Development of the feline lungworms *Aelurostrongylus abstrusus* and *Troglostrongylus brevior* in *Helix aspersa* snails

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SUMMARY

Aelurostrongylus abstrusus (Strongylida, Angiostrongylidae) and *Troglostrongylus brevior* (Strongylida, Crenosomatidae) are regarded as important lungworm species of domestic felids, with the latter considered an emerging threat in the Mediterranean region. The present study aimed to assess their concurrent development in the mollusc *Helix aspersa* (Pulmonata, Helicidae). Thirty snails were infested with 100 first-stage larvae (L1) of *A. abstrusus* and *T. brevior*, isolated from a naturally infested kitten. Larval development was checked by digesting five specimens at 2, 6 and 11 days post infestation. Larvae retrieved were morphologically described and their identification was confirmed by specific PCR and sequencing. All *H. aspersa* snails were positive for *A. abstrusus* and *T. brevior*, whose larval stages were simultaneously detected at each time point. In addition, snails were exposed to outdoor conditions and examined after overwintering, testing positive up to 120 days post infestation. Data herein presented suggest that *A. abstrusus* and *T. brevior* develop in *H. aspersa* snails and may eventually co-infest cats. Data on the morphology of both parasitic species in *H. aspersa* provide additional information on their development and identification, to better understand the population dynamics of these lungworms in receptive snails and paratenic hosts.

Key words: *Aelurostrongylus abstrusus*, *Troglostrongylus brevior*, feline lungworms, *Helix aspersa*, snails, larval development, morphological identification.

INTRODUCTION

The superfamily Metastrongyloidea comprises a number of roundworms, which infest the cardio-pulmonary system of vertebrate animals, and are primarily transmitted by pulmonate molluscs (Anderson, 2000). Some of these parasites may also have a zoonotic potential, as in the case of *Angiostrongylus cantonensis* and *Angiostrongylus costaricensis* (Strongylida, Angiostrongylidae), the causative agents of eosinophilic meningitis and ileitis, respectively (Wu *et al*. 1997; Wang *et al*. 2008). Among feline lungworms, *Aelurostrongylus abstrusus* (Strongylida, Angiostrongylidae) is regarded as the most prevalent species, whereas *Troglostrongylus brevior* (Strongylida, Crenosomatidae) has been reported in felids from Palestine (Gerichter, 1949), Italy (Brianti *et al*. 2012, 2013), and Spain (Jefferies *et al*. 2010). Clinically, feline bronchopulmonary strongyloses may range from subclinical to life-threatening conditions, featured by dyspnoea, mucoid-purulent nasal discharge, sneezing, depression and anorexia, which can lead to *exitus*, especially during concomitant infections or immunosuppressive conditions (Brianti *et al*. 2012). These lungworms share similar life cycles (Gerichter, 1949) in that, after mating, females lay eggs, from which first-stage larvae (L1) hatch, ascend the respiratory tract up to the pharynx, where they are swallowed, eventually leaving the definitive host throughout feces (Gerichter, 1949; Anderson, 2000). L1 of *A. abstrusus* and *T. brevior* may survive in the environment for up to 60 and 142 days, respectively, until they infest land or freshwater snails, and slugs (Gerichter, 1949; Ash, 1970; Gökpinar and Yildiz, 2010; Ramos *et al*. 2013). In the intermediate host, development to the second-(L2) and third-(L3) larval stage occurs in about 2 weeks, depending on the gastropod species involved, as well as environmental factors (Gerichter, 1949). The life cycle completes when a feline host ingests infested gastropod molluscs or paratenic hosts, harbouring infective L3 (Anderson, 2000). In addition, a direct route of transmission, from the queen to her kittens, has been speculated for *T. brevior*, via lactation or throughout the placenta (Brianti *et al*. 2013).

