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Research paper

Larval development of *Aelurostrongylus abstrusus* in experimentally infected *Rumina decollata* snails

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ABSTRACT

Aelurostrongylus abstrusus is a lungworm distributed worldwide that affects wild and domestic cats, causing bronchopneumonia of varying intensity. Snails serve as intermediate hosts. The aim of the present study was to assess the larval development of *A. abstrusus* in *R. decollata* snails and to investigate its potential as an intermediate host. For this purpose, first-stage larvae (L1) of *A. abstrusus* were obtained from the faeces of naturally infected cats. Doses of 500 L1/snail were given to 24 *R. decollata* snails, placed on the soil of the breeder chamber, and maintained under laboratory conditions. Three snails were killed at 8, 10, 12, 16, 22, 26, 45 and 55 days post-infection (dpi), and the muscular foot and visceral body were separately digested by an artificial digestion technique. The morphometric parameters of different larval stages were recorded. The mean number of larvae reaching the infective stage at the end of the study (L3) was 262 larvae/snail. The greatest development to L3 was recorded from days16 to 55 pi, during which the isolation was maximum. *A. Abstrusus* L3 were isolated from the viscera, but isolation from the snail foot was significantly higher. Our results showed for the first time the ability of *A. Abstrusus* larvae to develop in *R. decollata*, thus serving as a potential intermediate host.

1. Introduction

Aelurostrongylus abstrusus is a lungworm of cats distributed worldwide that causes bronchiolitis and interstitial pneumonia (Traversa et al., 2008). Cats can acquire the parasite by eating slugs and snails with infective third-stage larvae (L3) of *A. abstrusus.* Mice, birds and reptiles can act as paratenic hosts by the ingestion of infected snails (Giannelli et al., 2017; Hamilton, 1969; Hansen et al., 2017; Hobmaier and Hobmaier, 1935; Traversa and Di Cesare, 2016). In cats, L3 migrate to the lungs where they reach the adult stage and reproduce. The first-stage larvae (L1) are coughed up, swallowed and eliminated into host faeces where they can survive between 45 and 60 days (Dernegi and Turkish, 2010). When larvae reach the molluscs, they actively penetrate the foot integument and moult twice to L3 (Hobmaier and Hobmaier, 1935). Different species of gastropods have been reported as intermediate hosts for this nematode, including *Agriolimax agrestis* and *A. columbianus, Helminthoglypta californiensis* and *H. nickliniana, Helicella spp*. (Hobmaier and Hobmaier, 1935), *H. aspersa* (Di Cesare et al., 2013; Giannelli et al., 2013), *Mesodonthyroidus, Triodopsis albolabris, Biomphalaria glabrata* (Zottler and Schnyder, 2016), *Cernuella virgata* (López et al., 2005), *Achatina fulica* (Ohlweiler et al., 2010; Thiengo et al., 2008; Valente et al., 2017), and recently, *R. decollata* (Cardillo et al., 2014). L3have been demonstrated to survive for up to 2 years in *H. aspersa* snails (Hamilton, 1969), and transmission between two intermediate hosts (intermediesis) may occur (Colella et al., 2015) by shedding lungworm larvae within gastropod mucus in the environment (Giannelli et al., 2015).

R. decollata is a pulmonata land snail that belongs to the Subulinidae family (Rascop, 1960). This snail is native to and widely distributed in the countries around the Mediterranean Sea, southern Europe, northern Africa and western Asia (Batts, 1957; Neck, 1986), and it has been spread to other parts of the world (Matsukuma and Takeda, 2009; Prévot et al., 2015). In the 1970s, it was intentionally introduced into North America as a biological control agent of the garden snail *H. aspersa*, and then it was accidentally spread into the United States, Mexico, Bermuda, Cuba and Uruguay (Cowie, 2001; Selander and Kaufman, 1973). In Argentina, it has only been recorded in urban areas; it was first reported in 1988 in Buenos Aires province (Miquel, 1988)

