THE LIFE CYCLE OF PROTOSRONGYLUS STILESI IN BIGHORN SHEEP[1]

by

Ruth Ann Monson, Graduate Student
Colorado State University

ABSTRACT

The objective of this research was to complete the life cycle of the lungworm, *Protostrongylus stilesi*, in bighorn sheep through the experimental transmission of infection. First stage larvae of the lungworm were obtained from naturally infected animals in Rocky Mountain National Park, Colorado. Land snails of the species *Vallonia pulchella* were exposed to these larvae in the laboratory. The larvae were allowed to develop to the infective stage. The snails were then fed to bighorn x mouflon sheep which were lungworm-free. The animals were in isolation. Daily fecal samples were collected and analyzed to monitor the progress of the infection attempts. Three of four hybrid sheep have shown *Protostrongylus* larvae in their droppings after as little as a 60 day prepatent period. Adult *Protostrongylus stilesi* have been recovered and identified upon necropsy.

Lungworms are thought by some to be the reason for the decline in present populations of Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*). (Pillmore and Moser 1954, Moser 1962). Bighorn sheep losses have been described by a number of people (Rush 1928, Marsh 1938, Potts 1938, Packard 1939, Pillmore and Moser 1954). Some believe that lungworms such as *Protostrongylus stilesi* (Dikmans 1931) predispose animals to bacterial lung disorders, though the primary cause of the fatalities may be traced to malnutrition and insufficient winter range (Honess 1955, Buechner 1960). Parasitism is the natural condition in wild ungulates (Cowan 1951), and the *P. stilesi - O. canadensis* relationship is one of long standing (Pillmore 1958).

The life cycle of *Protostrongylus stilesi* in bighorn sheep (*Ovis canadensis*) has not been completely known. The adult nematode lives in the lung parenchyma of the definitive host. The eggs are laid in the lung, hatch into first stage larvae and eventually make their way into the intestinal tract, leaving the body of the host with the feces. The first stage larva must leave the sheep feces and enter an intermediate host where it develops to the infective stage. This stage must then enter the definitive host. This part of the cycle has been the subject of intensive experimentation (Pillmore 1955-1961, Post 1958).

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The life cycles of some members of the genus are known. All species of *Protostrongylus* with known life cycles require terrestrial molluscs as intermediate hosts. Therefore studies on the life cycle have been primarily focused on finding the correct terrestrial mollusc which would transfer the infection back to the definitive host.

Past research has shown several species of terrestrial molluscs which will accept first stage larvae of *P. stilesi*. Metamorphosis from first stage to third (probably the infective stage) has been observed on several occasions (Honess 1955, Pillmore 1955-1961, Post and Winter 1957, Post 1958). Attempts to complete the life cycle of the worm have met with inconclusive results or failure because lungworm-free bighorn sheep have not been available for experimentation. Therefore, genetic crosses of bighorn with domestic sheep or mouflon sheep (*Ovis musimon*) have been used as possible experimental definitive hosts of *P. stilesi*.

The purpose of the present research was to complete the life cycle of *P. stilesi* by transmission of the infective larvae from the intermediate host to the definitive host. *Vallonia pulchella* was used as the experimental intermediate host. Definitive hosts were mouflon-bighorn sheep hybrids.

MATERIALS AND METHODS

First stage larvae of *P. stilesi* (and possibly *P. rushi*) were obtained from the feces of wild-ranging bighorn sheep in Rocky Mountain National Park, Colorado. Fecal droppings were collected in bighorn sheep bedding areas on the crater rim of Specimen Mountain. These fecal droppings were air dried for maximum preservation of larvae. A Baermann apparatus (Baermann 1917) was used to separate first stage lungworm larvae from fecal samples.

Land snails of the species *Vallonia pulchella* were obtained from Laramie, Wyoming. Other snails (*Gastrocopta pellucida hordeacella*, *Pupilla muscorum* and *Zonitoides arboreus*) were collected from Pikes Peak and Larimer County, Colorado and from Rachelwood Wildlife Research Preserve, New Florence, Pennsylvania. Dr. H. Van der Schalie, Curator of Molluscs at the University of Michigan Museum of Zoology, identified all snails for this study.

