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SHORT COMMUNICATIONS

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Extracting *Protostrongylus* spp. Larvae from Bighorn Sheep Feces

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ABSTRACT: First-stage larvae of *Protostrongylus* spp. were more numerous in the core of bighorn sheep (*Ovis canadiensis canadiensis*) pellets than near the surface. As a result, only 22% could be extracted from whole pellets and the numbers collected did not reflect the total number of larvae present in samples. Crushing semi-dried pellets yielded seven times as many larvae and numbers collected were correlated with totals present. The use of tissue, in addition to a screen filter in a beaker extraction method, produced a cleaner sample and did not affect larval collection or the correlation. By comparison, most first-stage larvae of *Parelaphostrongylus tenuis* from white-tailed deer (*Odocoileus virginianus*) were near the surface of fecal pellets where they may be removed readily by water.

Key words: Beaker method, extracting nematode larvae, fecal examination, *Protostrongylus rushi*, *Protostrongylus stilesi*.

Infection of bighorn sheep (*Ovis canadiensis canadiensis*) by lungworms (*Protostrongylus stilesi* and *Protostrongylus rushi*) continues to warrant the attention of wildlife health researchers. Monitoring herd infection levels by assessing the prevalence and mean intensity of first-stage larvae traditionally has involved collecting fecal pellets, allowing them to dry in paper bags, and cracking or crushing them prior to suspension in water over porous paper or cheese-cloth in a Baermann funnel apparatus (Pillmore, 1961; Forrester, 1971; Uhazy et al., 1973; Samuel and Gray, 1982; Beane and Hobbs, 1983). However, the efficiency of this method has never been evaluated nor is it fully understood why cracking pellets increases the number of larvae recovered.

Forrester and Lankester (1997) recently demonstrated that the sloping sides of Baermann funnels retain variable numbers of *Parelaphostrongylus tenuis* larvae while a new method of submerging pellets over screening in straight-sided beakers improved yield and extracted a consistent proportion of larvae present. How applicable this new method might be for other parasite and host species is unknown. Its use may be limited to those nematodes whose larvae, like those of *P. tenuis* in white-tailed deer (*Odocoileus virginianus*), occur largely on or near the surface of feces (Forrester and Lankester, 1997). The purpose of this study was to determine where larvae of *Protostrongylus* spp. occur in the feces of bighorn sheep and to assess the efficiency of the new beaker method in extracting them.

Fecal samples from bighorn sheep containing first-stage larvae of *Protostrongylus* spp. were collected off snow near the town-site of Banff in Banff National Park (Alberta, Canada; 51°11′N, 115°30′W). Bighorn sheep in the area are known to be infected with both *P. stilesi* and *P. rushi* (Uhazy et al., 1973) and larvae recovered conformed to published morphometrics of these species (Pillmore, 1956). Fecal samples were used fresh or frozen at −14 C for ≤2 mo before examination. Preliminary examination of pooled fecal samples from 15 animals indicated that no more than 5% of the larvae collected were unidentified, dorsal-spined nematode larvae as had been reported previously from bighorn sheep in the Banff area by Samuel and Gray (1982).

The location of first-stage larvae in bighorn sheep pellets was first investigated by determining what proportion of those present could leave an immersed pellet within 1 hr. Ten replicate pellets were impaled individually on a pin and immersed

TABLE 1. Distribution of protostrongylid larvae in fecal material from white-tailed deer and bighorn sheep.

	White-tailed deer with *P. tenuis*		Bighorn sheep with *Protostrongylus* spp.	
Pellet	Near surface	Core	Near surface	Core
1	46[a]	0	7	40
2	102	10	64	87
3	42	17	20	67
4	23	0	36	80
5	74	0	38	52
6	29	25	37	73
7	100	12	21	84
8	87	12	18	32
9	95	25	31	64
10	92	17	18	40
Mean	60	12	29	63
SEM[b]	11	3	5	7
Mean%	86	14	31	69
SEM	5	5	3	3

[a] Larvae/g of feces.
[b] SEM = Standard error of the mean.

for 3, 3, 3, 10, 10 and 31 min (total 1 hr), in a series of six standard Petri dishes filled with water at room temperature. The Petri dishes had a manually etched grid (0.8 × 0.8 cm) on the bottom that facilitated in counting the number of larvae leaving after each time interval. After 1 hr, the number of larvae remaining in each pellet was determined by breaking it up finely and shaking in 200 ml of water before quickly pipetting a 25 ml subsample into a Petri dish for counting using a stereoscopic microscope at a magnification of 16 to 25×.

