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Prevalence of *Angiostrongylus vasorum, Aelurostrongylus abstrusus* and *Crenosoma vulpis* larvae in native slug populations in Germany

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Highlights:

- First survey elucidating *Angiostrongylus vasorum* infections in intermediate hosts in Germany.
- Highest risk of *A. vasorum* slug infection in autumn.
- Higher slug weight represents risk factor for *A. vasorum* infection.
- *A. vasorum* prevalence positively influenced by slug species *Arion lusitanicus.*
- *Deroceras reticulatum* associated with higher larval *A. vasorum* burden than *Arion lusitanicus*.

Abstract

Metastrongyloid parasites represent sparsely studied parasites of dogs and cats in Germany. Recent European surveys indicate that these parasites are spreading in Europe. Actual data on prevalence of *Angiostrongylus vasorum* in dogs and foxes reveal several endemic foci in Germany. However, actual data on the prevalence of *A. vasorum* and other metastrongyloid lungworm larvae in a wide range of slug and snail intermediate hosts, such as *Arion lusitanicus*, are missing for Germany. To fill this gap, we conducted an epidemiological survey on native German slugs in selected regions of Hesse and Rhineland-Palatinate. The focus was on slugs, because in study areas slugs appear to be more abundant than snails. Slugs were collected throughout different seasons of the year in areas that were previously proven to be hyperendemic for *A. vasorum* fox infections. Overall, a total of 2701 slugs were collected and examined for lungworm larvae via artificial digestion. The number of *A. vasorum* larvae per slug varied considerably (1-546 larvae per specimen). Some hotspot areas with high *A. vasorum* prevalence in slugs (up to 19.4%) were identified. The overall *A. vasorum* prevalence varied with season with largest number of slugs infected in summer (9.1%) and lowest number in winter (0.8%). The current study revealed a total *A. vasorum* prevalence of 4.7% in slugs based on microscopic analyses. Confirmation of lungworm species was made by specific duplex-real-time PCRs. Hence, these data demonstrate that final hosts are at a permanent risk for *A. vasorum* infections during all seasons when living in investigated areas. Besides *A. vasorum*, other lungworm larvae were also detected, such as *Crenosoma vulpis* (the fox lungworm, 2.3%) and *Aelurostrongylus abstrusus* (feline lungworm, 0.2%).

Note: Supplementary data associated with this article

Keywords*:* Gastropod-borne diseases, Metastrongyloidea, *Angiostrongylus vasorum, Crenosoma vulpis, Aelurostrongylus abstrusus,* lungworm, *Arion lusitanicus, Deroceras reticulatum.*

1. Introduction

Recently, metastrongyloid lungworms infecting canids and felids have become the focus of special attention in the scientific community due to their apparent emergence in domestic and wildlife populations. Besides their presence in endemic foci in many countries, *Angiostrongylus vasorum, Aelurostrongylus abstrusus* and *Crenosoma vulpis* have spread into previously non-endemic geographical areas in Europe, South America, Africa and North America (Bwangamoi 1972; Payo-Puente et al. 2008; Taubert et al. 2009; Traversa et al. 2010; Conboy 2011; Lucio-Forster and Bowman 2011; Helm et al. 2015; Latrofa et al. 2015; Spratt 2015; Tolnai et al. 2015; Di Cesare et al. 2016). The causes of the apparent emergence of these parasites are unknown. Factors such as global human travel, changes in intermediate host population dynamics, movements in populations of domestic animals and global warming are hypothesised to play a role in the rise of reports of these nematodes (Traversa et al. 2010; Giannelli et al. 2016; Maksimov et al. 2017). *A. vasorum* infections, the most pathogenic canid lungworm species, were reported from arid, tropical, subtropical as well as Nearctic, Palearctic and temperate climate zones (Bolt et al. 1994; Morgan et al. 2005; Jefferies et al. 2010) with a northward expansion (Härtwig et al. 2015; Taylor et al. 2015). Evidence of its spread beyond endemic areas was reported for the UK (Helm et al. 2009; Yamakawa et al. 2013), Denmark, Sweden (Ablad et al. 2003) and Germany (Maksimov et al. 2017), while the presence of this infection within fox populations was documented in the UK, Canada, Denmark, Ireland and Germany (Jeffery 2004; Saeed et al. 2006; Härtwig et al. 2015; Taylor 2015; McCarthy et al. 2016; Schug et al., 2018). Continuous reports on *A. vasorum* in dogs in Germany (Barutzki and Schaper 2009; Taubert et al. 2009; Maksimov et al. 2017), Netherlands (van Doorn et al. 2009), Switzerland (Staebler et al. 2005), Italy (Sasanelli et al. 2008) and Canada (Bourque et al. 2008) amongst others were published.

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A. vasorum is known for its spatial heterogeneity but the causes of this are unknown (Morgan et al. 2005; Ferdushy and Hasan 2010; Aziz et al. 2016). Spatial variation in intermediate host density and composition of gastropod communities are regarded as some potential factor (Aziz et al. 2016). *A. vasorum* has a relatively broad intermediate host range, including many slug and snail species (Simpson and Neal 1982; Barcante et al. 2003; Ferdushy et al. 2009; Helm et al. 2009; Jefferies et al. 2009; Koch and Willesen 2009; Patel et al. 2014). Furthermore, several species of wild canids are potential definitive hosts, including red foxes (*Vulpes vulpes*), golden jackals (*Canis aureus*), wolves (*Canis lupus*) and domestic dogs (Guilhon 1965; Bolt et al. 1994; Saeed et al. 2006; Gavrilović et al. 2017; Hermosilla et al. 2017, Schug et al. 2018). Due to rising fox populations and the increasing urbanisation of these animals (Gloor et al. 2001; Schweiger et al. 2007), there is an increased risk of infection for dogs (Morgan et al. 2005) since urbanising foxes might carry the parasite into the environment of domestic dogs (McCarthy et al. 2016; Schug et al. 2018).

Another reason for the strong concern about *A. vasorum* is the potential severe manifestation of angiostrongylosis in dogs (Bolt et al. 1994; Brennan et al. 2004). Systemic bleeding disorders (Denk et al. 2009; Schmitz and Moritz 2009) and sudden death are reported besides cardiopulmonary symptoms (Brennan et al. 2004; Mozzer and Lima 2012). For disease control and early diagnosis of this disease it is important that clinicians are aware of the risk areas for *A. vasorum* and other lungworm infections. In dogs, *A. vasorum* generally induces more severe manifestations (Barutzki and Schaper 2009; Taubert et al. 2009) than other lungworms, such as *C. vulpis* (Shaw et al. 1996). In cats of northern Europe, *Ae. abstrusus* is the main cause of lungworm infections inducing different severities of disease (Hamilton 1969; Lautenslager 1976; Ribeiro and Lima 2001; Tüzer et al. 2002; Payo-Puente et al. 2008; Traversa and Guglielmini 2008; Taubert et al. 2009).

Only few studies on the prevalence of metastrongyloid parasites in native gastropod populations exist for Europe. These were conducted in the UK and Denmark and show a geographically highly variable prevalence for *A. vasorum* slug infections. As such, prevalences were 1.6% in London, UK (Patel et al. 2014), 29.4% in Swansea, UK (Aziz et al. 2016), 6.7%

in Scotland (Helm et al. 2015) and up to 26% in Copenhagen, Denmark (Ferdushy et al. 2009). Investigations on prevalences of *A. vasorum, Ae. abstrusus* and *C. vulpis* in gastropod populations could help to estimate local risk for canine and feline infections (Helm et al. 2015). Consequently, further surveys on epidemiology, ecology and biology in addition to analyses on environment-intermediate host-parasite interactions are needed to develop new control and prevention strategies (Traversa and Guglielmini 2008; Giannelli et al. 2015; Giannelli et al. 2016; Maksimov et al. 2017).

The present study aims to survey canine and feline metastrongyloid lungworm infections in slug populations during different seasons in hyperendemic foci of the Federal States of Hesse and Rhineland-Palatinate, Germany.

2. Material and methods

For an overview of all steps of sample processing please see also Fig. 1.

2.1. Sampling areas and processing of samples

This epidemiological survey focused on slugs, since slugs appear to be more abundant than snails in the study areas. Slugs were collected in all seasons, i. e. in autumn and winter 2014 and in spring and summer 2015. Sampling areas were previously shown to be hyperendemic for *A. vasorum* in foxes with a prevalence > 75% (Schug et al. 2018). Four different sites (two in Hesse and Rhineland-Palatinate, each) were chosen via GPS tracking with Google maps (https://www.google.de/maps, Tab. 1). Selection criteria for sampling areas included proximity to sub-urban human settlements with forests and grassland (Tab. 1), with high potential for co-existence of foxes, slugs and dog owners and hence a high probability of lungworm infections. Since slugs are more active in the morning and at humid days, sampling was conducted on days of forecasted rainfall (www.wetter.com) starting at 6:00 in the morning for approximately 3 h. Although areas were chosen based on prevalence in foxes (Schug et al. 2018), these regions were also found to be endemic for lungworm infections in dog populations (Maksimov

et al. 2017). Data on climate conditions of cities close to sampling areas were obtained from www.wetterkontor.de (Suppl. file 1).

