International Journal for Parasitology: Parasites and Wildlife 4 (2015) 283–290

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Morphological and morphometric differentiation of dorsal-spined first stage larvae of lungworms (Nematoda: Protostrongylidae) infecting muskoxen (*Ovibos moschatus*) in the central Canadian Arctic

Pratap Kafle [a, *], Manigandan Lejeune [b], Guilherme G. Verocai [a, 1], Eric P. Hoberg [c], Susan J. Kutz [a, b]

[a] Department of Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta T2N 4N1, Canada

[b] Canadian Wildlife Health Cooperative – Alberta Node, Faculty of Veterinary Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta T2N 4N1, Canada

[c] U.S. National Parasite Collection, Animal Parasitic Diseases Laboratory, USDA, ARS, BARC East 1180, 10300 Baltimore Avenue, Beltsville, MD 20705, USA

ARTICLE INFO

Article history:
Received 14 April 2015
Received in revised form
14 May 2015
Accepted 18 May 2015

Keywords:
Arctic parasitology
Diagnostic parasitology
Morphology and morphometry
Umingmakstrongylus pallikuukensis
Varestrongylus eleguneniensis

ABSTRACT

Umingmakstrongylus pallikuukensis and *Varestrongylus eleguneniensis* are the two most common protostrongylid nematodes infecting muskoxen in the North American Arctic and Subarctic. First stage larvae (L1) of these lungworms have considerable morphological similarity that makes their differential diagnosis very difficult. Using light microscopy, we studied in detail the L1 of these two species and identified the key differences in morphological and morphometric attributes. Thirty L1 of each species from naturally infected muskox were heat-killed and then assessed for morphological and morphometric features that could be used for species-level differentiation. Key differentiating features include: length and morphology of the tail extension, curvature of the body, ventral post-anal transverse cuticular striations, and total body length. A laboratory guide for differentiation of L1 based on these species-specific characters was prepared and used by an experienced observer to identify an additional 35 L1 extracted from a different set of fecal samples from free-ranging muskoxen with mixed infections. The identities of these L1 were confirmed by sequence analysis of the ITS-2 region of the nuclear ribosomal DNA. Accuracy of morphological identification was 100 percent, reflecting the reliability of the proposed guide for differentiation. Using the guide, three minimally trained lab assistants each fixed and accurately identified 10 of 10 randomly selected L1. Ability to morphologically differentiate these facilitates the monitoring of overlapping range expansion of both parasites in the Canadian Arctic. Studies enabling species-level parasite identification are also critical for defining biodiversity, detecting mixed infections, and understanding host–parasite interactions. Morphological identification is a simple, reliable and cost-effective alternative to labor and equipment intensive molecular methods and can easily be performed in low resource settings.

1. Introduction

Wild ungulates of North America are host to numerous parasites encompassing several genera in the family Protostrongylidae (Kutz et al., 2012). In general, protostrongylids are parasites of the pulmonary, musculo-skeletal and nervous system of wild and domestic ruminants. They require gastropods as intermediate hosts in which the first stage larva (L1) undergoes temperature dependent development to the infective third stage larva (Kutz et al., 2001b; Jenkins et al., 2006). The parasites are transmitted to the definitive host through ingestion of the infected gastropods or third stage larvae that have emerged from these intermediate hosts. Protostrongylids are considered important parasites because of the pathogenicity of some species as well as their sensitivity to the climate warming which has led to disease emergence and range

* Corresponding author.
E-mail addresses: pkafle@ucalgary.ca (P. Kafle), mlejeune@ucalgary.ca (M. Lejeune), gverocai@gmail.com (G.G. Verocai), eric.hoberg@ars.usda.gov (E.P. Hoberg), skutz@ucalgary.ca (S.J. Kutz).
[1] Current address: Department of Global Health, College of Public Health, University of South Florida, 3720 Spectrum Boulevard, Tampa, Florida 33613, USA.

http://dx.doi.org/10.1016/j.ijppaw.2015.05.003

expansion to new geographical areas (Handeland and Slettbakk, 1994; Kutz et al., 2013; Hoberg and Brooks, 2015). The discovery of new species, new host-associations and new geographic records of protostrongylids in North American ungulates in the last two decades (Hoberg et al., 1995, 2002; Kutz et al., 2001a; Jenkins et al., 2005; Verocai et al., 2014a) highlight the degree to which our understanding of parasite diversity in the Arctic is still incomplete. A greater knowledge of parasite biodiversity and host–parasite interactions is vital to better understand the role of parasites in arctic ecosystems (Davidson et al., 2011; Hoberg et al., 2013; Johnson et al., 2013) and inform wildlife management decisions.

A major challenge in defining and tracking biodiversity of protostrongylids is the inability to morphologically differentiate the L1 stages of parasites that are retrieved through less invasive fecal analysis; L1 of many species have similar morphology and a single host species may be infected by multiple protostrongylid species (Kutz et al., 2007, 2012). Historically, diagnosis of protostrongylids was based on fecal examination using the Baermann method to recover L1 (Forrester and Lankester, 1997). Differentiating between L1 with dorsal spines (e.g. species within the protostrongylid subfamilies Muelleriinae, Elaphostrongylinae and Varestrongylinae) and without dorsal spines (e.g species within Protostrongylinae) can be easy, however, differentiation among species within the dorsal-spined group is challenging because of their considerable morphological similarity (Van Wyk et al., 2004; Verocai et al., 2014b). Current methods involve the sequencing of PCR amplified ITS-2 of nuclear ribosomal DNA of individual larvae where sequences have been validated relative to adult worms that had been authoritatively identified (Kutz et al., 2007; Asmundsson et al., 2008). Although this is effective and accurate, it may be limited by resources and technical complexity and may not detect co-infections if only small numbers of larvae are sequenced from a limited number of hosts Although techniques aimed at multi-species differentiation are being developed, morphological and/or morphometric identification of larvae is useful because it requires less equipment and expertise and is cost efficient. Identification based on unique larval features permits the examination of a large number of larvae in a short period of time and at a lesser cost.

