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Caudal Polymorphism and Cephalic Morphology among First- Stage Larvae of *Parelaphostrongylus odocoilei* (Protostrongylidae: Elaphostrongylinae) in Dall's Sheep from the Mackenzie Mountains, Canada

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CAUDAL POLYMORPHISM AND CEPHALIC MORPHOLOGY AMONG FIRST-STAGE LARVAE OF *PARELAPHOSTRONGYLUS ODOCOILEI* (PROTOSTRONGYLIDAE: ELAPHOSTRONGYLINAE) IN DALL'S SHEEP FROM THE MACKENZIE MOUNTAINS, CANADA

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ABSTRACT: We demonstrate polymorphism in the structure of the tail among first-stage larvae of *Parelaphostromylus odocoilei* (Protostrongylidae). Two distinct larvae, both with a characteristic dorsal spine, include (1) a morphotype with a kinked conical tail marked by 3 distinct transverse folds or joints and a symmetrical terminal tail spike and (2) a morphotype with a digitate terminal region lacking folds or joints and with an asymmetrical, subterminal tail spike. These divergent larval forms had been postulated as perhaps representing distinct species of elaphostromyline nematodes. Application of a multilocus approach using ITS-2 sequences from the nuclear genome and COX-II sequences from the mitochondrial genome confirmed the identity of these larvae as *P. odocoilei*. Additionally, based on scanning electron microscopy (low-temperature field emission), the cephalic region of these larvae consisted of a cuticular triradiate stoma surrounded by 6 single circumoral papillae of the inner circle, 10 papillae of the outer circle (4 paired and 2 single), and 2 lateral amphids. Our's is the first demonstration of structural polymorphism among larval conspecifics in the Metastrongyloidea and Strongylida. The basis for this polymorphism remains undetermined, but such phenomena, if discovered to be more widespread, may contribute to continued confusion in discriminating among first-stage larvae for species, genera, and subfamilies within Protostrongylidae.

In North American ungulates, species among 5 genera of protostrongylids, i.e., *Elaphostromylus* Cameron, 1931, *Parelaphostromylus* Boev and Schul'ts, 1950, *Muellerius* Cameron, 1927, *Umingmakstrongylus* Hoberg, Polley, Gunn and Nishi, 1995, and *Varestrongylus* Bhalerao, 1932, produce morphologically similar first-stage larvae (L1) with a characteristic dorsal spine (DSL) (Boev, 1975; Mason, 1995; Carreno and Hoberg, 1999). Other species of protostrongylids in the Nearctic (e.g., species of *Protostrongylus* Kamensky, 1905, and *Orthostromylus* Dougherty and Gobel, 1946) produce spike-tailed larvae clearly distinguishable from such DSL (Boev, 1975; Mason, 1995; Carreno and Hoberg, 1999). Although relative and absolute lengths for L1 and third-stage larvae (L3) among a diversity of protostrongylids has been applied in species-level identification, measurements may overlap among congeners or among larvae representing otherwise divergent genera (Platt, 1978; Kralka and Samuel, 1984; Pybus and Shave, 1984; Pybus et al., 1984; Gray et al., 1985). Intraspecific variation in length for L1 has commonly been recognized among a diversity of protostrongylids in ungulate hosts, including *Cystocaulus ocreatus* (Railliet and Henry, 1907), *Muellerius capillaris* (Mueller, 1889), and *Protostrongylus tauricus* Schul'ts and Kadenazii, 1949 (Boev, 1975); among Elaphostromylinae, such has been observed in *Parelaphostromylus odocoilei* (Hobmaier and Hobmaier, 1934), *P. andersoni* Prestwood, 1972, and *Elaphostromylus alces* Stéen, Chabaud and Rehbinder, 1989, from isolated geographic localities (e.g., Kontrimavichus et al., 1976; Platt, 1978; Lankester and Hauta, 1989; Lankester et al., 1998). Consequently, definitive identification of L1 among genera or

species based on either morphological or meristic data is often problematic due to extensive intraspecific variation (length) or minimal interspecific variation manifested in multiple genera with structurally similar larvae (Jenkins et al., 2005).