In total 2701 slugs were collected by hand. Each slug was identified based on morphological characteristics according to Nordsieck (2000). Slugs were weighed, cryo-euthanized and stored frozen at -20 °C until further processing. Specimens which could not unequivocally be identified by morphology, were identified by DNA sequencing as described below. Slugs were processed as described by Patel et al. (2014) via artificial HCl/pepsin digestion (Lange et al. 2017; Penagos-Tabares et al. 2018). Digested samples were stored at 4 °C until the pellets were re-suspended and examined via microscopy (Olympus BH-2, equipped with a SC30 digital camera, Olympus, Hamburg, Germany). Nematode larvae were identified morphologically, counted, collected by pipetting under microscopic control (Pasteur pipette, Hirschmann GmbH & Co. KG) and digital photos were taken.

“insert Tab. 1 here”

2.2. DNA-based identification of slug species

Slugs (*n* = 16) which could not be identified by their morphology, were further examined by nucleotide sequencing of two mtDNA fragments, viz. the cytochrome oxidase subunit I (COI) barcode (*n* = 14) and 16S ribosomal DNA (*n* = 16). These sequences were compared to reference sequences with the Basic Local Alignment Search Tool for nucleotides (blastn) of GenBank™ (16S) and the Barcode of Life Data-Identification System (IDS- BOLD) in BOLD (COI).

2.2.1. DNA extraction and PCR

Pieces of slug head tissue (2 mm³) were stored in 96% ethanol until analysis. Genomic DNA was extracted with the Nucleospin® Tissue Kit (Macherey-Nagel) according to the manufacturer’s instructions. The COI fragment was PCR amplified using the primers LCO1490 (forward) and HCO2198 (reverse) (Folmer et al. 1994) and 16S using the primers 16Sar (forward) and 16Sbr (reverse) (Palumbi 1996). PCR was carried out in 11 µl reaction volumes containing 1 µl of 2 mM of each dNTP, 1 µl of 2 µM of each primer, 0.05 µl of 5 U/µl Taq® DNA

Polymerase (Qiagen, Venlo, Netherlands), 1 µl of Taq Coloured Buffer® 10x (Qiagen, Venlo, Netherlands),1 µl of DNA extract and sterile ultra-pure water to a volume of 11 µl. All amplifications included positive (snails of the genus *Emoda*) and negative controls. The PCR cycling conditions follow the protocol ofRowson et al. (2014): (1) initial denaturation for 150 s at 94 °C, (2) 40 cycles of 30 s at 94 °C, 45 s at 47 °C, and 75 s at 72 °C, and (3) a final extension at 72 °C for 10 s. Amplification products were visualised under UV light on a 1% Midori Green stained agarose electrophoresis gel. Positive amplicons were purified using the ExoSap® kit (Applied BiosystemsTM) according to the manufacturer's instructions. DNA sequencing reactions (both directions) were performed using BigDyeTM Terminator Cycle Sequencing® Kit v3.1 (Applied BiosystemsTM) and an ABI 3130xl capillary DNA sequencer (Applied BiosystemsTM).

2.2.2. DNA sequence comparison and slug identification

DNA sequences were checked and assembled using CodonCode Aligner v. 6.0.2. Subsequently, they were corrected and trimmed in BioEdit v. 7.2.5 (Hall 1999). Using blastn of GenBank™, all new 16S sequences were compared to those in GenBank™. This was also done for all new COI sequences using IDS-BOLD (Identification System) in BOLD. Sequences were subsequently aligned in MAFFT v. 7 (Katoh and Standley 2013) for 16S and COI separately. Each dataset included all generated sequences, as well as a selection of sequences (depending on the number of available sequences) of congeneric species retrieved from GenBank™ and BOLD. P-distances and pairwise Kimura 2-parameter (K2P) distances were calculated in MEGA v. 7.0 to estimate the sequence divergence with pairwise deletion and to reconstruct Neighbor-Joining (NJ) trees with complete deletion of missing data and alignment gaps (Kumar et al. 2016). The reliability of tree nodes was assessed by bootstrapping with 1000 replicates. *Helix pomatia* (Helicidae) was used as outgroup. The DNA species identifications of slugs were based on the clustering position of each new sequence in the NJ trees relative to Genbank™ or BOLD reference sequences.

2.3. Morphological identification of lungworm species

Note: Supplementary data associated with this article

To obtain references for morphological identification of lungworm larval stages, *Ar. lusitanicus* slugs (bred in the Institute of Parasitology, JLU Giessen, Germany) were experimentally infected with either *Ae. abstrusus, A. vasorum* or *C. striatum* and artificially digested at different time points (5, 25, 40 days *post infectionem*) corresponding to L1, L2 and L3 development. Larvae of *C. striatum* originated from hedgehog faecal samples submitted to routine diagnostic at the Institute of Parasitology (JLU Giessen). Larvae of *Ae. abstrusus* were kindly provided by S. Rehbein, Boehringer Ingelheim, Germany, and larvae of *A. vasorum* were supplied by Helena Mejer of the University of Copenhagen, Denmark. A catalogue of images showing morphological characteristics of the individual stages was created (e. g. Fig. 2) and served as additional reference for morphological identification. Lungworm larvae were identified by typical morphometric characteristics (Wetzel 1940; Ash 1970; Guilhon and Cens 1973; Di Cesare et al. 2013; Giannelli et al. 2014, Colella et al. 2016). One general feature of metastrongyloid larvae is the non-rhabditiform oesophagus, which forms $1/3 - 1/2$ of the total larval length (Traversa et al. 2010). The lengths of metastrongyloid larvae vary according to lungworm species, developmental stage and size of the respective intermediate host (Ash 1970; Lopez et al. 2005; Traversa et al. 2010; Di Cesare et al. 2013; Giannelli et al. 2014). Thus, only the tail morphology was here used to differentiate different metastrongyloid species (Ash 1970). The tail of *Ae. abstrusus* L1 is notched and S-shaped (Fig. 2 A), while tails of L3 stages possess a characteristic rounded terminal knob (Fig. 2 C). *Angiostrongylus vasorum* tail morphology resembles that of *Ae. abstrusus* (see Fig. 2), but the L1 tails show a sinus wave curve and a dorsal spine (Fig. 2 D) and the third stage larvae (Fig. 2 F) has a conical tail end with a much smaller, digitiform appendage. Since larvae of *C. striatum* (hedgehog lungworm) and *C. vulpis* (fox lungworm) have a similar appearance (sharp pointed tail, Fig. 2 G – I), their morphological differentiation is difficult. *C. vulpis* L1 can be recognized by the pointed tip and L3 are characterized by a conical tail, whose posterior half is bent dorsally with a distinct narrowing anterior to the tail tip (Colella et al 2016). Second stage larvae of all three species (Fig. 2 B, E, H) share common features like the C-shaped position, ensheathment with external cuticles of the L1, dark granules around the gut and a shorter and pointed tail which

Note: Supplementary data associated with this article

is lacking typical characteristics of L3. L2 are therefore not suitable for species distinction. Given that less than 5% of all detected larvae were of L2 stage, these stages were considered as negligible.

2.4. DNA-based confirmation of lungworm species

All isolated larvae derived from one slug were pooled and DNA was extracted using a commercial kit (Qiagen DNeasy Blood and Tissue Kit®, Hilden, Germany), and eluted in a final volume of 50 µl. Lungworm species *A. vasorum, C. vulpis* and *Ae. abstrusus* were detected using species-specific nested PCRs which amplify the internal transcribed spacer 2 (ITS2) region of ribosomal DNA. The first PCR was performed with flanking universal nematode primers NC1 and NC2 (Gasser et al. 1993) in a reaction volume of 50 µl, HOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia) and 5 µl of DNA template. PCR conditions were as follows: initial denaturation/activation 95 °C 15 min, 35 cycles of denaturation 95 °C 30 s, annealing 52 °C 45 s and extension 72 °C 45 s, followed by a final elongation of 72 °C 5 min. PCR products were analysed by gel electrophoresis and used as templates for the following specific nested PCRs.