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and then in La Pampa and Mendoza provinces (Francesco and Lagiglia, 2006). *R. decollata* is an omnivore and a detritivore, feeding on organic matter such as animal faeces (Batts, 1957, and cited by Tupen and Roth, (2001). It also preys upon other land snails, worms and insects (Batts, 1957, and cited by Mc Donnell et al. (2016)). *R. decollata* is a highly invasive snail adapted to arid conditions, extreme temperatures and low relative humidity (Rascop, 1960; Batts, 1957). Despite this, it does not reach long distances. However, human activities and the lack of natural predators could lead to their rapid dispersal (Francesco and Lagiglia, 2006). The increase in the stray cat population in public places of the Autonomous City of Buenos Aires (Sommerfelt et al., 2006) may contribute epidemiologically to the spread of *A. abstrusus*. Together with the increase in the population of *R. decollata* snail (Cardillo et al., 2014; Miquel, 1988), this raises the question about the snail as a possible transmitter of *A. abstrusus* for cats. Cardillo et al. (2014) previously reported a high infection rate of *A. abstrusus* (average of 93.89 L3/pool) in 80% (20/25) of the pools of three *R. decollata* snails collected from the environment of a place in Buenos Aires city inhabited by a stray cat population. The study of this gastropod species' susceptibility as an intermediate host of metastrongyloids highlights its possible implication in the transmission and dispersion of parasites of medical and veterinary importance (Colella et al., 2015).

For this purpose, experimental infection of *A. abstrusus* in *R. decollata* was performed to study the infection rate and larval development, and therefore, elucidate the snaiĺs capability as an intermediate host in the parasités life cycle.

2. Materials and methods

2.1. Maintenance of snails

R. decollata snails were bred at the Institute of Parasitology (Facultad de Ciencias Veterinarias. Universidad de Buenos Aires) and thus had no previous contact with other parasites. Twenty-four adult snails were placed individually in plastic chambers with wet natural pre-sterilized soil. The upper part of the plastic box was covered with a net, which was wetted daily with a water sprayer to maintain proper ventilation and humidity in the box. They were kept in the laboratory in a temperature-controlled room (22 ± 2 °C) and fed adlibitum with lettuce and commercial cat food.

First-stage larvae of *A. abstrusus* were obtained by Baermann's technique (Lacorcia et al., 2009) from the faeces of a naturally infected cat and concentrated by centrifugation at 2000 rpm for 5 min. Larvae were identified morphologically and morphometrically according to previous descriptions (Ash, 1970; Di Cesare et al., 2013; Gerichter, 1947; Hobmaier and Hobmaier, 1935; Traversa and Di Cesare, 2016):a rounded head with a terminal oral opening and a kinked (s-shaped) tail with a small, finger-like projection at the tip of a cuticular spine as reviewed by Traversa and Di Cesare, 2013 (Fig. 2). Larvae were suspended in a sterile saline solution (0.9% NaCl). The sediment was homogenized, 3 aliquots of 20 µl were taken and larvae were counted. The mean number of larvae was calculated when the differences between the aliquots were under 20%; otherwise, the procedure was repeated. The final concentration of larvae per doses was adjusted to a final volume of 0.5 ml containing ~500 L1 *A. abstrusus*. Each infective dose (500 L1 of *A. abstrusus*) was placed on the wet soil of the chambers, and the larvae remained in contact with the snails until the day they were selected for larval isolation. The chambers were closed with a wet gauze cloth. The snails were sprayed daily, and laboratory maintenance according to the above descriptions was applied.

2.2. Larval recovery and morphological identification

Three snails were randomly selected at 8, 10, 12, 16, 22, 26, 45 and 55 days post-infection (dpi). They were cleaned by brushing the foot and shell, and then they were killed by immersion in tepid water for 24 h (López et al., 2005). The shells were removed, and the bodies were weighed. The muscular foot was separated from the rest of the viscera, and the two parts of the snail body were placed separately in small beakers (20 ml) and finely minced with scissors. Added to the digestion solution (Per 1 gr. of snail tissue) were 15 ml of tap water at 37 °C, 0.15 ml of HCl (1%) and 0.15 gr. of pepsin 1:1000 (Sigma-Aldrich, St. Louis, Missouri, United States). The digestion solution was stirred for approximately 1 h in a magnetic stirrer at 37 °C. Afterwards, the digestion solution was strained through a 170 µm sieve, collected in plastic tubes and centrifuged for 3 min at 1500 rpm. The supernatant was discharged, and the whole sediment (0,5 ml)was examined under a light microscope; larvae were morphologically identified according to developmental stages (L1, L2 and L3) and counted (Cardillo et al., 2014; Di Cesare et al., 2013; Giannelli et al., 2013; López et al., 2005). Ten snails were used as controls and processed to evaluate the presence of nematode larvae before starting the trial.

