Parasitology

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Genetic analyses of the parasitic nematode, *Parelaphostrongylus tenuis*, in Missouri and Kentucky reveal unexpected levels of diversity and population differentiation

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Research Article

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Abstract

Wildlife translocations, which involve the introduction of naive hosts into new environments with novel pathogens, invariably pose an increased risk of disease. The meningeal worm *Parelaphostrongylus tenuis* is a nematode parasite of the white-tailed deer (*Odocoileus virginianus*), which serves as its primary host and rarely suffers adverse effects from infection. Attempts to restore elk (*Cervus canadensis*) to the eastern US have been hampered by disease caused by this parasite. Using DNA sequence data from mitochondrial and nuclear genes, we examined the hypothesis that elk translocated within the eastern US could be exposed to novel genetic variants of *P. tenuis* by detailing the genetic structure among *P. tenuis* taken from white-tailed deer and elk at a source (Kentucky) and a release site (Missouri). We found high levels of diversity at both mitochondrial and nuclear DNA in Missouri and Kentucky and a high level of differentiation between states. Our results highlight the importance of considering the potential for increased disease risk from exposure to novel strains of parasites in the decision-making process of a reintroduction or restoration.

Introduction

Wildlife translocation remains a popular tool for re-establishing declining or extirpated species and the science guiding the application of this tool has progressed (Seddon *et al.*, 2007, 2012; Batson *et al.*, 2015). Despite increased knowledge and improved procedures, translocations, which involve the introduction of naive hosts into new environments with novel pathogens, invariably pose an increased risk of disease (Ballou, 1993; Viggers *et al.*, 1993; Gerhold and Hickling, 2016). It is possible to carefully consider and mitigate the disease risk for the translocated organisms at capture, captivity and pre-release stages, but the novel host–pathogen interactions that result post-release are often problematic and difficult to predict in advance (Cunningham, 1996; Ewen *et al.*, 2012; Hartley and Sainsbury, 2017). This is in part due to the large number of factors that can be involved in disease outcomes which can include genetic composition of the hosts (Acevedo-Whitehouse *et al.*, 2006; Savage and Zamudio, 2011), characteristics of parasites at the release site (LoGiudice, 2003), presence of other parasites in the host (Faria *et al.*, 2010), physiological stress (Parker *et al.*, 2012), altered host dynamics (Aiello *et al.*, 2014), host body condition (Mathews *et al.*, 2006) and habitat variables at the release site (Raskevitz *et al.*, 1991). These factors, in turn, can work singly or in combination to influence the ultimate success or failure of a restoration (Seddon *et al.*, 2007).

Parelaphostrongylus tenuis, commonly known as the meningeal worm, is a nematode parasite of the white-tailed deer (*Odocoileus virginianus*), which serves as its primary host and rarely suffers adverse effects from infection (Anderson, 1963). *Parelaphostrongylus tenuis* occurs in forested areas of eastern North America (Anderson, 1972; Wasel *et al.*, 2003), and is transferred *via* one of many possible intermediate gastropod hosts (Anderson, 1963, 1972; Lankester and Anderson, 1968). Attempts to restore elk (*Cervus canadensis*) to the eastern US have been hampered by disease caused by this parasite (Carpenter *et al.*, 1973; Severinghaus and Darrow, 1976; Eveland *et al.*, 1979). Infection with *P. tenuis* is a primary source of mortality, particularly for younger age classes (Samuel *et al.*, 1992) and can also make translocated elk susceptible to other sources of mortality including secondary infections, predation and vehicle collisions (Keller *et al.*, 2015). Though most elk suffer neurological damage from *P. tenuis*, some individuals can tolerate low levels of infection (Samuel *et al.*, 1992).

Infection rates for *P. tenuis* at elk release sites may be reduced though reductions in overlap among elk and white-tailed deer or reduced contact with intermediate hosts, which may promote the persistence of restored elk populations in the eastern US (Raskevitz *et al.*, 1991; Samuel *et al.*, 1992). However, ecological factors alone cannot explain the success of some restored populations (Bender *et al.*, 2005). Some authors suggest that persistent restored elk

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Table 1. Samples collected and analysed for this study from white-tailed deer (*O. virginianus*) and meningeal worms (*P. tenuis*) in Missouri and Kentucky

State	County	Sex of deer[a] (M:F:U)	*P. tenuis* collected	Deer in genetic study (M:F:U)	*P. tenuis* in genetic study
MISSOURI	Bollinger	0:1:0	1	0:1:0	1
	Butler	3:2:0	11	1:2:0	9
	Carter	7:6:0	34	3:1:0	20
	Dent	0:1:0	1	0:1:0	1
	Iron	1:0:0	2	1:0:0	2
	Madison	0:2:0	4	0:2:0	4
	Reynolds	2:4:0	18	1:4:0	15(14)[b]
	Ripley	3:1:0	10	1:1:0[c]	7(6)[b]
	Shannon	1:0:0	1	1:0:0	1
	Stoddard	0:1:1	6	0:1:1	6
	Wayne	8:11:2	46	3:2:1	23
KENTUCKY	n/a	0:0:22	68	0:0:22(21)[d]	64(62)[e]

[a]M = male, F = female, U = unknown.
[b]Two *P. tenuis* were removed from the 28S dataset as they did not yield readable sequence data.
[c]One male deer was removed from the mtDNA study, as the single sample did not yield readable sequence data.
[d]One deer was removed from the 28S dataset as the single sample did not yield readable sequence data.
[e]Four *P. tenuis* were removed from the mtDNA dataset and 6 *P. tenuis* were removed from the 28S dataset as they did not yield readable sequence data.

populations occur due to decreased susceptibility to *P. tenuis* over time (Anderson, 1972; Lankester, 2001; Larkin *et al.*, 2003; Bender *et al.*, 2005). Adaptation to the parasite may be beneficial to further elk translocation efforts since individuals that can survive at low levels of infection may have a better chance of surviving another exposure (Ewen *et al.*, 2012). However, infectivity and/or the severity of infection by a parasite can vary spatially due to local adaptation (Dybdahl and Storfer, 2003). Thus, the effects of parasitism by *P. tenuis* on translocated elk in the eastern US are difficult to predict, in part because little is known about the spatial variation of *P. tenuis*.