All 173 samples that microscopically appeared positive for the presence of lungworm larvae were further examined for *A. vasorum* and *C. vulpis* DNA by duplex real time PCR according to Schug et al. (2018). Final reaction volume was 20 µl and consisted of 400 nM of each primer, 200 nM of each probe, 10 µl of PerfeCTa FastMix II (Quantabio, Beverly, USA), and 1 µl template. Cycling protocol was 95 °C 10 min, 45 cycles of denaturation at 95 °C 10 s and annealing/extension at 60 °C 45 s with fluorescence detection in green (FAM) and yellow channel (HEX). As positive control *A. vasorum* and *C. vulpis* DNA in a concentration 5 pg/µl was used. A Ct value of ≤ 40 was regarded as positive. Additionally, inhibitor controls containing *Besnoitia besnoiti* DNA (*n* = 20) were performed and confirmed in 20% of the 173 samples an inhibitory effect. To account for this inhibitory effect, samples were diluted 10-fold in sterile water and re-tested as suggested by Jefferies et al. (2009) and Patel et al. (2014). *A. abstrusus* templates were also used with the preceded nested PCR (NC1/NC2) and were run in a duplex three step PCR with melt curve analysis amplifying partial ITS-2 region of 220 bp

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(*Ae. abstrusus*) and 370 bp (*Troglostrongylus brevior*). This PCR was conducted using the forward primers TrogloF, AeluroF and the single reverse primer MetR as described elsewhere (Annoscia et al. 2014). The final reaction volume of 20 µl consisted of 4 µl 5x HOT FIREPol® Evagreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), 250 nM of each primer, 13.5 ml sterile water and 1 µl template. As positive control, DNA isolated from first stage larvae of each cat lungworm species, kindly provided by Boehringer Ingelheim, was used. Cycling protocol was 95 °C 10 min, 35 cycles of denaturation 95 °C 20 s, annealing 59 °C 20 s, and extension 72 °C 30 s. All samples which did not test positive for any of the four mentioned lungworm species were additionally analysed using a conventional NC1/MetR PCR. Final reaction volume was 50 µl with the same composition as in NC1/NC2-based PCR and cycling protocol used was 95 °C 15 min, 37 cycles of denaturation 95 °C 20 s, annealing 56 °C 30 s, extension 72 °C 30 s. Samples which showed a sufficient amount of amplified DNA were gel-purified, cloned and sequenced by a commercial service (LGC Genomics, Berlin, Germany). Sequences were analysed by BLAST search of the GenBank™ database.

Sequences of *C. striatum* and one unknown *Crenosoma* sp. were extended by additional PCRs (not shown) and submitted to GenBank™ (accession numbers **KP941434-35**, **KR868714-16**, **KT257661-62**, **MG878893-94**).

If sufficient undigested tissue remained after DNA identification of slug species (*n* = 54), slug tissue (25 – 35 mg) was used for DNA isolation and directly tested for presence of *A. vasorum* and *C. vulpis* DNA using above mentioned methods of DNA isolation and nested duplex PCR.

2.5. Statistics

In order to analyse qualitative associations between the independent variables season, sampling location, slug species and log weight (logarithmic transformed because of the skewness towards the right of its statistical distribution), a multiple logistic regression was applied on dependent variables (lungworm species): *A. vasorum* and *C. vulpis* (prevalence of *Ae. abstrusus* was too low for statistical analysis). In this step of analysis dependent variables were dichotomised into negative and positive by presence or absence of examined lungworm

Note: Supplementary data associated with this article

species. Due to 397 unidentified slug species the sample size was reduced to 2304 samples, in statistical analyses. Only the two abundant slug species, *Ar. lusitanicus* [syn. *Ar. vulgaris* (Mabille, 1868)*, n* = 1563] and *Deroceras reticulatum* (*n* = 741), were considered for statistical analysis as other slug species, viz. *Tandonia rustica* (*n* = 1), *D. leave* (*n* = 34), *Limax maximus* (*n* = 25)*, L. cinereoniger* (*n* = 5)*,* and *Ar. ater* (*n* = 3) did not occur in statistically sufficient numbers. Because a large number of spring samples scored negative for presence of *C. vulpis*, calculation was repeated without consideration of season spring for *C. vulpis.* Calculations of logistic regression models were performed using the programme BMDPLR of the validated statistical package BMDP/Dynamic, Release 8.1 (Dixon 1993).

Besides the larval prevalence of lungworm infection in slugs we also analysed the larval burden. Because this variable is the result of a counting process an adequate count model was needed. Due to an accumulation of zero values and to avoid an overdispersion of model adaptation to the data set, a zero-inflated negative binomial event count model was used. For those situations commonly used Poisson regression analysis was not suitable due to an increased variance in the data. To analyse larval burden for the different species, a general linear model analysis with multiple zero-inflated negative binomial regression model was used. Regarding the variable larval burden in *A. vasorum*, calculations were performed with consideration of slug species, log weight, season and sampling area. Regarding *C. vulpis* the same multiple zero-inflated negative binomial regression was carried out. Nevertheless, the full model was not adaptable if season was included in this test. Therefore, the factor season had to be excluded from the calculation. In all cases the Wald-test was used to test statistical significance of regression parameters of the count model coefficients and the zero-inflated model coefficients. All these analyses of a generalized linear mixed effects model were performed with the statistic programme R, version 3.2.3 using the function ‘zeroinfl’ of the package ‘psych’. Significance levels in the analyses of larval prevalence and burden were set at a $p \leq 0.05$.

Note: Supplementary data associated with this article

3. Results

3.1. Prevalence of A. vasorum *and other lungworms in native slug populations*

In total, 2701 slugs were analysed from all sampling areas. In autumn 873 slugs were collected, in winter 254, in spring 791 and in summer 783. Overall, 6.4% (173/2701) of all slugs were positive for lungworm larvae based on microscopic analyses. The most common parasite species by far was *A. vasorum* (prevalence 4.7%, 127/2701), followed by *C. vulpis* (we refer here to *C. vulpis* since exclusively this species was identified by PCR) with a prevalence of 2.3% (62/2701) and *Ae. abstrusus* (0.2%, (6/2701, Tab. 2). . 29.0% of all detected larvae, were found as first stage (L1), whereas 4.7% of all larvae were L2 and 66.3% of all larvae were L3. All three lungworm species were confirmed via PCR and PCR detected four co-infected slugs (with *A. vasorum* and *C. vulpis*) which were not detected by microscopy. Of the 173 microscopically positive samples, 38 were lungworm positive by means of DNA-based identifications. Of these 38 positive samples 3 had an unexpected outcome in microscopy (Suppl. file 2). As such, one sample was positive for “insert Tab. 2 here”

Referring to the group of 173 lungworm-positive slugs, the highest number of mono-infections was recorded for *A. vasorum* (108/173 positive slugs, 62.4%), followed by *C. vulpis* (43/173 positive slugs, 24.9 %), whereas only 1 mono-infection with *Ae. abstrusus* was detected. Overall, co-infections were occasionally detected (21/173 positive slugs, 12.1%) with dual infections being observed for the combination of *A. vasorum* and *C. vulpis* (16/173 positive slugs, 9.2%), *Ae. abstrusus* and *C. vulpis* (1/173 positive slugs, 0.6%), and *Ae. abstrusus* and *A. vasorum* (2/173 positive slugs, 1.2%). In 1.2% (2/173 positive slugs) a triple infection was detected. For a detailed overview see also Fig. 3.

3.2. Seasonal and geographic variation of lungworm prevalence and larval burden

The prevalence of *A. vasorum* in slugs varied with seasons (Fig. 4) since the highest prevalence was detected in summer (9.1%), followed by autumn (4.7%), spring (1.1%) and winter (0.8%). The percentage of *C. vulpis* infections in slugs showed a seasonal peak in autumn (5.5%) and lowest prevalence was found in spring (0.3%), while *Ae. abstrusus* was

Note: Supplementary data associated with this article

only detected in summer (0.1%) and autumn (0.6%) (Fig. 4). The differences between the seasonal prevalences were statistically significant for *C. vulpis* ($p < 0.001$) and *A. vasorum* ($p = 0.004$) (Suppl. file 3). The highest risk of *C. vulpis* infection was in autumn by an odds ratio (OR) of 8.24 compared to winter and 11.9 compared to summer. Furthermore, in autumn there is also a higher chance (OR) of *A. vasorum* infection than in winter (OR = 7.02), spring (OR = 11.11) or summer (OR = 1.27).

Overall, the mean yearly geographical prevalence varied between 1.5% (Rockenhausen, Rhineland-Palatinate) and 9.9% (Otterberg, Rhineland-Palatinate) (Tab. 2). Broken down by seasons and areas, the overall highest prevalence for *A. vasorum* was found in Otterberg in summer (19.4%) (Tab. 2). For *C. vulpis* the prevalence varied between 0.3% (Eppstein, Hesse) and 5.9% (Weilburg, Hesse) (Tab. 2). The highest seasonal prevalence was detected in autumn in Weilburg (Hesse, 10%) (Tab. 2). *Ae. abstrusus* infections of slugs were exclusively found in Weilburg (Hesse) and Otterberg (Rhineland-Palatinate) with a low yearly prevalence of 0.5% and 0.4%, respectively (Tab. 2). The difference in prevalence between the four regions was highly significant for both, *A. vasorum* and *C. vulpis* infections ($p < 0.001$) (Suppl. file 3).