Hybrid sheep from a cross of bighorn and mouflon were provided by Rachelwood Wildlife Research Preserve. These animals harbored infections of *Muellerius* sp. and *Pneumostrongylus* sp. The sheep were treated with Tramisol for removal of these infections.

Snail cultures were maintained in two different ways. Cultures were started in fingerbowls on autoclaved soil. These snails were fed finely ground oatmeal and calcium oxide. Temperature of the cultures was kept at 29-32 C,at all times and the soil was kept moistened. However, cultures were found to thrive better in clay pots of mulch, moss, leaf debris and wood fragments. These snails were kept moist at all times. The clay pots were kept in an incubator at 25C.

Infection of snails with the first stage larvae of *Protostrongylus* was accomplished in several ways. Some snails were placed on moistened, infected bighorn sheep fecal pellets. Any movement of the snail then necessitated contact with the infected surface of the pellet. Baermannized fluid was used for infection of other snails. A drop of liquid containing a concentration of first stage larvae was placed on autoclaved soil in a fingerbowl and the snails placed directly on the moistened spot. This method produced higher levels of infection in individual snails.

Infections in the snails were detected and followed by use of a dissecting microscope at 15X and 25X magnification. Details of larval development were observed with a compound microscope at 40X and 100X.

Two experimental sheep were used at a time. These animals were kept in a 12' x 12' isolation shed set on a two-inch concrete pad. The concrete pad was surrounded by a cresol-filled trough. The trough was set in under the walls of the shed and extended six inches outside the walls. Snails and crawling insects were thus kept out of the isolation shed. Windows were designed to be insect-proof while providing ventilation for the animals.

Some fecal samples from experimental sheep were collected from the floor of the isolation shed. An isolation crate was used when 24 hour fecal samples were needed. This crate accommodated a single animal at a time and was used inside the isolation shed. The entire unit was set on cement blocks and a collecting screen was placed underneath. Fecal matter passed through the wire floor of the crate and onto the lower screen. Urine passed through both screens. Total fecal material was removed from the lower screen at 24 hour intervals. Three consecutive 24 hour collections were taken from each animal at regular intervals in order to evaluate production of larvae by the sheep during attempts at infection.

Infections in experimental sheep were induced by the oral administration of snails containing larvae which had been in the heavily chitinized, third stage for at least two weeks. Two weeks were allowed for further development in the snail in the event the third stage needed to mature somewhat before becoming infective. The snails were examined microscopically to determine number of larvae present. Snails were then put into gelatin capsules and fed to the sheep with a balling gun. Sheep #1 and sheep #2 each received 345 infective larvae in 14 doses. Sheep #3 and sheep #4 received 376 and 372 larvae respectively in 18 doses.

Fecal samples from experimental sheep were monitored daily after infective larvae had been administered to determine infection. Three aliquots of the 24 hour fecal collections were Baermannized to estimate the numbers of first stage larvae present. The entire collection was weighed. The number of larvae were counted in four gram aliquots and daily production of larvae and larvae per gram of feces were calculated.

Fecal samples of hybrid sheep not in isolation were irregularly examined. This was done as an experimental control measure. These

hybrid sheep had the same natural lungworm infections as did the sheep in isolation.

Proof of infection was obtained at necropsy. Lungs were minutely dissected with the aid of a dissecting microscope and all adult worms, larvae and nodules were carefully preserved. Adult nematodes were then measured and identified.

RESULTS

Three of four transmission attempts with hybrid sheep from bighorn x mouflon crosses were successful. The three successful attempts gave prepatent periods varying from 63 to 122 days (Table 1).

Table 1 - Results of transmission experiments with *P. stilesi* to hybrid sheep.

Animal number	No. larvae given (Oral administration)	Number of doses	Transmission	Days from first dose to first stage larvae production
1	345	14	+	119*
2	345	14	-	
3	376	18	+	122
4	372	18	+	63

*Time not reliable. See discussion p. 11.