In general, parasite distribution and geographic variation is related to the distribution of its primary host (Poulin, 2007). Population genetic differences of the host and environmental variation can result in spatial variation among the evolutionary strategies employed by the parasite; in other words, local adaptation (Dybdahl and Storfer, 2003). Restricted movement and dispersal of parasites results in genetic drift. Thus, by examining the population genetic structure of *P. tenuis*, the possibility of local adaptation and spatial variation of virulence can be inferred. In the case of *P. tenuis*, its primary host, the white-tailed deer, has been translocated throughout the eastern US and shows high neutral genetic diversity and low genetic differentiation at a broad scale (DeYoung *et al.*, 2003; Budd *et al.*, 2018). But at a finer scale, white-tailed deer may exhibit spatial genetic structure due to habitat discontinuities (Blanchong *et al.*, 2007) and/or social structure (Comer *et al.*, 2005; Cullingham *et al.*, 2011), which may also correlate to geographic barriers for *P. tenuis* (Jacques *et al.*, 2015). Factors relevant solely to parasite dispersal, such as gastropod densities and climatic conditions, may impact geographic variation as well. For instance, certain habitat types, such as grasslands, may not support *P. tenuis* at early larval growth stages (Shostak and Samuel, 1984), and thus may inhibit movements of *P. tenuis* across some ecoregions (Wasel *et al.*, 2003).

When translocated from Kentucky to Missouri in the springs of 2011–2013 (Dent, 2014), elk experienced high morbidity and mortality due to *P. tenuis* infections (Chitwood *et al.*, 2018). In Kentucky, the persistence of some elk with *P. tenuis* and no clinical signs of disease (Larkin *et al.*, 2003), and the observation of decreased mortality from *P. tenuis* (Slabach *et al.*, 2018) suggest that the elk herd has become less susceptible to the local variant of *P. tenuis*. Though translocation-induced stress was also considered a factor, exposure to a new genetic variant of *P. tenuis* in Missouri was suspected to have contributed to the high mortality rate in the translocated herd (Chitwood *et al.*, 2018).

We examined the hypothesis that elk translocated within the eastern US could be exposed to novel genetic variants of *P. tenuis* by detailing the genetic structure among *P. tenuis* taken from white-tailed deer and elk at a source (Kentucky) and a release site (Missouri). We employed DNA sequence data from mitochondrial and nuclear genes, both of which have proven useful for population genetic and phylogenetic studies of nematodes (Nadler, 1992; Blouin *et al.*, 1998). Our study represents one of the first examinations of the genetic structure and molecular variability of *P. tenuis*. Furthermore, our study represents an important and often underrepresented component of restoration programmes: post-release monitoring and evaluation (Ewen *et al.*, 2012).

Materials and methods

Sampling in Missouri

Parelaphostrongylus tenuis samples ($n = 140$) were collected from 57 hunter-harvested deer (25 males, 29 females, 3 unknown sex; 134 *P. tenuis*) and 5 elk (6 *P. tenuis*) natural mortalities in 11 counties (Table 1, Supplementary Table 1) during 2015 and 2016. Carcasses were collected as soon as possible after they were reported to the Missouri Department of Conservation (MDC). If it was not feasible to preserve the entire carcass, heads and tissue samples were preserved at −20°C until necropsies could be performed. Necropsies were conducted by trained MDC personnel or by veterinary personnel at the University of Missouri College of Veterinary Medicine. At necropsy, all observed *P. tenuis* were carefully collected and preserved in individual vials at room temperature in a 1:1 solution of glycerol and absolute ethanol. We tested for differences between host sexes with respect to the number of *P. tenuis* detected at necropsy using a Mann–Whitney *U* test.

For the genetic study, we selected 96 *P. tenuis* samples collected from 28 deer (11 males, 15 females, 2 unknown sex) and 5 elk. When selecting samples, our goals were to represent all

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counties, maximize the number of deer with multiple parasites, and analyse all available *P. tenuis* from elk.

Sampling in Kentucky

Parelaphostrongylus tenuis samples ($n = 71$) were collected from 22 hunter-harvested white-tailed deer and 2 elk in the Elk Restoration Zone by the Kentucky Department of Fish and Wildlife Resources in December 2016 (Table 1). The collecting locations and sexes of the hosts were not provided; thus, we analysed the state as a whole. Following the same procedure that was used for the Missouri samples, *P. tenuis* were carefully removed from heads and preserved at room temperature in a 1:1 solution of glycerol and absolute ethanol prior to DNA extraction.

Mitochondrial DNA analyses

We extracted genomic DNA from the Missouri and Kentucky *P. tenuis* samples using the Qiagen DNEasy Blood and Tissue Kit with the manufacturer's protocol (Qiagen, Valencia, CA). Adult worms were removed from the preservation buffer and washed in sterile water to remove residual buffer. For small worms, we extracted DNA from the entire worm. For larger worms, we used approximately one-third of the tail. We amplified and sequenced 876 bp of the mitochondrial (mtDNA) cytochrome oxidase I (COI) gene using the primers and conditions described by Asmundsson *et al.* (2008) in their study of the congeneric species *Parelaphostrongylus andersoni* found in western North America. Amplification products were sequenced in both directions in an ABI 3730xl DNA analyser (Applied Biosystems, Foster City, CA) at the University of Missouri DNA Core and sequences were aligned in Sequencher 5.4 (Gene Codes, Ann Arbor, MI) and collapsed into haplotypes in Fabox 1.41 (Villesen, 2007).

For all hosts with multiple *P. tenuis*, we examined the number of COI haplotypes present. We calculated the number of polymorphic sites and the nucleotide diversity (π) in Arlequin 3.5 (Excoffier and Lischer, 2010). Because each Missouri county was represented by a small number of non-randomly collected samples, we examined the distribution of haplotypes by county but did not compute the values of F_{ST} between counties.

To examine genetic differentiation between *P. tenuis* mtDNA haplotypes in Missouri and Kentucky, we calculated F_{ST} in Arlequin 3.5. To examine relationships among all haplotypes and published sequences from an individual *P. tenuis* detected in Gibson Island, Maryland, USA (EF173722, Asmundsson *et al.*, 2008) and from *P. andersoni* (EU052282, EU029988, Asmundsson *et al.*, 2008), we constructed a TCS network of unique haplotypes in POPART (Clement *et al.*, 2002; Leigh and Bryant, 2015). Networks were manually reconstructed for clarity in Inkscape (https://inkscape.org).

Nuclear 28s ribosomal RNA analyses

We amplified and sequenced 718 bp of the nuclear 28S ribosomal RNA gene using primers Pt28SF: CGCTGATCTTTCGATGTTAATC and Pt28SR: CGCAACCTGTACGCTCTACC, which we designed from GenBank accession #EU595594 (Asmundsson *et al.*, unpublished). The PCR was performed in 25 μL volumes including 1× Amplitaq Gold PCR buffer (Applied Biosystems), 0.4 μM each primer, 2.0 mM $MgCl_2$, 0.8 mM BSA, 0.5 U Amplitaq Gold polymerase (Applied Biosystems) and 15–20 ng of the extracted DNA. The PCR profile included an initial 10 min incubation at 95°C followed by 40 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min and a single final incubation at 72°C for 10 min. Amplification products were sequenced in an ABI 3730xl DNA analyser (Applied Biosystems) and the resulting sequences were aligned in Sequencher 5.4 (Gene Codes, Ann Arbor, MI). Genotypes were determined by manual comparisons.

