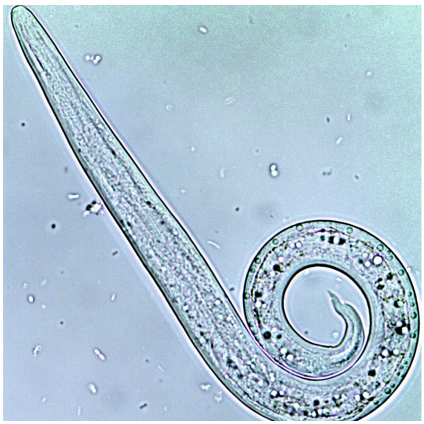
Malin Katharina Lange

Untersuchungen zu *Angiostrongylus vasorum* und anderen Lungenwürmern von Hund und Katze im Zwischenwirt Nacktschnecke



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5. Penagos-Tabares, F; Gutiérrez, J; Vélez, J; Piedrahita, D; **Lange, M K**; Hermosilla, C; Taubert, A; Chaparro-Gutiérrez J J (2016). First report of *Angiostrongylus* spp. infections in African giant snails (*Achatina fulica*) in Valle del Cauca and Antioquia, Colombia. 4th Bayer Angiostrongylosis Forum, 12. Juli 2016, Wien, Österreich, *Proceedings* p 30

6. Lange, M K; Penagos-Tabares, F; Muñoz-Caro, T; Magdowski, G; Gärtner, U; Mejer, H; Schaper, R; Hermosilla, C; Taubert, A (2016). First characterisation of gastropod haemocyte extracellular trap formation in response to different lungworm

larvae. 4th Bayer Angiostrongylosis Forum, 12. Juli 2016. Wien, Österreich, *Proceedings* p 21

7. Lange, M K; Penagos-Tabares, F; Hermosilla, C; Schaper, R; Taubert, A (2016). *Angiostrongylus vasorum* in its intermediate hosts: an epidemiological survey in Germany. 4th Bayer Angiostrongylosis Forum, 12. Juli 2016, Wien, Österreich, *Proceedings* p 6

8. Lange, M K; Raue, K; Strube, C; Schaper, R; Hermosilla, C; Taubert, A (2017). Vector competence of three slug species as intermediate hosts of *Angiostrongylus vasorum*. Deutsche Veterinärmedizinische Gesellschaft "Parasitologie und Parasitäre Krankheiten", 12.-14. Juni 2017, Hannover, *Proceedings* pp 148-149

9. Lange, M K; Penagos-Tabares, F; Vélez, J; Gutiérrez, J; Piedrahita, D; Hermosilla, C; Taubert, A; Chaparro-Gutiérrez, J J (2017). Prevalence of metastrongyloid lungworm larvae in Colombian giant African snails (*Achatina fulica*). Deutsche Veterinärmedizinische Gesellschaft "Parasitologie und Parasitäre Krankheiten", 12.-14. Juni 2017, Hannover, *Proceedings* pp 146-147

10. Lange, M K; Penagos-Tabares, F; Hermosilla, C; Schaper, R; Taubert, A (2017). Epidemiological survey on the occurrence of *Angiostrongylus vasorum* stages in natural slug populations in Germany. The 26th International Conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP), 4.-8. September 2017, Kuala Lumpur, Malaysia, *Proceedings* of the Bayer Animal Health Symposium p 11

11. Lange, M K; Penagos-Tabares, F; Vélez, J; Gutiérrez, J; Piedrahita, D; Hermosilla, C; Taubert, A; Chaparro-Gutiérrez, J J (2017). Prevalence of metastrongyloid lungworm larvae in Colombian giant African snails (*Achatina fulica*). 10th Annual GGL Conference on Life Sciences, 27.-28. September 2017, Giessen, *Proceedings* p 15

12. Van Bourgonie, Y R; Backeljau, T; **Lange, M K**; Penagos-Tabares, F; Hirzmann, J , Hermosilla, C; Taubert, A (2017). Identification of intermediate slug hosts in the framework of an epidemiological survey in Germany. The 24th zoology congress 2017 "Genotype-phenotype map: from model systems to ecosystems", 23.-24. November 2017, Wageningen, the Netherlands

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14. Lange, M K; Penagos-Tabares, F; Raue, K; Strube, C; Schaper, R; Hermosilla, C; Taubert, A (2018). Spontaneous and provoked larval shedding in *A. vasorum*- infected slug intermediate hosts. Deutsche Veterinärmedizinische Gesellschaft "Parasitologie und Parasitäre Krankheiten", 2.-4. Juli 2018, Gießen, *Proceedings* p 4

