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Spontaneous Shedding of Metastrongyloid Third-Stage Larvae by Experimentally Infected *Limax maximus*

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Abstract

Felids and canids acquire infection of most metastrongyloids by the ingestion of infective third-stage larvae (L3) in the tissues of gastropod intermediate hosts (IMH) or paratenic hosts. Direct ingestion of L3 shed by gastropods may be an alternative route of exposure. The significance of this route in natural infections is unknown. Larval shedding after experimental infection of *Limax maximus* with 5 metastrongyloids was tested in this study. First-stage larvae (L1) of *Aelurostrongylus abstrusus*, *Angiostrongylus vasorum* and *Crenosoma vulpis* (400–1445 L1/slug) were fed to laboratory raised *L. maximus* in separate exposure groups of 12–42 slugs. A mixture of *Oslerus rostratus*-*Troglostrongylus wilsoni* (90%/10%) and *T. wilsoni*-*O. rostratus* (95%/5%) L1 was used to infect 30 and 22 *L. maximus* (1600 L1/slug), respectively. Slug faeces was examined 2x/week by modified Baermann to detect L3. A small number of L3 (1.3–2.8% of the total) was shed in all infected groups beginning 20–32 days PI. Weekly shedding levels ranged from 0–3.3 L3/slug. Longevity was tested by placing L3 of *A. vasorum, C. vulpis* and *T. wilsoni*-mix on lettuce and held at 16°C/75% humidity. Motile *A. vasorum* and *T. wilsoni*-mix L3 were recovered up to 12–16 days post-deposit (PD). Motile L3 of *C. vulpis* were still present 120 PD. Detection in all 5 parasite species indicates that spontaneous shedding of L3 into the environment is likely a general characteristic of the metastrongyloids. Prolonged survival of L3 indicates that exposure through environmental contamination may play a role in natural infection transmission with these parasites.

Introduction

The Superfamily Metastrongyloidea is a diverse group of nematode parasites consisting of 7 families with most species occurring in the lungs of various mammals and some occurring in the pulmonary artery, veins, or frontal sinuses (Anderson, 2000). Excepting species infecting marine mammals or swine, the exposure route for most involve the ingestion of infective third-stage larvae (L3) contained in the tissues of a gastropod intermediate host or also in some species a paratenic host. Spontaneous emergence of infective third-stage larvae (L3) from infected gastropod intermediate hosts has been reported for various metastrongyloids, most prominently in the protostrongylids (*Cystocaulus ocreatus, Muellarius capillaris, Parelaphostrongylus odocoilei, Protostongylus boughtoni, Protostongylus davtyani, Protostongylus kamenskyi, Protostongylus pulmonalis, Protostongylus rufesens, Protostongylus stilesi, Protostongylus tauricus, Umingmakstrongylus pallikuukensis*) and angiostrongylids (*Aelurostrongylus abstrusus, Angiostrongylus cantonensis, Angiostrongylus costaricensis, Angiostrongylus vasorum*) (Barcante et al. 2003, Boev 1975, Bonetti et al. 1998, Giannelli et al. 2015, Heyneman and Lim 1967, Jenkins et al. 2006, Kralka and Samuel 1984, Kutz et al. 2000, Monson and Post 1972, Ubelaker et al. 1980). Recently gastropod L3 shedding has also been reported for a crenosomatid (*Troglostrongylus brevior*) (Giannelli et al. 2015). Detection of L3 in mucus of slugs and semi-slugs has been reported for *A. cantonensis* and *A. costaricensis*, although in small numbers and able to survive for only short periods of time outside the gastropod host, bringing into question what role it may play as a source of exposure in natural infections (Ash, 1976, Bonetti et al. 1998, Cowie, 2013, Prociv et al. 2000, Qvarnstrom et al. 2007). L3 of *A. cantonensis* may also be released by land snails and slugs immersed in water suggesting that contaminated drinking water could be a potential route of exposure for human infection (Cheng and Alicata, 1964). Gastropod shedding of L3 has been implicated by cases of human infection with *A. cantonensis* where exposure appeared to be through consumption of fresh produce or handling gastropods (Chen et al. 2005, Cowie 2013, Wan and Weng 2004, Waugh et al. 2005). At present the epidemiological significance of L3 shedding by gastropods is unknown. If sufficient numbers of L3 are shed into the environment by infected gastropods and those larvae are able to survive for an extended period of time once outside the intermediate host this may represent a significant exposure route of infection to the definitive host. The objective of this study was to determine and quantify the spontaneous shedding of L3 for 5 metastrongyloid parasites of canids (*Angiostrongylus vasorum, Crenosoma vulpis*) and felids (*Aelurostrongylus abstrusus*, *Oslerus rostratus, Troglostrongylus wilsoni*) by experimental infection of a laboratory raised slug species, *Limax maximus*.

Materials and Methods

Laboratory Gastropod Colony

Limax maximus collected from a suburban garden in Charlottetown, Prince Edward Island, Canada were housed in petri-dishes (150 mm diameter x 25 mm) lined with damp paper towel, fed Romaine lettuce and kept in an incubator at 16°C and 75–80 % humidity. Eggs were removed and placed into separate petri-dishes and housed under the same conditions. After hatching, immature slugs were fed on Romaine lettuce. When juvenile slugs had attained about the weight of 0.5 g they were housed in lock-top plastic food storage containers (3.9 L; 28 cm x 21.5 cm x 7.5 cm) on a damp paper towel substrate, fed Romaine lettuce and kept at 16°C and 75 % humidity.