The phylogenetic relationships among *P. tenuis* genotypes from Missouri and Kentucky were inferred using a heuristic search with the maximum likelihood criterion in Paup* V. 4.0a166 (Swofford, 2002). A published sequence from *P. tenuis* (EU595594) was added for comparison, and sequences from *P. andersoni* (EU595597), *Parelaphostrongylus odocoilei* (AY292803) and *Elaphostrongylus rangiferi* (EU595596) were added as outgroups for this analysis. Support for each node was assessed using 10 000 bootstrap replicates in Paup*.

Results

Prior to the genetic study, we compared the number of *P. tenuis* collected from male and female deer in Missouri. We detected 54 *P. tenuis* in 25 males (average 1.8 ± 1.3 s.d.) and 75 *P. tenuis* in 29 females (average 3.0 ± 2.5 s.d.). Although this does not represent a random sample of individuals in Missouri and thus we cannot extrapolate our results to the population as a whole, we found no significant difference between males and females with respect to the number of *P. tenuis* detected at necropsy ($U = 285.5$, $z = -1.327$, $P = 0.184$).

Mitochondrial DNA

Missouri

We successfully recovered COI sequences from 95 *P. tenuis* from 27 deer and 5 elk (Table 2, Supplementary Table 1). One sample could not be sequenced reliably and was eliminated from the mtDNA study. After confirming that all recovered sequences were most similar to published sequences of *P. tenuis* using a nucleotide BLAST (Basic Local Alignment Search Tool) search (https://blast.ncbi.nlm.nih.gov), we translated them in Sequencher and found no evidence of stop or nonsense codons. Twenty unique haplotypes were detected based on 31 polymorphic sites [26 Transitions (Ts):5 Transversions (Tv)]. Fifteen were found only in deer, 1 was found only in elk and 4 were found in both species.

Nucleotide diversity was 0.0082 ± 0.0043 (s.d.), and gene diversity was 0.8990 ± 0.0167 (s.d.).

Of the 27 deer, 21 had multiple parasites and 17 of those individuals had parasites from multiple mitochondrial lineages. Of the deer with multiple parasites, females ($n = 10$) had an average of 2.9 ± 1.2 (s.d.) mitochondrial lineages, while males ($n = 9$) had an average of 2.0 ± 0.7 (s.d.) mtDNA lineages. For the two deer of unknown sex, one had parasites from 2 lineages while the other had parasites from only 1 lineage. The most common haplotype was Hap8, 19 samples were found in deer and 2 in elk. This haplotype had a wide geographic distribution, being found in 7 of the 11 counties included in this study (Table 2).

Kentucky

Of the 71 *P. tenuis* samples provided, we successfully recovered sequences from 67 samples from 22 deer and 2 elk (Table 1, Supplementary Table 1). Four could not be sequenced reliably and were removed from this part of the study. Two sequences (KY12E and KY18D) were removed from the dataset when replicated sequences from each were translated in Sequencher and stop codons were consistently found, suggesting that they represented nuclear copies (numts, Lopez *et al.*, 1994). An additional two sequences (KY16A and KY38B, both found only in deer) were found to be only 91 and 90% similar to published sequences for this species, respectively, making them substantial outliers. Because these sequences could represent numts or parasites that

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Table 2. Numbers and geographic distribution of *P. tenuis* mitochondrial DNA haplotypes detected in samples from deer and elk in Missouri (MO) and Kentucky (KY)

		MO deer by county											MO	KY	KY
		Bollinger	Butler	Carter	Dent	Iron	Madison	Reynolds	Ripley	Shannon	Stoddard	Wayne	elk	deer	elk
Hap#	Samp#	*n* = 1	*n* = 9	*n* = 20	*n* = 1	*n* = 2	*n* = 4	*n* = 15	*n* = 7	*n* = 1	*n* = 8	*n* = 21	*n* = 6	*n* = 62	*n* = 3
1	KY1A													2	
2	KY40E													23	
3	G162A		1	2				1				1			
4	R277B			2			2	2					1		
5	KY38B													1	
6	G192C							5	3						
7	KY25B													8	2
8	R287G	1		7		1	1	2			1	6	2	7	
9	KY16A													1	
10	KY34E													2	
11	ELK1322												1		
12	KY13A													1	
13	G180E			2											
14	KY12D													1	
15	KY36B													1	
16	KY34B													1	
17	W9C			2		1	1	1			3	9			
18	G153B		4									2	1	2	
19	W10E								2						
20	W10G		1					1	1						
21	G198B			1				1			2				
22	G187			3						1					
23	G162C							1			1		1		
24	G180D			1											
25	KY34C													5	
26	KY35													1	
27	R287D											1			
28	G142A										1				
29	KY13B													1	
30	W22F		1		1			1							

31	ELK4							1	
32	KY12F						1		
33	KY20A						1		
34	R287F					2			
35	KY12H						1		
36	KY6E						2		
37	P4A				2				
38	W10C							1	

had been misidentified as to species, we analysed the Kentucky mtDNA data with and without these sequences.

When the two highly divergent sequences were included, we detected 20 haplotypes in 65 samples based on 114 polymorphic sites (97 Ts: 27 Tv). Eighteen were found only in deer, 1 was found only in elk, and 1 was found in both. Without these samples, we detected 18 haplotypes in 63 samples, 16 of which were found only in deer. Only 2 haplotypes (Hap8 and Hap18) were shared with the Missouri deer. The most common haplotype was Hap2, found in 23 deer (Table 3).

Including the two divergent haplotypes, the nucleotide diversity was 0.0278 ± 0.0137 (S.D.) and gene diversity was 0.8399 ± 0.0357 (S.D.). Of the 22 deer, 15 had multiple parasites and 12 of those individuals had parasites from multiple mitochondrial lineages. Of the deer with multiple parasites, the average number was 2.3 ± 1.2 (S.D.). Without the divergent haplotypes, the nucleotide diversity was 0.0238 ± 0.0118 (S.D.) and gene diversity was 0.8295 ± 0.0372 (S.D.). One of the two elk had 2 parasites with 2 different mtDNA haplotypes. (Supplementary Table 1).

mtDNA differentiation

The pairwise value of F_{ST} between Missouri and Kentucky was 0.180, which was found to be significant ($P < 0.001$) based on a permutation test (110 permutations) in ARLEQUIN 3.5. The minimum spanning network produced using our mtDNA sequences revealed a star-like structure (Fig. 1), with many haplotypes differing by only a few base pairs (bp). It illustrates the strong differentiation between the two divergent Kentucky haplotypes, each of which differs by more than 60 bp from the most similar haplotypes, but only differ from each other by 3 bp. Three haplotypes (KY12F, KY20A and KY25B) were found to be more similar to *P. tenuis* sequences from Maryland and *P. andersoni* sequences than to other sequences from Missouri and Kentucky (Fig. 1).