Muskoxen (*Ovibos moschatus*) are a culturally, economically and ecologically important species (Gunn et al., 1990) found naturally in the Canadian and Greenland Arctic and in introduced populations in Alaska, Scandinavia, and Russia (Gunn and Forchhammer, 2008). Five protostrongylid species have been identified in muskoxen globally. *Umingmakstrongylus pallikuukensis* Hoberg, Polley, Gunn and Nishi, 1995, *Varestrongylus eleguniensis* (Verocai, Kutz, Simard and Hoberg, 2014) and *Protostrongylus stilesi* Dikmans, 1931 are reported in the North American Arctic (Kutz et al., 2012) and *Muellerius capillaris* Mueller, 1889 and an unconfirmed species of *Elaphostrongylus* are reported to infect introduced muskoxen in Sweden and Norway (Holt et al., 1990; Davidson et al., 2014). *Umingmakstrongylus pallikuukensis* is a cyst-forming parasite that infects only muskoxen and is geographically restricted to the west-central Canadian Arctic (Hoberg et al., 1995; Kutz et al., 2013). In contrast, *V. eleguneniensis* has a broader host range occurring primarily in caribou, but also muskoxen, and rarely moose, and occupies an extensive geographical range from Alaska to Labrador, including areas of the boreal forest (Kutz et al., 2007; Verocai et al., 2014a). *Protostrongylus stilesi* is also found in muskoxen from North America, but only in introduced populations in areas of sympatry with Dall's sheep (*Ovis dalli dalli*) (Hoberg et al., 2002). This species is easily distinguishable from *U. pallikuukensis* and *V. eleguneniensis* by its spike-tailed larvae lacking a dorsal spine (Hoberg et al., 2002). *M. capillaris* and *Elaphostrongylus* sp. have not been detected in muskoxen from North America.

Herein we focus on two protostrongylid lungworms, *U. pallikuukensis* and *V. eleguneniensis*, infecting muskoxen of northern Canada. These parasites are important pathogens of muskoxen, as reflected by their increasing prevalence and intensity of infection, and expanding geographic range over the past decades (Hoberg et al., 1995; Kutz et al., 2013; Verocai et al., 2014a, b; Kafle et al., unpublished data). Previously confined to the North American mainland, *U. pallikuukensis* and *V. eleguneniensis* were discovered in muskoxen on Victoria Island in the Canadian Arctic Archipelago for the first time between 2008 and 2010 (Kutz et al., 2013). These two parasites infecting muskoxen, singly or in co-infections, are undergoing continuous geographic expansion and increasing in prevalence and intensity of infection across Victoria Island (Kutz et al., 2013; Hoberg and Brooks, 2015; Kafle, Kutz, Leclerc unpublished data). This recent invasion, establishment and rapid range expansion on the Arctic Archipelago is a potential conservation concern, thus reliable and efficient methods that can diagnose and differentiate these lungworms are needed to effectively track changes and inform management decisions.

The aim of this study was to identify morphological characters that can be used to reliably differentiate L1 of *U. pallikuukensis* and *V. eleguneniensis*. We examine the hypothesis that these two species of lungworms can be differentiated based on morphology and morphometry. Further, we had an opportunity for direct comparisons to L1 of *Cystocaulus ocreatus* Railliet and Henry, 1907, a representative of the sister-group for *Umingmakstrongylus* (see Carreno and Hoberg, 1999) as an initial basis for understanding the generality of some diagnostic attributes among these genera. The direct application of this study is for use in ongoing monitoring of range expansion of these lungworms in the Arctic, however, the general principle of improving morphological diagnosis of L1 in this family of parasites has broader implications.

2. Materials and methods

2.1. Source of larvae

Umingmakstrongylus pallikuukensis L1 used in this study were obtained from fecal samples collected from naturally infected muskox populations near Lady Franklin Point (68.50 N, 112.71 W), Victoria Island, Nunavut in 2007, and *V. eleguneniensis* L1 from Nunavik region, northern Quebec (58.75 N, 68.55 W) in 2008. L1 obtained from feces of muskoxen from these populations had previously been identified through sequencing of the ITS-2 and only *U. pallikuukensis* had been found in Lady Franklin Point samples (Kutz et al., 2013) and only *V. eleguneniensis* in northern Quebec (Verocai et al., 2014a). Prior to the start of the present study, an additional subset of L1 ($n = 10$) from each of these two populations was recovered from the archived feces by the beaker Baermann method (Forrester and Lankester, 1997) and subjected to DNA extraction, PCR, and sequencing of ITS-2 as described by Verocai et al. (2013) to confirm species identity. Larvae attributable to *C. ocreatus* were confirmed morphologically and genetically (See Kutz et al., 2007).

Voucher specimens of larval *Umingmakstrongylus* and *Varestrongylus* were deposited and archived in the Museum of Southwestern Biology (MSB), Division of Parasites, University of New Mexico, Albuquerque, NM, USA. (**MSB:Para:20798** and **MSB:Para:20799** respectively). Selected frozen specimens were also deposited in frozen-tissue archives at the same location (**MSB:Para:20802** and **MSB:Para:20800**). DNA sequences were deposited at the GenBank (Accession numbers: **KR057219** and **KR057220**).