15. Lange, M K; Penagos-Tabares, F; Vélez, J; Gutiérrez, J; Taubert, A; Chaparro-Gutiérrez, J J; Hermosilla, C (2018). Prevalence of metastrongyloid lungworm larvae in neozoan giant African snails (*Achatina fulica*) from Colombia. 6th European *Dirofilaria* and *Angiostrongylus* Days (SiEDAD), 5.-7. Juli 2018, Belgrad, Serbien, *Proceedings* p 9

16. Lange, M K; Penagos-Tabares, F; Raue, K; Strube, C; Schaper, R; Hermosilla, C; Taubert, A (2018). Slug intermediate hosts of *Angiostrongylyus vasorum*: Vector competence and larval shedding capacity. 6th European *Dirofilaria* and *Angiostrongylus* Days (SiEDAD), 5.-7. Juli 2018, Belgrad, Serbien, *Proceedings* p 25

ABKÜRZUNGSVERZEICHNIS

L1 – erstes Larvenstadium

L2 – zweites Larvenstadium

L3 – drittes Larvenstadium

L4 – viertes Larvenstadium

L5 – fünftes Larvenstadium

ELISA – *Enzyme-linked immunosorbent assay*

PCR – Polymerase-Kettenreaktion

IFAT – *indirect fluorescent antibody test*

ET – *Extracellular traps*

DNA – Desoxyribonukleinsäure

INHALTSVERZEICHNIS

1. Einleitung

In der Vergangenheit waren Lungenwurminfektionen bei Hund und Katze oftmals nicht im Fokus der praktizierenden Tierärzte, obwohl sie zu schweren Erkrankungen führen können, die u. a. mit respiratorischen Erscheinungen und ggf. systemischer Ausbreitung und Tod einhergehen können (Bolt et al. 1994, Brennan et al. 2004). Bei Hunden ist *Angiostrongylus vasorum* der pathogenste Lungenparasit (Barutzki und Schaper 2009), weniger schwere Erkrankungen werden v. a. durch *Crenosoma vulpis*-Infektionen verursacht (Shaw et al. 1996). Bei der Katze spielt in Deutschland vor allem *Aelurostrongylus abstrusus* als Lungenparasit eine wichtige Rolle.

A. vasorum-, C. vulpis- und *A. abstrusus*-Infektionen wurden in diversen Ländern Europas, Süd- und Nordamerikas und Afrikas beschrieben. Das Vorkommen von *A. vasorum* erstreckt sich auf klimatisch variable Regionen, zu denen aride, tropische, subtropische sowie auch nearktische und paläarktische Klimazonen gehören (Bolt et al. 1994, Morgan et al. 2005, Jefferies et al. 2010a), was für eine hohe Anpassungsfähigkeit des Parasiten spricht.

Neuere Studien zeigen einen Trend zur Ausbreitung von *A. vasorum,* sowohl in bisher freie Gebiete innerhalb endemischer Länder Europas als auch in bisher nicht betroffene Länder (Morgan et al. 2005, Bourque et al. 2008, Traversa und Guglielmini 2008, Barutzki und Schaper 2009, Helm et al. 2009, Yamakawa et al. 2013). Dies wurde auch für Deutschland bestätigt (Barutzki et al. 2017, Maksimov et al. 2017). Die Ursachen dieser Ausbreitungstendenz sind bisher nicht klar. Neben Klimawandel und verstärkter Urbanisierung von Füchsen als Reservoirwirten, vermehrter Reisefreudigkeit von Hundebesitzern mit ihren Haustieren, stellt auch die Einfuhr und akzidentielle Verbreitung von Gastropoden eine Risikoquelle dar (Gloor et al. 2001, Morgan und Shaw 2010, Schnyder 2015b). Bezüglich der Ausbreitung der Zwischenwirte sind insbesondere klimatische Veränderungen hervorzuheben, da Gastropoden hochempfindlich auf Temperatur- und Feuchtigkeitsveränderungen reagieren. Mittels eines Klimamodells wurde gezeigt, dass schon heute die ökologischen und epidemiologischen Bedingungen zur Ausbreitung von *A. vasorum* in diversen, bisher nicht-endemischen Gebieten gegeben sind, was eine zukünftige Ausbreitung erwarten lässt (Morgan et al. 2009).