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The global impact of feline bronchopulmonary strongyloses, coupled with the worldwide spread of several mollusc-borne diseases (e.g. human angiostrongyliasis, schistosomiasis, bilharziasis) (Wang *et al*. 2008; Majoros *et al*. 2010; Soldánová *et al*. 2013), spurred the interest of the scientific community on the biology of *A. abstrusus* and *T. brevior* (Di Cesare *et al*. 2011; Barutzki and Schaper, 2013; Brianti *et al*. 2012). From this perspective, the knowledge on the mollusc species involved in the epidemiology of feline lungworms is crucial to their prevention and control. The morphology of *A. abstrusus* and *T. brevior* larvae in molluscs has been poorly investigated (Gerichter, 1949) and mostly restricted to the former lungworm species (López *et al*. 2005; Di Cesare *et al*. 2013). Accordingly, the limited data available on *T. brevior* is due to the fact that L1s cannot be easily differentiated from those of *A. abstrusus* (Brianti *et al*. 2012, 2013; Otranto *et al*. 2013). Interestingly, the occurrence of co-infestation (Jefferies *et al*. 2010) indicates that both species occupy the same ecological niches, thus potentially challenging the proper diagnosis and therapeutic schedule of feline infestations (Brianti *et al*. 2012, 2013).

Hence, the present research aimed to provide insights into the development of *A. abstrusus* and *T. brevior* in *Helix aspersa* snails, and morphological information of larval stages.

MATERIALS AND METHODS

Maintenance of snails

In December 2012, 50 farmed specimens of *H. aspersa* were purchased from a snail farming centre from Teramo (Abruzzo region, central Italy), where this species is bred for human consumption. The absence of natural infections by any metastrongyloid larvae was assessed by microscopic examination of 10 snails at 5 days before the infestation, and of all snail specimens that died naturally during the observation period. The molluscs were put in plastic boxes, filled with fresh soil, which was changed and humidified every 3 days. Feeding of snails occurred every 2 days with potatoes, lettuce and water *ad libitum*. In addition, the upper part of the vivarium was covered with a net, which was wetted every 8 h with a water sprayer, in order to maintain proper ventilation and humidity in the box. The vivarium was kept in a temperature-controlled room (20 ± 1 °C), where all procedures below were carried out.

Larval collection and infestation procedures

L1 of *A. abstrusus* and *T. brevior* were isolated from the feces of a 50-day-old naturally infested female kitten. The cat was referred to the Department of Veterinary Medicine of the University of Bari (southern Italy), due to a persistent cough and dyspnoea. Larvae were retrieved by Baermann technique, as previously described (MAFF, 1986). The solution containing fecal sedimentation and larvae was firstly centrifuged at 600 ***g*** for 5 min, the supernatant removed, and the sediment observed under light microscopy (Leica®, DM LB2). Nematode species were differentiated on the basis of the size and tail morphology and then molecularly identified (see below). Single infective doses of 100 L1s each were kept in small tubes, until used for snail infestation.

Helix aspersa snails ($n = 30$) were deprived of food 24 h before the infestation. Then, they were placed individually in a plastic infestation chamber, composed of six circular wells, containing a potato slice (1 cm wide) (Fig. 1A), which was contaminated with the infective dose (Fig. 1B). The infestation chamber was covered with a wet gauze cloth and secured with rubber bands, after ascertaining that snails had their foot on the tuber slice, to maximize contact with larvae (Fig. 1C). Specimens were left in the infestation chamber for 48 h and then removed into their vivarium.

Larval isolation and morphological identification

Larval development of *A. abstrusus* and *T. brevior* was assessed using 5 snail specimens for each of the following time points: 2 (T1), 6 (T2) and 11 (T3) days post infestation at 20 ± 1 °C. In addition, 15 of the infested *H. aspersa* snails were exposed to outdoor conditions from December to April 2013 (mean temperature: 10·6 °C; min–max: 6·6–15·0 °C), and finally examined 120 days post infestation (T4), when snails recovered from natural hibernation, as described in the following.