2.2. Morphological and morphometric analysis of L1

L1 were isolated from feces using the beaker Baermann technique (Forrester and Lankester, 1997), individually placed on a clean glass slide in a drop of water (50 μL of tap water), and heat killed by passing the slide 10 times over the Bunsen burner flame (Kutz et al., 2001b). Once killed, 30 L1 of each species were examined microscopically in detail under bright-field and differential interference contrast (DIC) settings (Olympus BX53 fitted with digital camera, Olympus DP73, Olympus®) at 400 × magnification. Photomicrographs and measurements were taken using special software (CellSens; Olympus Soft Imaging Solutions GmbH). Detailed morphological observation was done to identify any consistent differences between the two species. Anatomically important larval features were located (see Fig. 1.) and measurements were done including: total body length, total tail length (from anus), tail extension length, length of dorsal spine and the maximum width of esophagus and body (Table 1). The anatomical features used for measurement were based on the original description of *U. pallikuukensis* (Hoberg et al., 1995) and similar studies by Kutz et al. (2001b) (Fig 1). Comparative specimens of *C. ocreatus* were not evaluated morphometrically, but were examined relative to qualitative structural attributes of the caudal region considered of importance in identification.

2.3. Development and testing a guide for L1 differentiation

Based on detailed morphological observations and morphometry, consistent differences between the species were identified and a guide to differentiate L1 of *U. pallikuukensis* and *V. eleguneniensis* was developed. We used this guide to identify L1 extracted from a different set of muskox fecal samples with known mixed infections collected during springs of 2013 and 2014 on south-central Victoria Island (68.26 N, 106.90 W to 70.09 N, 108.20 W). A total of 35 larvae were recovered individually using the Baermann method, heat fixed and examined at 400 × magnification by an experienced observer and species were identified based on attributes summarized in the guide. Each larva was then collected and sequenced (as above) to determine if the morphological identification was accurate.

To assess if morphological criteria were of use to laboratory personnel with minimal training, we had three undergraduate students as test users apply this guide in the identification of larvae. These students had no prior knowledge or experience with this family of parasites, but had a basic knowledge of routine laboratory procedures and light microscopy. The test users were given a short presentation explaining the key defining features for larval identification. After this training and familiarization with the guide, they were each asked to identify 10 L1. The larvae were selected randomly from different animals and the test users were asked to follow the protocol from heat killing through to microscopic examination in a consistent manner and work independently using the guide to identify the larvae. The identities were then checked and confirmed by the experienced observer.

2.4. Statistical analysis

Data analysis was carried out with Statistical Package for Social Sciences (SPSS Version 9.0). The morphometric data between the two species were compared using Student's *t*-test. The statistical significance was assessed at $P \leq 0.01$.

3. Results

We identified several morphometric and morphological characteristics for differentiating L1 of *U. pallikuukensis* and *V. eleguneniensis*. All measured characters (except esophagus maximum width) were significantly greater in *U. pallikuukensis* than in *V. eleguneniensis* (Table 1), with total body length and the length of tail extension identified as the most practical morphometric features for species differentiation.

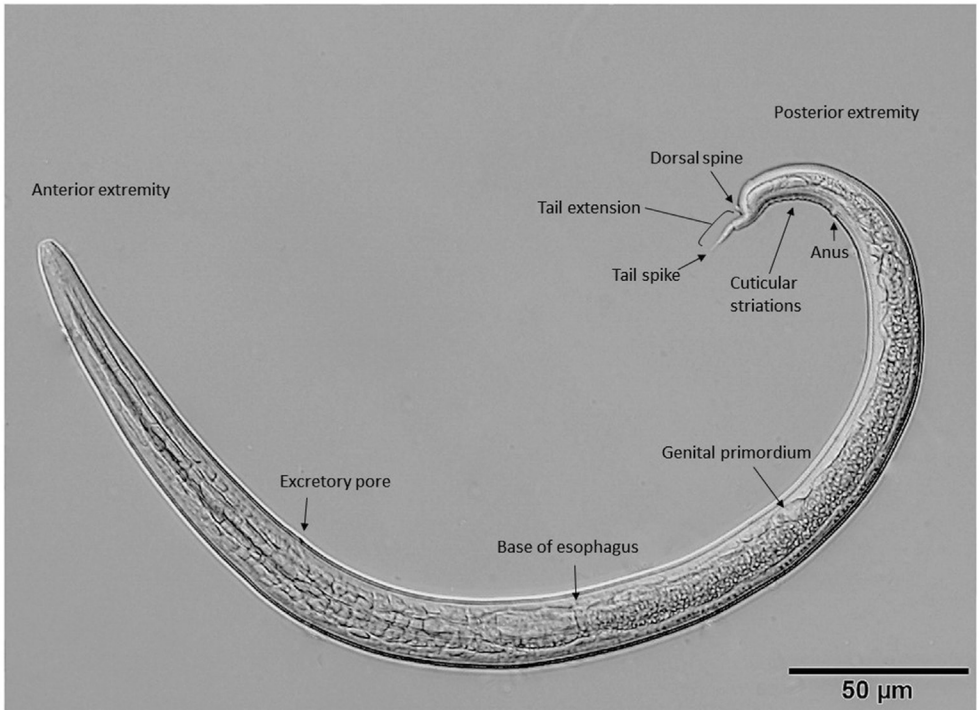


Fig. 1. Morphology of first stage larva (L1) of *Umingmakstrongylus pallikuukensis*. Photomicrograph of *U. pallikuukensis* L1 taken at 400 × magnification in differential interference contrast indicating the location of relevant important anatomical structures.