2.3. Data analysis

A statistical analysis was performed using InfoStat program version 2015p; Universidad Nacional de Córdoba (FCA-UNC). The following parameters were considered in studying the larval development of *A. abstrusus* in *R. decollata* snails: a. Infection rate: mean number of total larvae (L1 + L2 + L3)/infective dose), b. Differences between L1 and L2 stage isolation *versus* the L3 stage across time points, and c. Differences in total L3 stage recuperation and L3 recovered from the foot and viscera between time points. The Kruskal-Wallis chi-squared approximation was used to compare the number of different larval stages isolated across time points. The averages were compared using the Dunn test using 5% probability. Differences with $p < 0.05$ were considered statistically significant. A correlation analysis to verify the association between mean larval isolations and time points and a simple regression analysis were performed to verify the correlation between the variables.

3. Results

No larval nematodes were isolated from the *R. decollata* control group. *A. abstrusus* larvae were isolated from all snails experimentally infected at each sampling point. The number and developmental stages of larvae isolated from the foot and viscera of each *R. decollata* snail at different days post infection (DPI) are shown in Table 1.

The average infection rate was 172.17 larvae/snail (95% IC: 134.56–209.77), 34.43% of the infective dose (500), and the mean total larvae reaching L3 at the end of the study (55 dpi.) was 262 larvae/snail (95% IC: 188.43–335.57), with a maximum of 293 larvae/snail, between 52.4–58.6% of the inoculation dose.

The mean length of L1 was 354 ± 17.6 µm, presenting the posterior end with a typical notched, S-shaped tail and a notched dorsal spine (Fig. 1).

The L2 larvae measured 475 ± 22 µm in length, characterized by the presence of dark granules surrounding the gut and a short tail ending straight and sharply pointed (Fig. 2).

The L3 larvae were 581.78 ± 18.3 µm in length, presenting a stiletto in the anterior end, a rounded knob tip tail in the posterior end, a lateral line throughout the body and the cuticle of moulting larvae (Fig. 3).

Significant differences were found in the isolation of the different larval stages among dpi ($X^2 = 20.37$; $p < 0.01$), and this could be explained by a significant linear tendency. L1 and L2 isolations were high at the beginning of the infection and then decreased gradually over time ($R2 = 0.19$; $p = 0.0029$), while L3 isolation increased progressively ($R2 = 0.89$; $p < 0.01$) (Fig. 4). This behaviour could be explained by a significant negative correlation ($p < 0.001/rs = -0.71$) between both L1 and L2 isolations and the different dpi. In contrast, L3 isolation showed a significant positive correlation ($rs = 0.94$; $p < 0.001$) between dpi.

Table 1
Number of different developmental larval stages (L1, L2 and L3) of *A. abstrusus* recovered from muscular foot and visceral body of *R. decollata* snails, at different days post infection.

DPI	Foot			Viscera			Total			
	L1	L2	L3	L1	L2	L3	L1T	L2T	L3T	Total L/snail
8	48	156	1	27	70	0	75	226	1	302
	36	184	0	20	54	0	56	238	0	294
	14	84	0	21	33	0	35	117	0	152
10	7	88	2	34	10	3	41	98	5	144
	1	105	0	12	13	2	13	118	2	133
	4	90	0	4	6	0	8	96	0	104
12	29	127	0	7	12	0	36	139	0	175
	7	128	0	4	6	0	11	134	0	145
	39	125	0	13	31	0	52	156	0	208
16	10	122	1	7	97	2	17	219	3	239
	27	131	1	10	30	0	37	161	1	199
	7	107	2	15	11	0	22	118	2	142
22	2	24	16	0	0	0	2	24	16	42
	1	30	2	4	17	0	5	47	2	54
	2	41	11	0	0	0	2	41	11	54
26	4	11	13	0	0	0	4	11	13	28
	4	23	6	0	16	23	4	39	29	72
	8	40	31	3	24	18	11	64	49	124
45	0	23	115	0	12	23	0	35	138	173
	0	32	127	0	13	22	0	45	149	194
	0	47	207	0	11	23	0	58	230	288
55	0	32	234	0	2	59	0	34	293	327
	0	24	222	0	0	37	0	24	259	283
	0	17	217	0	5	17	0	22	234	256

DPI (Days post-infection).