Snails were pooled and digested in a solution of 1% HCl (150 mL) and 1·2 g powdered pepsin (A/S N Foss Electric, Hillerod, Denmark), after their foot was separated from the body and cut into small pieces (1–2 mm). The suspension was stirred on a magnetic, heated plate at 37 °C for 35 min, strained through a 180 μm sieve to remove undigested material, collected in plastic tubes, centrifuged at 600 ***g*** for 5 min, before re-suspending the pellet in 5 mL tap water. Three replicates (100 μL each) of the suspension were microscopically examined and larvae were morphologically and morphometrically identified according to species and developmental stage (Gerichter, 1949; Euzeby, 1981), using an analyser program (Leica LAS® AF 4.1). Drawings were made with an optical microscope (Olympus® BX51) equipped with a camera lucida. Microscopic images and measures were taken using a digital image processing system (AxioVision rel. 4.8, Carl Zeiss®, Germany).

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Fig. 1. *Helix aspersa* infestation with feline lungworms larvae. Snails were placed individually in a plastic infestation chamber (A), containing a potato slice, which was contaminated with the infective dose (B). The infestation chamber was covered with a wet gauze cloth and closed with several rubber bands (C).

Table 1. *Aelurostrongylus abstrusus* and *Troglostrongylus brevior* developmental larval stages detected in *Helix aspersa* foot, following the pepsin-HCl digestion (300 μL), at different time points post infestation

	Aelurostrongylus abstrusus			*Troglostrongylus brevior*		
Time points	L1	L2	L3	L1	L2	L3
T1 (+2 days)	8	–	–	12	–	–
T2 (+6 days)	6	4	–	4	10	–
T3 (+11 days)	–	4	5	–	3	8
T4 (+120 days)	–	–	21	–	–	6

Molecular procedures

Single larval specimens of *A. abstrusus* and *T. brevior* ($n = 10$ per each species), at different developmental stages, were isolated from the medium using a 10 μL micropipette and stored in plastic vials containing phosphate buffer saline (PBS) at −20 °C, until analysed. Briefly, the DNA was extracted using a commercial kit (DNeasy Blood & Tissue Kit, Qiagen, GmbH, Hilden, Germany), in accordance with the manufacturer's instructions, and a partial 18S ribosomal RNA gene sequence (1708 bp) was amplified from individual larvae using primers NC18SF1 (5′-AAAGATTAAGCCATGCA-3′) and NC5BR (5′-GCAGGTTCACCTACAGAT-3′), as previously described (Patterson-Kane *et al*. 2009). Sequences were determined from both strands, using the same primers individually as for the PCR, and the electropherograms verified by eye. In order to ensure open reading frames, the nucleotide sequence was conceptually translated into amino acid sequence, using the invertebrate mitochondrial code MEGA5 software. Sequences were compared with those available in the GenBank™ database by Basic Local Alignment Search Tool (BLAST) (Altschul *et al*. 1997).

RESULTS

All specimens of *H. aspersa* ($n = 10$) digested 5 days before the infestation, as well as those that died naturally during the pre-infestation period ($n = 10$), were negative for nematode larvae. Conversely, all pooled snails were positive for *A. abstrusus* and *T. brevior*. In particular, of 91 larvae retrieved from the *H. aspersa* examined, 48 (52·7%) were identified as *A. abstrusus* and 43 (47·3%) as *T. brevior*. Up to 27 larvae were detected in a single sample with a mean concentration of 22·75 larvae/sample (Table 1). The morphological identification was confirmed as the 18S rRNA sequences from larvae showed a 100% overall nucleotide BLAST homology with those of *A. abstrusus* and *T. brevior* (accession numbers

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Table 2. Measurements (mean length and width ± S.D.; expressed in μm) of *Aelurostrongylus abstrusus* and *Troglostrongylus brevior* larvae

	First-stage larvae		Second-stage larvae		Third-stage larvae	
Species	Length	Width	Length	Width	Length	Width
Aelurostrongylus abstrusus	384·5 ± 33·4	17·7 ± 2·6	479·4 ± 53·6	27·6 ± 4·5	538·9 ± 51·8	26·7 ± 1·9
Troglostrongylus brevior	347·3 ± 12·4	16·1 ± 1·5	380·7 ± 18·6	24·9 ± 2·7	432·1 ± 15·3	20·9 ± 1·5