Structural polymorphism among L1 representing single species is considered uncommon among the Protostrongylidae (Boev, 1975; Kontrimavichus et al., 1976; Lankester, 2001), although it has been recognized, but not extensively described, in the structure of the tails and tail spikes among DSL of some Elaphostromylinae, such as *P. odocoilei* and *P. andersoni* (Lankester et al., 1976; Pybus and Shave, 1984; Kutz et al., 2001). Pybus and Shave (1984) reported extensive variation in caudal structure among populations of DSL in fecal samples from mule deer, *Odocoileus hemionus* (Rafinesque), and mountain goats, *Oreamnos americanus* (de Blainville), infected with adult *P. odocoilei* from Alberta. Subsequently, Kutz et al. (2001) briefly described 2 distinctive forms of L1 based on specimens in fecal samples from Dall's sheep, *Ovis dalli dalli* Nelson, with patent infections of *P. odocoilei* from the Mackenzie Mountains, Northwest Territories. They suggested that 1 of these morphotypes could represent an undescribed species with adult nematodes sequestered in an unknown site, perhaps in the vasculature. Meristic differences, however, were not demonstrated between morphotypes of larvae, and adult nematodes were all consistent with *P. odocoilei* (Platt and Samuel, 1978; Kutz et al., 2001); other elaphostromylinae were not discovered in the vasculature or central nervous system of definitive hosts.

General observations of morphological variation in L1 of various protostrongylids and the specific reports of structurally divergent larvae associated with patent infections of *P. odocoilei* emphasize the challenges involved in identification and diagnostics for these nematodes (e.g., Boev, 1975; Kontrimavichus et al., 1976; Lankester et al., 1976; Platt, 1978; Pybus and Shave, 1984; Pybus et al., 1984). In the current study, we applied molecular methods and multilocus sequence analyses of nuclear (ITS-2) and mitochondrial (COX-II) DNA in a comparison of known adults and distinct morphotypes of DSL representing putative *P. odocoilei*. Our protocols paralleled those

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developed by Jenkins et al. (2005), in which ITS-2 sequences for unknown larvae extracted from feces were validated against those from definitively identified adults of *P. odocoilei*. We examine the hypothesis that these distinct forms do not represent separate species, but are representative of dimorphic phenotypes. Additionally, we provide new information for structural characteristics of protostrongylid L1 based on scanning electron microscopy (SEM), as advocated by Carreno and Hoberg (1999).

MATERIALS AND METHODS

Specimens examined

Specimens of adult males and females of *P. odocoilei* were derived from natural sources and identified based on definitive morphological characters consistent with this elaphostrongyline (Kutz et al., 2001). Representative physical vouchers are in the U.S. National Parasite Collection (USNPC) and the Canadian Museum of Nature-Parasitology (CMNP). Specimens from 2 naturally infected Dall's sheep prepared for molecular analysis included (1) 1 male (USNPC 89194, 94330–94333) in *Ovis d. dalli* collected 7 April 1999 at Loretta Canyon, Mackenzie Mountains, Northwest Territories, 65°01'N, 127°50'W and (2) 3 females in *Ovis d. dalli* with the same collection data (USNPC 94329, 94334; CMNP 2000–0021–22).

Specimens of DSL examined by SEM and by comparative molecular analyses were extracted from fresh fecal samples from naturally infected Dall's sheep collected by A. Veitch and R. Popko on 8 April 2000 at Cache Creek Falls in the Katherine Creek study site, 65°01'N, 127°35'W, (USNPC 94335, 95217). These live DSL were sorted into 2 morphotypes (20 of type 1 and 20 of type 2) based on structural characteristics of the tail (Kutz et al., 2001) and prepared for molecular analyses.

Comparative morphology and SEM

Larvae were examined using temporary preparations with light microscopy (Zeiss Axiophot, differential interference contrast) and oil magnification to $\times 1,600$; specimens were alive in water, or heat-killed, and were not cleared. Protocols for low-temperature field emission (LTSEM) were modified from Carta et al. (2003). Live specimens in distilled water were concentrated by sedimentation and transferred by pipette onto squares (approximately 5.0 by 5.0 mm) of Whatman No. 1 qualitative filter paper that had been positioned on a copper plate specimen holder. Excess water was wicked from the side and was followed by an additional period of 1 min for air drying of the surface of the specimen. Samples were frozen by contact freeze-immobilization in liquid nitrogen by placing the prepared specimen on the precooled surface of a brass metal bar (-196 C) and then were transferred to the cryopreparation chamber, Oxford CT-1500HF Cryotrans System (Gatan Inc., Warrendale, Pennsylvania). Additional water was removed by sublimation through etching at -90 C for about 2 min. Recooling below -100 C was followed by coating with a thin layer of platinum using a magnetron sputter coater. Samples were examined and photographed at an accelerating voltage of 2 kV on the cold stage (< -160 C) of a Hitachi S-4100 LTSEM. Micrographs were recorded on Polaroid type 55 P/N film; negatives of representative images were later scanned and digitized.