Table 1
Summary (Mean ± SD) of measurements (in micrometers) of 30 L1 each of *U. pallikuukensis* and *V. eleguneniensis*. Measurements of the excretory pore, genital primordium and anus were taken from the cephalic extremity. Maximum width was measured at the base of esophagus, tail length was measured from the anus to the tip of tail, and tail extension was measured from base of tail to the tail tip. The standard for measurement was based on earlier works by Hoberg et al. (1995) and Kutz et al. (2001b).

Dimensions	*Umingmakstrongylus pallikuukensis*	*Varestrongylus eleguneniensis*	*P* value
Total Length	427.89 ± 20.73 (396.70–467.13)	378.98 ± 9.84 (355.03–393.71)	<0.001
Maximum width	19.37 ± 0.95	17.98 ± 0.83	<0.001
Esophagus maximum width	12.67 ± 1.14	12.20 ± 1.24	>0.01
Excretory pore	115.90 ± 5.59	97.34 ± 3.48	<0.001
Genital primordium	272.45 ± 21.13	244.15 ± 5.38	<0.001
Anus	380.52 ± 17.91	341.32 ± 9.32	<0.001
Tail length[a] (from anus to tail tip)	47.36 ± 4.07	37.65 ± 4.19	<0.001
Tail extension length (3 folds)	12.82 ± 1.10 (10.28–14.55)	8.47 ± 0.82 (6.89–10.62)	<0.001
Dorsal spine	2.42 ± 6.65	1.70 ± 0.34	<0.001
Esophagus %	46.18 ± 2.24	43.90 ± 1.70	<0.001
Tail %	2.99 ± 0.24	2.23 ± 0.21	<0.001

[a] (As illustrated in Fig. 1) all measurements in micrometers (μm).

The most consistent and remarkable differences occurred in the caudal region of the body, primarily on the ventral aspect of the tail (region posterior to anus) (Fig. 1). The tail region of both the species has the same general structure. The tail extension of both *U. pallikuukensis* and *V. eleguneniensis*, like other protostrongylids, has three distinct cuticular folds, proximal, middle and distal with a dorsal spine originating at the base of the tail extension (Hoberg et al., 1995, 2005; Carreno and Hoberg, 1999). On detailed observation of the tail extension, *U. pallikuukensis* has a long and slender tail spike (tip of the tail) (Fig. 2A), whereas the tail spike of *V. eleguneniensis* is relatively shorter and angled ventrally giving a "vulture beak" appearance (Fig. 2B). These features were consistently characteristic across all L1 examined of each of the species and were identified as the primary character for differentiation. Similarly, the degree of ventral curving of the distal one third portion of heat killed L1 was also identified as one of the characteristic differences. The distal one third of *V. eleguneniensis* is more tightly curved than *U. pallikuukensis* (Fig. 2C and D). The third consistent feature was the difference in the appearance of surface cuticle anterior and posterior to the anus. The post-anal ventral cuticular surface in *U. pallikuukensis* has prominent transverse striations (Fig. 2E); this is not apparent in specimens of *V. eleguneniensis* (Fig. 2F). The other morphological feature that generally differed was the appearance of the intestinal granules. The intestinal granules appeared rougher and larger in *V. eleguneniensis* than in *U. pallikuukensis*. This feature, although seen in most of the larvae, was not appreciable in 100% of the larvae, so it could potentially be used only in support of the other identification characters.

Distal tail morphology in specimens of *C. ocreatus* resembled that of *U. pallikuukensis* with the presence of an elongate tail-spike. The presence of transverse striations in the ventral post-anal region was also a prominent character in specimens of *C. ocreatus* (Fig. 3).

Three minimally trained test users (undergraduate students) accurately discriminated L1 based on the developed guide. 10 randomly selected L1 prepared by heat-fixation (following the protocol described above) were correctly identified by each observer and subsequently verified by the experienced observer. The ease of using the keys, identification of L1 in a relatively short period of time, and 100% accuracy, reflected the simplicity, reliability and accuracy of the guide in rapid differential diagnosis.

4. Discussion

Our observations show that L1 of *U. pallikuukensis* and *V. eleguneniensis* can be differentiated with certainty based on key morphological and morphometric differences. The tail region was a key feature for differentiation of these two protostrongylids, and this is consistent with other nematodes and nematode stages where the morphological and morphometric variations in the caudal region of the larva have been important for species identification (Van Wyk et al., 2004; Hoberg et al., 2005). The distal segment of the tail extension of *U. pallikuukensis* is longer and straighter than the basal segments, ending in a slender spike (Figs. 1 and 2A). In contrast, for *V. eleguneniensis*, the distal segment is relatively much shorter and curved towards the body giving a "vulture beak" appearance of the tail spike (Fig. 2B). The straighter and longer distal segment of *U. pallikuukensis* L1 observed in our study is consistent with observations by Hoberg et al. (1995) in the first description of the parasite. Similarly, Kutz et al. (2007) commented on an apparently longer basal portion of the tail extension than distal tip in *V. eleguneniensis* compared to *Umingmakstrongylus* or species of *Parelaphostrongylus* Boev and Schulz, 1950. The morphology of this tail extension is so distinct and consistent that larval differentiation between *U. pallikuukensis* and *V. eleguneniensis* could potentially be based solely on this attribute. We believe that this major character, when supported by other features, improves the reliability and accuracy of identification.

We observed a unique pattern of curving of the distal one third region for each of these species. The distal extremity of heat fixed *V. eleguneniensis* was more curved ventrally (Fig. 2D) compared to *U. pallikuukensis*, which assumes more or less a J/C shape (Fig. 2C). The curving of the posterior part of the larva in protostrongylids has been mentioned previously (Lankester et al., 1976, 1998) but the degree of curving has not been reported as a diagnostic character. The storage time and conditions, and the handling and processing methods were the same for both species in our study, excluding their potential influence. This supports a species-specific appearance and shape of heat-killed larvae that is potentially diagnostic for *U. pallikuukensis* and *V. eleguneniensis*.