Fig 1. First-stage larva of *A. abstrusus:* arrow (a) indicates the anterior rounded extremity end; arrow (b) shows the kinked S-shape tail with the small projection at the dorsal spine (45x). A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM04695.

From the first week p.i. (8 dpi), the three larval stages were isolated, but the number of immature larvae (L1 and L2) was the highest and most significantly different ($p < 0.05$) from the infective stage larvae (L3) obtained until 16 dpi, after which L1 also decreased. By day 22 pi., the isolation of L1 was significantly lower than L2 ($p < 0.05$);afterwards, a significant reduction of larvae per snail was observed, and the number of L3 began to increase progressively. By day 26, no significant differences in the number of isolated larvae were noted between three larval stages ($P > 0.05$). From 45 dpi, no L1 were observed, and by 55 dpi., significant differences were found between L2 and L3 isolation ($p < 0.05$) in which L2 decreased and the mean value of L3 was the highest, reaching 262 L3/snail (95% IC: 188.43–335.57).

The isolation rate of the different larval stages of *A. abstrusus* from *R. decollata* snail foot was significantly higher than that from the viscera

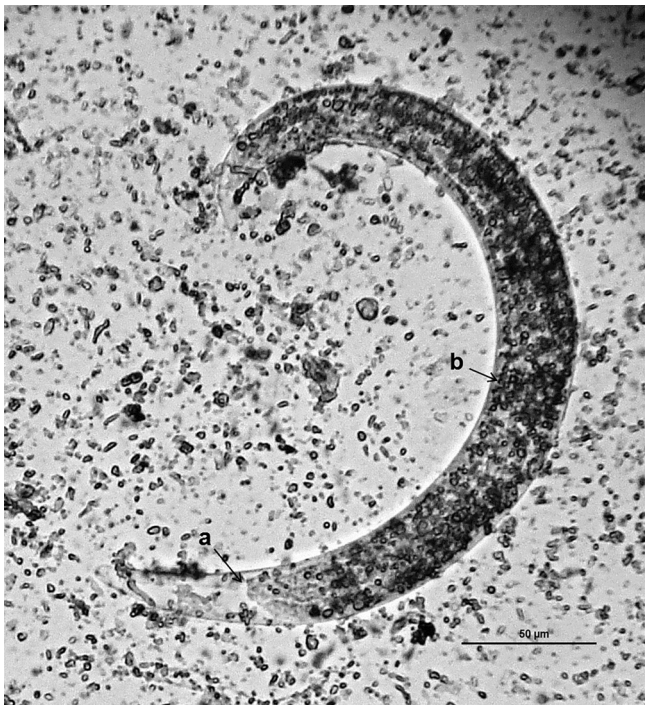

Fig. 2. Second-stage larva (L2), a. Anterior region, b. details of dark granules around the gut(45x). A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM04696.

($X2 = 5.56$; $p = 0.018$) (Fig. 5).

4. Discussion

The susceptibility of molluscs to a protostrongylid parasite is defined by the ability of L1 to penetrate the foot, by the possibility of developing into infective-stage larvae (L3) and by the time required to complete this process (Urban, 1980). All of these aspects were demonstrated in the present study in *R. decollata* snail experimentally infected with *A. abstrusus*.

The infection rate observed in this work was 34.43% of the infective dose with a maximum of 65.4% (293 larvae/snail), and the total larvae reaching L3 at the end of the study (day 55 pi.) was between 52.4–58.6% of the inoculation dose. Our results showed a high parasite development in the *R. decollata* snail, similar to that reported by Di Cesare et al. (2013) in *H. aspersa* (i.e., 47.9%) but higher than that reported by Hamilton and Mccaw, 1967, in *H. aspersa* snails (3.16% L3/snail), by López et al. (2005), in *C. virgata* (4.78% L3/snail), and by Zottler and Schnyder (2016), in *B. glabrata* (0,4%). However, in the present study, L1 may have been infectious in the soil of the infection chamber during the whole study period, remaining in permanent contact with the snails. Thereby, the snails at the end of the study may have been exposed to larvae for a longer time than the ones investigated at the beginning. The increase in the recovery of larvae from the snails over time may explain the moulting rate being higher than the average infection rate found. It could be due to the long survival capability of *A. abstrusus* L1, which can be as long as 7 weeks in tap water (Gerichter, 1947) and more than a month in faeces (Dernegi and Turkish, 2010).