DNA extraction

DNA was extracted from adult nematodes and individual DSL using a Qiagen DNeasy (Valencia, California) protocol for animal tissues employing 3 washes with AW1 buffer and a final elution in 100 μ l AE buffer after 10 min of incubation. No-template controls, comprised solely of water and extraction reagents, were used to establish that reagents did not become contaminated during the extraction procedure.

DNA amplification

Polymerase chain reactions were performed to amplify ITS-2 and COX-II. To amplify ITS-2, primers NC1 (5'-ACG TCT GGT TCA GGG TTG TT-3') and NC2 (5'-TTA GTT TCT TTT CCT CCG CT-3') were

used (Ellis et al., 1986; Gasser et al., 1993). To amplify COX-II, primers MTD16 (5'-ATT GGA CAT CAA TGA TAT TGA-3') and MTD18 (5'-CCA CAA ATT TCT GAA CAT TGA CCA-3') were used (Simon et al., 1994).

The standard protocol for Platinum High Fidelity Taq polymerase (Invitrogen, Carlsbad, California) was used for each 20- μ l PCR reaction. Each reaction was comprised of 1 \times PCR buffer, 0.6 mM of MgSO₄, 0.2 mM dNTP mixture, 0.5 μ M of each primer, 0.25 units of Platinum High Fidelity Taq polymerase, and 2–2.5 μ l of template.

Amplification of ITS-2 employed an initial 94 C denaturation for 3 min followed by 35–45 cycles of 94 C for 1 min, 55 C for 1 min, and 72 C for 2 min. Cycle parameters used for the COX-II consisted of an initial 3-min denaturation at 94 C followed by 40–45 cycles of 30 sec at 94 C, 30 sec at 45–50 C, and 30 sec at 72 C. Each assay included a terminal extension step of 72 C for 10 min and was followed by cooling to 4 C. Each experiment included reactions containing no template, PCR (reagents only), and extraction negative controls to detect potential contamination. Reactions were analyzed by electrophoresis using a 1.4% agarose gel with ethidium bromide staining.

Sequencing

To prepare PCR products for direct fluorescent sequencing, 1.6 μ l of ExoSap-IT[®] (USB Corp., Cleveland, Ohio) was added to 4 μ l of the PCR product to remove excess primers and dNTPs. Samples were then incubated at 37 C for 15 min and then heated to 80 C for 15 min. To complete the sequencing reaction, 4 μ l of BigDye[®] Terminator, version 3.0 or 3.1 (Applied Biosystems, Foster City, California), and 3.2 pmol primer were added before cycle sequencing. When possible, PCR products were sequenced in both directions using the ITS-2 or the COX-II primers. Samples were then electrophoresed on an ABI 3100 or ABI 3730 capillary sequencer.

Data analyses

Sequence chromatograms were aligned and edited using Sequencer 4.1 (GeneCodes Corp., Ann Arbor, Michigan) software. Aligned sequence chromatograms were inspected for the occurrence of polymorphic sites and consensus sequences used in subsequent phylogenetic analyses including homologous sequences from the congener *Parella-phastrongylus tenuis*. The relationships among all individual COX-II haplotypes were inferred by means of neighbor-joining phylogenetic reconstruction based on Kimura 2-parameter genetic distances using PAUP* (Swofford, 2001). Sequences were manually examined for species-specific differences and polymorphic sites. Further, ITS-2 sequences were assessed in reference to data from a broad-based geographic survey of *P. odocoilei* in western North America (Jenkins et al., 2005).