Mean larval burdens of *C. vulpis* were significantly different between locations ($p < 0.001$). They were higher in slugs from Weilburg compared to Rockenhausen ($p < 0.001$) and lower in Weilburg compared to Eppstein ($p = 0.001$) (Suppl. file 4).

Differences in mean overall larval burdens of *A. vasorum* were statistically significant between seasons ($p = 0.001$) and between locations ($p < 0.001$) (Suppl. file 4). *A. vasorum* larval burden was significantly higher in autumn than in winter ($p = 0.016$) and spring ($p < 0.001$) (Suppl. file 4), whereas differences between autumn and summer were not statistically significant. At Weilburg, *A. vasorum* larval burdens were significantly lower than in Otterberg ($p < 0.001$) but higher than in Eppstein ($p = 0.033$) (Suppl. file 4). Regarding the negative samples for *C. vulpis*, the influence of the different locations was highly significant ($p = 0.0002$) (zero-inflated model coefficients, Supplementary file 2), whilst this was not the case for *A. vasorum* ($p = 0.2531$).

3.3. Slug weight and A. vasorum *infection risk of slugs*

Note: Supplementary data associated with this article

The raw correlation of slug weight with *A. vasorum* prevalence showed that 77.2% of positive slugs had a weight of < 5 g (Fig. 5). In case of *C. vulpis* and *Ae. abstrusus,* 42.4% and 66.7% of positive slugs weighed less than 5 g, respectively (Fig. 5). Overall, the slug showing highest *A. vasorum* larval burden (546 larvae, Fig. 5) was a *D. reticulatum* of 0.9 g. Slug weight correlated significantly withprevalence with a positive coefficient for both lungworm species, i. e. *C. vulpis* ($p < 0.001$, 1.76 ± 0.5) and *A. vasorum* ($p = 0.007$, 1.03 ± 0.37, Suppl. file 3). This implies an odds ratio (OR) of 5.81, which means that probability of infection with *C. vulpis* would theoretically rise 5.81-fold if weight of slugs increased 10-fold (Supplementary file 3). The chance of *A. vasorum* infection would hence increase 2.8-fold if slug weight increased 10-fold (Supplementary file 3).

In addition to lungworm prevalence, also larval burden of *A. vasorum* was significantly correlated withslug weight ($p = 0.002$), but not for *C. vulpis* larval burden (Suppl. file 4). Accordingly, a 10-fold (log weight, Suppl. file 4) increase of slug weight would increase the mean larval burden per slug in case of *A. vasorum* by a factor 9.4 (e^2.243 = 9.424, exponential function as inverse function of coefficient in Suppl. file 4). While the correlation between logarithmic weight and proportion of negative samples was not significant for *A. vasorum* ($p = 0.322$), it was highly significant for *C. vulpis* infections ($p < 0.001$) (Suppl. file 4).

Overall, the number of lungworm larvae per slug varied considerably. Thus there were 1-546 *A. vasorum* larvae per specimen*,* with highest larval burdens in summer (546, 230, 195 larvae) and autumn (301 larvae) (Fig. 5, Suppl. file 2). Larval burden ranged from 1 to 92 larvae per slug for *C. vulpis* and from 1 to 13 larvae per slug for *Ae. abstrusus* (Fig. 5, Suppl. file 2). Higher larval burdens were particular evident in summer and autumn, paralleling prevalence data of the three lungworms. However, most of positive slugs showed low parasite burden and harboured fewer than 10 larvae (*A. vasorum*: 78.9%, *C. vulpis*: 83.3% and *Ae. abstrusus*: 66.6%, Fig. 6), while 15.5 %, 15% and 33.3% of *A. vasorum-, C. vulpis- and Ae. abstrusus*-positive slugs contained more than 11-49 larvae*,* respectively*.* Larval burdens of more than 50 larvae were only found for *A. vasorum* (2.4%) and *C. vulpis* (1.7%) infections. Only 3.2% of *A. vasorum*-positive slugs harboured more than 100 larvae.

Note: Supplementary data associated with this article

3.4. The slug species Ar. lusitanicus (= Ar. vulgaris) *was a better suited intermediate host than* D. reticulatum *in native infections*

Arion lusitanicus (*n* = 1587) was the most abundant slug species in the current study, followed by *D. reticulatum* (*n* = 699). Both species were present in all sampling regions (Suppl. file 5). Only few specimens of *Limax maximus* (*n* = 25)*, L. cinereoniger* (*n* = 5), *D. laeve* (*n* = 34), *Ar. ater* (*n* = 3) and *T. rustica* (*n* = 1) were found (Suppl. file 5), but none of these were positive for lungworm larvae. The slug samples identified by DNA sequencing that were most similar to *Ar. vulgaris* sequences in GenBank™ and BOLD had an average p-distance of 0.7% (range: 0.0 - 2.3%) for 16S and an average of 0.6% (range: 0.0 - 1.8%) for COI with *Ar. vulgaris*. The average p-distance between *Ar. vulgaris* and the *Ar. ater* / *Ar. rufus* species complex is 10.6 % (range: 10.1 - 11.4%) for 16S and 11.9 % (range: 10.7 - 13.1%) for COI. The slug samples identified by DNA sequencing that were most similar to *D. reticulatum* sequences in GenBank™ and BOLD had an average p-distance of 1.5% (range: 0.0 - 2.4%) for 16S and an average of 2.8% (range: 0.2 - 5.5%) for COI with *D. reticulatum*. With *D. laeve* there was an average p-distance of 13.4% (range: 13.3 - 13.4%) for COI. Other *Deroceras* species showed even larger p-distances for COI. Species identifications were consistent between the two markers 16S and COI. Compiled alignments used to infer NJ trees involved 21 16S reference sequences of 13 species from GenBank™ and 63 COI reference sequences of 34 species from BOLD. The NJ trees clustered 14 new 16S sequences with references sequences of *Ar. lusitanicus* and 2 new 16S sequences with reference sequences of *D. reticulatum*. New COI sequences of 12 samples clustered with reference sequences of *Ar. lusitanicus* and new 16S sequences of 2 samples with reference sequences of *D. reticulatum*. Clustering results thus confirmed the identifications of *Ar. lusitanicus* and *D. reticulatum.*

Overall, 6.3% of *Ar. lusitanicus* slugs were infected with *A. vasorum,* 3.8% with *C. vulpis* and 0.4% with *Ae. abstrusus,* whilst for *D. reticulatum* the infection rates were only 4.0%, 0.1% and 0%, respectively (Tab. 3). Thus, *A. vasorum* prevalence was significantly higher in *Ar. lusitanicus* than in *D. reticulatum* (*p* = 0.038), yet the prevalence of *C. vulpis* did not differ significantly between both slug species (*p* = 0.076) (Suppl. file 3). Infected *D. reticulatum*

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showed a significantly higher larval burden of *A. vasorum*- and *C. vulpis*- than *Ar. lusitanicus* (both: $p < 0.001$) (Suppl. file 4). However, higher *C. vulpis* larval burdens were more likely to appear in *Ar. lusitanicus* (negative coefficient -2.93 ± 0.65) than in *D. reticulatum*. Although *A. vasorum* prevalence was positively influenced by the slug species *Ar. lusitanicus*, larval burden was likely to be higher in *D. reticulatum* (positive coefficient 3.66 ± 0.80, Suppl. file 3, 4).

"insert Tab. 3 here"

4. Discussion

In the current epidemiological study we analysed for the first time prevalence of several lungworm species in native slug populations in four selected areas in Germany. These analyses were based on morphological characteristics which allowed discrimination of lungworm larvae from other typical slug nematodes, such as *Phasmarhabditis hermaphrodita* or *P. neopapillosa* (Mengert et al. 1953), by observing oesophagus type, tail morphology and parasite size [length and width (Mengert et al., 1953)]. Different *Crenosoma* species (i. e. *C. striatum, C. vulpis*) are too similar at the level of L1 to L3 to be distinguished via microscopy. Thus, misidentification of these species cannot be excluded. However, *C. striatum* was neither detected via PCR, nor via DNA sequencing. Nevertheless, two samples contained DNA of a *Crenosoma* species of which no sequence is listed in GenBank™.