As demonstrated by Giannelli et al. (2015), in *H. aspersa* snails experimentally infected with *A. abstrusus*, the immersion of infected *R. decollata* in tap water to be euthanized may have caused the emergence of L3 from the gastropod. The authors reported 1.2% L3 in mucus discharge and 5.7% in water of the total L3 recovered from the snails (including from digestion). Unfortunately, we did not evaluate the sediment after the euthanasia of *R. decollata,* and some L3 could have been released to the water. Anyway, the amount of L3 recovered from

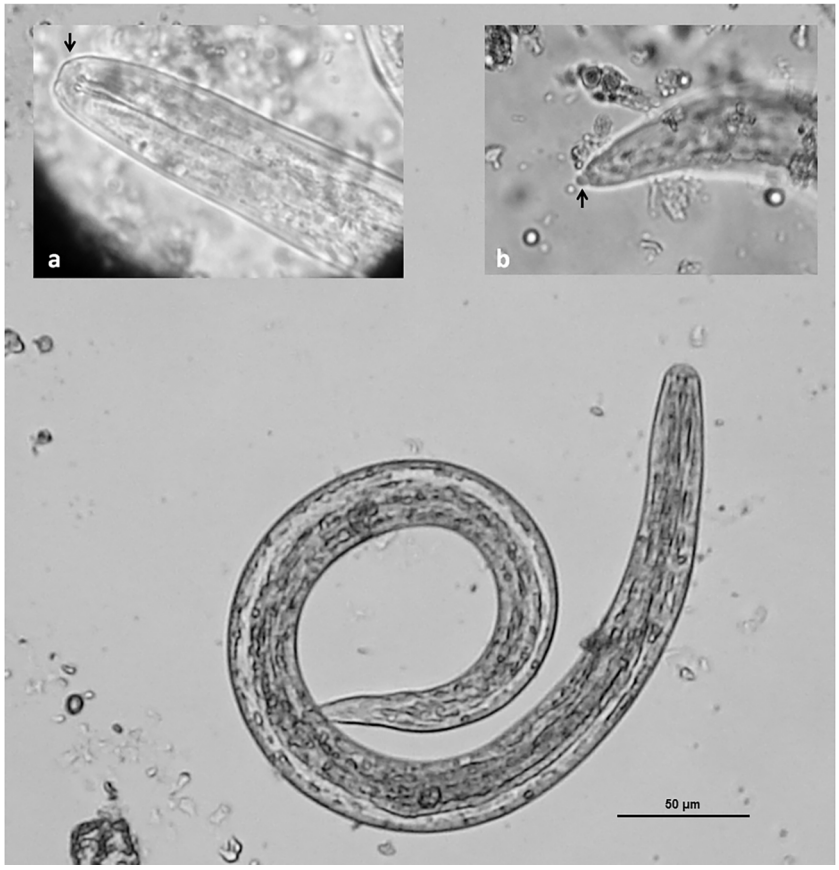

Fig. 3. Infective third-stage larva (L3) of *A. abstrusus* larva 3 (45x), a. Anterior region. b. Rounded knob tip tail. A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM04697.

R. decollata through digestion was high (262 L3/snail), and it shows that this snail may play an important role as an intermediate host of *A. abstrusus*.

Several factors may influence the development time and the larval burdens in intermediate hosts (Di Cesare et al., 2013), such as the snail species, size and age, their defence mechanisms which can destroy larvae after penetration (López et al., 2005; Zottler and Schnyder, 2016), the infective dose, and environmental conditions such as temperature (Di Cesare et al., 2013; Gerichter, 1947; Giannelli et al., 2013). Gerichter (1949) found in different mollusc species experimentally infected with *A. abstrusus* that the optimum temperature for the development of larvae is approximately 30 °C; at lower temperatures development proceeds more slowly (Di Cesare et al., 2013). In the present study, the environmental conditions remained constant and similar to that reported in previous experimental studies performed on different species of molluscs (20 ± 2 °C). Nevertheless, different stages