Parasites

Angiostrongylus vasorum first-stage larvae (L1) were acquired from the faeces of a naturally infected dog from Newfoundland-Labrador, Canada. The

larvae were recovered from the faeces by a modified Baermann technique. A 24 gram fecal sample was divided in half and each half (12 grams) was placed in a double-layer of cheese cloth and put into a glass Baermann funnel containing warm water. The samples were left overnight (> 12 hrs) after which 50-ml of the water was drawn from each funnel into screw-top plastic graduated centrifuge tubes. The samples were centrifuged (800 g) for 10 minutes, the supernatant was discarded and the volume was adjusted to a total of 5 ml. The pellet was re-suspended by vortexing for 20 seconds. The L1 concentration was determined through counting all of the larvae in duplicate 50 microliter subsamples placed on a slide with coverslip and examined using a compound microscope under the 10x-objective.

Crenosoma vulpis first-stage larvae were acquired from adult worms recovered by lung flush (modified Inderbitzen method: Oakley, 1980) from red fox carcasses obtained from local fur-trappers within 48-hrs of pelting. The animals were obtained as a by-catch of the trapping harvest; foxes were trapped in accordance with the laws and regulations of the province of Prince Edward Island and no animals were trapped specifically for the purposes of this study. The animals were frozen upon submission and stored for 4 months at -20 °C prior to parasite recovery. The carcasses were thawed at 4 °C for 24 hrs prior to processing. Heart and lungs were removed from the thoracic cavity and an incision was made into the right ventricle. Plastic tubing connected to the water tap was inserted into the incision and while holding the lungs on a 150-micron geologic sieve, the water was slowly turned on making sure the water outflow from the trachea passed through the sieve. The lungs were held for 2 minutes after reaching full inflation. The sieve was rinsed with isotonic saline (0.85 %) into screw-top 100-ml plastic sample collection containers. Female worms were removed and placed in a small petri-dish (60 mm diameter x 15 mm) and chopped into small pieces using a razor blade. The water and worm fragments were rinsed onto a double-layer of cheese cloth in a Baermann funnel containing warm water, held overnight and L1 were collected and counted (as above).

Oslerus rostratus and *Troglostrongylus wilsoni* L1 were recovered from a small piece of lung tissue from a naturally co-infected bobcat from New Brunswick, Canada. Adult *T. wilsoni* were recovered from the bronchi, placed in a small petri-dish (60 mm diameter x 15 mm) and chopped into fine pieces with a razor blade, placed into a Baermann (as above) and L1 were recovered and counted (as above). The sample appeared to be a mix of L1 with the majority (about 95 %) being *T. wilsoni* and the remainder (5 %) were *O. rostratus*. After removal of the adult *T. wilsoni*, the lung tissue containing coiled *O. rostratus* in the parenchyma was placed into a Baermann funnel in warm water overnight (> 12 hrs) and L1 were recovered and counted (as above). This sample also appeared to be a mix with the majority (about 90 %) being *O. rostratus* and the rest (10 %) *T. wilsoni*.

Aelurostrongylus abstrusus L1 were acquired from the faeces of a naturally infected cat from Nova Scotia, Canada. The larvae were recovered from the faeces by Baermann technique (as above).

Gastropod Exposures

Small squares of lettuce were placed in the bottom of each well in a 6-well-flat bottom tissue culture plate (12.5 cm x 8.5 cm x 2 cm). After vortexing for 10–20 seconds, 150 microliters of the L1 solution was placed on top of the lettuce. A slug (0.7–2.6 g) was added to each well with the provided lettuce as the only source of food for 48–72 hrs. After the majority of the lettuce had been consumed by the slugs, they were housed in the 3.9L plastic lock-top food storage containers on damp paper towel, fed Romaine lettuce and kept in an incubator at 16 °C and 75 % humidity.

Larval Shedding Monitoring

L3 shedding was detected and quantified by use of a modified Baermann technique. Twice per week, slug faeces was collected from the plastic containers

and placed on a double layer of cheese cloth. The cheese cloth was placed in a 50-ml screw-top centrifuge tube containing warm tap water. The cap was screwed on to the tube catching a small part of the cheese cloth to keep it in place suspended at the top of the tube. The samples were left overnight (> 12 hrs). The cheese cloth and faeces was discarded, the tubes centrifuged (800g) for 10 minutes, the supernatant discarded and the pellet was re-suspended by vortexing for 10–20 seconds. The fluid was pipetted into a square grid-marked petri-dish (9 cm x 9 cm x 1.5 cm) and the sample was examined using a dissecting microscope for the presence of L3. The recovered L3 were either fixed in 95 % ethanol or hot (65 °C) 2 % formalin.

Gastropod Digestion

Slugs were artificially digested to obtain total L3 counts at weeks 4–7 Post-Infection (PI) for each infection (except *A. abstrusus*) and then to terminate each study at week 14 PI for *A. abstrusus, O. rostratus* and *T. wilsoni* and at 27–33 weeks PI for *A. vasorum* and *C. vulpis*. Gastropods were beheaded and placed in a double layer of cheese cloth and suspended in a digest solution (10g pepsin; 13 ml concentrated hydrochloric acid; 1667 ml distilled water) in 50-ml screw-top centrifuge tubes for 3–4 hrs at 37 °C. The remnants of the gastropod were discarded, the tube centrifuged (800g) for 10 minutes, the supernatant discarded and the number of L3 counted (as above for L3 shedding).

Longevity of L3

L3 of *A. vasorum* and *T. wilsoni*-Mix recovered from *L. maximus* by digestion were placed on squares of lettuce (approximately 180–210 L3/well) in the bottom of 6-well tissue culture plates and kept at 75 % humidity and 16 °C. A single 6-well plate was set-up for *A. vasorum* and duplicate plates were done for the *T. wilsoni*-Mix. The lettuce from 2 wells was examined for the presence of L3 by Baermann technique at 2, 8 and 12 days (*A. vasorum*) or 5 and 16 days (*T. wilsoni*-Mix) post-deposit. Recovered larvae were considered alive if moving or they were tightly coiled; larvae were considered dead if they showed no movement and were not tightly coiled. No attempt was made to determine the species composition of the *T. wilsoni*-Mix L3 recovered from the lettuce.