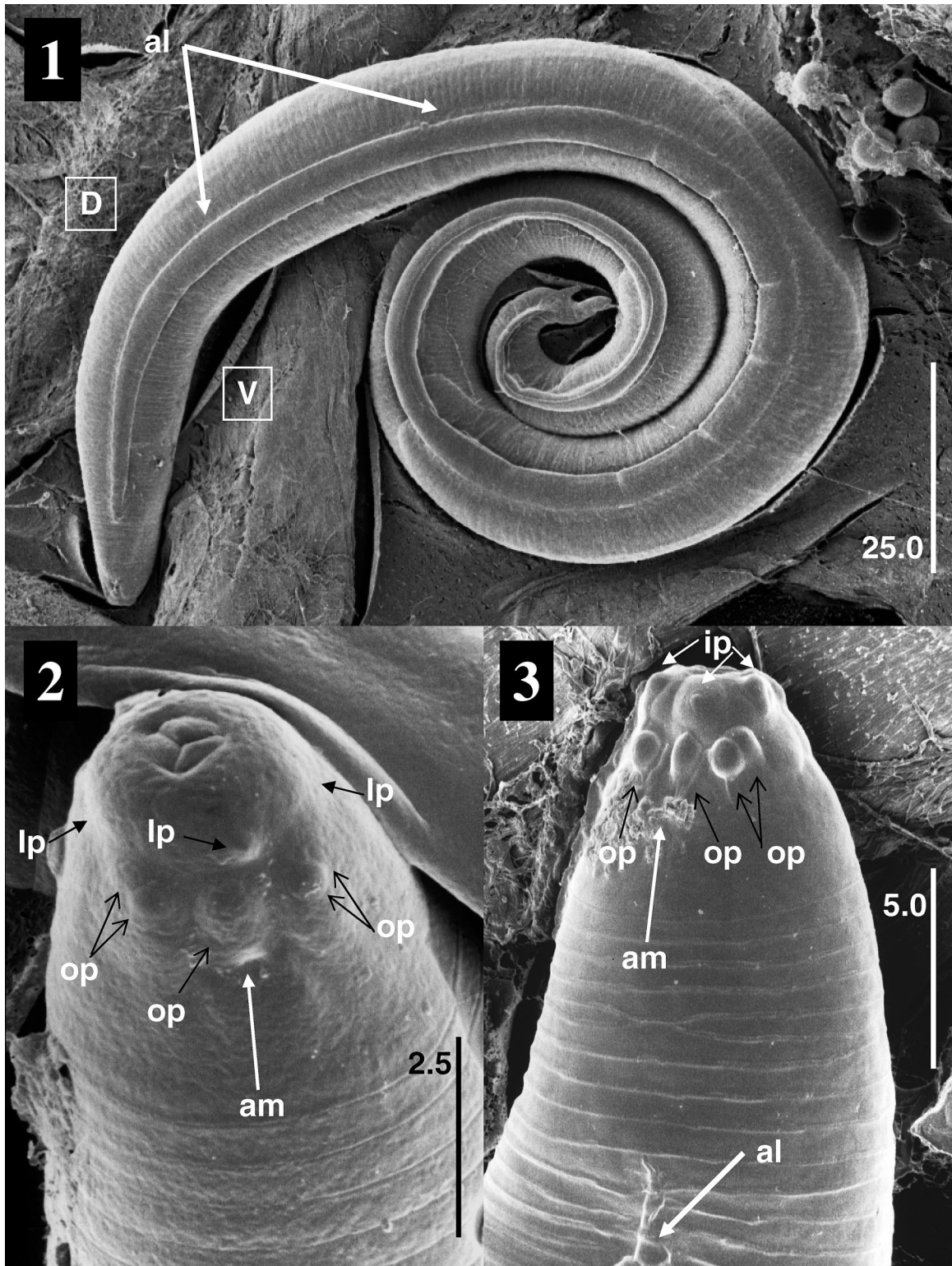
RESULTS

Polymorphism in dorsal-spined larvae

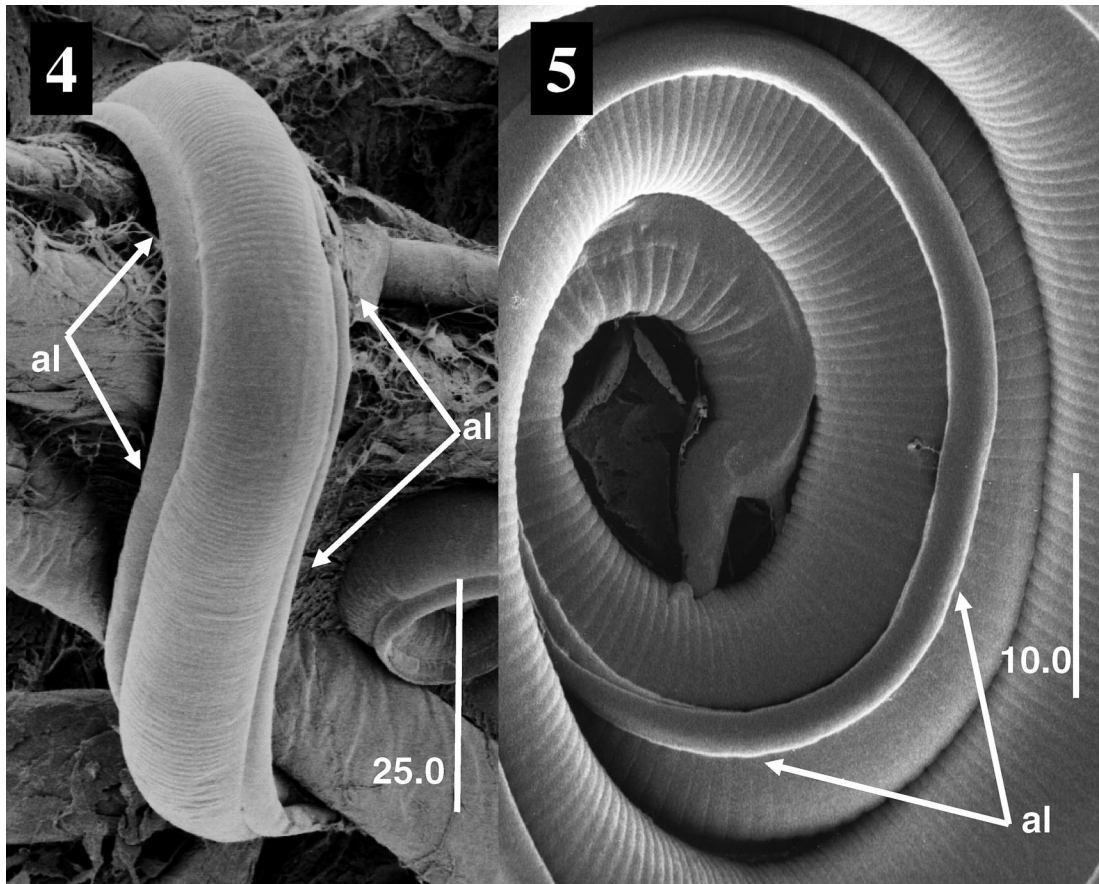
Polymorphism among DSL representing putative *P. odocoilei* has been noted regularly during parasitological surveys based on fecal samples from thornhorn sheep across the Mackenzie Mountains. Meristic data have been presented and discussed previously for these DSL and are consistent with the original description and subsequent redescrptions for *P. odocoilei* (summarized in Kutz et al., 2001).

