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Experimental Life-Cycle of *Varestrongylus eleguneniensis* (Nematoda: Protostrongylidae) in a Captive Reindeer (*Rangifer tarandus tarandus*) and a Muskox (*Ovibos moschatus moschatus*)

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ABSTRACT: The life-cycle of a recently described protostrongylid lungworm, *Varestrongylus eleguneniensis*, which infects caribou, muskoxen, and moose from Arctic and boreal regions of North America, was completed experimentally for the first time. A native North American slug species, *Deroceras laeve*, was infected with the first-stage larvae (L1) isolated from the feces of wild muskoxen to generate third-stage larvae (L3). These were administered to a captive reindeer calf (250 L3) and an adult captive muskox (380 L3). The prepatent periods for the reindeer and muskox were 56 and 72 days, respectively. Patency lasted for only 19 days in the reindeer, and fecal larval counts were very low (0.09–1.53 larvae per gram of feces). Patency in the muskox was at least 210 days, and likely over 653 days, and the fecal larval counts were higher (0.06–17.8 larvae per gram of feces). This work provides the first experimental completion of the life-cycle of *V. eleguneniensis*.

Varestrongylus eleguneniensis Verocai, Kutz, Simard and Hoberg, 2014 (Nematoda: Protostrongylidae) is a recently described multi-host lungworm that infects caribou (*Rangifer tarandus* sspp.), muskoxen (*Ovibos moschatus* sspp.) and moose (*Alces americanus* sspp.) across boreal, sub-Arctic, and Arctic latitudes of North America (Verocai et al., 2014b). The small (1–2 cm) adult nematodes reside in the terminal bronchioles of the lungs, but the associated pathology and impacts on hosts, as well as broader aspects of its life-cycle and transmission dynamics, remain unknown.

Species of *Varestrongylus*, similar to all species within the family Protostrongylidae Leiper, 1926 (Strongylida: Metastrongyloidea), have indirect life-cycles requiring a gastropod intermediate host for development and transmission. First-stage larvae (L1) are shed in the feces of the definitive hosts. Once in the environment, the L1 enters gastropod intermediate hosts where they undergo a temperature-dependent development to third-stage larvae (L3). The definitive host becomes infected through the ingestion of gastropods containing L3 or, for some protostrongylid species, L3 that have emerged from the intermediate host and are present free in the environment (Kutz et al., 2000).

The transmission dynamics of protostrongylid nematodes in Arctic and sub-Arctic Canada are highly influenced by climatic conditions and season length (Kutz et al., 2005; Jenkins et al., 2006b). It has been hypothesized that recent warming in the Arctic is a major driver for the recent range expansion of *V. eleguneniensis* and the related muskox lungworm *Umingmakstrongylus pallikuukensis* Hoberg, Polley, Gunn and Nishi, 1995 onto Victoria Island in the Arctic Archipelago (Kutz et al., 2013). Defining key features of the life-cycle, including prepatent and patent periods as well as patterns of larval output, are critical to understanding the transmission dynamics of *V. eleguneniensis* in an increasingly changing environment. Additionally, in a multi-host system, investigating differences in the parasite's life history characteristics among hosts provides valuable insights into the ecology of parasites (Holt et al., 2003; Dobson, 2004). The objective of this study was to elucidate the life cycle of *V. eleguneneniensis* in its 2 main definitive hosts, caribou and muskoxen.

The experimental trials were done with the approval of the Animal Care Committee of the University of Calgary (Animal Care Protocol BI09R-14). Fecal samples collected from wild, naturally infected muskoxen near Kuujjuaq (58°45′00′′N, 68°33′29′′W), Nunavik Region, Quebec, Canada, were used as a source of L1, which were extracted using the beaker Baermann technique (Forrester and Lankester, 1997). L1 were pooled together and stored in the refrigerator at 4 C for up to 3 days (until infection of gastropods). A pilot study using the garden slug, *Deroceras reticulatum*, as an intermediate host resulted in slow larval development (L3 first observed in 52 days post-infection [DPI]) and poor recovery of L3. Exposure of 1 reindeer (*Rangifer tarandus tarandus*) to 30 L3 and 1 muskox (*Ovibos moschatus wardi*) to 51 L3 recovered from *D. reticulatum* did not result in patent infections.