In a second trial of longevity, 4000 L3 of *C. vulpis* recovered from *L. maximus* by digestion were divided between 4 wells in each of two 6-welled tissue culture plates. The L3 (approximately 500 L3/well) were placed on squares of lettuce in the bottom of each of the 4 wells/plate. In one plate, the 4 wells were lined with moist paper towel. The plates were kept at 75 % humidity and 16 °C. The plate cover and wells were lightly sprayed with a mist of distilled water 2–3 times/week. At 120 days post-deposit, the remnants of the lettuce or the lettuce-paper towel were removed and placed on a double layer of cheese cloth and held in 50-ml centrifuge tubes containing slug digest solution at 37 °C for 2 hours. All 4 wells were combined into a single digest for each of the two plates. Warm digest solution was then added to each of the wells and the plate was examined using a dissecting microscope for the presence of remaining L3. After the 2-hr incubation, the cheese cloth-lettuce-paper towel was removed and the tubes were centrifuged at 800 g for 10 minutes. The supernatant was poured off and the pellet was re-suspended in distilled water, placed in square grid marked petri dishes (9cm x 9 cm x 1.5 cm) and L3 were counted and collected into small petri dishes (60 mm diameter x 15 mm). The majority of the water was removed by pipette and warm (37 °C) slug digest solution was added. A differential count was done on the first 100 L3 to determine the percent that were moving, tightly coiled or not moving-not coiled (ie presumed dead).

Morphological Identifications of Infective Third-stage Larvae

Temporary wet mounts for the morphological identifications of fresh or hot 2 % formalin-fixed L3 were made by attaching small fragments of broken 18 mm square coverslips on a large (75 x 50 mm)

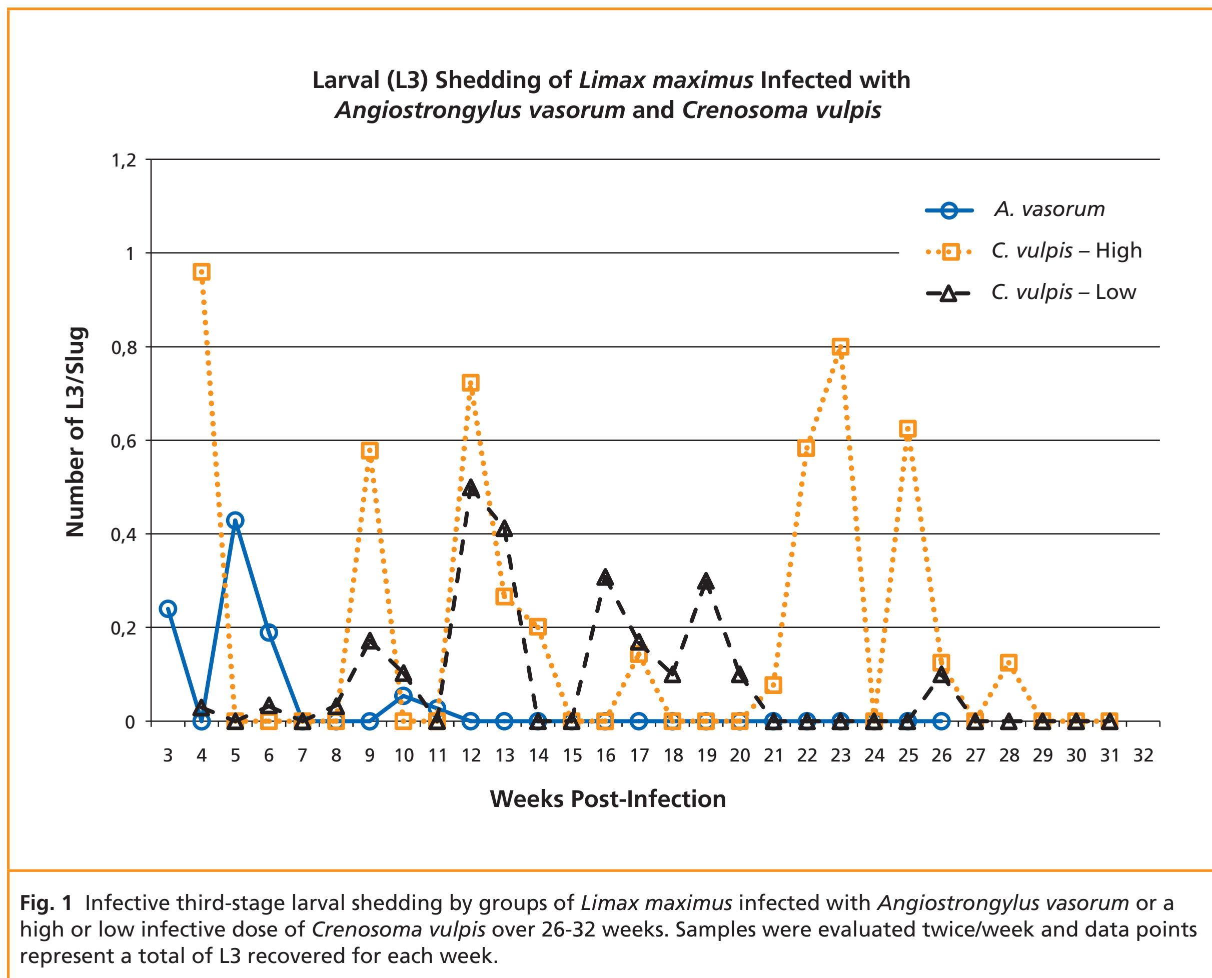

Fig. 1 Infective third-stage larval shedding by groups of *Limax maximus* infected with *Angiostrongylus vasorum* or a high or low infective dose of *Crenosoma vulpis* over 26-32 weeks. Samples were evaluated twice/week and data points represent a total of L3 recovered for each week.

microscope slide with a small drop of Canada Balsam such that the fragments would support the weight (at the edges) of a 30 x 22 mm coverslip. The formalin-fixed larvae were placed directly on the slide between the attached coverslip fragments. Fresh larvae were placed on the slide along with a drop of dilute iodine (diluted 1:10 with distilled water) to kill and immobilize the L3 to allow thorough examination. A 30 x 22 mm coverslip was placed on the drop and the edges of the coverslip were sealed with clear cosmetic nail polish to prevent evaporation. This method allowed examination of the larvae without the potential for distortion of morphological features due to the weight of the coverslip and also allowed the examination of the larvae using the oil-immersion objective.

