruled out, due to small sample sizes of some grasshoppers. These results may provide valuable insight into the temporal patterns and spatial distribution associated with parasitic infection that may result from the ecology of these grasshoppers. For instance, prevalence of

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Table 2. Percentage of samples from the suborder Ensifera positive for *A. pennula* larvae (*N* = number of samples tested).

Crickets and katydids	*N*	%
Grylloidea		
Gryllinae		
Gryllus texensis	2	0
Nemobiinae		
Allonemobius fasciatus	4	0
Oecanthinae		
Oecanthus argentinus	19	0
Oecanthus sp.	44	0
Tettigonioidea		
Phaneropterinae		
Amblycorypha sp.	1	0
Dichopetala brevihastata	5	0
Scudderia texensis	1	0
Conocephalinae		
Conocephalus strictus	3	0
Tettigoniinae		
Neobarrettia victoriae	3	0
Pediodectes sp.	9	0

Table 3. Percentage of positive samples from samples of the suborder Caelifera with *A. pennula* larvae present (*N* = number of samples tested).

Grasshoppers	*N*	%
Gomphocerinae	45	18
Ageneotettix deorum	13	38
Boopedon nubilum	13	0
Mermiria bivittata	5	40
Opeia obscura	10	0
Psoloessa sp.	4	25
Melanoplinae	2	50
Melanoplus differentialis	1	0
Melanoplus sanguinipes	1	100
Oedipodinae	7	29
Trimerotropis sp.	3	67
Xanthippus corallipes	4	0
Total	54	20

Echinococcus multilocularis in coyotes was dependent on local variation of the intermediate hosts, and peak infection within the intermediate host determined peak infection within the coyote (Liccioli *et al.*, 2014). Similar results were also seen in European eels infected with *Partenuisentis ambiguus* (Sures & Streit, 2001). This dependence on intermediate host abundance was potentially demonstrated with *A. pennula* as well. Henry *et al.* (2017) observed increased infection levels of *A. pennula* in March 2017 compared to March 2016. It was speculated that this resulted from above-average rainfall and a subsequent increase in arthropod intermediate hosts that facilitated infection in late autumn.

As it has been demonstrated that grass-feeding grasshopper density will likely increase with more precipitation (Lenhart *et al.*, 2015), the potential intermediate hosts identified in this study may increase the prevalence of *A. pennula*. Furthermore, Guo *et al.* (2009) showed that temperature increases can expand grasshopper range northward, possibly expanding the distribution of *A. pennula*. Because precipitation and temperatures are expected to increase in North America as a result of climate change and increase in the frequency of extreme climatic events (Harvell *et al.*, 2002; Trenberth, 2011), there is concern that this will facilitate an increase in the distribution of *A. pennula*. These climate changes are also expected to result in more epizootic events and prolong their duration and severity in temperate regions (Harvell *et al.*, 2002; Hudson *et al.*, 2006). Additionally, Hudson *et al.* (2006) speculated that these epizootic events will result from the synchronized emergence of parasites from arrested development, which is associated with unfavourable conditions such as extreme climatic events.

The potential alterations to parasite prevalence and distribution in families related to *A. pennula*, such as Ascarididae and Anisakidae (Kalyanasundaram *et al.*, 2017), due to climate change, are already being noted. For example, the ascarid *Toxocara canis* is becoming more common at higher latitudes (Jenkins *et al.*, 2011). The latter authors found that 5% of dogs are now infected with *T. canis*, while <1% were infected previously. Kim *et al.* (2012) determined that increased temperatures accelerate the development of another ascarid, *Ascaris suum*, and potentially increases its prevalence. Because *A. suum* and *Ascaris lumbricoides*, a parasite that shares a close relationship with *A. pennula* (Kalyanasundaram *et al.*, 2017),

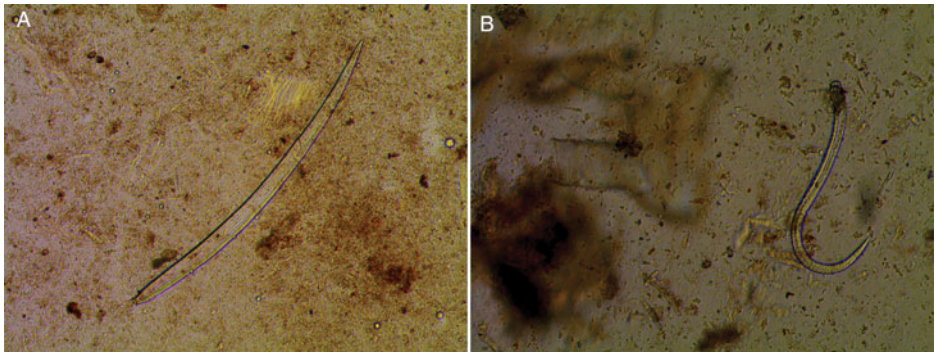

Fig. 1. Third-stage *A. pennula* larvae from extra tissue of grasshoppers (40×).

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are closely related (Kim *et al.*, 2012), it is possible that increased temperatures will also increase the prevalence of *A. pennula*. Additionally, it is expected that parasites infecting terrestrial animals will expand their range northward due to climate change (Harvell *et al.*, 2002). However, the effects of climate change on Subuluridae have not been investigated to our knowledge, and future studies on subulurids and climate change may give insight on the effects climate change will have on *A. pennula*.

Moreover, it will be important to expand the investigation of the intermediate hosts of *A. pennula* to other orders, because some parasites with low intermediate host specificity are known to utilize more than 60 species from seven orders (Shostak, 2014). The different intermediate hosts utilized by *A. pennula* will influence timing and intensity of infection, as well as how it will change with global warming. Because all species that tested positive were primarily grass-feeding, further research should consider insects with similar feeding strategies. Additionally, investigating the potential for Coleoptera and Dermaptera to serve as intermediate hosts may be valuable, because it has already been shown that they are utilized as an intermediate host in another subulurid, *S. brumpti* (Alicata, 1939). The identification of intermediate hosts is also important for epidemiological studies, understanding timing of larval development and accurately simulating infection in the laboratory (Otranto *et al.*, 2004, 2005; Kistler *et al.*, 2016a). The species identified in this study may be useful for laboratory studies investigating the life cycle *A. pennula*, such as larval development.

In this study, it was demonstrated that PCR may be a valuable tool for determining intermediate hosts. This is also the first study to document potential intermediate hosts of *A. pennula*, but additional studies are needed to determine whether these species are capable of transmitting *A. pennula* to the bobwhite. This will expand our knowledge of the life cycle of *A. pennula*, which will be key for determining how *A. pennula* varies in time and space.

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Conflict of interest. None.

Ethical standards. All bobwhites were trapped and handled according to Texas Parks and Wildlife permit SRP-0715-095 and consistent with Texas Tech University Animal Care and Use Committee protocol 16071-08.

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