A second approach determined the location of *Protostrongylus* spp. larvae by comparing the number present near the surface of the pellet with that in the core. The core was removed using a 6.5 mm diameter cork borer; a 1 mm section was cut from each end and added to the surface material. The core and surface material was separately weighed, macerated, and each placed in a 200 ml beaker filled with water. The contents of each beaker were thoroughly suspended by stirring before three 25 ml aliquots were removed and examined for larvae. All pellets used were approximately 9 × 11 mm and 0.5 to 0.95 g. For comparison, fecal pellets from white-tailed deer infected with *Parelaphostrongylus tenuis* from Grand Marais, Minnesota (USA; 47°41′N, 90°35′W) were treated in an identical fashion.

Using the beaker method of Forrester and Lankester (1997), we compared the efficiency of three variations for extracting *Protostrongylus* spp. larvae from bighorn sheep feces. These included using (1) whole pellets in screen envelopes, (2) crushed pellets in screen envelopes, and (3) crushed pellets in screen envelopes wrapped in a single layer of tissue (Kimberly-Clarke Kimwipes, Mississauga, Ontario, Canada) with irregular pore sizes up to 0.4 × 0.6 mm. Envelopes were made of vinyl window screening with a mesh opening of 1.0 × 1.4 mm.

Fecal samples of 21 pellets (~9 mm × 11 mm) were divided equally into thirds with seven pellets used in each method. All samples were allowed to dry overnight at room temperature to approximately 50% of their original fresh weight to facilitate crushing into five to eight pieces. Screen envelopes containing samples were submerged in 250 ml beakers filled with water and removed after 24 hr. Larvae that had left the pellets were allowed to settle for 1 hr before the solution was siphoned off to a residual volume of 50 ml, and ex-

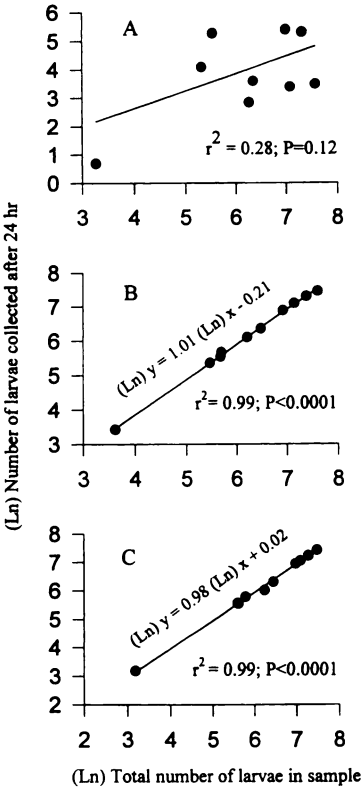

FIGURE 1. Linear regression of the number of *Protostrongylus* spp. larvae collected from bighorn sheep fecal pellets in a beaker after 24 hr verses the total number in fecal samples. A. Using whole pellets over vinyl screening. B. Using crushed pellets over vinyl screening. C. Using crushed pellets over vinyl screening wrapped in tissue paper.

amined. To determine the number of larvae remaining on or in feces, the fecal material in each envelope was macerated, suspended in 1,000 ml of water and six 30 ml aliquots of solution were removed and examined. Any larvae trapped in the screening or tissue paper filters were collected by soaking the filters in 200 ml of water in a beaker for 24 hr and siphoning the supernatant down to a volume of 50 ml before counting. Each method was replicated ten times. All glassware was washed between trials with soap and water and rinsed with 70% ethyl alcohol (Whitlaw and Lankester, 1995).

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) (SPSS Inc., Chicago, Illinois, USA). Larval counts were normalized where necessary using a natural log transformation. Larval counts were compared using Student's *t*-test or one-way analysis of variance. The relationship between the number of larvae collected and the total in samples for each method was analyzed using regression analysis (Zar, 1984). *P*-values ≤ 0.05 were considered significant.

In this study, only 22% of *Protostrongylus* spp. larvae left whole bighorn sheep pellets immersed in water on a pin for 1 hr while up to 89% of *P. tenuis* larvae were able to leave white-tailed deer feces in the same period of time (Forrester and Lankester, 1997). The likelihood that larvae of the two species are distributed differently in fecal material of their respective hosts was confirmed by comparing the number of larvae near the surface with that in the pellet core (Table 1). Only 31% of *Protostrongylus* spp. larvae occurred near the surface compared to 86% of *P. tenuis*. In the core of bighorn sheep pellets there were twice as many *Protostrongylus* spp. larvae/g of fecal material as near the surface ($t = 4.08$; 18 df; $P < 0.05$), whereas in white-tailed deer with *P. tenuis* there were five times as many larvae/g near the surface compared to that in the core ($t = 3.23$; 18 df; $P < 0.05$) (Table 1).