Results

No L3 were recovered from the faeces or the digests of unexposed control *L. maximus* at any point in the study.

Angiostrongylus vasorum

A total of 29 L3 were shed from day 20 to 77 PI (>50% of the L3 were shed by day 41 PI). Peak shedding/slug occurred at week 5 PI (Fig. 1). The weekly shedding rate ranged from 0–0.43 L3/slug. Two of the 42 exposed slugs died during the 191 days of the study. Slugs digested at week 5 PI had 26–99 L3 (Mn = 59.2 L3; total = 296 L3). The remainder of the slugs (n = 35) digested at weeks 26–27 PI had 29–145 L3 (Mn = 60.8 L3; total = 2127 L3). Shedding

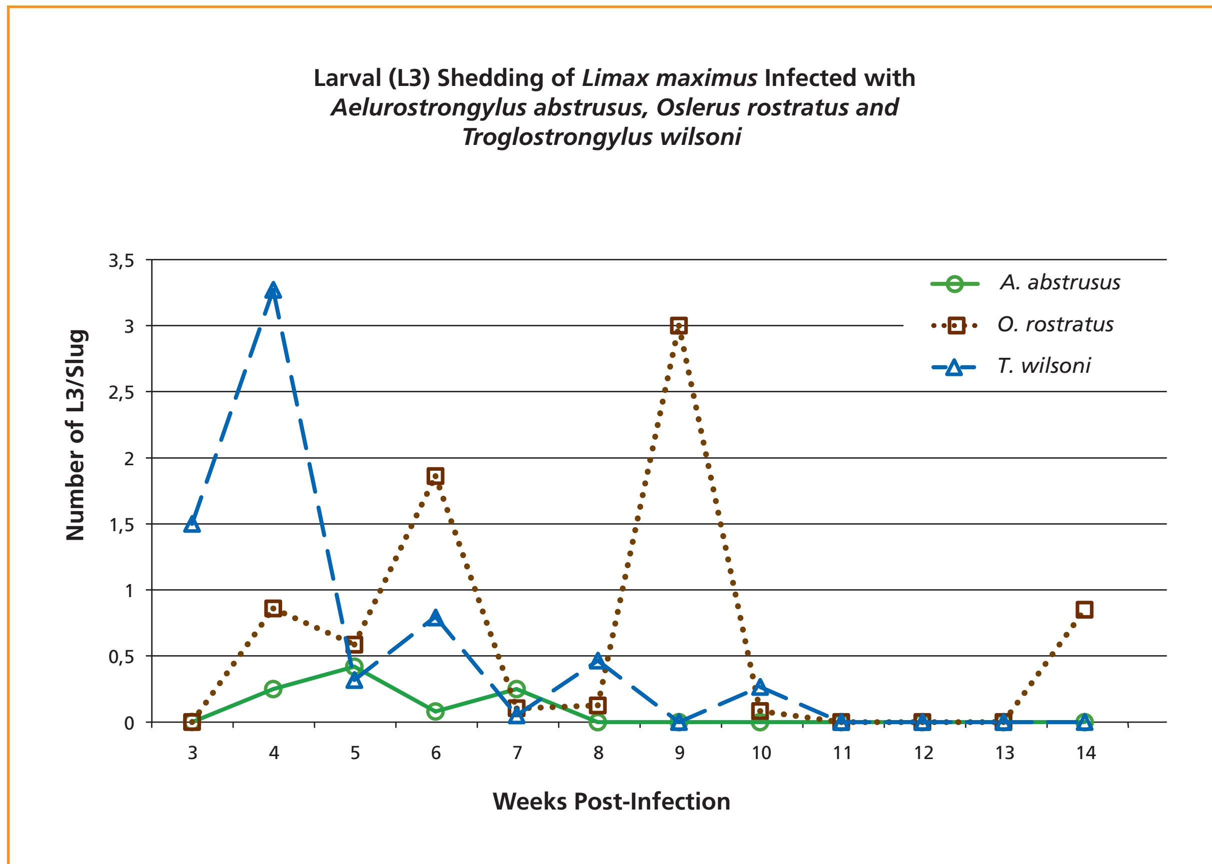

Fig. 2 Infective third-stage larval shedding by *Limax maximus* infected with *Aelurostrongylus abstrusus* or a mix of *Troglostrongylus wilsoni* (90 %)-*Oslerus rostratus* (10 %) or *O. rostratus* (90 %)-*T. wilsoni* (10 %) over 14 weeks. Samples were evaluated twice/week and data points represent a total of L3 recovered for each week.

29 L3 represents 1.3 % of the total L3 recovered from the infected slugs. The *A. vasorum* L3 (Fig. 3) measured (n = 5) 523–590 x 24–28 microns (Mean = 556.6 ± 23.1 x 26.0 ± 1.3). A rounded knob occurred at the termination of the tail (Fig. 4).