Nuclear 28s ribosomal RNA

Missouri

We successfully recovered sequences from 93 samples collected from 28 deer and 6 elk (Table 1, Supplementary Table 1). We detected 41 unique genotypes based on 9 polymorphic base pairs, 35 of which were found only in deer, 3 of which were found only in elk and 3 of which were shared between species. Of the 28 deer, 21 had multiple parasites and of those 20 had parasites with differing nuclear genotypes. Of the deer with multiple parasites, females ($n = 10$) had an average of 3.8 ± 1.6 (S.D.) different nuclear genotypes, while males ($n = 9$) had an average of 2.6 ± 1.2 (S.D.) different nuclear genotypes. Two deer of unknown sex each had 2 parasites with 2 different nuclear genotypes. The most common genotype was Genotype25; 18 samples with this genotype were found in deer and 2 in elk. This genotype had a wide geographic distribution, being found in 8 of the 11 counties included in this study (Table 3).

Kentucky

We successfully recovered sequences from 62 samples collected from 20 deer and 2 elk (Table 1, Supplementary Table 1). We detected 10 genotypes based on 5 polymorphic base pairs, 8 of which were found only in deer and 2 of which were shared between deer and elk. Of the 20 deer, 15 had multiple parasites and 12 had parasites with differing nuclear genotypes. Deer with multiple parasites had 3.6 ± 1.5 (S.D.) parasites with an average of 2.5 ± 1.1 (S.D.) different nuclear genotypes. One of the two elk had only one parasite while the other had 2 parasites with different genotypes. Seven of the 10 genotypes were shared with Missouri deer and elk. The most common genotype was

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Table 3. Numbers and geographic distribution of *P. tenuis* 28S ribosomal RNA genotypes (GT) detected in samples from deer and elk in Missouri (MO) and Kentucky (KY)

		MO deer by county											MO	KY	KY
		Bollinger	Butler	Carter	Dent	Iron	Madison	Reynolds	Ripley	Shannon	Stoddard	Wayne	elk	deer	elk
GT#	Samp#	*n* = 1	*n* = 9	*n* = 20	*n* = 1	*n* = 2	*n* = 4	*n* = 14	*n* = 6	*n* = 1	*n* = 6	*n* = 23	*n* = 6	*n* = 59	*n* = 3
1	G160A											1			
2	G162A							1							
3	G160C			1								2			
4	G162B							1							
5	W16A							1							
6	G153B											1			
7	G142A										1				
8	C3A		1	1			1				1	2			
9	G197C		1	5								1			
10	G180B			1											
11	G151							1							
12	ELK1622-1												1		
13	G168A						1								
14	W22D		1												
15	W22B		1												
16	G160B									1		1			
17	KY13B			1										1	
18	G135C							2			1				
19	W16B							1							
20	G192E							1							
21	W10E		1						1						
22	C3D			3								2			
23	R287F											1			
24	ELK1322												1		
25	G142B		1	3	1		1	4	4		1	3		25	1
26	G180D			1		1						3		4	
27	R281								1						
28	ELK1622-2			1									1		
29	G148A											1			
30	P4A		1											1	

31	W25A				1							
32	W9B								1			
33	G135A							1				
34	W9E								1			
35	G162D						1					
36	G135D		2	2			1	1	1		10	
37	KY10										5	
38	KY36A										1	
39	ELK1514	1								1		
40	ELK1627									1		
41	R277B					1						
42	G198B			1					1		1	
43	KY6D								1	1	6	
44	ELKKY2A										5	2

Genotype25, which was found in 25 deer samples and 1 elk sample (Table 3).

Nuclear 28s ribosomal RNA differentiation

Phylogenetic analyses revealed that *P. tenuis* did not form a monophyletic group with respect to the single *P. andersoni* sample included as an outgroup (Fig. 2). Although bootstrap analyses supported only the group that included both *P. tenuis* and *P. andersoni*, that group included two major sub-groups, one of which contained the majority of genotypes found in Missouri as well as two of the most common shared genotypes between states (G142B, G180D). The second clade contained all 3 of the unique Kentucky genotypes and two commonly shared genotypes (G135D, KY4A, Fig. 2).

Discussion

Although its definitive host is the white-tailed deer, a number of studies have confirmed that *P. tenuis* can parasitize many different hosts, including elk, moose (*Alces alces*), llamas (*Lama glama*), alpacas (*Vicugna pacos*), goats (*Capra hircus*), cattle (*Bos taurus*), horses (*Equus caballus*), bison (*Bison bison*), sika deer (*Cervus nippon*) and guinea pigs (*Cavia porcellus*; Anderson, 1972; Lankester, 2001, 2010; Weiss *et al.*, 2008; Whitehead and Bedenice, 2009; Gerhold *et al.*, 2010; Tanabe *et al.*, 2010; Mitchell *et al.*, 2011; Southard *et al.*, 2012; Dobey *et al.*, 2014; Gerhold and Hickling, 2016). To date, the majority of these studies have focused on genetic confirmation of host infection and identification of morphologically indistinguishable dorsal-spined larvae in Elaphostrongyline species (Gajadhar *et al.*, 2000; Kutz *et al.*, 2001; Gerhold *et al.*, 2010; Dobey *et al.*, 2014). Other than an unpublished study by Gerhold *et al.* (2016), we are not aware of studies of this parasite at the population or regional level.

Given previous suggestions that *P. tenuis* has little intraspecific diversity or population structure (Gerhold *et al.*, 2016), we were surprised to find high levels of diversity at both mitochondrial and nuclear DNA in Missouri and Kentucky. While we found that 2 of the 38 mtDNA haplotypes and 7 of the 44 nuclear genotypes we detected were shared between states, there were significant frequency differences between states. These frequency differences and the presence of unique mtDNA haplotypes and nuclear genotypes in each state contributed to the high levels of diversity within and the high level of differentiation between states. Further, we found that the previously published mtDNA haplotype of *P. tenuis* from Maryland, USA, differed at 30 or more bp from the majority of our Missouri and Kentucky haplotypes and that three haplotypes detected in Kentucky clustered with the Maryland haplotype. Taken as a whole, our results support previous suggestions that genetic differences among populations across the range of this parasite may have contributed to differences in susceptibility to infection between hosts that have acquired immunity to parasite lineages in their home ranges (Chitwood *et al.*, 2018). Translocation of such locally adapted individuals risked exposing them to strains to which they were immunologically naive.