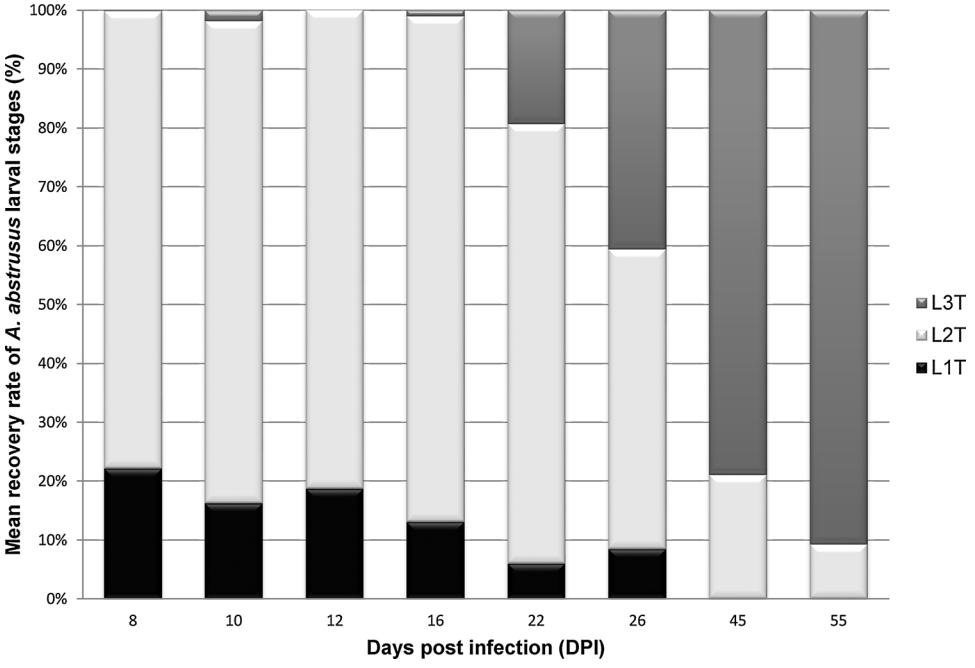

Fig. 4. *A. abstrusus* developmental larval stages (L1, L2 and L3) recovered from *R. decollata* snails at different days post infection (for each time points, 3 snails were analysed).

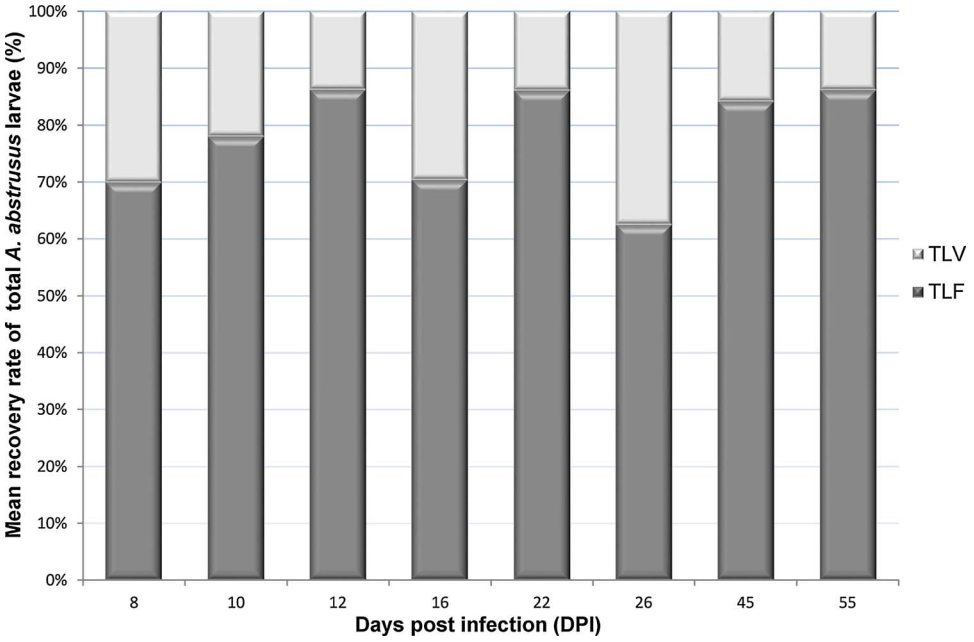

Fig. 5. Mean recovery rate of *A. Abstrusus* larvae from viscera and foot of *R. decollata* snails at different days post infection.