The meadow slug, *Deroceras laeve* (Müller, 1774), collected within the town limits of Kugluktuk (67°49′32″N, 115°05′42″W) and near the hamlet of Cambridge Bay (69°07′04″N, 105°03′28″W) in Nunavut, Canada, was used to obtain L3 for the experimental infections. *Deroceras laeve* collected from Kugluktuk were assumed to be free of infection by *V. eleguneniensis* or any other protostrongylid nematode because live ungulates do not enter the town and all hunted animals are butchered in the field. Similarly, specimens collected near Cambridge Bay were considered free from any protostrongylids because wild muskoxen and caribou were rare to absent at the collection sites. To further confirm that slugs were free of prior protostrongylid infection, we inspected their feet for characteristic lesions (Kutz et al., 2000). No foot lesions suggestive of protostrongylid infection were found in any slugs.

Gastropods were maintained in sealed containers with kale, carrots, and moist paper towels at 2–4 C prior to infection. The slugs were infected in groups of 5 and maintained in contact with

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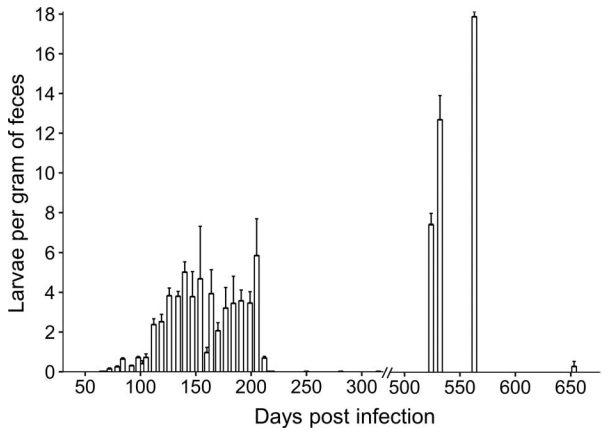

FIGURE 1. Pattern of larval shedding for a muskox infected with *Varestrongylus eleguneniensis* third-stage larvae (L3). Each bar represents the mean larval count (per gram of feces) ± SE averaged for a week.

L1 for 3 hr. The infected slugs were then kept in the same dishes overnight before being transferred to a clean dish with food. In all cases, slugs were maintained at room temperature (~20 C). Dates of digestion were pre-determined (18–24 days) based on our preliminary trials and published studies (Kutz et al., 2001; Jenkins et al., 2006a). Third stage larvae were stored in tap water in 25-ml Erlenmeyer flasks covered with Parafilm (Sigma-Aldrich, St. Louis, Missouri) and refrigerated at 4 C until administration (maximum 3 days later) to the reindeer and the muskox. Prior to administration of L3, all larvae were inspected for mobility, counted, and transferred to 2.0-ml Eppendorf tubes containing water.