Clearly, neither species of larvae is uniformly distributed in fecal material; the larvae of *P. tenuis* are concentrated near the surface and those of *Protostrongylus* spp. are concentrated in the core. The implications of this observation for the transmission of the two parasites to terrestrial gastropod intermediate hosts are unknown. Most *P. tenuis* larvae are probably dispersed in soil by melting snow and rain and encounters with gastropods may be entirely by chance. If *Protostrongylus* spp. take longer to leave bighorn sheep pellets, then species of gastropods which are attracted to fecal material may be important in transmission.

The total number of *Protostrongylus* spp. larvae in bighorn sheep feces varied

TABLE 2. Comparisons of numbers of *Protostrongylus* spp. larvae collected from bighorn sheep feces using three variations of the beaker method.

	Whole pellets over screening				Crushed pellets over screening				Crushed pellets over screening wrapped in tissue			
Sample	I[a]	II[b]	III[c]	IV[d]	I[a]	II[b]	III[c]	IV[d]	I[a]	II[b]	III[c]	IV[d]
1	194	3	54	251	257	1	12	270	289	8	0	297
2	59	0	144	203	319	1	7	327	254	0	36	290
3	36	0	530	566	540	3	89	632	575	5	71	651
4	192	9	50	251	251	0	24	275	213	5	18	236
5	29	1	1,160	1,190	1,025	3	48	1,076	975	5	36	1,016
6	17	0	506	523	405	1	109	515	445	1	54	500
7	201	2	1,291	1,494	1,372	3	70	1,445	1,485	16	95	1,596
8	2	0	24	26	24	0	0	24	31	0	6	37
9	32	0	1,898	1,930	1,648	3	125	1,776	1,722	17	262	2,001
10	219	1	862	1,082	1,144	3	48	1,195	1,203	2	60	1,265
Mean*	98[f]	1.6[h]	652[j]	752[l]	698[g]	1.8[i]	53[k]	754[l]	719[g]	6[i]	64[k]	789[l]
SEM[e]	29	0.9	202	202	175	0.4	14	185	186	2	24	207
Mean%	24	<1	78		93	<1	7		91	<1	9	
SEM	29		10		6		7		1		5	

* Means followed by the same letter are not significantly different ($P<0.05$).
[a] Number of larvae collected after 24 hr.
[b] Number of larvae trapped in filter.
[c] Number of larvae remaining in feces.
[d] Total number of larvae in the sample.
[e] Standard error of the mean.

among pellets from the same group (30 to 83 larvae/pellet; mean ± SE = 59 ± 6.6). Similarly, some pellets from white-tailed deer had twice as many first-stage larvae as others in the same pellet group (Forrester and Lankester, 1997). For this reason, single pellets should never be used to quantify the intensity of protostrongylid larvae in feces of these hosts.

When we extracted *Protostrongylus* spp. larvae from whole bighorn sheep pellets using the beaker method, only 24% (95% CI = 3 to 45%) of the total number of larvae present left the pellets after 24 hr (Table 2) and the numbers collected did not correlate with total numbers present ($r^2 = 0.28$; $P = 0.12$) (Fig 1A). However, when pellets were crushed the numbers of larvae collected were increased seven fold (Table 2). Crushed pellets over screening and over screening wrapped in tissue yielded 93% (95% CI = 88 to 97%) and 91% (95% CI = 87 to 94%) of the total numbers present, respectively and counts were correlated with totals present ($r^2 = 0.99$; $P < 0.0001$) (Fig. 1B and C). When Beane and Hobbs (1983) used crushed bighorn sheep feces they obtained only a four fold increase in yield of *Protostrongylus* spp. larvae and they did not determine what portion of the total number of larvae was collected. Their extraction method utilized the Baermann funnel which has been shown to retain large numbers of protostrongylid larvae on the sloping glass sides (Forrester and Lankester, 1997). Our results suggest that using crushed pellets suspended in beakers is superior to using Baermann funnels to collect *Protostrongylus* spp. larvae from bighorn sheep pellets.

The addition of tissue paper wrapped around the screening produced a cleaner sample that was easier to examine for larvae and did not reduce larval yield (Table 2). Less than 1% of the total number of *Protostrongylus* spp. larvae in samples were retained by either screening alone or screening wrapped in a layer of tissue. On the other hand, *P. tenuis* larvae were more likely to be caught in the filter material; up to 15% became trapped in tissue paper

(Forrester and Lankester, 1997). Direct observation gave the impression that *Protostrongylus* spp. larvae were more mobile than those of *P. tenuis*, possibly explaining why fewer are detained in filters.

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