Comparative morphology, description of the L1

First-stage larvae are coiled ventrally in life and possess a surface cuticle with transverse striations except on the tail (Fig. 1). The lateral alae are prominent and inflated, extending from slightly posterior to the cephalic extremity, and terminate near the level of the tail (Figs. 1–5). A rounded cephalic extremity contains an oval mouth that is defined by a cuticular triradiate stoma (Fig. 2). Adjacent to the mouth are situated the 6 simple circumoral inner labial papillae; broadened circular regions, or perityls, which may be inflated, are located at the base of each



FIGURES 1–3. First-stage larvae of *Parelaphostrongylus odocoilei* as viewed in scanning electron microscopy (LTSEM); scale bars in micrometers. **1.** Dorsal-spined larva showing ventral coiling when alive; note prominent lateral alae (al) extending to near level of dorsal spine. D = dorsal; V = ventral. **2.** Cephalic structure of DSL in right lateral view showing 3 labia, circumoral inner papillae (ip), lateral outer papillae (op), and lateral amphid (am); note paired papillae in the outer circle. **3.** Cephalic region, lateral view, showing inflated inner papillae (ip), outer papillae (op), position of amphid (am), and origin of lateral ala (al).



FIGURES 4–5. First-stage larvae of *Parelaphostrongylus odocoilei* as viewed in scanning electron microscopy; scale bars in micrometers. **4.** Body of DSL in dorsal view showing structure of inflated alae (al). **5.** Body of DSL in right lateral view showing structure of inflated ala (al) and prominent cuticular striations. Note position of dorsal spine and structure of sinuous, kinked tail.

papilla (Fig. 3). The outer papillae are 10 in number and are situated in single right and left lateral rows posterior to the inner papillae; dorsally and ventrally disposed papillae are paired; 1 simple papilla resides adjacent to each amphid (Figs. 2, 3). Amphids are prominent and pit like, with a crescent shape, and lacking in ornamentation and are situated laterally and posterior to respective right and left median outer papillae.

Type 1 and type 2 larvae typically have a sinuous tail with a prominent triangular dorsal spine posterior to the level of the ventral anus. Among type 1 DSLs, the conical terminal region of the tail is kinked and has 3 distinct folds or joints (Figs. 6, 8); the tail spike occurs as a symmetrical terminal extension of the caudal extremity. In contrast, type 2 larvae have a digitate terminal region lacking folds or joints (Figs. 7, 9); the tail spike occurs as an asymmetrical, subterminal, and ventral extension of the caudal extremity.