Adult *Protostrongylus* recovered at necropsy from experimentally infected sheep are described in Table 2. The measurements given for these sepcimens are compared with measurements given by Dikmans (1931) for *P. stilesi*. The specimens from the present study were identified as *P. stilesi* by comparison of measurements.

Numbers of adult *P. stilesi* recovered upon necropsy were compared to first stage larval production of the infected animal (Table 3).

DISCUSSION

The experimental sheep used in this study were hardier and more easily handled than bighorn sheep. Rocky Mountain bighorn sheep are known to be a susceptible definitive host of *P. stilesi*. Mouflon sheep have also been found to harbor natural infections of the same parasite (Howe 1965). Therefore the hybrid sheep were expected to be suitable definitive hosts. Successful transmission of infections to the hybrid sheep proved that they were susceptible.

Precautions were taken to prevent accidental infections of any kind from becoming established in the experimental sheep. The cresol-filled trough and double screened windows were the major means of

Table 2 - Measurements of *P. stilesi* recovered from experimental sheep compared to Dikmans (1931).

	Present study Average (Range)	Dikmans (1931)
Males		
number recovered*	16 (1 complete)	none complete
body length	19.8 mm	8 mm
body width in front of bursa	85.6μ (72.6-103.5)	150-160μ
esophagus	212μ (172.5-239.2) x 38.8μ (29.9-53)	235-270 x 50μ
spicules	332.2μ (290-368)	300-340μ
accessory pieces		
proximal	54.8μ (41.4-63)	58μ
distal	85.7μ (71.3-98.9)	96μ
bursa	short	short
gubernaculum	present	present
Females		
number recovered*	6 (none complete)	none complete
body length	uncertain	uncertain
body width	77.3μ at vulva (64.6-92.4)	100μ at vagina
anus to tip of tail	64.1μ (46.0-83.7)	67-75μ
anus to vulva	124.5μ (103.5-165)	190-200μ
provagina	prominent	prominent
vagina	340μ (290-390)	475μ
eggs in vagina	76.0μ (59.8-85) x 34.8μ (27.6-50)	85-90μ x 30-38.5μ
Eggs		
number measured	100	
length	90.7μ (60-110)	
width	48.8μ (35-65)	
Larvae (first stage)		
Number measured	50	
length	251.1μ (197.8-368.0)	
width	15.7μ (9.8-24.6)	
tail	23.6μ (18.4-32.2)	

*Only ♂ and ♀ parts were counted.

Table 3 - Relationship of first stage larvae in feces to numbers of adult *P. stilesi* in the definitive host.

	Sheep #1	Sheep #3	Sheep #4
Fecal samples	1-10 larvae/ gram		
Average of samples		0.4 larvae/ gram	9.4 larvae/ gram
sample No. 1*		0.25 larvae/ gram	5.3 larvae/ gram
sample No. 2		2.75 larvae/ gram	0.9 larvae/ gram
sample No. 3		0.75 larvae/ gram	0.33 larvae/ gram
sample No. 4		0.25 larvae/ gram	0.5 larvae/ gram
sample No. 5		0.37 larvae/ gram	0.0
Necropsy Results			
Fecal	few	------------	2.75 larvae/ gram
Larvae in lungs	---	6,910 larvae	23,100 larvae
Adults recovered (only ♂ and ♀ parts were counted)			
Male	5	2	9
Female	1	0	5
Seen in Histological sections of lungs		3	3
Total	6	5	17

*Samples 1 through 5 were 24 hour collections taken the 5 days prior to necropsy of the sheep.

keeping out possible parasitic vectors. Despite these precautions, insects on occasion got into the isolation shed. Nevertheless, isolation was felt to be sufficient to have prevented infections from sources other than those intended with this study.

Maintenance of snail cultures required constant attention to moisture and to temperature conditions. Incubation at 25 C was the most successful temperature used for snail production.