Our finding of two highly divergent mtDNA haplotypes is especially interesting. While we cannot rule out the possibility that these haplotypes represent nuclear copies of the COI gene, we found no evidence of stop codons and a NCBI-BLAST search found that they are most similar to previously published sequences of *P. tenuis.* However, the sequences of haplotype KY16A and KY38B were found to be only 90–91% similar to published sequences (EF173722-23) for this species. In the haplotype network, they differ from other *P. tenuis* sequences by a minimum of 62 bp, or 7% of the COI sequence. The 28S sequences for these samples were found to match two of the most common genotypes

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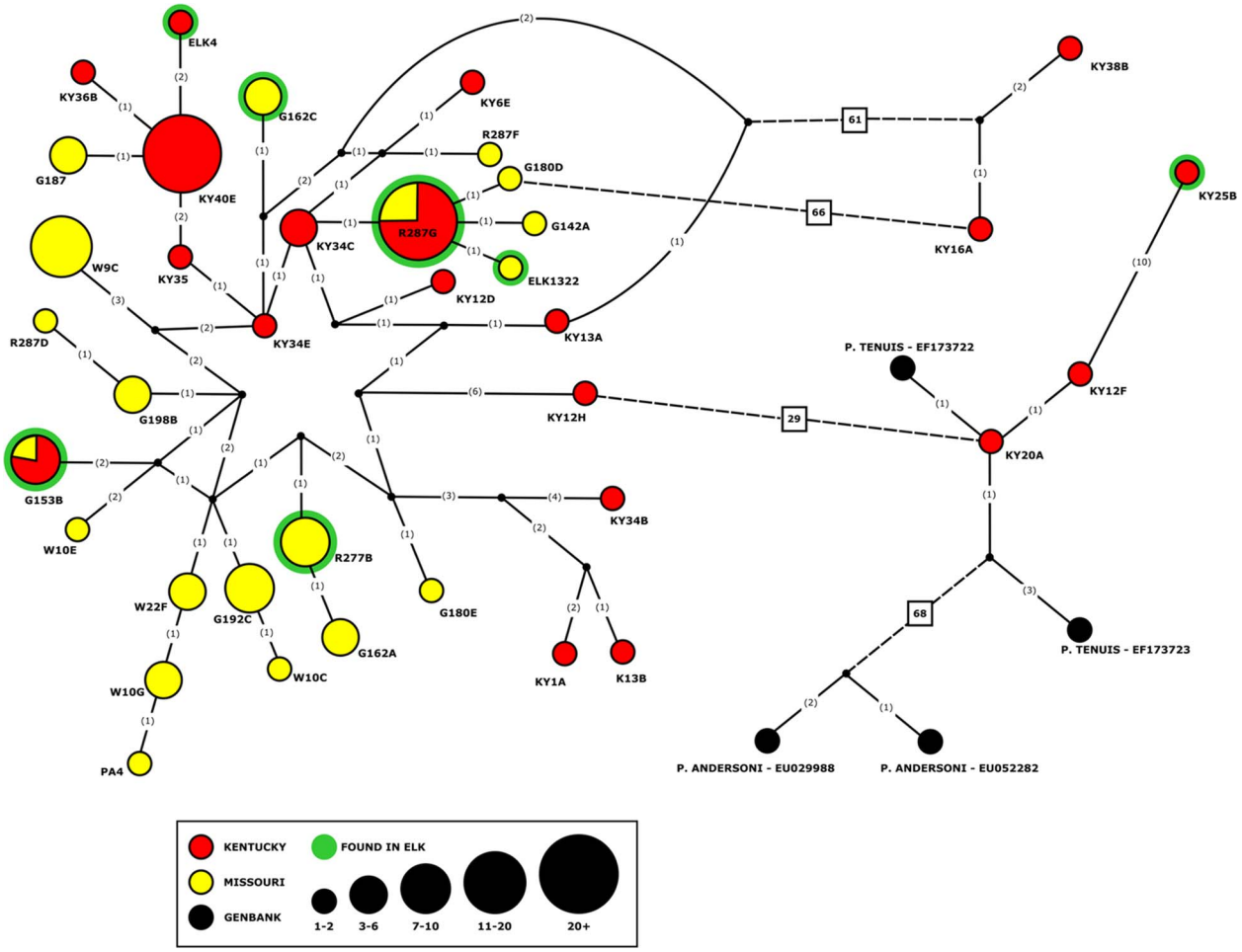

Fig. 1. TCS network based on 876 bp of mitochondrial cytochrome oxidase I (COI) sequences in samples of *P. tenuis* from Missouri and Kentucky, USA. One *P. tenuis* sample from Maryland, USA (EF173722) and two samples of the closely related species *P. andersoni* (EU029988 and EU052282) were included for comparison.

in both states, G142B and G180D, supporting their identification as *P. tenuis*. Collectively, our mitochondrial and nuclear results suggest there may be previously unrecognized lineages present in *Parelaphostrogylus* in Missouri and Kentucky, similar to the novel protostrongylid described by Dobey *et al.* (2014), who detected the DNA in a goat with lesions that suggested infection with *P. tenuis*.

Although we found a relatively large number of nuclear genotypes, they were based on only 9 polymorphic base pairs. As in Carreno *et al.* (2012), our nuclear data did not clearly resolve differences between species as it could not differentiate the *P. andersoni* sequence we included as an outgroup from our *P. tenuis* sequences. Thus, our results support the recommendations of Blouin (2002), who demonstrated that mitochondrial DNA had much higher levels of sequence divergence within nematodes than the nuclear ITS-1 and ITS-2 regions and suggested that the high substitution rate of mtDNA makes it a much more potent tool for detecting cryptic lineages of nematodes than nuclear DNA.

High levels of genetic diversity in nematodes have been attributed to high substitution rates and large effective population sizes (Blouin *et al.*, 1995, 1998). But it should also be acknowledged that the history of introductions of the white-tailed deer hosts in Missouri and Kentucky may have contributed to the high levels of *P. tenuis* genetic diversity observed in both Missouri and Kentucky. In Missouri, the host population declined to near extirpation by the late 1800s. A combination of hunting limitations, predator eradication and translocations from Wisconsin, Michigan and Minnesota between 1925 and 1957 restored the Missouri population, which has expanded to an estimated size today of 1.4 million (Bennitt and Nagel, 1937; McDonald and Miller, 2004; Budd *et al.*, 2018). Similarly, the Kentucky population was supplemented by deer from Wisconsin in the 1920s, then driven down to approximately 100 individuals by around 1935. Between 1935 and 1953, the population recovered through natural recruitment as well as the translocation of deer from Wisconsin, Oklahoma and Tennessee (Gassett, 2001). Today, white-tailed deer in both Missouri and Kentucky have high levels of genetic diversity and low levels of differentiation across the landscape (Doerner *et al.*, 2005; Budd *et al.*, 2018), consistent with rapid population growth and the mixing of multiple lineages derived from recovering native populations and translocated individuals. Our data suggest that these processes also resulted in high levels of genetic diversity within state populations and genetic differentiation between states in the parasitic nematode *P. tenuis*.