Crenosoma vulpis

Low dose – A total of 42 L3 were shed from days 30 to 184 PI (> 50 % of the L3 were shed by day 90 PI). Peak shedding/slug occurred at week 12 PI (Fig. 1). The weekly shedding rate ranged from 0–0.50 L3/slug. Twenty-six of the 40 slugs died during the 229 days of the study. Slugs (n = 5) digested at 4 weeks PI had 45–187 L3 (Mn = 131.4 L3; total = 657 L3). The remainder (n = 9) digested at week 32 had 57–480 L3 (Mn = 218.6 L3; total = 1967 L3). The 42 L3 shed represents 1.6 % of the total L3 recovered from the infected slugs.

High dose – A total of 80 L3 were shed from days 28 to 196 PI (> 50 % of the L3 were shed by day 90 PI). Peak shedding/slug occurred at week 4 PI (Fig. 1). The weekly shedding rate ranged from 0–0.96 L3/slug. Eighteen of the 30 slugs died during the 231 days of the study. Slugs (n = 5) digested at 4 weeks PI had 88–227 L3 (Mn = 166.0 L3; total = 830 L3). The remainder (n = 7) digested at week 33 had 117–438 L3 (Mn = 282.1 L3; total = 1975 L3). The 80 L3 shed represents 2.8 % of the total L3 recovered from the infected slugs. The *C. vulpis* L3 (Fig. 5) measured (n = 6) 535–600 x 24–28 microns (Mean = 560.2 ± 21.1 x 25.5 ± 0.9). The tail terminates in a simple point (Fig. 6).

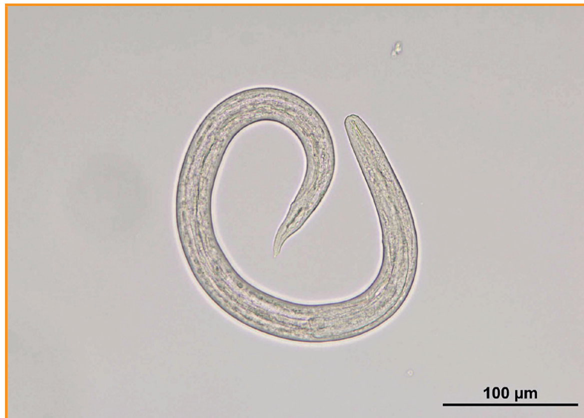

Fig. 3 Infective third-stage larvae of *Angiostrongylus vasorum* shed by an infected *Limax maximus* kept at 16° C (hot fixed in 2 % formalin; temporary wet mount; viewed under 20 x objective).

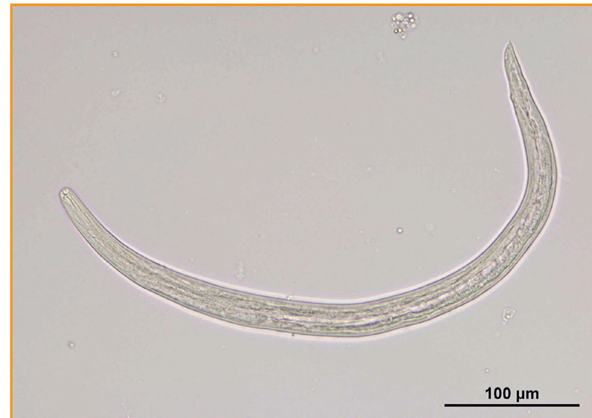

Fig. 5 Infective third-stage larvae of *Crenosoma vulpis* shed by an infected *Limax maximus* kept at 16° C (hot fixed in 2 % formalin; temporary wet mount; viewed under 20x objective).

Fig. 4 Tail of an infective third-stage larvae of *Angiostrongylus vasorum* shed by an infected *Limax maximus* (hot fixed in 2 % formalin; temporary wet mount; viewed under oil-immersion objective). Note the tail terminates as a rounded knob similar in appearance to the L3 of *A. abstrusus*.

Fig. 6 Tail of an infective third-stage larvae of *Crenosoma vulpis* shed by an infected *Limax maximus* kept at 16° C (hot fixed in 2 % formalin; temporary wet mount; viewed under oil-immersion objective). The tail terminates as a simple point.

Aelurostrongylus abstrusus

A total of 12 L3 were shed from days 32 to 55 PI (> 50 % of the L3 were shed by day 35 PI). Peak shedding/slug occurred at week 5 PI (Fig. 2). The weekly shedding rate ranged from 0–0.42 L3/slug. None of the exposed slugs died during the 98 days of the study. Slugs (n = 12) digested at week 14 had 6–114 L3 (Mn = 45.9 L3; total = 551 L3). The 12 L3 shed represents 2.1 % of the total L3 recovered from the infected slugs. The *A. abstrusus* L3 (Fig. 7) measured (n = 5) 543–600 x 25–29 microns (Mean = 564.9 ± 19.4 x 27.8 ± 1.2). A rounded knob occurred at the termination of the tail (Fig. 8).

Oslerus rostratus-Mix

A total of 193 L3 were shed from days 30 to 98 PI (> 50 % of the L3 were shed by day 55 PI). Peak shedding/slug occurred at week 9 PI (Fig. 2). The weekly shedding rate ranged from 0–3.00 L3/slug. Five of the 30 slugs died during the 105 days

Fig. 7 Infective third-stage larvae of *Aelurostrongylus abstrusus* shed by an infected *Limax maximus* kept at 16° C (hot fixed in 2 % formalin; temporary wet mount; viewed under 20 x objective).