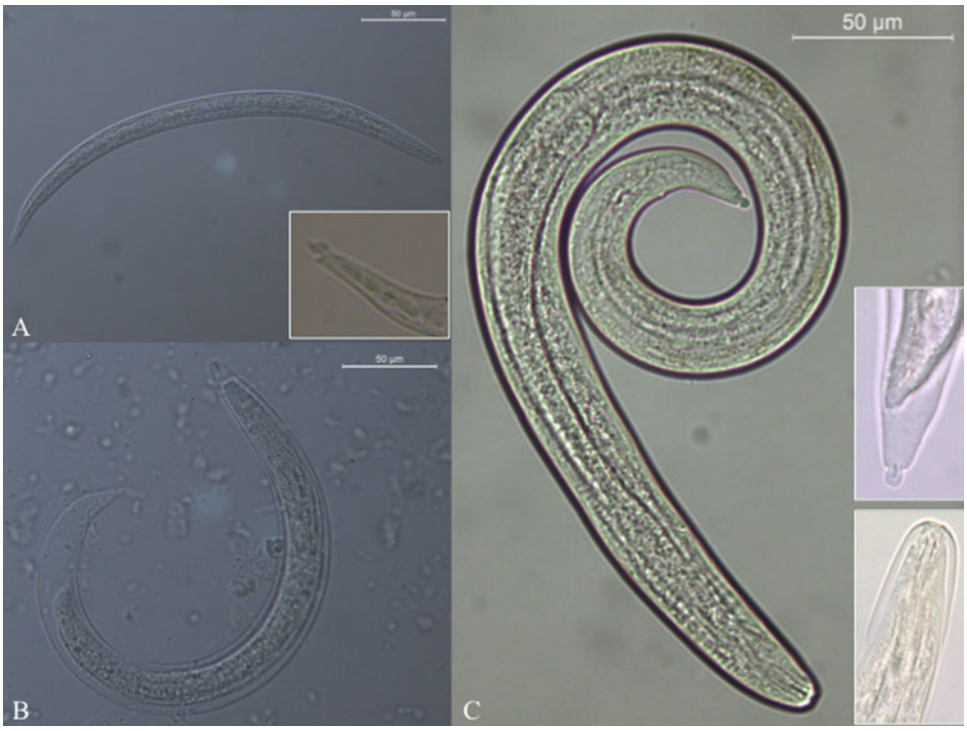

Fig. 2. *Aelurostrongylus abstrusus*. (A) First-stage larva (L1) and detail of the tail; (B) Second-stage larva (L2); (C) Third-stage larva and details of the anterior extremity and tail, ending into a rounded projection.

AJ920366 and JX290562, respectively), previously deposited in GenBank™ (Table 2). At each time point, larvae of both species were at the same developmental stage with L1 found at T1, L2 at T2 and L3 at T3. After the overwintering period (T4) only *A. abstrusus* and *T. brevior* L3 were detected (Table 1).

Aelurostrongylus abstrusus *larvae*

L1 (Fig. 2A) measured 384·5 ± 33·4 μm in length and 17·7 ± 2·6 μm in width, and were featured by a narrowed anterior extremity, with several granules on the middle part of the body. The tail, which appeared forked-like, was bent in the form of S, with a small short appendage split on its dorsal side. The shape of the tip of the tail was constant in all specimens examined.

L2 (Fig. 2B) were 479·4 ± 53·6 long and 27·6 ± 4·5 wide, being incorporated into an external cuticle, which appeared empty at both end portions. The anterior extremity was rounded and the metacorpus filled by numerous refractive food granules. The tail was conoid, resembling that of the previous stage.