At the time of the white-tailed deer translocations, managers were not yet fully aware of the risks of translocating parasites and pathogens along with their hosts (Mathews *et al.*, 2006). After translocation, parasites are subject to the same evolutionary pressures as their host, including population bottlenecks, hybridization between locally adapted lineages, and adaptation to novel environmental conditions, including new host species. With their large population sizes and rapid generation times, parasites have the capacity for rapid evolution. The star-like pattern we detected in mtDNA, as well as the different frequencies of nuclear genotypes we detected in *P. tenuis* suggest both the introduction of

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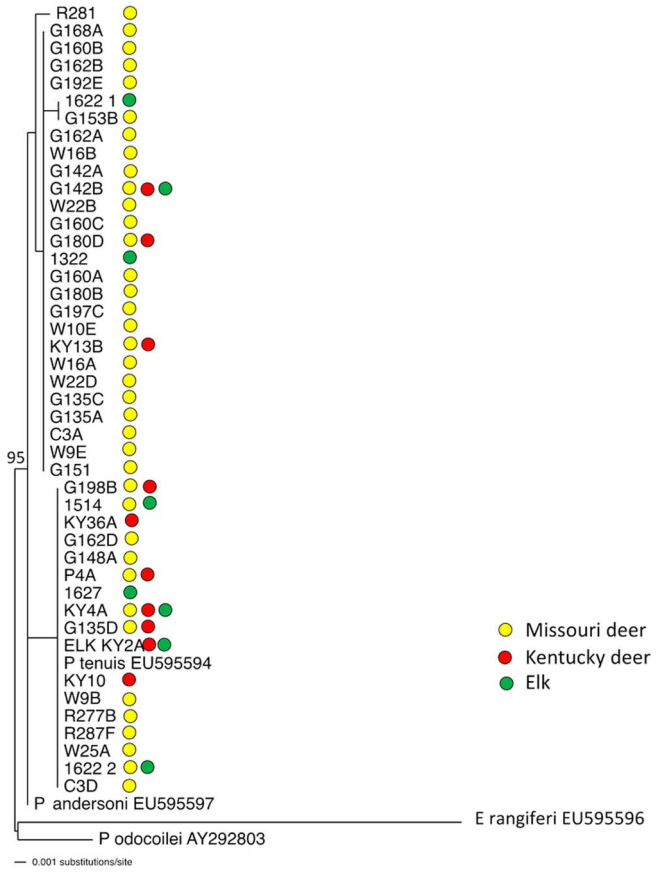

Fig. 2. Maximum likelihood tree based on 718 bp of the 28S ribosomal RNA gene in samples of *P. tenuis* from Missouri and Kentucky, USA. One published *P. tenuis* sequence (EU595594) was included for comparison and sequences from related species *P. andersoni* (EU595597), *P. odocoilei* (AY2928030) and *Elephostrongylus rangiferi* (EU595596) were included as outgroups.

new parasite lineages in Missouri and Kentucky and the rapid diversification of those lineages since introduction. This combination could have resulted in *P. tenuis* lineages in Missouri to which translocated elk from Kentucky were unable to mount an effective immune response.

Since *P. tenuis* is as ubiquitous in eastern North America as its primary host and is an important source of disease and mortality for elk (Keller *et al.*, 2015) and moose (Lankester, 2010), we suggest that conservation and restoration efforts for these ungulates would benefit from an expanded understanding of the spatial genetic variability of *P. tenuis*. Our study has provided evidence that spatial variability exists in a central region of the eastern US, and we suspect that a broader survey of the parasite would be informative. In addition to a broader knowledge base, we recommend that translocation plans incorporate analyses of *P. tenuis* strains when evaluating the suitability of a release site. Our study has provided a framework for such an evaluation. If a large amount of genetic differentiation exists among source and release sites, the potential for increased disease risk from exposure to a novel strain of *P. tenuis* should be considered in the decision-making process of a reintroduction or restoration.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182020001912.

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Conflicts of interest. None.

Ethical standards. None, the project did not concern vertebrates.

References

Acevedo-Whitehouse K, Spraker TR, Lyons E, Melin SR, Gulland F, Delong RL and Amos W (2006) Contrasting effects of heterozygosity on survival and hookworm resistance in California sea lion pups. *Molecular Ecology* **15**, 1973–1982.

Aiello CM, Nussear KE, Walde AD, Esque TC, Emblidge PG, Sah P, Bansal S and Hudson PJ (2014) Disease dynamics during wildlife translocations: disruptions to the host population and potential consequences for transmission in desert tortoise contact networks. *Animal Conservation* **17**, 27–39.

Anderson RC (1963) The incidence, development, and experimental transmission of *Parelaphostrongylus tenuis* Dougherty (Metastrongyloidea: Protostrongylidae) of the meninges of the white-tailed deer (*Odocoileus virginianus borealis*) in Ontario. *Canadian Journal of Zoology* **41**, 775–792.

Anderson RC (1972) The ecological relationships of meningeal worm and native cervids in North America. *Journal of Wildlife Diseases* **8**, 304–310.

Asmundsson IM, Mortenson JA and Hoberg EP (2008) Muscleworms, *Parelaphostrongylus andersoni* (Nematoda: Protostrongylidae), discovered in the Columbian white-tailed deer from Oregon and Washington: implications for biogeography and host associations. *Journal of Wildlife Diseases* **44**, 16–27.

Ballou JD (1993) Assessing the risks of infectious diseases in captive breeding and reintroduction programs. *Journal of Zoo and Wildlife Medicine* **24**, 327–335.

Batson WG, Gordon IJ, Fletcher DB and Manning AD (2015) Translocation tactics: a framework to support the IUCN guidelines for wildlife translocations and improve the quality of applied methods. *Journal of Applied Ecology* **52**, 1598–1607.

Bender LC, Schmitt SM, Carlson E, Haufler JB and Beyer Jr DE (2005) Mortality of Rocky Mountain elk in Michigan due to meningeal worm. *Journal of Wildlife Diseases* **41**, 134–140.

Bennitt R and Nagel WO (1937) A survey of the resident game and furbearers of Missouri. *University of Missouri Studies* **XII**, 77–85.