In contrast to L1 and L3, the L2 stages of lungworm larvae lack specific morphological characteristics for their discrimination within the metastrongyloid superfamily. Given that only 4.7% of all detected larvae were in L2 stage (data not shown), this uncertainty appeared negligible. The discrepancy between microscopic and DNA-based species identifications (Suppl. file 2) might, however, be caused by non-detected co-infections of L2 stages. Furthermore, some of the larvae were in poor condition, probably due to immunological degradation and elimination processes in living gastropod hosts. These larvae could not be identified via microscopy alone and might add another source of error. Nevertheless, the applied PCR techniques allowed to detect four cases of co-infections by *A. vasorum* and *C.*

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vulpis, which otherwise would have remained undetected. We initially thought to confirm each microscopic diagnosis via PCR, but the prolonged contact of samples with pepsin at low temperature before microscopy may have sometimes degraded the DNA (Shigei et al. 2001; Liu et al. 2015; Zhang et al. 2016), thus probably explaining negative results of PCR analysis (NC1/NC2 PCR, Suppl. file 2). PCR-based identifications were only applied to larvae of microscopically positive samples. Whether or not more positive samples could have been detected if PCR was carried out on slug tissue samples and microscopically negative samples remains uncertain. Although PCR might be more sensitive than microscopy, the actual relevance of these findings remains questionable when no larvae could be found via microscopy. It is imaginable that PCR even might detect DNA traces of larvae, which had been eliminated by the gastropod innate immune system (Lange et al. 2017, Penagos-Tabares et al. 2018) and thus do no longer represent an infection risk for final hosts. This could also be true for the sample in which *H. contortus* was identified via sequencing but not via microscopy. Since this species does not infect gastropods it can be assumed that it was just coincidentally passing the slug's intestine.

The overall prevalence of *A. vasorum* in slugs (of 4.7%) confirms the occurrence of this parasite in areas which are hyperendemic for this parasite in wild fox populations (prevalence > 75% in foxes, Schug et al 2018) and dogs (Maksimov et al., 2017). So far, only limited data exist on the prevalence of *A. vasorum* in intermediate hosts in other countries, where prevalences in slugs vary between 1.6% and 43% depending on sampling areas (Ferdushy et al. 2009; Jefferies et al. 2009; Patel et al. 2014; Aziz et al. 2016). This seems in line with current findings and with the distribution of *A. vasorum* infections involving hyperendemic regions in close proximity to areas of low prevalence (Morgan et al. 2005; Ferdushy and Hasan 2010; Aziz et al. 2016; Maksimov et al. 2017; Schug et al. 2018). Thus, we here identified a hotspot for *A. vasorum*-slug infections (9.9% in Otterberg, Rhineland-Palatinate) and regions with a rather low prevalence (1.5% in Rockenhausen, Rhineland-Palatinate, Tab. 2) in close geographical proximity. Ferdushy et al. (2009) also reported strongly varying prevalences (4 - 26%) within a sampling area close to Copenhagen, Denmark. In agreement with this, Aziz et

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al. (2016) described a rural-urban gradient of *A. vasorum* prevalence in slugs with higher prevalences in suburban areas in the UK. The causes of the patchiness of the *A.vasorum* distribution are still unknown (Patel et al., 2014; Aziz et al. 2016), but may include spatial variation in the intermediate host density, environmental factors, and local dispersal of infected intermediate hosts spreading from areas of new parasite introductions (Lahodny and Allen 2013).

Although study areas were chosen because of their high *A. vasorum* prevalence in foxes (Schug et al. 2018), they were most likely also endemic for lungworm infections in dogs. For example, Maksimov et al. (2017) showed that dogs from regions close to Weilburg/Eppstein and Rockenhausen/Otterberg had an *A. vasorum* prevalence of 0.01% – 1.42% and 1.43% - 2.54%, respectively and a prevalence of *C. vulpis* varying between 2.68% - 7.79% and 0.01% - 1.56%, respectively. Moreover, these authors observed a rise of *A. vasorum* prevalence in dogs from Hesse and from Rhineland-palatinate during the last 7 years. Based on eco-climatic information, the potential future distribution of *A. vasorum* in Germany predicts higher suitability for parasite transmission in the western part of Germany, including Rhineland-Palatinate (Morgan et al. 2009). This may be in line with the highest observed *A. vasorum* prevalence of 19.4% in Otterberg (Tab. 2).

Since sampling areas were chosen based on the prevalence of *A. vasorum* only, low prevalences of *C. vulpis* (2.3%) and *Ae. abstrusus* (0.2%) are not surprising and suggest that more research on the prevalence of these species in Germany is needed. Co-infections of *A. vasorum* and *C. vulpis* have been reported in both, foxes (Jeffery et al. 2004; Saeed et al. 2006; Schug et al. 2018) and dogs (Barutzki and Schaper 2009; Taubert et al. 2009; Maksimov et al. 2017), but not yet in slugs. In this study we describe, to our best knowledge, for the first time co-infections of *A. vasorum*, *C. vulpis* and *Ae. abstrusus* in naturally infected slugs.

Seasons are hypothesized to have a strong influence on the development and infection rate of metastrongyloid larvae. For example, higher ambient temperatures increase larval development speed, viability and vitality of *Ae. abstrusus* in the snail *Helix aspersa* (Di Cesare

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et al. 2013). Similar results were reported for *A. vasorum* development in *Ar. lusitanicus* with higher infection rates at higher temperature (100% at 10 °C and 15 °C), whereas at lower temperature (5 °C) only 73.3 – 86.7% of slugs were infected (Ferdushy et al. 2010). Moreover, slugs as obligate intermediate hosts, themselves prefer humid and temperate conditions (Willis et al. 2006). Thus, highest slug abundance and largest sample sizes occurred in autumn. During that season between 115.9 and 175.3 l/m^2 precipitation was measured, with mean temperatures between 11.1 and 12.3 °C and with 202.5 and 244.9 h sunshine, depending on the region (Suppl. file 1). This abundance of slugs in autumn coincided with a high *A. vasorum* prevalence. Conversely, low precipitation of 52.8 - 105.6 l/m^2 and many hours of sunshine (595.3 - 618.3 h, Suppl. file 1) in spring might partly explain low prevalence of *A. vasorum* (0 - 3.5%, Tab. 2) since larvae in faeces are vulnerable to desiccation (Costa Dias 2012) and thus are less able to infect slugs. The highest *A. vasorum* prevalence in slugs was found in summer (9.1%) followed by autumn (4.7%, Fig. 4). However, in contrast to the raw data, statistical analysis favoured autumn to be positively associated with the prevalence of *A. vasorum* (Suppl. file 3). The discrepancy between seasonal observations of highest infection risk from raw correlations and thorough statistical analysis could be due to the interference of other factors during seasons such as higher slug abundance in autumn leading to bias for raw correlations. Moreover, autumn prevalence was evaluated in 2014, but summer prevalence in 2015. Differences between the two seasons could therefore possibly mirror either a general increase of prevalence between the years 2014 and 2015 or a pure chance phenomenon, instead of a true seasonal pattern. To resolve this uncertainty, surveys of lungworm prevalences in slug populations over longer time periods are needed. However, our observations correspond well with other authors speculating that the highest infection risk for dogs may be in late summer and early autumn because the onset of clinical signs in infected dogs most often occurs in winter (Barutzki and Schaper 2009; Taubert et al. 2009; Maksimov et al. 2017). In addition, the greatest abundance of slugs is expected in autumn in Europe (Morgan and Shaw 2010), which may also influence seasonal epidemiology of *A. vasorum* infections. Climatic conditions in autumn also match well with experimental infections

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suggesting that larvae survival rate of *A. vasorum* is highest with a temperate (5 °C) and humid weather (Ferdushy et al. 2010; Morgan and Shaw 2010; Dias and Dos Santos Lima 2012). Despite higher infection risk during summer and autumn the current data suggest that dogs can in principle be infected at any season in the analysed sampling areas. This has to be kept in mind in case of atypical onset of clinical signs in canine *A. vasorum* infection.

Regarding larval burden, we found up to 546 *A. vasorum* larvae/slug which obviously poses a risk of severe infection on dogs in case of slug consumption (Bolt et al. 1994). Similarly, high larval burdens of up to 392 larvae per slug were reported by Ferdushy et al. (2009) in *Ar. lusitanicus*. Yet, these authors reported that 14% of slugs harboured more than 100 larvae per specimen, while in the current study only 3.3% of slugs harboured more than 100 larvae. The majority of slugs harboured only few *A. vasorum* larvae (78.9% carried <10 larvae/slug), which is in line with other reports on *A. vasorum* and *A. costaricensis* (Laitano et al. 2001; Ferdushy et al. 2009), where 82% and 51% of investigated slugs harboured few larvae, respectively. The two other lungworm species also showed a majority of larval burdens of less than 10 larvae per slug (*C. vulpis* 83.3% and *Ae. abstrusus* 66.6%, Fig. 6). This sort of overdispersion (Anderson and Gordon 1982), as reported for *A. vasorum* by Ferdushy et al. (2009), was also observed in *A. costaricensis* (Laitano et al. 2001). The high proportion of slugs harbouring few larvae may explain why infections in dogs and foxes often remain subclinical (Ferdushy et al. 2009; Di Cesare et al. 2014). It is tempting to speculate that low larval burdens in slugs may also result from an intact gastropod innate immune system. This hypothesis matches well with recent reports on slug innate immune reactions showing efficient formation of so-called invertebrate extracellular phagocyte traps (InEPTs) in response to metastrongyloid larvae (Lange et al. 2017). This study showed that haemocytes of *Ar. lusitanicus* and *L. maximus* attack and firmly entrap *A. vasorum, Ae. abstrusus* and *T. brevior* larvae *in vivo* and *in vitro*, fixing up to 41% of *A. vasorum* larvae. However, further detailed analyses are needed to evaluate the influence of these gastropod InEPTs on development and final larval burden.