The ventral post-anal cuticular striations of *U. pallikuukensis* were more pronounced than those striations anterior to anus, which was not the case in *V. eleguneniensis*, where striations of cuticular surface were subtle and similar, anterior and posterior to the anus. This feature was surprisingly consistent in each of the species. Similar differences in appearance of ventral cuticular striations anterior and posterior to the anus were also observed in *Cystocaulus* (putative sister species of *U. pallikuukensis*; subfamily – Muelleriinae) collected from Uzbekistan (Fig. 3). Of possible phylogenetic significance, the general overall similarity of caudal morphology in *Umingmakstrongylus* and *Cystocaulus*, as shown in our specimens, may provide another unique structural attribute (synapomorphy) that defines the sister-group relationship for these genera (Carreno and Hoberg, 1999). If these characters are also confirmed for species of *Muellerius* Cameron, 1927, such would

Morphological and morphometric guide for differentiation of first stage larva (L1) of *Umingmakstrongylus pallikuukensis* and *Varestrongylus eleguneniensis*

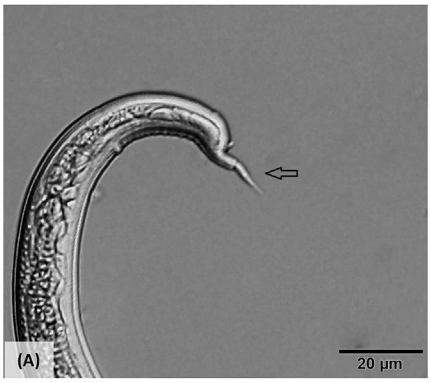
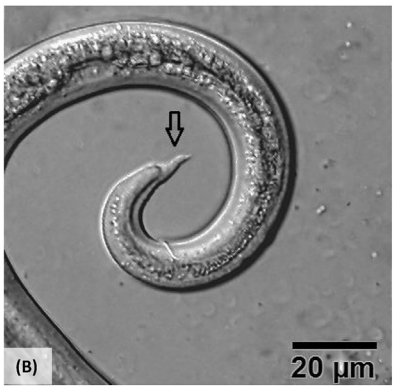
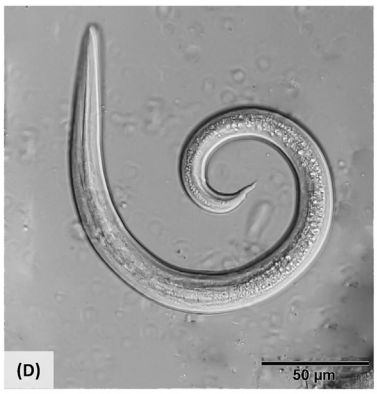
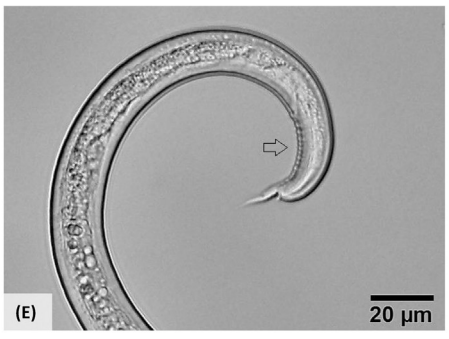
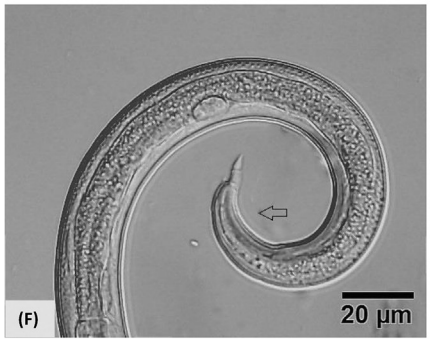
Characters	*Umingmakstrongylus pallikuukensis*	*Varestrongylus eleguneniensis*
	Main characters	
Tail spike morphology	Long and slender 	Small and curved tail spike assuming 'Vulture beak appearance" 
Caudal curvature	Less curved, assuming approximately J/C shape 	More curved towards the body at the caudal region 
Post-anal cuticular striations	More prominent cuticular striations posterior to anus 	No marked difference in cuticular striations anterior and posterior to anus 
	Supporting characters	
Tail extension length	Longer (>12 µm)	Smaller (<12 µm)
Total body length	Longer (> 400 µm)	Smaller (< 400 µm)

Fig. 2. Laboratory guide for the differentiation of L1 of *U. pallikuukensis* and *V. eleguneniensis*. The guide is based on key morphological features supported by morphometric data of heat killed L1. These features were characteristic of each of the species as visible under 400 × magnification.

Fig. 3. Morphology of *Cystocaulus ocreatus* first stage larva (L1). Photomicrograph of *C. ocreatus* L1 collected from Uzbekistan (US National Parasite Collection No. 95144) taken at 400 × magnification in differential interference contrast showing ventral post-anal cuticular striations and overall elongated structure of the tail spike similar to *U. pallikuukensis*.

provide further support for a phylogenetic diagnosis of the Muelleriinae as a discrete group within the protostrongylids.

Our observation of consistent differences in the prominence of ventral surface cuticular striations anterior and posterior to anus in *U. pallikuukensis*, but not in *V. eleguneniensis* provides a valuable visual clue for differential diagnosis of these two species. This feature was not specifically discussed in earlier studies of L1 of these parasites (Hoberg et al., 1995; Verocai et al., 2014a), nor were observations of the ventral tail region mentioned in scanning electron microscopy of a related protostrongylid, *Parelaphostrongylus odocoilei* (Hobmaier and Hobmaier, 1934) (Hoberg et al., 2005). Although it is hard to generalize only based on this study and requires further evaluation, this feature could be conserved in Muellerinae and potentially be used in differential diagnosis among other subfamilies of protostrongylids.