Blanchong JA, Samuel MD, Scribner KT, Weckworth BV, Langenberg JA and Filcek KB (2007) Landscape genetics and the spatial distribution of chronic wasting disease. *Biology Letters* **4**, 130–133.

Blouin MS (2002) Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. *International Journal for Parasitology* **32**, 527–531.

Blouin MS, Yowell CA, Courtney CH and Dame JB (1995) Host movement and the genetic structure of populations of parasitic nematodes. *Genetics* **141**, 1007–1014.

Blouin MS, Yowell CA, Courtney CH and Dame JB (1998) Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. *Molecular Biology and Evolution* **15**, 1719–1727.

Budd K, Berkman LK, Anderson M, Koppelman J and Eggert LS (2018) Genetic structure and recovery of white-tailed deer in Missouri. *Journal of Wildlife Management* **82**, 1598–1607.

Carpenter JW, Jordan HE and Ward BC (1973) Neurologic disease in wapiti naturally infected with meningeal worms. *Journal of Wildlife Diseases* **9**, 148–153.

Carreno RA, Caporaso D, Beade MS, Marull C, Uhart MM, Markwardt DD and Nadler SA (2012) Discovery of an undescribed protostrongylid nematode from the endangered pampas deer (*Ozotoceros bezoarticus celer*) in Argentina. *Journal of Wildlife Diseases* **48**, 724–731.

Chitwood MC, Keller BJ, Al-Warid HS, Straka K, Hildreth AM, Hansen L and Millspaugh JJ (2018) Meningeal worm (*Parelaphostrongylus tenuis*) as a cause of mortality in the elk (*Cervus canadensis*) population in Missouri, USA. *Journal of Wildlife Diseases* **54**, 95–100.

Clement M, Snell Q, Walke P, Posada D and Crandall K (2002) TCS: estimating gene genealogies. *Proceedings of the 16th International Parallel and Distributed Processing Symposium 2*, 184.

Comer CE, Kilgo JC, D'Angelo GJ, Glenn TC and Miller KV (2005) Fine-scale genetic structure and social organization in female white-tailed deer. *Journal of Wildlife Management* **69**, 332–344.

Cullingham CI, Merrill EH, Pybus MJ, Bollinger TK, Wilson GA and Coltman DW (2011) Broad and fine-scale genetic analysis of white-tailed deer populations: estimating the relative risk of chronic wasting disease spread. *Evolutionary Applications* **4**, 116–131.

Cunningham AA (1996) Disease risks of wildlife translocations. *Conservation Biology* **10**, 349–353.

Dent R (2014) *Elk Restoration 2010–2013*. Jefferson City, MO: Missouri Department of Conservation, 682 pp.

Deyoung RW, Demarais S, Honeycutt RL, Rooney AP, Gonzales RA and Gee KL (2003) Genetic consequences of white-tailed deer (*Odocoileus virginianus*) restoration in Mississippi. *Molecular Ecology* **12**, 3237–3252.

Dobey CL, Grunenwald C, Newman SJ, Muller L and Gerhold RW (2014) Retrospective study of central nervous system lesions and association with *Parelaphostrongylus* species by histology and specific nested polymerase chain reaction in domestic camelids and wild ungulates. *Journal of Veterinary Diagnostic Investigation* **26**, 748–754.

Doerner KC, Braden W, Cork J, Cunningham T, Rice A, Furman BJ and McElroy D (2005) Population genetics of resurgence: white-tailed deer in Kentucky. *Journal of Wildlife Management* **69**, 345–355.

Dybdahl MF and Storfer A (2003) Parasite local adaptation: red queen versus suicide king. *Trends in Ecology & Evolution* **18**, 523–530.

Eveland JF, George JL, Hunter NB, Forney DM and Harrison RL (1979) A preliminary evaluation of the ecology of the elk in Pennsylvania. In Boyce MS and Hayden-Wing LD (eds), *North American elk: Ecology, Behavior, and Management*. Laramie, Wyoming: University of Wyoming, pp. 145–151.

Ewen JG, Acevedo-Whitehouse K, Alley MR, Carraro C, Sainsbury AW, Swinnerton K and Woodroffe R (2012) Empirical consideration of parasites and health in reintroduction. In Ewen JG, Armstrong DP, Parker KA and Seddon PJ (eds), *Reintroduction Biology: Integrating Science and Management*. Oxford, UK: Blackwell Publishing Ltd., pp. 290–335.

Excoffier L and Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**, 564–567.

Faria PJ, van Oosterhout C and Cable J (2010) Optimal release strategies for captive-bred animals in reintroduction programs: experimental infections using the guppy as a model organism. *Biological Conservation* **14**, 35–41.

Gajadhar A, Steeves-Gurnsey R, Kendall J, Lankester M and Stéen M (2000) Differentiation of dorsal-spined elaphostrongyline larvae by polymerase chain reaction amplification of ITS-2 of rDNA. *Journal of Wildlife Diseases* **36**, 370–373.

Gassett JW (2001) Restoration of white-tailed deer in Kentucky: from absence to overabundance. In Maehr DS, Noss RF and Larkin JL (eds), *Large Mammal Restoration: Ecological and Sociological Challenges in the 21st Century*. Washington, DC, USA: Island Press, pp. 119–123.

Gerhold R and Hickling G (2016) Diseases associated with translocations of captive cervids in North America. *Wildlife Society Bulletin* **40**, 25–31.

Gerhold RW, Keel MK, Arnold K, Hotton D and Beckstead RB (2010) *Parelaphostrongylus tenuis*-associated meningoencephalitis in a sika deer (*Cervus Nippon*). *Journal of Wildlife Diseases* **46**, 287–290.

Gerhold R, Grunenwald C, Muller L and Su C (2016) Genetic characterization of the meningeal worm *Parelaphostrongylus tenuis* from multiple host species and across spatial scales. July, 2016, 65th Annual International Conference of the Wildlife Disease Association, Cortland, NY, USA. Available at http://programme.exordo.com/wda2016/.

Hartley M and Sainsbury A (2017) Methods of disease risk analysis in wildlife translocations for conservation purposes. *EcoHealth* **14**, 16–29.

Jacques CN, Jenks JA, Grovenburg TW, Klaver RW and Dubay SA (2015) Influence of ecologic factors on prevalence of meningeal worm (*Parelaphostrongylus tenuis*) infection in South Dakota, USA. *Journal of Wildlife Diseases* **51**, 332–340.

Keller BJ, Montgomery RA, Campa III HR, Beyer Jr DE, Winterstein SR, Hansen LP and Millspaugh JJ (2015) A review of vital rates and cause-specific mortality of elk *Cervus elaphus* populations in eastern North America. *Mammal Review* **45**, 146–159.