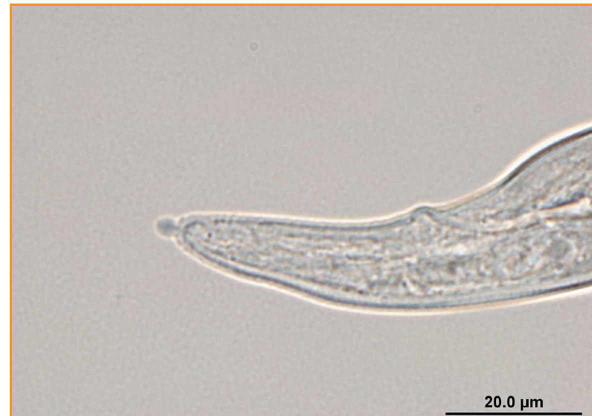

Fig. 8 Tail of an infective third-stage larvae of *Aelurostrongylus abstrusus* shed by an infected *Limax maximus* kept at 16° C (hot fixed in 2 % formalin; temporary wet mount; viewed under oil-immersion objective). Note the tail terminates as a rounded knob similar in appearance to the L3 of *A. vasorum*.

of the study. Slugs (n = 5) digested at 7 weeks PI had 172–644 L3 (Mn = 343.2 L3; total = 1716 L3). The remainder (n = 20) digested at week 15 had 145–799 L3 (Mn = 357.6 L3; total = 7152 L3). The 193 L3 shed represents 2.1 % of the total L3 recovered from the infected slugs. Results of identifications based on morphology (n = 119) indicated 90.8 % of the L3 recovered by digestion were *O. rostratus* and 9.2 % were *T. wilsoni*. The *O. rostratus* L3 (Fig. 9) measured (n = 9) 469–511 x 24–28 microns (Mean = 494.7 ± 14.2 x 26.3 ± 1.3). The tail terminates as a simple point but there are small serrations on the surface visible just before the end of the tail (Fig. 10).

Troglostrongylus wilsoni-Mix

A total of 140 L3 were shed from days 27 to 70 PI (> 50 % of the L3 were shed by day 30 PI). Peak shedding/slug occurred at week 4 PI (Fig. 2). The weekly shedding rate ranged from 0–3.27 L3/slug. Two of the 22 slugs died during the 103 days of the study. Slugs (n = 5) digested at 7 weeks PI had 187–331 L3 (Mn = 250.8 L3; total = 1254 L3). The remainder (n = 15) digested at week 14 had 128–1077 L3 (Mn = 468.9 L3; total = 7033 L3). The 140 L3 shed represents 1.7 % of the total L3 recovered from the infected slugs. Results of L3 identifications based on morphology (n = 89) indicated 94 % of the L3 recovered by digestion were *T. wilsoni* and 6 % were *O. rostratus*. The *T. wilsoni* L3 (Fig. 11) measured (n = 9) 409–449 x 24–30 microns (Mean = 427.1 ± 15.0 x 26.7 ± 1.5). There are 2 indentations along the ventral surface of the tail which terminates in a sharp point (Fig. 12).

Longevity

Vigorously motile *A. vasorum* L3 were recovered at each sample time (2, 8, 12 days post-deposit). A total of 109 L3, 110 L3 and 91 L3 were recovered representing 61.9 %, 62.5 % and 51.7 % of the L3 placed on the lettuce at days 2, 8 and 12, respectively. Moving or tightly coiled larvae (ie considered alive) comprised 95.4 %, 89.1 %, and 74.7 % of the recovered L3 at 2, 8, and 12 days post-deposit, respectively. Vigorous movement was still observed although in very few L3 from each well by day 12; the lettuce squares were in an advanced degree of spoilage by day 12.

Vigorously moving *T. wilsoni*-Mix L3 were recovered at both sample times (5 and 16 days post-deposit). At day 5, 434 L3 were recovered (36.2 %

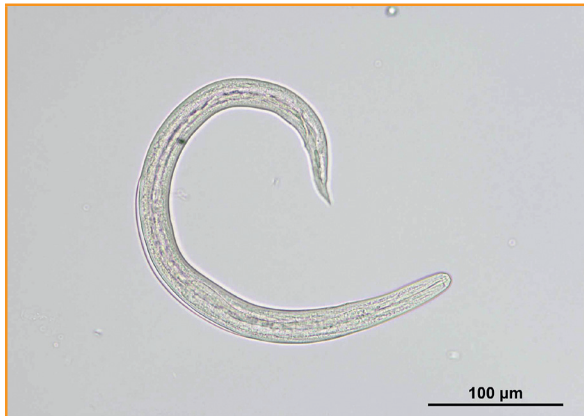

Fig. 9 Infective third-stage larvae of *Oslerus rostratus* shed by an infected *Limax maximus* kept at 16° C (hot fixed in 2 % formalin; temporary wet mount; viewed under 20 x objective).

Fig. 10 Tail of an infective third-stage larvae of *Oslerus rostratus* shed by an infected *Limax maximus* (hot fixed in 2 % formalin; temporary wet mount; viewed under oil-immersion objective). The tail terminates in a simple point but note the fine serrations between the anus and the point of the tail.

of the L3 deposited in those wells) with 63.9 % showing vigorous movement, 24.3 % tightly coiled and 11.8 % appeared dead. At day 15, 442 L3 were recovered (35.2 % of the L3 deposited in those wells) with 40.0 % showing movement (vigorous movement only in a few); 20.1 % coiled and 39.8 % appeared dead. Lettuce was in an advanced stage of spoilage by day 16.

Vigorously moving *C. vulpis* L3 were recovered from both plates 120 days post-deposit. A total of 151 L3 (7.6 % of the 2000 L3 placed in the wells; 27 % moving, 17 % coiled and 56 % not moving-not coiled) were recovered from the plate that had contained only lettuce as substrate. A total of 164 L3 (8.2 % of the 2000 L3 placed in the wells; 17 % moving; 81 % coiled and 2 % not moving-not coiled) were recovered from the plate that had both paper towel and lettuce substrate.