Sequence identity

Sequences of ITS-2 were derived and compared from 10 larvae of type 1 and 5 of type 2, along with 4 adult nematodes identified as *P. odocoilei* based on morphological characters from *O. d. dalli*. In our sample, no fixed polymorphisms distinguished the adults or either of the 2 morphological forms of DSL. Instead, the ITS-2 of these specimens derived from the Mackenzie Mountains corresponded perfectly to sequences pre-

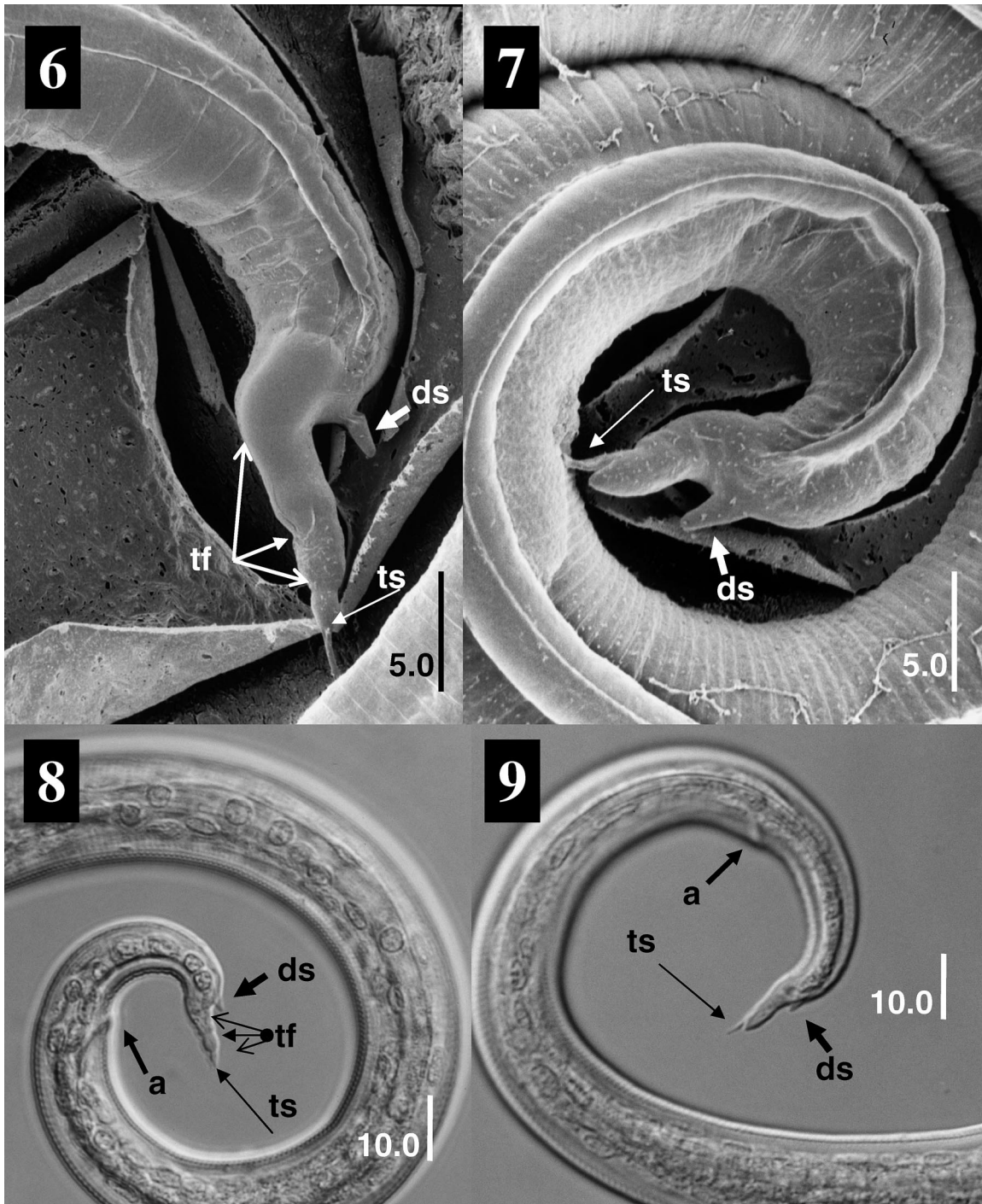
viously reported from populations of *P. odocoilei* sampled across its range from California to Alaska and the Northwest Territories (Jenkins et al., 2005). Notably, individual larvae and adults possessed copies of ITS-2 that differed at the same 5 nucleotide positions previously identified as variable within the genomes of individual specimens of *P. odocoilei* (Jenkins et al., 2005). These sequences differed from those of congeneric species and from species of *Muellerius*, *Umingmakstrongylus*, and *Varestrongylus* (Jenkins et al., 2005).