L3 (Figs 2C and 4A) were recovered enclosed into the two cuticles or without the external sheaths, and showed a slender body 538·9 ± 51·8 μm long, with a maximum body width of 26·7 ± 1·9 μm, at about mid-body. The anterior end was blunt and beard lateral alae, extending from 40–65 μm (mean 53 μm) to mid-tail, interrupted by the excretory pore at 87–96 μm (mean 92 μm) from the anterior end. The stoma, 19 μm long, was characterized by slightly cuticularized cheilorhabdion (4–5 μm long) followed by stylet-like organ with prominent barb-like points, extending into insertion in upper oesophagus (Fig. 2C). The muscular oesophagus, 183–210 μm long (mean 202 μm) and 7–10 μm wide (mean 8 μm) was almost cylindrical to the level of excretory pore and, subsequently increased in diameter up to 13–16 μm (mean 14 μm). The relative length of oesophagus to body length was 0·37–0·44 (mean 0·41) and the nerve ring was indistinctly

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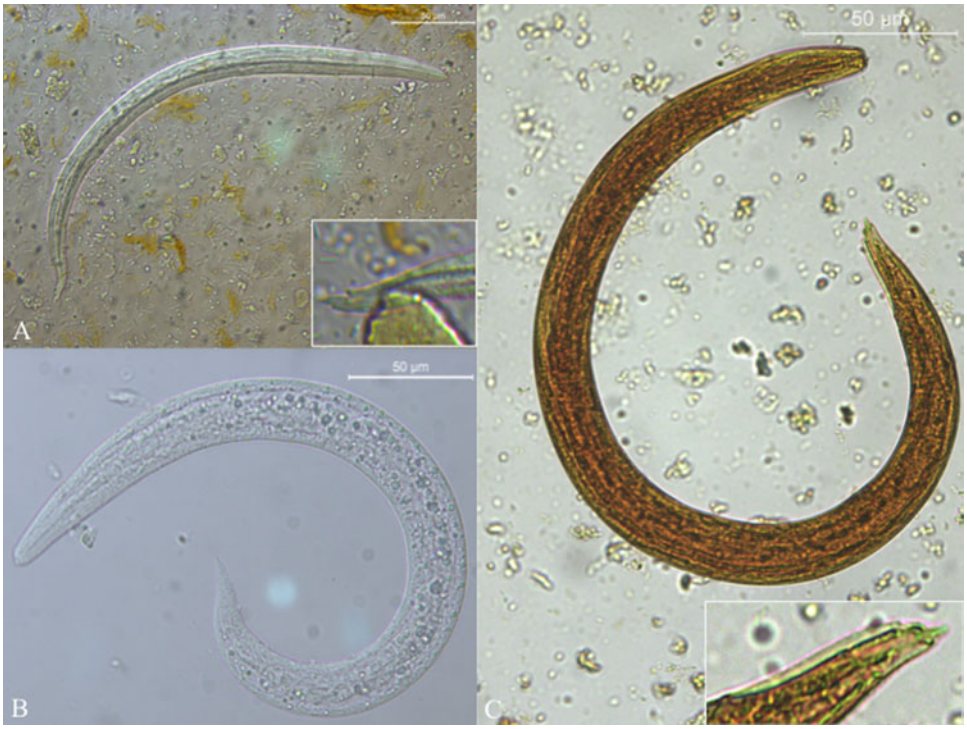

Fig. 3. *Troglostrongylus brevior*. (A) First-stage larva (L1) and detail of the tail; (B) Second-stage larva (L2); (C) Third-stage larva (L3) and details of the anterior extremity and tail, ending in four terminal appendages.

observed at 75–80 μm from the anterior end. The tail was conical with rounded projection, measuring 34–40 μm in length (mean 36 μm) and 15–18 μm in width at anus level, with relative tail length to body length being 0·067–0·080 (mean 0·073) (Figs 2C and 4A).

Troglostrongylus brevior *larvae*

L1 (Fig. 3A) were 347·3 ± 12·4 μm long and 16·1 ± 1·5 μm wide, with a pointed anterior extremity and with granules confined to the second half of the body. The tail gradually tapered to the extremity, being featured by a deep incision, which divided the appendage into a shallower ventral spine and a conspicuous dorsal appendix, further divided near its tip. Individual variations of the shape of the caudal extremity were observed, with the tail more or less squared (Fig. 3A).