Whether the presence of lungworm larvae in combination with high larval burdens impacts intermediate host population health in endemic regions cannot be assessed with the current

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data. Information on slug health impairment after *A. vasorum* infection is scarce. Yet, in experimental infections a weight loss was observed, which did not occur in non-infected control gastropods (Mozzer et al. 2015; M.K. Lange personal communication). Furthermore, *A. vasorum*-infected slugs lay more eggs per egg clutch, but have a lower hatching rate than non-infected slugs (Mozzer et al 2015). These findings indicate that *A. vasorum* infections might indeed negatively affect gastropod intermediate host population health.

In terms of prevalences, *Ar. lusitanicus* is clearly the better slug intermediate host for *A. vasorum* and *C. vulpis* than smaller *D. reticulatum* (6.3% vs. 4.0% and 3.8% vs. 0.1%, respectively). However, *A. vasorum*- and *C. vulpis*-positive *D. reticulatum* harboured significantly higher larval burdens than *Ar. lusitanicus*. In addition, the probability of infection increases with increasing slug weight. Nevertheless, the raw correlations (Fig. 5) suggest that the majority of *A. vasorum* positive slugs have a rather low weight. Since raw correlations do not consider other factors which could influence the relationship between parasite prevalence and slug weight, the statistical results are assumed to provide a more accurate picture since they considered other factors such as slug species and season. Thus, a higher slug weight seems to truly influence the chance of lungworm infection, if all factors are considered (Suppl. file 3). Nonetheless, it cannot be excluded that if slugs of the current study had lived longer and gained more weight, they might also have presented higher prevalences and higher larval counts. Statistical estimation that mean larval burden would rise 9.4-fold if slugs were 10-fold heavier is in line with experimental data on *A. vasorum* in slugs showing that larval development is not only influenced by climatic factors but also by size and age of intermediate hosts (Yousif and Lämmler 1975; Barcante et al. 2003; Ferdushy et al. 2010; Patel et al. 2014). To summarise, increased slug size and higher ambiental temperatures may increase larval burden per slug. Correspondingly, Aziz et al. (2016) described *Ar. rufus* as efficient intermediate host (prevalence 41%) and larger slug species are assumed to be more competent intermediate hosts for *A. vasorum* (Patel et al. 2014; Schnyder 2015). In contrast, Helm et al. (2015) observed no significant difference between slug species in prevalence but nevertheless speculated that larger specimens are more likely to draw the attention of final

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hosts. In contrast, smaller slugs are most probably more often prone to accidental consumption by domestic dogs (Schnyder 2015). In this context, the coprophagic activity of certain slug species (Patel et al., 2014; Schnyder 2015; Aziz et al. 2016) should clearly increase the risk of individual slug infection. Additionally, the long life span and carnivorous activity, which allows to accumulate larvae via gastropod-to-gastropod transmission – *intermediesis* (Colella et al. 2015) of certain slug species, i.e. *L. maximus*, may explain the high larval burdens found by Ferdushy et al. (2009) and Rollo (1983). In the current study, however, *L. maximus* was not infected by lungworm larvae, but this may be due to the small sample size (*n* = 25) (Suppl. file 5). Like *Angiostrongylus cantonensis* (Chen et al. 2011), *A. vasorum* shows a significant positive correlation between slug weight and larval burden. Moreover, the high larval burdens for *A. vasorum* in summer and autumn coincide with a high prevalence in these two seasons. However, the overall highest larval burden was found in a slug weighting only 0.9 g, while the majority of positive slugs had a body weight less than 5 g (Fig. 5). So far, it remains unclear whether the slug age might also influence prevalence and larval burden.

5. Conclusions

To our knowledge, this is the first large scale epidemiological survey of *A. vasorum*, *Ae. abstrusus* and *C. vulpis* infections in indigenous intermediate hosts in selected areas of Germany. *Ar. lusitanicus* was clearly the slug species with highest prevalence of *A. vasorum*. The current data demonstrate that in the analysed study areas dogs are at a permanent risk for *A. vasorum* infections throughout the year. The study also contributes to a better understanding of the temporal dynamics of canine angiostrongylosis but also for related lungworms, and to further evidencing the necessity to investigate in depth epidemiological factors under standardized conditions (i.e. temperature, humidity, nutrition, light) that might impact the endogenous development of metastrongyloid parasites in gastropods.

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Declarations

Ethics approval and consent to participate

This survey does not involve living vertebrates or cephalopods. The slugs dealt with do not belong to the specially protected fauna regulated by the Act on Nature Conservation and Landscape Management (Federal Nature Conservation Act – BNatSchG, Germany).

Consent for publication

Not applicable

Conflicts of interest:

none

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Note: Supplementary data associated with this article

Availability of data and material

All data generated or analysed during this study are included in this published article [and its supplementary information files]

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Legends to figures

"Figure 1 and Figure 2 should be printed in colour in online journal only"

Figure 1: Processing of samples and overview of analyses conducted.

In total, 2701 slugs were processed and samples were analysed following the here depicted steps. Slugs were cut into small pieces, processed via artificial HCl/ pepsin digestion and samples were stored at 4 °C until further use. Pellets were re-suspended and examined microscopically. Larvae were identified morphologically, counted, collected by pipetting under microscopic control and larvae were further analysed via nested duplex PCRs.

Figure 2. Morphological characteristics for the identification of larval stages of *Aelurostrongylus abstrusus*, *Angiostrongylus vasorum, Crenosoma striatum*.

Key for morphological identification of the three metastrongyloid lungworms *Aelurostrongylus abstrusus* (A – C), *Angiostrongylus vasorum* (D - F) and *Crenosoma striatum* (G – I). All stages present in gastropods are described: L1 stage (A, D, G), L2 stage (B, E, H) and L3 stage (C, F, I). First stage larvae of *Ae. abstrusus* are characterized by a notched S-shaped tail (A), the tail of *A. vasorum* appears with a sinus wave curve and a dorsal spine (D). L1 of *C. striatum* show a very pointed and straight tail. Second stage larvae of all three species (B, E, H) share nearly identical morphology, lying in an enrolled position being ensheathed with an external cuticle of L1 with dark granules around the gut and the tail being shorter and pointed, lacking the typical tail characteristics of L3 (in case of *Ae. abstrusus* and *A. vasorum*). Third stage larvae of all three lungworms (C, F, I) only have few dark granules around the gut and are only occasionally sheathed with an external cuticle of previous larval stages. *Ae. abstrusus* (C) can be recognised by a tail with a terminal, evident rounded knob. The L3 of *A. vasorum* possess a tail which ends in a much smaller knob than *Ae. abstrusus,* with a short, digitiform appearance (F). The tail of *C. striatum* L3 (I) resembles the tail of previous stages, only size and proportion of L3 changed in comparison to L1 and L2 of this species.

Note: Supplementary data associated with this article

Figure 3. Mono-, co- and triple infections with *Angiostrongylus vasorum*, *Crenosoma vulpis* and *Aelurostrongylus abstrusus*

Of the 2701 slugs collected, microscopy revealed 173 lungworm larvae-positive samples. Those were analysed for mono- and co-infections with the different lungworm species via microscopy and PCR.

Figure 4. Seasonal prevalence of *Aelurostrongylus abstrusus*, *Angiostrongylus vasorum* and *Crenosoma vulpis*) in German slugs.

Of the 2701 slugs collected throughout the season in different areas of Germany, 873 slugs were collected in autumn, 254 in winter, 791 in spring and 783 slugs in summer. Here, the data were assigned to the different seasons.

Figure 5. Correlation of slug weight and *Angiostrongylus vasorum*, *Crenosoma vulpis* and *Aelurostrongylus abstrusus* larval burden

The larval burden was correlated with the slug weight. X and Y axis are shown as a nonlinear logarithmic scale.

Figure 6. Larval burden categories for slug *Aelurostrongylus abstrusus*, *Angiostrongylus vasorum* and *Crenosoma vulpis* infections.

Here, the proportion of slugs harbouring <10, 10-49, 50-100 and >100 lungworm larvae per specimen is depicted.