Sequences of *COX-II* were derived and compared from 6 type 1 and 4 type 2 larvae and 4 adult specimens of *P. odocoilei*. The *COX-II* of all sequenced adults and larvae was identical at 301 of 303 sequenced nucleotides, representing 101 amino acid codons. The first of 2 polymorphisms represented a GTC/GTT synonymous transition (Val) and the second represented a GTT/ATT transitional substitution of Val for Ile (GenBank accessions DQ297378–DQ297389). At neither position was either nucleotide variant exclusively associated with either larval morphotype; each was clearly distinguished from the homologous locus of *P. tenuis*.

DISCUSSION

Larval polymorphism

Sequence homogeneity at each of 2 genetic loci support the conclusion that dorsal-spined larvae of 2 distinct morphological



FIGURES 6–9. First-stage larvae of *Parelaphostrongylus odocoilei* in scanning electron microscopy (Figs. 6, 7) and differential interference contrast microscopy (Figs. 8, 9; modified from Kutz et al., 2001), depicting structure of tail in type 1 and type 2 DSL; scale bars in micrometers. **6, 8.** Sinuous tail of typical type 1 DSL in lateral view showing position of dorsal spine (ds), 3 tail folds (tf), tail spike (ts), and position of anus (a). **7, 9.** Sinuous digitate tail of type 2 DSL in lateral view showing position of dorsal spine (ds), tail spike (ts), and anus (a). Note absence of prominent tail folds.

forms are conspecific and correspond to vouchered adults of *P. odocoilei*. Absence of fixed variation in sequences between type 1 and type 2 larvae confirms they are both referable to *P. odocoilei* (Kutz et al., 2001). Characteristic features of the ITS-2 sequences for these dimorphic L1 differentiate these specimens from those of other congeneric species also producing DSL

found in North American cervids and bovids. Thus, we demonstrate unequivocally the occurrence of structural polymorphism among first-stage larvae in a single species of the Protostrongylidae. Polymorphism may be geographically widespread among populations of *P. odocoilei* distributed at boreal to subarctic latitudes based on prior records and likely is not

limited with respect to nematodes in Dall's sheep, but may be expected among mountain goats and mule deer (Pybus and Shave, 1984; Kutz et al., 2001).

The basis for polymorphism in DSL's of *P. odocoilei* and whether similar larval morphological polymorphism occurs in other protostrongylids is unknown. It is intriguing to speculate if such differences may reflect specific characters linked to male and female genders.

Morphology and LTSEM

Our study represents a description of fine-scale morphology for the L1 of *P. odocoilei*. First-stage larvae have been described for many species among the protostrongylids (e.g., Boev, 1975; Kontrimavichus et al., 1976; Hoberg et al., 1995; Lankester et al., 1998); however, detailed information about cephalic and caudal structure and specifically that gained by application of SEM has remained elusive (Carreno and Hoberg, 1999). Resolution of the smallest structures, such as cephalic papillae, has exceeded the capacity of light microscopy. As a consequence, there is no comparative framework that may reveal biologically or phylogenetically important attributes among L1 across the diversity of genera and species of protostrongyles (Carreno and Hoberg, 1999). Low-temperature field emission (LTSEM) provided the opportunity to examine live or hydrated specimens that were not degraded through dehydration in ethanol and by critical point drying (Carta et al., 2003). Specimens observed with LTSEM, particularly those that are very small or with fluid-filled body cavities, such as larval and adult nematodes, retain cuticular structures and surface morphology as seen in life (Carta et al., 2003).

Cephalic morphology

It was predicted that L1 among the Protostrongylidae should have similar cephalic structures consistent with conservation of the arrangement for reduction and fusion of labia and papillae among crown strongylate nematodes (Chitwood and Chitwood, 1950; Maggenti, 1981; Blaxter et al., 1998). Cephalic structure among adult protostrongyles and their larvae, however, has remained largely unknown.

Cephalic structure, specifically the number and distribution of papillae, demonstrated for L1 of *P. odocoilei* (Figs. 2, 3), is consistent with that for adults of *Parelaphostrongylus* and *Umingmakstrongylus*, although it appears that elements of either the outer or inner circles were incompletely described in some studies. Further, it appears that this pattern is retained during the series of molts from L1 through adult in at least the elaphostrongyline and muelleriine and possibly other related metastrongyloids (Kontrimavichus et al., 1976). Anderson (1963) described cephalic morphology for L1 and L3 of *P. tenuis*, where the mouth is surrounded by 6 single papillae of the inner circle, with 4 submedian bulges constituting the outer circle and 2 lateral amphids; in adults, there are obscure but additional single ventro-ventral papillae situated lateral to each amphid, and the submedian bulges each contain 2 papillae. Platt (1978) used SEM to show cuticular morphology for L1 and L3 of *P. odocoilei*, but did not examine cephalic structure in detail. Gibbons et al. (1991) described and depicted a system consisting of 6 outer labial papillae, each bordered by a cuticular ridge or depression, 4 submedian papillae, and 2 lateral amphids among