V. pulchella was a suitable intermediate host for *P. stilesi*. *Pupilla muscorum* may also be a suitable host since a single experimental infection was obtained. Infection in *P. muscorum* was difficult to detect due to conformation and thickness of the shell. *V. pulchella* was used for all infection attempts since it proved to be better suited to microscopic examination and laboratory propagation.

The present study yielded infected snails as early as three days after exposure to *P. stilesi* larvae. Second stage larvae were seen in as little as six days following exposure. Third stage larvae were observed at 14 days post-exposure. Pillmore (1955-1961) found that rate of development of *P. stilesi* in snails of species of *Pupilla*, *Vallonia* and *Vertigo* was quite variable. Larvae were first noted in the foot of the snail in 4 to 10 days after exposure. The second stage was reached in 8 to 30 days. The second molt occurred in 11 to 60 days after exposure. These times agree closely with those given above for the present study.

The 119 day prepatent period (days from first feeding of infective larvae to first stage larval production) given for sheep #1 (Table 1) may have been considerably shorter because larvae of *Protostrongylus* may have been present in the feces before they were noted. Fecal samples were collected in Pennsylvania and mailed to Colorado where they were found to be negative, yet similar fecal samples were later found to be positive when examined in Pennsylvania. The disappearance of the larvae in transported fecal samples is unexplained.

Prepatent periods for sheep #3 and #4 were 63 and 122 days respectively. This is considerably longer than those given by Pillmore (1959) for species in rabbits and mule deer. The general prepatent period was given as 25-60 days. The longer periods observed in this study may reflect specific differences in the parasites or the hosts. The fact that the definitive hosts were hybrid animals may have had an effect on the length of time necessary for the development of the infective larvae to the sexually mature adults.

Adult *Protostrongylus* recovered from sheep #1, #3 and #4 were identified as *P. stilesi*. These experimental specimens compared favorably with measurements of specimens given by Dikmans (1931) and Honess (1942). Body widths were somewhat less in the present study but variation was great in the earlier descriptions and the discrepancy in widths was not thought to be significant.

Hybrid sheep in pens adjoining the isolation shed were used as experimental control animals. The experimental sheep had been taken from this group. Examinations of fecal pellets from this group were conducted during the transmission experiments. All examinations of the non-isolated hybrid sheep revealed infections of *Muellerius* and *Pneumostrongylus*. *Protostrongylus* larvae were not found in any samples.

The results of the present study prove that the life cycle of *P. stilesi* does indeed involve a mollusc as an intermediate host. *V. pulchella* has been shown to be a suitable intermediate host for the development of the parasite to the infective stage. Transmission from this intermediate host to the Rocky Mountain bighorn sheep, the definitive host, is now considered to be possible. The activity of infective stage *P. stilesi* in bighorn x mouflon hybrids is strongly indicative that similar results do occur in bighorn sheep. There may be other variations of the life cycle which exist in the bighorn sheep. Prenatal transmission may also be possible. However, the present research has shown that bighorn sheep-terrestrial snail-bighorn sheep is an actual mode of transmission of *P. stilesi*. To that extent, the life cycle is now known.

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DISCUSSION

QUESTION BY DICK PILLMORE, BSF&W, COLORADO: In one of your slides, you showed one of the ensheathed infective stage larva and indicated that it had left the snail in that form. In sheep, did you ever get ensheathed larvae leaving the snail in that form?

REPLY BY MONSON: Yes, we did see several larvae with the sheath leave the snail and then exsheath.

REPLY BY SARA MCGLINCHY, CSU: We sat and watched one particular snail over an hour period and watched approximately three infective stage larvae actively migrate out of the snail's foot.

REPLY BY PILLMORE: I have recovered the infective larvae from inside the vials that I have had snails in. These have left the snails, but in no case did I ever see the ensheathed larvae come out. That's why I ask.

QUESTION BY CHARLES HIBLER, CSU: Ruth, you said that Tramisol did not show the efficacy I would like. Would you like to point out our previous discussions relevant to this. I do not want the public mis-lead that Tramisol is not an effective compound. American Cyanamid

has taken Dr. Rubin and myself apart to a certain extent on this already. It is not as efficient against *Trichostrongylus* as it is against some of the other parasites, but please clarify what we discussed yesterday.