Although all the commonly used relevant anatomical features for larval differentiation were measured in this study, species differentiation was simplified by considering only total body length and tail length of the larvae, which are easily identifiable and measureable. Morphometrically, total body length and tail extension length of *V. eleguneniensis* were significantly shorter than *U. pallikuukensis* (Table 1), consistent with previous studies (Hoberg et al., 1995; Kutz et al., 2001b, 2007). Although Kutz et al. (2007) recorded shorter total body length of *V. eleguneniensis* in muskoxen from Nunavik (281–374 μm) compared to those from Aklavik, Northwest Territories (348–400 μm), this may reflect differences in fixation, with the former being fixed in ethanol and the latter heat killed in water. The measurements of heat killed *V. eleguneniensis* were, however, similar to our measurements for the same species. This reinforces the importance of standardizing fixation techniques when doing morphometric comparisons.

Currently, there are no other species of protostrongylids with dorsal-spined larvae reported in muskoxen from the North American Arctic. However, the possibility of transmission to muskoxen of species having similar larval morphology (e.g., *Parelaphostrongylus andersoni* Prestwood, 1972 and others) that infect assemblages of sympatric hosts including Caribou, moose, white-tailed deer, mule deer, and Dall's sheep cannot be ruled out. The dissolution of ecological barriers due to climate change, and natural and anthropogenic animal movement, may lead to changing patterns of host distribution and contact, potentially resulting in host switching (Hoberg et al., 2002; Kutz et al., 2009, 2012; Hoberg et al., 2012; Hoberg and Brooks, 2015). It is, therefore, relevant to complete similar studies to evaluate potential morphological differences between *V. eleguneniensis* and *U. pallikuukensis*, and those currently sympatric (*P. andersoni*) and those species that may expand northward with accelerating climate change (e.g., *P. odocoilei*, *Varestrongylus alpenae*) (Dikmans, 1935). Previous studies suggest that L1 length for *P. odocoilei* (334–428 μm), *P. andersoni* (308–382 μm) and *V. alpenae* (310–380 μm) (Prestwood, 1972; Mason, 1995; Kutz et al., 2001a; Verocai et al., 2014b) overlap with *U. pallikuukensis* and *V. eleguneniensis*, highlighting the value of investigation of morphological/structural differences.

At present, L1 of *U. pallikuukensis* and *V. eleguneniensis* are discriminated using molecular methods that usually sequence few larvae (often only individuals) from a larger subset or population within a host or at a particular locality. Estimates of diversity based on such protocols may be misleading because these species of protostrongylids differ in their fecundity. Field and experimental data demonstrate that *V. eleguneniensis* is considerably less fecund and, based on experimental trials, has a much shorter patency period (Kafle, Sullivan, Verocai and Kutz, unpublished) compared to *U. pallikuukensis*, which is highly fecund and long-lived (Kutz et al., 1999). Fecal surveys, based on direct observation and microscopy, also indicate that L1 of *U. pallikuukensis* generally outnumber *V. eleguneniensis* in co-infections (Kafle et al., unpublished data). Although different multiplex nucleic acid amplification technologies like species-specific random amplified polymer (RAPD)

markers, species-specific PCR, nested PCR, species-specific oligonucleotide probe, restriction length polymorphism analysis, DNA sequence comparison etc., are designed to detect mixed infections, they are also limited by their technical complexities, high sensitivity to impurities and higher costs (Perkins et al., 2011; Cunha and Inácio, 2015). In contrast, microscopy is far less resource and equipment intense and can be much more rapid, yet very effective. For example, a trained observer can examine and identify up to 100 L1 in roughly 2 min. This enables higher numbers of larvae to be examined and greatly reduces the chance of missing or underestimating the occurrence of less fecund species in a population of hosts (in our case, *V. eleguneniensis*). The influence of storage, handling and processing of larvae on morphology and morphometry, however, must always be taken into account, and the defining characters may not be consistent if methods are not standardized with the protocols followed in this study.

The significance of this study is emphasized by its relative advantage over currently employed identification methods (sequencing ITS-2 of individual larvae) by providing simple yet reliable alternatives and at the same time, improving parasite diagnosis by increasing accuracy and efficiency. This ultimately helps in better monitoring of current, continuous, and simultaneous range expansion of both species. The simplicity and low technical expertise of species diagnosis based on comparative morphology, supported by morphometry, makes it broadly relevant in parasite surveillance in general, and especially in resource poor settings. Our observations highlight the point that molecular methods are not always a panacea for challenging problems in parasite diagnostics. Further, we emphasize the fundamental importance of sound training in comparative morphological methods which remain at the core of integrated approaches in parasite systematics and diagnostics. Although we focused on only two species of protostrongylids of interest, this study can act as a model to stimulate detailed studies of other protostrongylids and reveal consistent and reliable morphological differences that will facilitate efficient and low cost definition and tracking of species diversity.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgments

This work was supported by scholarships to PK from the NSERC CREATE Host–Parasite Interactions Grant and Department of Ecosystem and Public Health, Faculty of Veterinary Medicine of University of Calgary and scholarship to GV from Alberta Innovates Health Solutions. Funding for research was provided through NSERC RTI, NSERC Discovery, and NSERC Northern Supplement grants to SK as well as funding from the Nunavut General Monitoring Program for sample collections. We thank Mathieu Dumond, and Lisa-Marie Leclerc of the Government of Nunavut, Manon Simard and the late Bill Doidge with the Nunavik Research Centre of the Makivik Corporation, Kamala Sapkota, Shruti Madhusudhan, Auguste Condemine, Mickael Combes for their help in the laboratory analysis and all the members of the Kutz lab for feedback, comments and support. Specimens of *Cystocaulus* were originally collected and made available for prior studies (e.g., Kutz et al., 2007) by Dr. Abdurakhim Kuchboev from the Laboratory of Parasitology, Institute of Zoology, Uzbekistan Academy of Sciences.