We used a captive-bred domestic reindeer and a muskox as experimental definitive hosts. The domestic reindeer was considered a suitable surrogate for wild caribou because *V. eleguneniensis* naturally infects 3 wild caribou subspecies in North America, 2 of which (*Rangifer tarandus groenlandicus* and *Rangifer tarandus granti*) are genetically closer to Eurasian reindeer than are woodland caribou (*Rangifer tarandus caribou*) (Banfield, 1961; Flagstad and Røed, 2003; Yannic et al., 2014). A reindeer calf (male, age 5 mo) was manually restrained in a handling chute and the muskox (male, age 8.5 yr) was chemically immobilized with an intramuscular injection of medetomidine (Vetlabs, Calgary, Canada) 25 mg, azaperone (Elanco, Greenfield, Indiana) 20 mg, and alfaxalone (Jurox, New South Wales, Australia) 20 mg. L3 were administered orally with a dosing syringe (reindeer) or by gastric tube (muskox). The dosing syringe and gastric tubes were flushed with 60 ml of water, 3 times, and then with 60 ml of air to minimize larval loss. Animals were maintained in pens, with access to grass and water ad libitum, and fed concentrates daily. Fresh fecal samples were collected at different intervals (Fig. 1) and assessed for the presence of L1 using the beaker Baermann technique (4–8 repetitions of ~5 g of feces per sampling day, depending on fecal availability). The species identity of recovered L1 was determined morphologically (Kafle et al., 2015) and by PCR amplification and sequencing of the ITS-2 region of the nuclear ribosomal DNA using published protocols (Kutz et al., 2007). It was not deemed necessary to euthanize the animals for adult parasite recovery as (1) the success of the infection had been established through definitive identification of larvae as indicated above, (2) adult parasite recovery for this species is extremely labor intensive (washing and sieving of lungs) and the sensitivity of the technique for quantifying parasite establishment is unknown, yet likely very poor, thus data on percent establishment would not be reliable, and (3) obtaining and maintaining these host species in captivity is extremely difficult, and as such, the experimental animals are highly valuable research animals and euthanasia for this study could not be justified.

Live L3 were first recovered from the slugs at 18 DPI. Administration of 250 L3 (reindeer) and 380 L3 (muskox) resulted in patent infections. Larvae recovered from feces of both hosts were confirmed as *V. eleguneniensis* morphologically as per Kafle et al. (2015) and based on the sequences at the ITS-2 region (GenBank KY498641-KY498644). Larvae were deposited as vouchers at the United States National Parasite Collection (USNPC) under accession numbers USNPC 106345–106347.

The prepatent period of *V. eleguneniensis* in the reindeer was 56 days and the patency period was 19 days (Table I). Larval counts were extremely low throughout the reindeer patency (0.09–1.53 larvae per gram [LPG]), peaking at 2 wk after patency. The prepatent period in the muskox was 72 days; L1 counts in the feces were higher than in the reindeer (0.06–13.2 LPG) and peaked at 9 wk after patency was established (Fig. 1). The muskox shed L1 continuously from 72 to 210 DPI, and then no L1 were detected on subsequent weekly monitoring for 90 days, at which point the trial was considered completed (Fig. 1). During a routine health check, the muskox was retested at 524 DPI and L1 were found. Subsequent testing on days 532, 563, and 653 also revealed the presence of L1, and the highest larval count was observed on 563 DPI (17.84 ± 0.48 LPG). All L1 examined were morphologically confirmed to be *V. eleguneniensis* (Kafle et al., 2015). No L1 were detected during opportunistic fecal testing at 711, 713, 720, and 743 DPI, and fecal sampling was stopped (Fig. 1). It is not clear whether the resumption of shedding was due to the initial infection followed by the reduction of larval shedding below the sensitivity threshold of the test over winter and subsequent resumption; or if the muskox had become re-infected through ingestion of infected gastropods. The latter was considered unlikely for 2 reasons: First, the larval counts were low and the muskox ranged over 5 acres of pasture, thus very low

TABLE I. Experimental infections of a reindeer and a muskox with *Varestrongylus eleguneniensis*. Abbreviations: PPP: pre-patent period; PP: patent period.

Animal	L3 dose	Infection date*	Experiment end date†	Monitoring period	PPP	PP
Reindeer	250	29 September 2011	19 June 2012	9 mo	56 days	19 days
Muskox	380	18 November 2013	2 December 2015	2 yr	72 days	~ 2 yr

* Day 0 of experimental infection trials with *V. eleguneniensis* L3.
† Last day of fecal collection.

environmental contamination occurred. Secondly, the pasture was not typical of a 'good' gastropod habitat and, while it is possible that they were present, the density would be very low. However, we still cannot preclude the possibility of re-infection.