Kutz SJ, Veitch AM, Hoberg EP, Elkin BT, Jenkins EJ and Polley L (2001) New host and geographic records for two protostrongylids in Dall's sheep. *Journal of Wildlife Diseases* **37**, 761–774.

Lankester MW (2001) Extrapulmonary lungworms of cervids. In Samuel WM, Pybus MJ and Kocan AA (eds), *Parasitic Diseases of Wild Mammals*. Ames, IA, USA: Iowa State University Press, pp. 228–278.

Lankester MW (2010) Understanding the impact of meningeal worm, *Parelaphostrongylus tenuis*, on moose populations. *ALCES* **46**, 53–70.

Lankester MW and Anderson RC (1968) Gastropods as intermediate hosts of *Pneumostrongylus tenuis* Dougherty of whitetailed deer. *Canadian Journal of Zoology* **46**, 373–383.

Larkin JL, Alexy KJ, Bolin DC, Maehr DS, Cox JJ, Wichrowski MW and Seward N (2003) Meningeal worm in a reintroduced elk population in Kentucky. *Journal of Wildlife Diseases* **39**, 588–592.

Leigh JW and Bryant D (2015) PopART: full-feature software for haplotype network construction. *Methods in Ecology and Evolution* **6**, 1110–1116.

LoGiudice K (2003) Trophically transmitted parasites and the conservation of small populations: raccoon roundworm and the imperiled Allegheny woodrat. *Conservation Biology* **17**, 258–266.

Lopez JV, Yuhki N, Modi W, Masuda R and O'Brien SJ (1994) Numt, a recent transfer and tandem amplification of mitochondrial DNA in the nuclear genome of the domestic cat. *Journal of Molecular Evolution* **39**, 174–190.

Mathews F, Moro D, Strachan R, Gelling M and Buller N (2006) Health surveillance in wildlife reintroductions. *Biological Conservation* **131**, 338–347.

McDonald JS and Miller KV (2004) *A History of White-Tailed Deer Restocking in the United States 1878 to 2004*. Bogart, GA, USA: The Quality Deer Management Association.

Mitchell KJ, Peters-Kennedy J, Stokol T, Gerhold RW, Beckstead R and Divers T (2011) *Parelaphostrongylus tenuis* infections as a cause of meningomyelitis in 5 calves. *Journal of Veterinary Internal Medicine* **23**, 1097–1103.

Nadler SA (1992) Phylogeny of some ascaridoid nematodes, inferred from comparison of 18S and 28S rRNA sequences. *Molecular Biology and Evolution* **9**, 932–944.

Parker KA, Dickens MJ, Clarke RH, Lovegrove TG, Ewen JG and Armstrong DP (2012) The theory and practice of catching, holding, moving and releasing animals. In Ewen JG, Armstrong DP, Parker KA and Seddon PJ (eds), *Reintroduction Biology: Integrating Science and Management*. Oxford, UK: Blackwell Publishing Ltd., pp. 105–137.

Poulin R (2007). *Evolutionary Ecology of Parasites*, 2nd Edn. Princeton, NJ, USA: Princeton University Press.

Raskevitz RF, Kocan AA and Shaw JH (1991) Gastropod availability and habitat utilization by wapiti and white-tailed deer sympatric on range enzootic for meningeal worm. *Journal of Wildlife Diseases* **27**, 92–101.

Samuel WM, Pybus MJ, Welch DA and Wilke CJ (1992) Elk as a potential host for meningeal worm: implications for translocation. *Journal of Wildlife Management* **56**, 629–639.

Savage AE and Zamudio KR (2011) MHC Genotypes associate with resistance to a frog-killing fungus. *Proceedings of the National Academy of Sciences, USA* **108**, 16705–16710.

Seddon PJ, Armstrong DP and Maloney RF (2007) Developing the science of reintroduction biology. *Conservation Biology* **21**, 303–312.

Seddon PJ, Strauss WM and Innes J (2012) Animal translocations: what are they and why do we do them. In Ewen JG, Armstrong DP, Parker KA and Seddon PJ (eds), *Reintroduction Biology: Integrating Science and Management*. Oxford, UK: Blackwell Publishing Ltd., pp. 1–32.

Severinghaus CW and Darrow RW (1976) Failure of elk to survive in the Adirondacks. *New York Fish and Game Journal* **23**, 98–99.

Shostak AW and Samuel WM (1984) Moisture and temperature effects on survival and infectivity of first-stage larvae of *Parelaphostrongylus odocoilei* and *P. tenuis* (Nematoda: Metastrongyloidea). *Journal of Parasitology* **70**, 261–269.

Slabach BL, Hast JT, Murphy SM, Bowling WE, Crank RD, Jenkins G, Johannsen K and Cox JJ (2018) Survival and cause-specific mortality of elk *Cervus canadensis* in Kentucky, USA. *Wildlife Biology* **2018**, wlb-00459. doi: 10.2981/wlb.00459.

Southard T, Bender H, Wade SE, Grunenwald C and Gerhold RW (2012) Naturally occurring *Parelaphostrongylus tenuis*-associated choriomeningitis in a guinea pig with neurologic signs. *Veterinary Pathology* **50**, 560–562.

Swoffford DL (2002) *PAUP*: Phylogenetic analysis using parsimony (and other methods), version 4.0 b10*. Sunderland, UK: Sinauer Associates.

Tanabe M, Gerhold RW, Beckstead RB, de Lahunta A and Wade SE (2010) Molecular confirmation of *Parelaphostrongylus tenuis* infection in a horse with verminous encephalitis. *Veterinary Pathology* **47**, 759.

https://doi.org/10.1017/S0031182020001912 Published online by Cambridge University Press

Viggers KL, Lindenmayer DB and Spratt DM (1993) The importance of disease in reintroduction programmes. *Wildlife Research* **20**, 687–698.

Villesen P (2007) FaBox: an online toolbox for fasta sequences. *Molecular Ecology Notes* **7**, 965–968.

Wasel SM, Samuel WM and Crichton V (2003) Distribution and ecology of meningeal worm, *Parelaphostrongylus tenuis* (Nematoda), in northcentral North America. *Journal of Wildlife Diseases* **39**, 338–346.

Weiss RB, Sarver CS, Thilsted J and Wolfe BA (2008) Clinical *Parelaphostrongylus tenuis* infection in two captive American bison (*Bison bison*). *Journal of the American Veterinary Medical Association* **233**, 1127–1130.

Whitehead CE and Bedenice D (2009) Neurologic diseases in llamas and alpacas. *Veterinary Clinics of North America Food Animal Practice* **25**, 385–405.

https://doi.org/10.1017/S0031182020001912 Published online by Cambridge University Press