References

Asmundsson, I.M., Mortenson, J.A., Hoberg, E.P., 2008. Muscleworms, *Parelaphostrongylus andersoni* (Nematoda: Protostrongylidae), discovered in Columbia white-tailed deer from Oregon and Washington: Implications for biogeography and host associations. J. Wildl. Dis. 44, 16–27.

Carreno, R.A., Hoberg, E.P., 1999. Evolutionary relationships among the Protostrongylidae (Nematoda: Metastrongyloidea) as inferred from morphological characters, with consideration of parasite-host coevolution. J. Parasitol. 638–648.

Cunha, M.V., Inácio, J., 2015. Overview and challenges of molecular technologies in the veterinary microbiology laboratory. In: Veterinary Infection Biology: Molecular Diagnostics and High-Throughput Strategies. Springer, pp. 3–17.

Davidson, R., Simard, M., Kutz, S.J., Kapel, C.M., Hamnes, I.S., Robertson, L.J., 2011. Arctic parasitology: why should we care? Trends Parasitol. 27, 239–245.

Davidson, R.K., Amundsen, H., Lie, N.O., Luyckx, K., Robertson, L.J., Verocai, G.G., Kutz, S.J., Ytrehus, B., 2014. Sentinels in a climatic outpost: Endoparasites in the introduced muskox (*Ovibos moschatus wardi*) population of Dovrefjell, Norway. Int. J. Parasitol. Parasit. Wildl. 3, 154–160.

Forrester, S.G., Lankester, M.W., 1997. Extracting protostrongylid nematode larvae from ungulate feces. J. Wildl. Dis. 33, 511–516.

Gunn, A., Adamczewski, J.Z., Elkin, B., 1990. Commercial harvesting of muskoxen in the Northwest Territories. In: Renecker, L.A., Hudson, R.J. (Eds.), Proceedings from the Second International Wildlife Ranching Symposium, Edmonton, Alberta, pp. 197–204.

Gunn, A., Forchhammer, M.C., 2008. *Ovibos moschatus*. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1. (Ed. IUCN), IUCN. http://www.iucnredlist.org (accessed 13.02.15.).

Handeland, K., Slettbakk, T., 1994. Outbreaks of clinical cerebrospinal elaphostrongylosis in reindeer (*Rangifer tarandus tarandus*) in Finnmark, Norway, and their relation to climatic conditions. J. Vet. Med. Ser. B 41, 407–410.

Hoberg, E.P., Brooks, D.R., 2015. Evolution in action: climate change, biodiversity dynamics and emerging infectious disease. Theme issue – climate change and vector-borne diseases. Philos. Trans. B 370, 20130554.

Hoberg, E.P., Galbreath, K.E., Cook, J.A., Kutz, S.J., Polley, L., 2012. Northern host-parasite assemblages: history and biogeography on the borderlands of episodic climate and environmental transition. In: Rollinson, D., Hays, S.I. (Eds.), Advances in Parasitology, 79. Elsevier, pp. 1–97.

Hoberg, E.P., Jenkins, E.J., Rosenthal, B., Wong, M., Erbe, E.F., Kutz, S.J., Polley, L., 2005. Caudal polymorphism and cephalic morphology among first-stage larvae of *Parelaphostrongylus odocoilei* (Protostrongylidae: Elaphostrongylinae) in Dall's sheep from the Mackenzie mountains, Canada. J. Parasitol. 91, 1318–1325.

Hoberg, E.P., Kutz, S.J., Cook, J.A., Galaktionov, K., Haukisalmi, V., Henttonen, H., Laaksonen, S., Makarikov, A., Marcogliese, D.J., 2013. Parasites in terrestrial, freshwater and marine systems. In: Meltofte, H. (Ed.), Arctic Biodiversity Assessment – Status and Trends in Arctic Biodiversity, Conservation of Arctic Floral and Fauna. Arctic Council, Akureyi, Iceland, pp. 476–505.

Hoberg, E.P., Kutz, S.J., Nagy, J., Jenkins, E., Elkin, B., Branigan, M., Cooley, D., 2002. *Protostrongylus stilesi* (Nematoda: Protostrongylidae): ecological isolation and putative host-switching between Dall's sheep and muskoxen in a contact zone. Comp. Parasitol. 69, 1–9.

Hoberg, E.P., Polley, L., Gunn, A., Nishi, J.S., 1995. *Umingmakstrongylus pallikuukensis* gen nov et sp nov (Nematoda: Protostrongylidae) from muskoxen, *Ovibos moschatus*, in the central Canadian Arctic, with comments on biology and biogeography. Can. J. Zool. 73, 2266–2282.

Holt, G., Berg, C., Haugen, A., 1990. Nematode related spinal myelomeningitis and posterior ataxia in muskoxen (*Ovibos moschatus*). J. Wildl. Dis. 26, 528–531.

Jenkins, E.J., Appleyard, G.D., Hoberg, E.P., Rosenthal, B.M., Kutz, S.J., Veitch, A.M., Schwantje, H.M., Elkin, B.T., Polley, L., 2005. Geographic distribution of the muscle-dwelling nematode *Parelaphostrongylus odocoilei* in North America, using molecular identification of first-stage larvae. J. Parasitol. 91, 574–584.