adults of the 3 species of *Elaphostrongylus*; structure in either L1 or L3 was not assessed. Carreno and Lankester (1993) described 6 inner circumoral papillae, each centered within an oval depression, 4 paired outer papillae, and lateral elliptical amphids for adults of *Parelaphostrongylus* and *E. rangiferi*. Hoberg et al. (1995) described adults of *Umingmakstrongylus pallikuukensis* Hoberg, Polley, Gunn and Nishi, 1995, with a triradiate stoma, 6 pedunculate circumoral papillae of the inner circle, 4 paired dorsal and ventral papillae of the outer circle, and a pair of laterally disposed amphids, each with an adjacent, miniscule, single papilla of the outer circle; en face views of cephalic structure for L1 and L3 were beyond the resolution of light microscopy. Additionally, Boev (1975), in summarizing available data for other protostrongyles, described for adults of *P. raillieti* (Shul'ts, Orlov and Kutas, 1933) a mouth surrounded by 3 labia (likely in reference to the triradiate structure of the stoma) and a row of 6 circumoral papillae, comprised of 2 dorsal, 2 ventral, and a pair of lateral amphids; in *Spiculocaulus austriacus* (Gebaur, 1932), 3 labia are present; these latter descriptions are likely to be incomplete and now have minimal value (Chitwood and Chitwood, 1950).

The inner circle in L1 of *P. odocoilei* consists of single papillae, with each projecting from a slightly broadened circular base or perityl that may appear inflated depending on the state of activity of individual live specimens (Figs. 2, 3). It appears that variation in the descriptions of such perityls may reflect the relative degree of expansion or contraction of the cephalic region of individual adult or larval worms at the time of fixation or preparation for examination by either light microscopy or SEM (Carta et al., 2003). Anderson (1978) considered the typical cephalic structures in metastrongyloids to consist of 6 perityls, essentially flattened sensory receptors surrounding the mouth that may be homologous to the 6 lips characteristic of genera such as *Heterostrongylus* Travassos, 1925, and *Didelphostrongylus* Prestwood, 1976, in the Angiostrongylidae. Platt (1978, 1984) postulated that the presence of perityls was symplesiomorphic among the species of *Parelaphostrongylus* and that adults of *Elaphostrongylus* were diagnosed by the absence of these labial organs. Gibbons et al. (1991) clearly depicted the perityls in redescribing adults of *E. cervi*, *E. rangiferi*, and *E. alces*, but may not have completely demonstrated the number and distribution of outer papillae (shown as being single and 4 in number). Further, Carreno and Lankester (1993) also observed the presence of perityls in adults of *Parelaphostrongylus* spp. (including *P. odocoilei*) and *E. rangiferi* based on studies using SEM, but did not completely define the pattern and distribution of outer papillae.

Caudal morphology of L1

Among Protostrongylidae, the major distinguishing feature in caudal structure for L1 is the presence of a dorsal spine typical of the elaphostrongyline, muelleriine, and species of *Varesstrongylus* versus a conical, spike tail, as exemplified by species of *Protostrongylus* (Boev, 1975; Kontrimavichus et al., 1976; Carreno and Hoberg, 1999). It had been assumed that there was a general level of uniformity in caudal structure for L1 and L3 representing a particular species, although morphological variation in tail structure of third-stage larvae (L3) is commonly recognized among North American elaphostrongyline (*P. ten-*

uis, *P. andersoni*, and *P. odocoilei*) (Ballantyne and Samuel, 1984; Lankester and Hauta, 1989). Polymorphism among L1 conspecifics, however, such as that noted for *P. odocoilei*, where discrete larval forms are present, has not been recognized previously among any of the Protostrongylidae or among other Metastrongyloidea and the Strongylida.

The basis for this polymorphism remains undetermined, but such phenomena, if discovered to be more widespread, may contribute to continued confusion in discriminating among first-stage larvae for species, genera, and subfamilies within Protostrongylidae. Consequently, we recommend comparative sequencing for adults, in which identity has been determined based on morphology, and L1 that represent putative conspecifics from single hosts, to provide an unequivocal diagnosis (Jenkins et al., 2005). Among the protostrongylids, an extension of these methods to L3 either in gastropod intermediate hosts or in the environment presents an opportunity for detailed studies of epidemiology and transmission dynamics in field-based situations where definitive hosts cannot be collected. Applications in conservation and wildlife biology are readily apparent and demonstrate the power of an integration of morphological and molecular protocols (Jenkins et al., 2005).

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