Table 2
Comparison of development times (DPI) of different *A. abstrusus* larval stages detected in mollusk species, from the present work and previous studies.

	Gerichter (1949) (dpi)	Lopez et al. (2005) (dpi)	Di Cesare et al. (2013) (dpi)	Giannelli et al. (2014) (dpi)	Zottler and Schnyder (2016) (dpi)	Present study (dpi)
L2	11 – n.e.	12 – 52	15 – 58	6 – 11	14 – 56	8–55
L3	18 – n.e.	18–52	21–58	11–120	28–182	22–55
Snail species	Different terrestrial snails	*C. virgata*	*H. aspersa*	*H. aspersa*	*B. glabrata*	*R. decollata*

dpi, days post infection; n.e., not evaluated.

of development were found at the same time; the first stage was observed until day 26 pi., a shorter time than that cited (until days 35–51) by Di Cesare et al. (2013), López et al. (2005), Zottler and Schnyder (2016) but longer than that reported by Giannelli et al. (2013) (Table 2).

In the same way, high isolation of L2 was obtained from day 8 pi., a shorter developmental time than that found by Di Cesare et al. (2013), López et al. (2005), Zottler and Schnyder (2016), indicating that larvae development in *R. decollata* may have started before that time and may occur faster than previously reported in other molluscs. In this study, the greatest development to L3 started between 16–22 days pi and was similar to that recorded by Di Cesare et al. (2013), Gerichter (1947), Giannelli et al. (2013) and López et al. (2005), (i.e., between 11–18 dpi); it was shorter than that found by Hamilton (1969), (i.e., 27 dpi) and Zottler and Schnyder (2016) (i.e., 28 dpi) (Table 2). At 55 dpi., most of the larvae were L3, and isolation probably may have been longer if the study had continued, as was reported by Giannelli et al. (2013), Zottler and Schnyder (2016), and by Hamilton (1969) (Table 2).

It has been suggested that the L1 of *A. Abstrusus* are able to penetrate the foot of *H. aspersa* and migrate into the viscera or develop in the muscular tissues (Hamilton, 1969). High development of larvae in snail visceral tissues was previously reported in *H. aspersa* by Di Cesare et al. (2013) (31. 8%) and by Hamilton (1969) (36%). However, in the present work, the mean isolation rate of the different larval stages of *A. abstrusus* from the foot of *R. decollata* was significantly higher (45. 13% ± 15. 2) than that from the viscera (12. 6% ± 7) (Fig. 5). In this sense, Hobmaier and Hobmaier (1935) proposed that *A. abstrusus* L1 may die after being ingested by different molluscs. In contrast, other metastrongyloids, e.g., *Angiostrongylus cantonensis*, may infect snails by ingestion (Richards and Merritt, 1967). Even so, in an experimental infection with the metastrongyloid of canids, *Crenosoma vulpis* in the common garden snail *Cornu aspersum*, a higher larval recovery was obtained from the visceral tissues (68.7%) than from the foot (31.3%) (Colella et al., 2016). Our study shows that *A. abstrusus* larvae can develop in visceral tissues but much less than in the muscular foot even considering the coprophagus habits of *R. decollata* (Cardillo et al., 2016; Tupen and Roth, 2001). Further studies are required to understand the implications of this finding.

5. Conclusions

The environmental dissemination of *R. decollata* might impact the distribution of the feline lungworm *A. abstrusus*. The results from this study show that *R. decollata* could be considered a suitable intermediate host for *A. abstrusus* since high infection rates were found and fast larval development to L3 was observed. The coprophagous habits of *R. decollata* and their slow movements could be relevant when snails feed on cat faeces, allowing *A. abstrusus* L1 to actively penetrate the snail foot and even be ingested. All of this, along with the predatory habits over other snails, shows the potential of *R. decollata* as a suitable intermediate host above others. For this reason, it is important to encourage the control of the *R. decollata* snail population to avoid the spread and maintenance of different pathogens in the environment. Furthermore, it is necessary to study the implications of this snail for paratenic hosts such as birds and rodent in addition to cats as definitive hosts, since it has been demonstrated that well-fed domestic cats continue hunting natural prey just as wild cats do (Ogan and Jurek, 1997).

Ethical approval

Not applicable.

Conflict of interest

All authors contributed to the interpretation of findings, approved the final manuscript and declare that they have no conflicts of interest.

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