Jenkins, E.J., Kutz, S.J., Hoberg, E.P., Polley, L., 2006. Bionomics of larvae of *Parelaphostrongylus odocoilei* (Nematoda: Protostrongylidae) in experimentally infected gastropod intermediate hosts. J. Parasitol. 92, 298–305.

Johnson, P.T., Preston, D.L., Hoverman, J.T., LaFonte, B.E., 2013. Host and parasite diversity jointly control disease risk in complex communities. Proc. Natl. Acad. Sci. 110, 16916–16921.

Kutz, S., Hoberg, E.P., Polley, L., 1999. Experimental infections of muskoxen (*Ovibos moschatus*) and domestic sheep with *Umingmakstrongylus pallikuukensis* (Nematoda: Protostrongylidae): parasite development, population structure, and pathology. Can. J. Zool. 77, 1562–1572.

Kutz, S., Veitch, A., Hoberg, E., Elkin, B., Jenkins, E., Polley, L., 2001a. New host and geographic records for two protostrongylids in Dall's sheep. J. Wildl. Dis. 37, 761–774.

Kutz, S.J., Asmundsson, I., Hoberg, E.P., Appleyard, G.D., Jenkins, E.J., Beckmen, K., Branigan, M., Butler, L., Chilton, N.B., Cooley, D., Elkin, B., Huby-Chilton, F., Johnson, D., Kuchboev, A., Nagy, J., Oakley, M., Polley, L., Popko, R., Scheer, A., Simard, M., Veitch, A., 2007. Serendipitous discovery of a novel protostrongylid (Nematoda : Metastrongyloidea) in caribou, muskoxen, and moose from high latitudes of North America based on DNA sequence comparisons. Can. J. Zool. 85, 1143–1156.

Kutz, S.J., Checkley, S., Verocai, G.G., Dumond, M., Hoberg, E.P., Peacock, R., Wu, J.P., Orsel, K., Seegers, K., Warren, A.L., Abrams, A., 2013. Invasion, establishment, and range expansion of two parasitic nematodes in the Canadian Arctic. Glob. Change Biol. 19, 3254–3262.

Kutz, S.J., Ducrocq, J., Verocai, G.G., Hoar, B.M., Colwell, D.D., Beckmen, K.B., Polley, L., Elkin, B.T., Hoberg, E.P., 2012. Parasites in ungulates of arctic North america and Greenland: a view of contemporary diversity, ecology, and impact

in a world under change. In: Advances in Parasitology, vol. 79. Elsevier Academic Press Inc, San Diego, pp. 99–252.
Kutz, S.J., Hoberg, E.P., Polley, L., 2001b. *Umingmakstrongylus pallikuukensis* (Nematoda : Protostrongylidae) in gastropods: larval morphology, morphometrics, and development rates. J. Parasitol. 87, 527–535.
Kutz, S.J., Jenkins, E.J., Veitch, A.M., Ducrocq, J., Polley, L., Elkin, B., Lair, S., 2009. The Arctic as a model for anticipating, preventing, and mitigating climate change impacts on host–parasite interactions. Vet. Parasitol. 163, 217–228.
Lankester, M.W., Crichton, V.J., Timmermann, H.R., 1976. A protostrongylid nematode (Strongylida: Protostrongylidae) in woodland caribou (*Rangifer tarandus caribou*). Can. J. Zool. 54, 680–684.
Lankester, M.W., Olsson, I.-M.C., Stéen, M., Gajadhar, A.A., 1998. Extra-mammalian larval stages of *Elaphostrongylus alces* (Nematoda: Protostrongylidae), a parasite of moose (*Alces alces*) in Fennoscandia. Can. J. Zool. 76, 33–38.
Mason, P.C., 1995. *Elaphostrongylus cervi* and its close relatives; a review of protostrongylids (Nematoda, Metastrongyloidea) with spiny-tailed larvae. Surveillance 22, 19–24.
Perkins, S., Martinsen, E., Falk, B., 2011. Do molecules matter more than morphology? Promises and pitfalls in parasites. Parasitology 138, 1664–1674.
Prestwood, A.K., 1972. *Parelaphostrongylus andersoni* sp. n. (Metastrongyloidea: Protostrongylidae) from the musculature of white-tailed deer (*Odocoileus virginianus*). J. Parasitol. 897–902.
Van Wyk, J.A., Cabaret, J., Michael, L.M., 2004. Morphological identification of nematode larvae of small ruminants and cattle simplified. Vet. Parasitol. 119, 277–306.
Verocai, G.G., Kutz, S.J., Simard, M., Hoberg, E.P., 2014a. *Varestrongylus eleguneniensis* sp. n. (Nematoda: Protostrongylidae): a widespread, multi-host lungworm of wild North American ungulates, with an emended diagnosis for the genus and explorations of biogeography. Parasit. Vect. 7, 556.
Verocai, G.G., Hoberg, E.P., Vikøren, T., Handeland, K., Ytrehus, B., Rezansoff, A.M., Rezansoff, R.K., Davidson, J.S., Kutz, S.J., 2014b. Resurrection and redescription of *Varestrongylus alces* (Nematoda; Protostrongylidae), a lungworm of the Eurasian moose (*Alces alces*), with report on associated pathology. Parasit. Vect. 7, 557.
Verocai, G.G., Lejeune, M., Finstad, G.L., Kutz, S.J., 2013. A Nearctic parasite in a Palearctic host: *Parelaphostrongylus andersoni* (Nematoda; Protostrongylidae) infecting semi-domesticated reindeer in Alaska. Int. J. Parasitol. Parasit. Wildl. 2, 119–123.