Discussion

Both natural and experimental infections of *A. vasorum* and experimental infections with *O. rostratus* in *L. maximus* have been reported previously (Ferdushy et al. 2009, Klewer 1958, Lange et al. 2017). This is the first report of the suitability of *L. maximus* as an intermediate host for *A. abstrusus, C. vulpis* and *T. wilsoni*. Excepting *T. wilsoni* for which this is the first gastropod intermediate host reported, the list of susceptible intermediate hosts for the other 4 metastrongyloid species studied is relatively long including both aquatic and terrestrial gastropods (Anderson 2000, Grewal et al. 2003). The addition of *L. maximus* as an intermediate host for these metastrongyloids is consistent with the view that this group of parasites lack gastropod intermediate host specificity.

The morphology of the L3 was consistent with that reported previously for 4 of the species (*A. abstrusus, A. vasorum, C. vulpis, O. rostratus*) (Ash 1970, Colella et al. 2016, Gerichter 1949, Giannelli et al. 2015). The morphology of the *T. wilsoni* was similar to that reported for *T. breviour* (Gerichter 1949). The size of the *A. vasorum* L3 was consistent with that reported by others (Ash 1970, Rosen et al. 1970). The size of the *A. abstrusus* L3 reported here was slightly larger than that reported by Ash (1970) but consistent with that of Zottler and Schnyder (2016). The size of the *C. vulpis* L3 (560 x 26 microns) was much larger than that reported by Wetzel (1940) and more recently by Colella et al. (2016). Both of

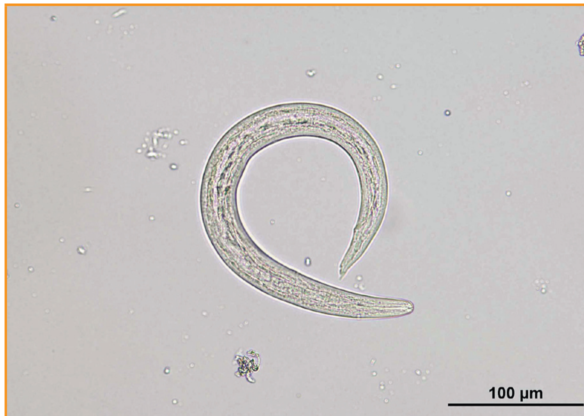

Fig. 11 Infective third-stage larvae of *Troglostrongylus wilsoni* shed by an infected *Limax maximus* kept at 16oC (hot fixed in 2 % formalin; temporary wet mount; viewed under 20x objective).

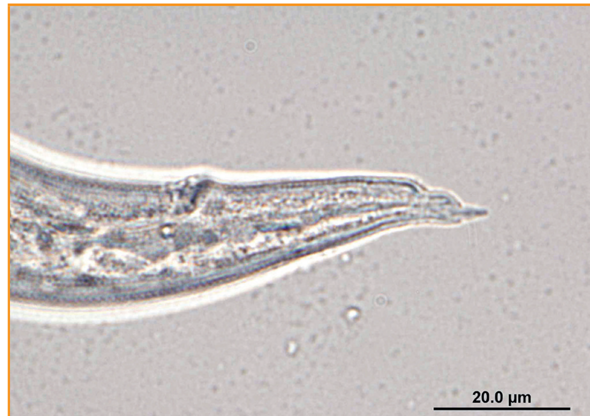

Fig. 12 Tail of an infective third-stage larvae of *Troglostrongylus wilsoni* shed by an infected *Limax maximus* (hot fixed in 2 % formalin; temporary wet mount; viewed under oil-immersion objective). Near the termination of the tail there are 2 indentations along the ventral surface and the tail ends in a fine point.

these researchers were working with European source material and therefore there may be a geographic difference in the species. All of the *C. vulpis* L3 recovered in this study were from L1 that had survived freezing for 4–5 months in the red fox carcasses. Recovery of motile L1 from adult female worms in raccoons frozen up to 14 months has been reported for *Crenosoma goblei* (Snyder 1985). The infectivity for the gastropod intermediate host of *Crenosoma* spp. L1 that have survived freezing has now been confirmed. Somewhat surprisingly, the L3 of *O. rostratus* from this study were significantly smaller than that reported elsewhere. The *O. rostratus* L3 were 495 x 26 microns as opposed to the 578 x 29 microns reported by Ash (1970). Hawaii was the source of the material Ash worked with. Whether this reflects geographic differences or the fact that the material used in this study came from a co-infection is unknown. The metastrongyloid L3 of this study were readily differentiated from each other based on distinctive tail morphology except for those of *A. abstrusus* and *A. vasorum*. Ash (1970) in his classic still frequently cited paper described the termination of the *A. vasorum* tail as "Digitiform, not fine point" and that of *A. abstrusus* as "Rounded knob". The tails of the 2 species appeared too similar to allow reliable accurate differentiation (Figs. 4 and 8). Morphological identification is likely insufficient as the sole means of identification of L3 recovered in a field survey of metastrongyloid infection in gastropods in any area endemic for both *A. vasorum* and *A. abstrusus*. It is also possible that there are other metastrongyloid species, where the L3 have not yet been adequately described, that may also confound the identification due to morphological similarities.

Gastropod shedding of L3 in the faeces was detected in all 5 of the metastrongyloid species tested. The 5 species in this study represent 3 different families within the Metastrongloididea: Angiostrongylidae (*A. abstrusus*, *A. vasorum*), Crenosomatidae (*C. vulpis, T. wilsoni*) and Filaroididae (*O. rostratus*). Of the 5 metastrongyloid families in which gastropods serve as intermediate hosts, only in the poorly studied Skrjabingylidae has larval shedding not been reported. Spontaneous larval shedding may be a common feature in all gastropod-borne metastrongyloids. Larval shedding in gastropods infected with *C. vulpis, T. wilsoni* or *O. rostratus* has not been reported previously. Gastropod shedding of *A. vasorum* L3 has been reported in

Table 1 Metastrongyloid species, exposure levels and number of *L. maximus* infected.