REPLY BY MONSON: The animals had a gastro-intestinal infection, *Ostertagia* and *Trichuris*. After administration of Tramisol, the animals still had *Ostertagia* and *Trichuris*. Due to some form of pre-immunity, the parasites involved may not have been the same adult worm, but different adult worms. The infections were still there after the dosage of the drug had been given.

REPLY BY HIBLER: There is no such thing as retarded development of parasitic nematodes, especially gastro-intestinal nematodes. What Ruth is trying to say is that sheep, by virtue of their eating habits, are constantly ingesting these things. *Ostertagia* goes into the abomasum, develops and comes out as a sexually mature individual. But all the rest of the *Ostertagia*, would pour into here while retarded. Therefore, when she removed the adult population of *Ostertagia*, this released the retarded population. And if you're not on your toes, if you're not examining these animals consistently, you'll miss the fact that you have wiped out one adult population and released a sub-adult population. Now I might ask you if Tramisol is effective against *Protostrongylus stilesi* -- if it will kill the adults.

REPLY BY MONSON: No.

QUESTION BY C. E. WILLIAMSON, USFS, COLORADO: Is there any indication from your studies as to whether or not an effective time can occur whereby removal of one of the hosts of this parasitic lungworm will cause a reduction in the population in a given area? For instance, where the bed grounds are infected and the land snails are infected and then by removal of the sheep, would there be a reduction in the parasite?

REPLY BY MONSON: I don't believe there would be a reduction in the parasite with reduction of the sheep. The infection will remain in the snail and the first stage larvae will remain in the droppings of the sheep. The range will not be cleared of first stage larvae each year. They will overwinter and will remain on the range. Infective larvae will remain in the snail. We have seen them in the snail for as long as six months.

REPLY BY WILLIAMSON: You mentioned that a mouflon sheep can also carry this lungworm parasite. Can domestic sheep of our western ranges also carry this?

REPLY BY MONSON: I don't know if domestic sheep would be susceptible to *Protostrongylus stilesi*. They haven't been infected with it. There is another *Protostrongylus* species that occurs in domestic sheep, *P. rufescens*, and I don't know whether or not *P. stilesi* will live in domestic sheep. Dick Pillmore has done some work with trying to infect domestic sheep but has not been able to cause infection. Maybe he can answer that better than I can.

REPLY BY PILLMORE: It's never been recorded in domestic sheep. In one instance, with a hybrid bighorn x domestic, we recovered first stage larvae over a period of time, but we were not able to recover the adult worms. At the same time, we had one mouflon ram that we repeatedly exposed but we never established an infection in it. I think that, in time, it wouldn't be surprising to have it turn up in domestic sheep, but it's not an important or a normal host.

QUESTION BY DON BAILFIELD, USFS, COLORADO: To carry Mr. Williamson's question a bit further, if we eliminated the sheep population for a number of years, could we break the cycle?

REPLY BY MONSON: Dr. Thorne from Wyoming could tell you more about this. They've kept fecal samples with first stage larvae in them which are still viable after 10 years.

REPLY BY DR. THORNE: Even longer than ten. I can't remember exactly, but it's much longer than 10. These are frozen. They are extremely hearty and I don't think it would be feasible to take the sheep off the range with the hopes of later re-introducing them and avoiding an infection. In the first place, I don't know where you'd get the sheep to start with that don't have the lungworm. In the second place, I think it would be extremely hard to assure yourself that you'd eliminated the infective larvae and the larvae there on the range. It would take quite a long time. I'd sure hate to exterminate a herd of sheep with that in mind.

QUESTION BY DOUGLAS GILBERT,CORNELL UNIVERSITY: One question, Ruth, how did you administer the snails to the sheep?

REPLY BY MONSON: Infective stage larvae in the snails were counted. Then the snails were put into gelatin capsules and fed to the sheep with a balling gun.