L2 (Fig. 3B) measured 380·7 ± 18·6 μm in length and 24·9 ± 2·7 μm in width. Similarly to the L2 of *A. abstrusus*, they were enclosed into an external cuticle, although specimens without the sheath were also observed. The anterior extremity was narrowed, widening progressively to the metacorpus, which intestinal cells were packed with many food granules. The tail tapered into an appendage similar to that of the previous stage.

L3 (Figs 3C and 4B) measured 432·1 ± 15·3 μm in length, with the maximum body width of 20·9 ± 1·5 μm, observed at about mid-body. Lateral alae were absent and the rounded anterior end was featured by a slightly cuticularized stoma, 3 μm long, which opened in a 121–145 μm long (mean 135 μm) muscular oesophagus (Fig. 4B). This was characterized by a 5–6 μm wide procorpus, a slightly swollen metacorpus, a long isthmus and basal bulb, 10–12 μm wide (mean 11 μm). The relative length of oesophagus to body length was 0·32–0·42 (mean 0·37). The excretory pore and the nerve ring were detected at 50–79 μm (mean 70 μm) and 60–67 μm (mean 65 μm) from anterior end, respectively. The tail, 30–38 μm long (mean 33 μm) and 13–14 μm wide at the anus level, ended in four terminal appendages (Figs 3C and 4B), with a relative length to body length of 0·081–0·103 (mean 0·090).

DISCUSSION

Results of the present study indicate that *A. abstrusus* and *T. brevior* may develop simultaneously in *H. aspersa*, reaching the third-larval stage in about 11 days post infestation. In addition, larvae of both metastrongyloids may overwinter in this snail species, remaining viable for at least 120 days. Data on the biology of these nematodes in the intermediate hosts are of relevance, considering that the information available is limited to the development of *A. abstrusus* in land (e.g. *Helicella* spp., *Achatina fulica, Monacha syriaca* and *Theba pisana*) and freshwater snails (i.e. *Biomphalaria glabrata*) or slugs (e.g. *Agrolimax* spp. and *Limax flavus*) (Gerichter, 1949; Ash, 1970; Ohlweiler *et al*. 2010). *Helix aspersa* has been herein confirmed as

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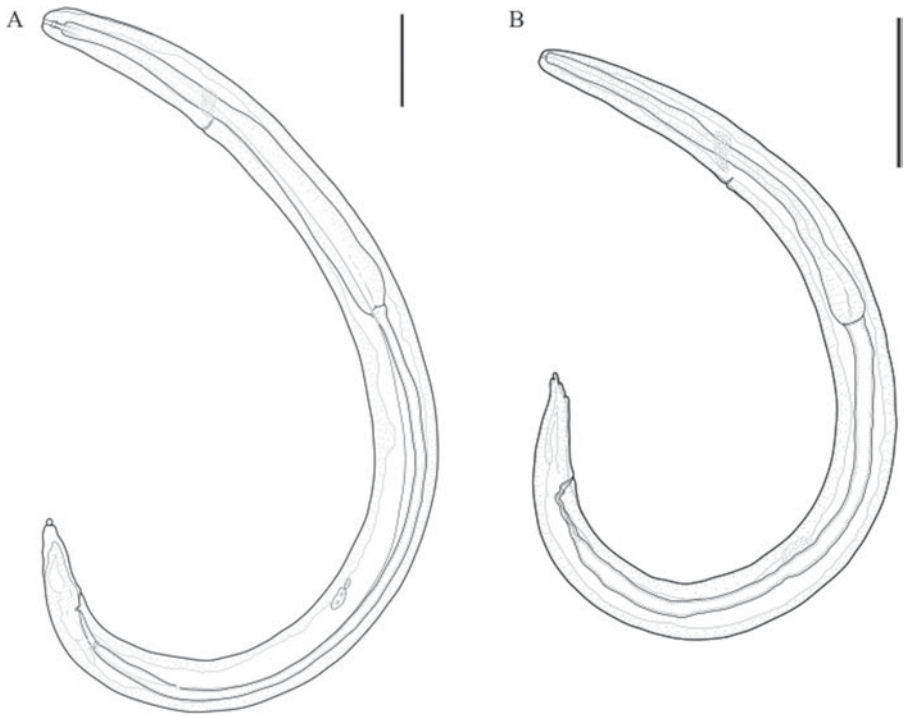

