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

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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...

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expansion to new geographical areas (Handeland and Slettbak, 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 Varestraeleninae) and without dorsal spines (e.g. species within Protostrongylinae) can be elusive, however differentiation among species within the dorsal-spined group is challenging because of the 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; Asmundson 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 multispecies 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.

Musoxen (Ovis 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 musoxen globally. Umingmakstrongylus pallikuukensis Hoberg, Polley, Gunn and Nishi, 1995, Varestraelongus eleguneniensis (Verocai, Kutz, Simard and Hoberg, 2014) and Protostrongylus stilesi Dikmans, 1931 are reported in the North American Arctic (Kutz et al., 2012) and Muelleriella etiosa Mueller, 1889 and an unconfirmed species of Elaphostrongylus are reported to infect introduced musoxen in Sweden and Norway (Holt et al., 1990; Davidson et al., 2014). Umingmakstrongylus pallikuukensis is a cyst-forming parasite that infects only musoxen and is geographically restricted to the western-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 musoxen, 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 musoxen from North America, but only in introduced populations in areas of sympathy 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 musoxen from North America.

Herein we focus on two protostrongylid lungworms, U. pallikuukensis and V. eleguneniensis, infecting musoxen of northern Canada. These parasites are important pathogens of musoxen, 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; Kafel et al., unpublished data). Previously confined to the North American mainland, U. pallikuukensis and V. eleguneniensis were discovered in musoxen on Victoria Island in the Canadian Arctic Archipelago for the first time between 2008 and 2010 (Kutz et al., 2013). These two parasites infecting musoxen, 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; Kafel, 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 morphtmometry. 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 muskox 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 ≤ 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.](image-url)
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 (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 ± 0.65                     | 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 |

* (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, this 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 other group relationships 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* |
|-------------------------------------|-------------------------------------|---------------------------------|
| 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 x magnification.
provide further support for a phylogenetic diagnosis of the Muellerinae 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, 1993) (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 musk-oxen from Nunavik (281–384 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*, *Var-estrostrongylus 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. elegenienisii*). 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.

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