Note: Supplementary data associated with this article

Slug collection in selected areas in all 4 seasons

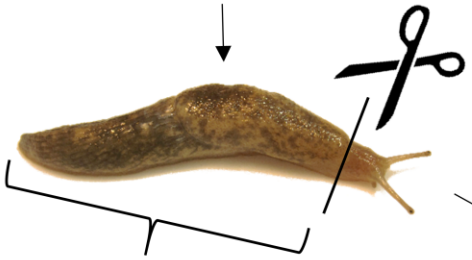
↓

Weighting, morphological slug species identification

↓

Cryo-euthanazation, storage at – 20 °C

↓



Cutting into small pieces (ca. 0.5 cm)

↓

Artificial digestion, sieving, microscopy

↓

Pipetting of all larvae of 1 slug into 1 tube, storage at 4 °C

↓

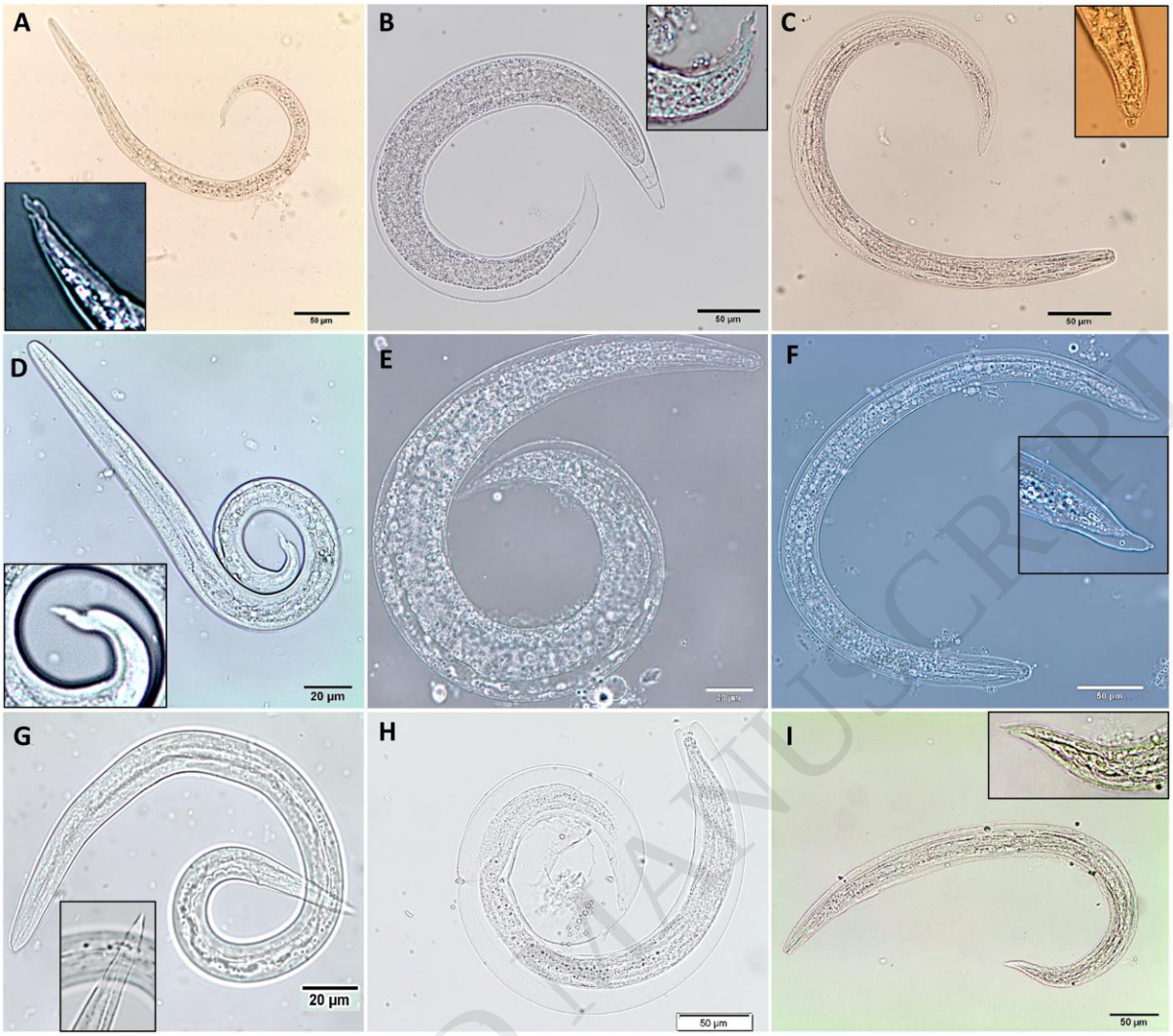
Nested duplex PCRs for dog and cat lungworm larvae, sequencing

Tissue of head part for DNA-based slug species identification

↓

If tissue remained it was used for nested duplex lungworm PCR of slug tissue

Note: Supplementary data associated with this article



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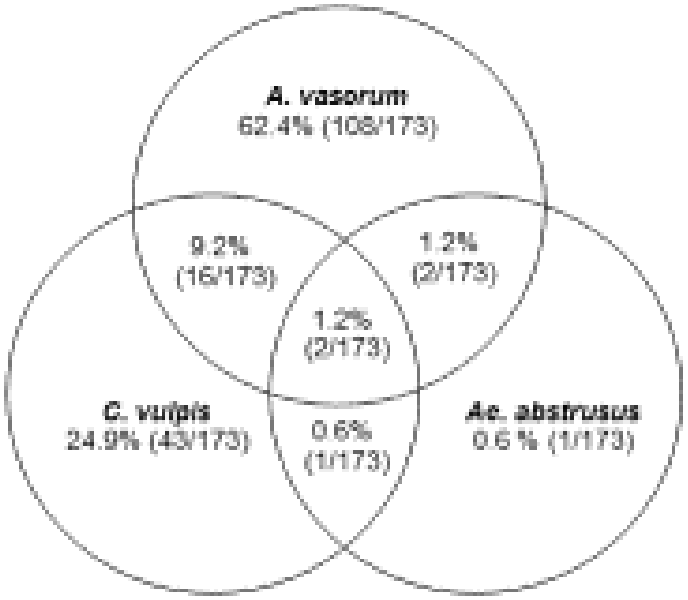

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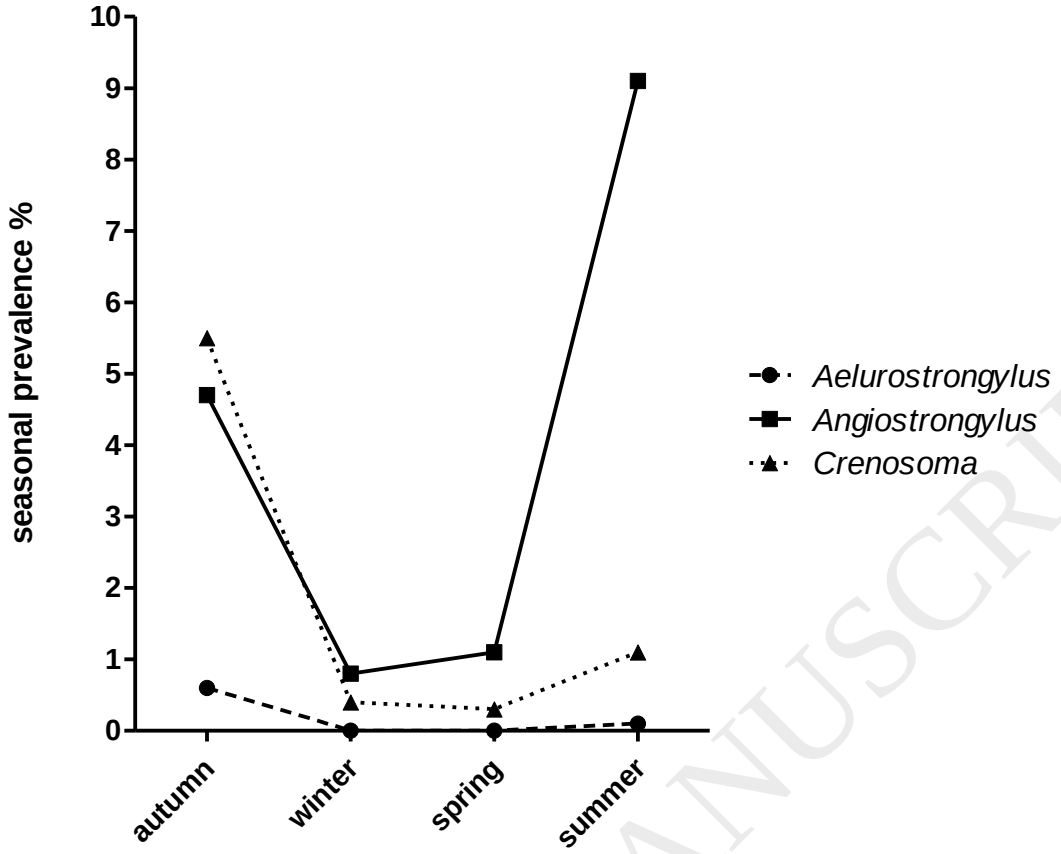