We have successfully completed the life-cycle of *V. eleguneniensis* under experimental conditions in a reindeer and a muskox. Comparisons of life-cycle parameters for *V. eleguneniensis* to other species of *Varestrongylus* are limited as data are only available for 2 out of the 10 valid species within this genus. The prepatent period of *Varestrongylus alpenae* (Dikmans, 1935), a lungworm of the white-tailed deer, *Odocoileus virginianus*, was 54 and 55 DPI in 2 white-tailed deer infected with 200–300 L3 and was between 43 and 49 DPI in 4 mule deer, *Odocoileus hemionus*, infected with a higher number of L3 (750–1,500) (Gray et al., 1985). In contrast, the prepatent period of *Varestrongylus sagittatus* (Mueller, 1890), a lungworm of the red deer, *Cervus elaphus*, is 134 days (Boev, 1975). The shorter prepatent period of *V. eleguneniensis* is perhaps more consistent with that of *V. alpenae*, which could be explained by their closer phylogenetic relationship to each other compared to the Eurasian species *V. sagittatus* (Verocai et al., 2014a).

This study demonstrates that the slug *D. laeve* is a suitable intermediate host species for *V. eleguneniensis*, as evidenced by rapid larval development in the slug at room temperature, good L3 recovery, and the subsequent establishment of the parasite in both the definitive hosts. *Deroceras laeve* has been found naturally infected with *V. eleguneniensis* and is considered a main intermediate host for several other North American protostrongylids (Hoberg et al., 1995; Kutz et al., 2001; Jenkins et al., 2006a). Another widespread but introduced gastropod species, *D. reticulatum* (Chichester and Getz, 1969), has been found to be a suitable intermediate host for some North American protostrongylids; for example, *Parelaphostrongylus odocoilei* (Hobmaier and Hobmaier, 1934) and *U. pallikuukensis* (Kutz et al., 2001), but results from our pilot study suggests that *D. reticulatum* is a poor intermediate host for *V. eleguneniensis*. While the failure of establishment of *D. reticulatum*-derived L3 might be related to low infection doses, studies on other protostrongylids have demonstrated good establishment at low doses (Pybus et al., 1992; Duffy et al., 2004).

Although our work is based on infections of only 1 individual of each host species, these infections occurred under similar conditions, and a few characteristics of the life-cycles observed here are worthy of note in relation to what is observed in wild populations. For example, where the parasite is established in muskoxen in the wild, prevalence approaches 100% and larva per gram of feces (LPG) ranges from 0.2 to 283. In contrast, in caribou, prevalence ranges from 0–27% and LPG ranges from 0.17–148 (Kutz et al., 2012; Verocai, 2015; P. Kafle, unpubl. data). Based on the low sample size, it is difficult to draw conclusions on differences on patency between the 2 hosts; however, the long patency observed in the experimentally infected muskox corroborates patterns observed in the wild. Phylogenetic studies suggest that *V. eleguneniensis* is a primary caribou parasite that colonized North America along with caribou and subsequently underwent several independent host-switching events to muskoxen in contact zones from, at minimum, the late Pleistocene until modern times (Verocai et al., 2014b; Verocai, 2015). The strong evidence of a higher success rate of *V. eleguneniensis* in muskoxen in the wild, and apparently in our experimental infections, may reflect the difference between a longer-standing host–parasite association with caribou vs. a more recent colonization of muskoxen. Host-switching events for a variety of other parasites to muskoxen have occurred including the protostrongylids *Protostrongylus stilesi* (Dikmans, 1931) and *Muellerius capillaris* (Mueller, 1889) through contact with Dall's sheep (*Ovis dalli*) and domestic sheep (*Ovis aries*), respectively (Alendal and Helle, 1983; Hoberg et al., 2002; Davidson et al., 2014).

Varestrongylus eleguneniensis is currently undergoing considerable northward range expansion in the Canadian Arctic (Kutz et al., 2013; P. Kafle, unpubl. data). While this study was limited to a single infection in each definitive host species, it has provided valuable new data on the life history of this parasite with suggestions that, not surprisingly, life history characteristics may vary between host species. These data, together with experimental studies to establish key thermal tolerances of *V. eleguneniensis* in the environment and in its intermediate hosts, will be used in predictive models to understand the current and future transmission dynamics, distribution, and impacts of this multi-host parasite in a rapidly changing environment (Hoberg et al., 2008; Kutz et al., 2009, 2014).

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