Fig. 4. Morphology of third stage larvae (L3) of *Aelurostrongylus abstrusus* (A) and *Troglostrongylus brevior* (B).

intermediate host for *A. abstrusus* (Hobmaier and Hobmaier, 1935; Hamilton, 1969; Di Cesare *et al.* 2013), and, for the first time, for *T. brevior*. Indeed, the known host range for the latter lungworm species was restricted to a small number of molluscs (i.e. *Helicella barbesiana, Helicella vestalis joppensis, Chondrula septemdentata, M. syriaca, Retinella nitellina, T. pisana* and *L. flavus*), but no information was available on their larval developmental times (Gerichter, 1949).

From an epidemiological viewpoint, the environmental dissemination of this snail might impact on the distribution of feline lungworm infestations. Indeed, *H. aspersa* has been already reported from several areas previously considered non-endemic for this snail (e.g. Oceania and South Africa), as well as in northern European regions (Guiller and Madec, 2010). In some of these areas (e.g. Germany), the infestation rate by feline lungworm was shown to range from 0·5 to 15·6% (Barutzki and Schaper, 2013). Considering that larvae of *A. abstrusus* do not develop in snails at 4–8 °C (Gerichter, 1949; Di Cesare *et al.* 2013) but, at the same low temperature, *T. brevior* can reach the infective stage (Gerichter, 1949), lungworm infestations in central and northern Europe could be due predominantly to *T. brevior*. In contrast, at mild temperatures, such as those of the Mediterranean area, both species may occur in sympatry. Based on these findings, *H. aspersa* may play an important role in the dissemination of both nematodes, maintaining infective larvae throughout the winter in hibernating molluscs.

The larval development time reported in the present study for *A. abstrusus* is shorter than that reported in *H. barbesiana* (11 and 18 days, at 30 °C) and *Cernuella virgata* (12 and 18 days, at 20 °C) (Gerichter, 1949; López *et al.* 2005), whereas the development of *T. brevior* in *H. aspersa* is similar to that recorded in *H. barbesiana* and *H. vestalis joppensis*, with L2 and L3 detected 5 and 8 days post infestation, respectively (Gerichter, 1949). Overall, data suggest that the occurrence of *T. brevior* and *A. abstrusus* in their intermediate hosts might display seasonal variations in relationship with the seasonal dynamic of their intermediate hosts. Indeed, snails are most prevalent in middle spring/early autumn when most kittens are born (Little, 2011). Importantly, the interactions between snails and paratenic hosts (e.g. birds, rodents and reptiles) should also be taken into account when considering the ecology of these parasites (Brianti *et al.* 2012, 2013).

Data on the morphology of *A. abstrusus* and *T. brevior* herein presented do not concur with those previously reported in a case of co-infestation in a kitten from Spain (Jefferies *et al.* 2010), as larvae of both species were clearly inverted in their identification. Molecular tools may assist and expedite the proper identification of these nematodes, both in vertebrate and mollusc hosts. Nonetheless, further studies are needed to simultaneously diagnose *A. abstrusus* and *T. brevior*, instrumentally to proper therapeutics (Annoscia *et al.* 2013). Indeed, while active compounds have been licensed for the prevention and treatment of *A. abstrusus*, it is not the case for *T. brevior*. Overall these data may improve the current understanding on the risk period for cats to acquire the infestation from snails, as well the

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population dynamics of these nematodes in receptive snails. Indeed, *A. abstrusus* and *T. brevior* co-evolve in *H. aspersa*, potentially co-infesting the definitive hosts. Practitioners should be aware of the occurrence of this crenosomatid and perform proper diagnostic techniques when a bronchopulmonary strongyloses in a feline patient is suspected.

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