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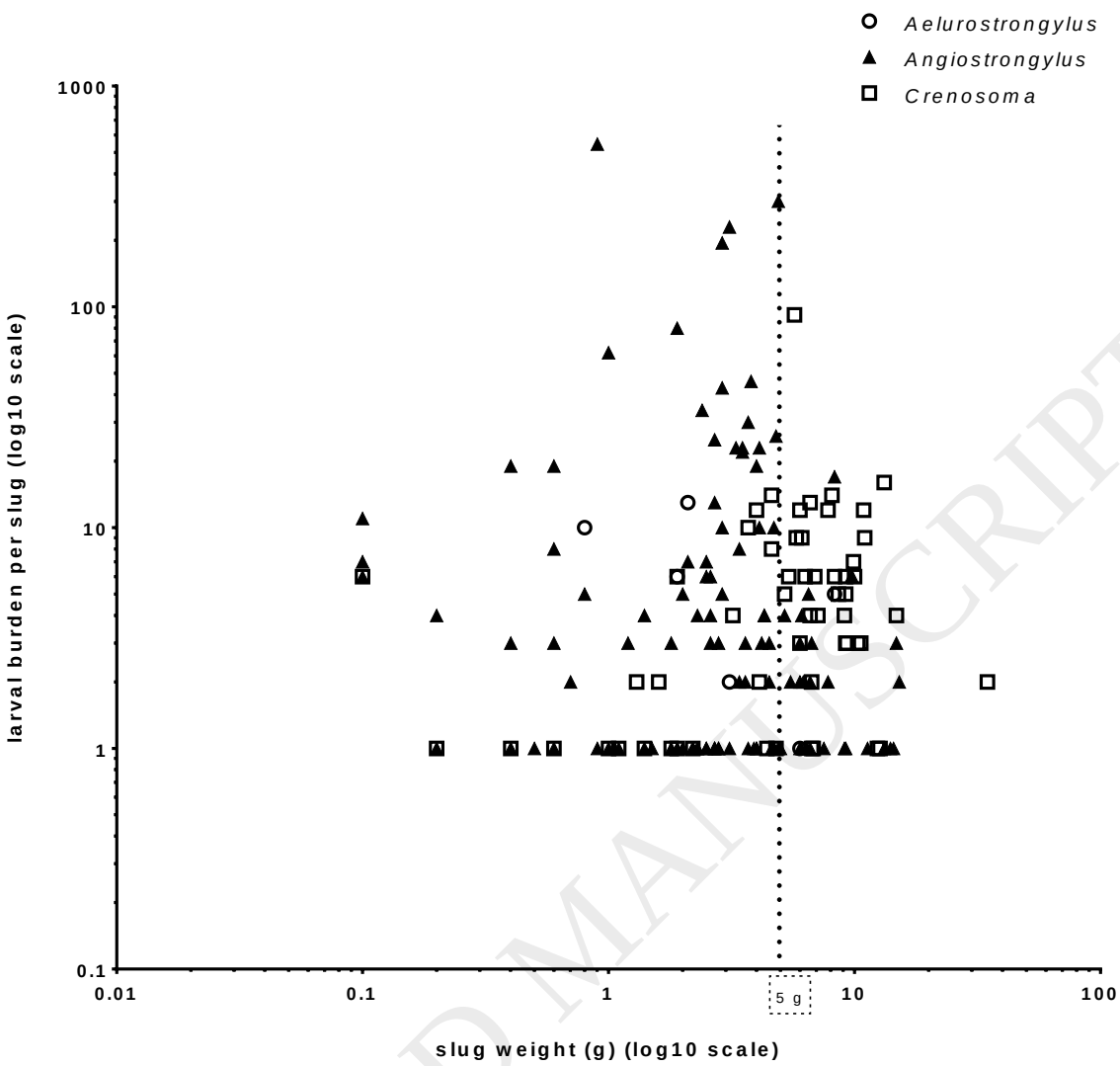

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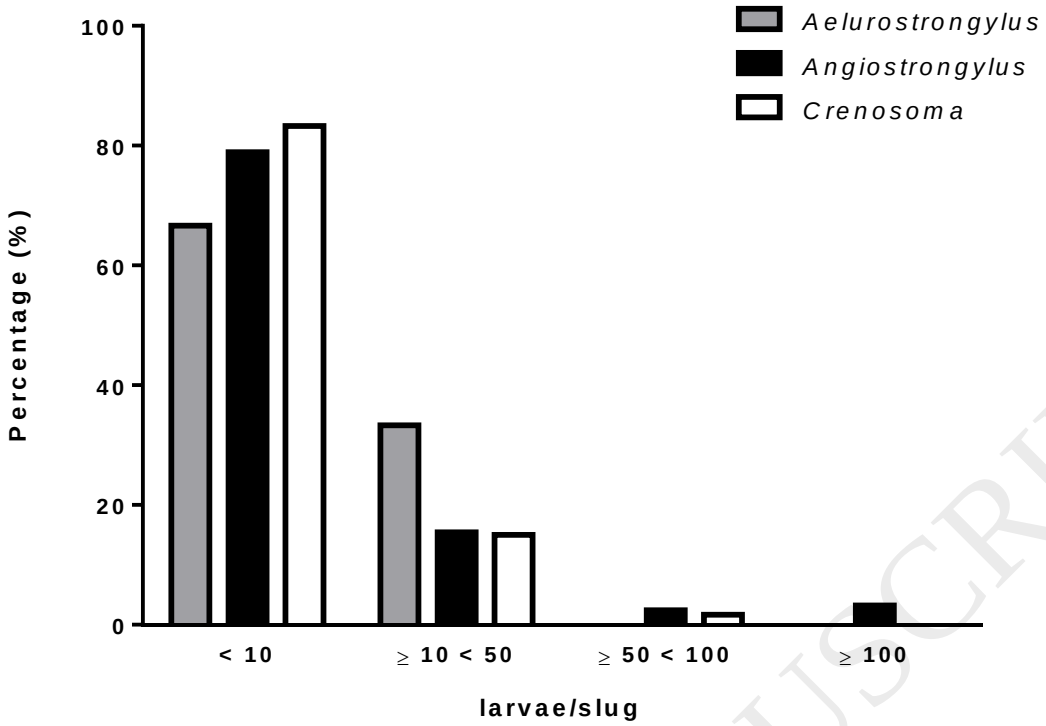

Table 1: Description of the regions of slug collection in Hesse and Rhineland-Palatinate, Germany.

Regions of slug collection

Regions	Description	Latitude	Longitude
Weilburg, Hesse	Meadow, forest	50°28'56.86''N	8°15'2.21''E
Eppstein, Hesse	Meadow, fruit trees	50°9'0.18''N	8°23'0.31''E
Rockenhausen, Rhineland-Palatinate	Meadow, Acre, Creek	49°37'57.02''N	7°49'39.47''E
Otterberg, Rhineland-Palatinate	Meadow, creek	49°29'41.94''N	7°46'22.63''E

Note: Supplementary data associated with this article

Table 2: Prevalences of *Aelurostrongylus abstrusus*, *Angiostrongylus vasorum*, and *Crenosoma vulpis* in all four seasons of the year in the different sampling areas.
Prevalences (%) and number of positive slugs compared to total number of slugs in corresponding region (*n* = /) based on results of microscopy.

		Parasite prevalence (%)		
Region	Season	*Aelurostrongylus abstrusus*	*Angiostronglus vasorum*	*Crennosoma vulpis*
Weilburg (He)	sp	-	-	-
	su	0.5	8.8	0.9
	au	1.3	18.0	10.0
	wi	-	1.7	0.8
	total	**0.5, *n* = 4/785**	**5.7, *n* = 45/785**	**6.1, *n* = 48/785**
Eppstein (He)	sp	-	-	-
	su	-	3.9	-
	au	-	1.3	1.3
	wi	-	-	-
	total	**0.0**	**1.8, *n* = 11/609**	**0.3, *n* = 2/609**
Rockenhausen (RP)	sp	-	1.0	-
	su	-	2.2	0.9
	au	-	1.5	0.4
	wi	-	-	-
	total	**0.0**	**1.5, *n* = 11/742**	**0.4, *n* = 3/742**
Otterberg (RP)	sp	-	3.5	1.4
	su	-	19.4	2.3
	au	0.8	7.4	0.8
	wi	-	-	-
	total	**0.4, *n* =2/565**	**9.9, *n* = 56/565**	**1.4, *n* = 8/565**

He = Hesse; RP = Rhineland-Palatine; sp = spring; su = summer, au = autumn; wi = winter

Note: Supplementary data associated with this article

Table 3: Prevalences of lungworm species in the intermediate host populations of *Angiostrongylus lusitanicus* and *Deroceras reticulatum.*

Prevalences are based on microscopic positive samples, number of lungworm positive slugs in brackets.

	intermediate host species	
lungworm species	*Arion lusitanicus* *n* = 1587	*Deroceras reticulatum* *n* = 699
Aelurostrongylus abstrusus	0.4% (*n* = 6)	0.0%
Angiostrongylus vasorum	6.3% (*n* = 100)	4.0% (*n* = 28)
Crenosoma vulpis	3.8% (*n* = 60)	0.1% (*n* = 1)

Note: Supplementary data associated with this article