Parasite	No. slugs exposed	No L1/slug
Angiostrongylus vasorum	42	800
Crenosoma vulpis **– Low dose**	40	800
– High dose	30	1445
Aelurostrongylus abstrusus	12	400
***Oslerus rostratus*-mix**	30	1600
***Troglostrongylus wilsoni*-mix**	22	1600

laboratory aquatic snails (*Biomphalaria glabrata*) but this is the first report in a terrestrial slug (Barcante et al. 2003). Consistent with the findings of others excepting those studying the protostrongylids, the shedding level was relatively low (1–3 % of the worm burden). Whether any environmental factor(s) influence or stimulate shedding in naturally infected gastropods to higher levels than those reported here remains unknown. Shedding levels in experimentally infected protostrongylids tend to be considerably higher ranging from 21–82 % of the worm burden released by the gastropods (Jenkins et al. 2006, Kralka and Samuel, 1984, Kutz et al. 2000). In general, the shedding levels did not appear to be dose dependent, however, between species comparisons in this study are probably not valid. However, two different exposure levels were used in the *C. vulpis* infected slugs with a higher shedding level (2.8 % vs 1.6 %) associated with the higher infection intensity (282.1 L3/slug vs 218.6 L3/slug).

Release of L3 via gastropod faeces has not been reported previously. Detection of metastrongyloid L3 in mucus trails or exiting directly through the body surface of slugs has been reported (Ash, 1976, Bonetti et al. 1998, Gianelli et al. 2015, Heyneman and Lim 1967, Jenkins et al. 2006, Kralka and Samuel 1984, Kutz et al. 2000, Qvarnstrom et al. 2007). No L3 were detected in the mucus of *A. cantonensis* infected *Limax flavus* (Campbell and Little, 1988). Whether fecal shedding represents an additional mode of exit or the mode of L3 exit varies and is species (parasite or gastropod) dependent is unknown. Both faeces and mucus may offer protection to L3 from adverse environmental elements by providing an organic matrix. It is also a certainty that some of the faeces examined in this study would have been contaminated with mucus. As would be expected, there is a wide range in reported longevity for various species of L3 shed by aquatic or terrestrial gastropods held under many different experimental conditions. Protostrongylid L3 held at 0–4 °C survived for 6 months to over 1 year (Jenkins et al. 2006, Kutz et al. 2000). Much shorter timeframes (72 hrs to 36 days) have been reported for the L3 of *A. vasorum*, *A. abstrusus* and *T. brevior* held in water (Cheng and Alicata 1964, Colella et al. 2015). The L3 used to test longevity in this study were recovered by digestion of infected *L. maximus*. Whether there are differences in survivability between digested L3 and those that have spontaneously been released is unknown. Previous work in our laboratory had indicated that all motility would be absent in digest recovered *C. vulpis* or *A. vasorum* L3 held in water at 4–24 °C within 5–7 days (Conboy unpublished). In an attempt to provide an organic matrix that would better mimic natural conditions, the L3 in this study were placed on fresh lettuce. Monitoring was conducted only up to 12–16 days for the *A. vasorum* and *T. wilsoni* L3 due to the predicted degree of deterioration of the lettuce over the sampling timeframes. Placing the lettuce on paper towel allowed a longer storage time for *C. vulpis*. However, a more important difference was the additional step of using warm artificial digest solution to help determine L3 viability.

The L3 held in storage appear to enter into a quiescent state. Placing them in warm digest solution induces many that would have been misidentified as "dead" to show vigorous movement or to tightly coil. Although only 7–8 % of the *C. vulpis* L3 were recovered after 120 days, 98 % were considered alive (17 % moving; 82 % coiled). Whether the same or similar results would have been found with *A. vasorum* or *T. wilsoni* L3 held under the same conditions is not known.

Gastropod shedding of metastrongyloid L3 may be (as speculated) just an aberration of experimental infection models with no relevance to natural infection of these parasites (Ash 1976, Prociv et al. 2000, Cowie 2013). Experimental infections tend to occur at higher levels than those generally found in gastropods under natural conditions (Kralka and Samuel, 1984). However, at least with the protostrongylids, shedding still occurs even at very low worm burdens and to a degree where the individual gastropod may shed its entire burden (Jenkins et al. 2006, Kutz et al. 2000). The results of the present study and the previous work reported in the literature leave us with a conundrum–if spontaneous gastropod shedding of metastrongyloid L3 is insignificant as a route of exposure then why is it so widespread amongst the species of this group? Furthermore, if most (if not all) definitive host animals acquire natural infections by ingesting gastropod intermediate hosts or various paratenic hosts, then how have L3 adapted to acquire the ability to survive outside of the gastropod in the environment for prolonged periods of time? The L3 would not be expected to have developed the capability for such survival if it never occurs naturally outside of a host. It is possible that some set of natural conditions of which we are currently unaware affect the gastropod shedding levels such that our experimental models underestimate the magnitude. It is also possible that previous attempts to determine longevity of L3 outside of the host have under-estimated the survival time due to mistaking quiescent larvae for dead. The significance of environmental contamination as a potential alternative route of exposure for these parasites is to greatly increase the risk of infection for definitive and paratenic hosts through a wider availability of the infective dose. Metastrongyloid L3 may have a much more durable and long-lasting presence in an ecosystem than we previously have appreciated.

Ethical Standards

All applicable international and institutional guidelines for the care and use of animals were followed. All procedures performed in the study involving animals were in accordance with the ethical standards of the University of Prince Edward Island. The research protocol was approved by the Biosafety Committee of the University of Prince Edward Island. Since the experimental infections involved only invertebrate animals, no animal care approvals were required.

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Conflict of Interest

One of the co-authors (R. Schaper) is an employee of Bayer Animal Health. He provided input into the research design of the study and in the writing of the manuscript. One of the co-authors (G. Conboy) has received research funding support from the pharmaceutical industry in the past.

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