In die Lebenszyklen der genannten Lungenwürmer sind Schnecken als Zwischenwirte zwingend eingebunden. Welche Immunreaktionen seitens der Schnecken hier eine Rolle spielen, ist derzeit völlig ungeklärt. Die Infektion der Endwirte erfolgt in aller Regel über die perorale Aufnahme der Zwischenwirte. Als zusätzliche Infektionswege werden sowohl die perorale Aufnahme paratenischer Wirte als auch die Aufnahme von Larven aus der Umgebung diskutiert (Hobmaier und Hobmaier 1935, Hamilton 1963a, Bolt et al. 1993, Anderson 2000, Barcante et al. 2003, Lopez et al. 2005, Jefferies et al. 2009a).

Bisher liegen nur sehr wenige epidemiologische Untersuchungen zu Lungenwurminfektionen bei Zwischenwirten vor. Diese beziehen sich ausschließlich auf England und Dänemark. Hier wurde in hochendemischen Gebieten eine Schnecken-bezogene, von starken regionalen Schwankungen geprägte Prävalenz von bis zu 29,4 % in Swansea (Süd-Wales, Aziz et al. 2016) und bis zu 26 % im Raum Kopenhagen (Ferdushy et al. 2009) nachgewiesen. Für Deutschland sind solche Daten bisher nicht verfügbar. Daher war ein Ziel dieser Arbeit, epidemiologische Befunde zu Lungenwurminfektionen in natürlichen Schneckenpopulationen in den Bundesländern Hessen und Rheinland-Pfalz zu erheben, um daraus ein potenzielles Infektionsrisiko für die einheimische Hundepopulation ableiten zu können. Um zudem eine epidemiologische Lücke für Kolumbien zu schließen, sollten im Rahmen von Forschungsreisen Untersuchungen zum Vorkommen der genannten Lungenwurmarten in natürlichen Schneckenpopulationen in Kolumbien durchgeführt werden. Nach Etablierung von Schneckenkulturen und experimentellen Infektionssystemen war es außerdem ein Ziel dieser Arbeit, immunologische Reaktionen von Nacktschnecken auf Infektionen mit Lungenwürmern zu analysieren.

2. Literaturübersicht

2.1 Lungenwürmer bei Karnivoren und deren Bedeutung für Hund und Katze in Deutschland

Diverse Nematoden- und Trematodenarten durchwandern im Verlauf ihrer Entwicklung unter anderem die Lunge und können entsprechend Lungenschädigungen, einschließlich Pneumonien und granulomatöser Entzündungsprozesse, auslösen (Barutzki 2013). Klassische Lungenwürmer suchen jedoch den Respirationstrakt des Endwirtes nicht nur zur Passage auf, sondern vollenden dort ihre Entwicklung zu Adulten und bilden Reproduktionsprodukte (Barutzki 2013). Zu diesen gehören als Parasiten von Hund und Katze zwei unterschiedlichen Nematodengruppen, die trichuriden und metastrongyliden Nematoden (Barutzki 2013). Die trichuriden Nematoden, auch Lungenhaarwürmer genannt, leben in den Bronchien und der Trachea (*Eucoleus aerophilus,* Synonym *Capillaria aerophila*) oder in der Nase (v. a. in *Sinus nasales*; *Eucoleus boehmi*) des Endwirtes (Bowman 2000a, Bowman 2000b). Da trichuride Lungennematoden monoxene Parasiten darstellen und somit für ihre Entwicklung nicht auf einen Zwischenwirt angewiesen sind, wird diese Parasitengruppe hier nicht näher behandelt. Gleiches gilt für *Filaroides osleri* (Synonym *Oslerus osleri*) und *Filaroides hirti/milksi* (Familie: Filaroididae; Superfamilie: Metastrongylidae). Beide Spezies wurden nur in Einzelfällen bei Hunden nachgewiesen; bei letzterem steht die endgültige Speziesbestimmung noch aus (Geisel 1979, Schuster und Hamann 1993, Kresken et al. 1996).

Einige Spezies aus der Superfamilie der Metastrongyloidea infizieren Hunde und Katzen und kommen auch in Deutschland vor (Tab. 1). Von diesen sind Vertreter der Familien Angiostrongylidae und Crenosomatidae auf Gastropoden als Zwischenwirt angewiesen (Tab. 1). Im Folgenden werden diese beiden Familien eingehend behandelt und ihre Bedeutung für Hund (*Canis lupus familiaris*) und Katze (*Felis silvestris catus*) in Deutschland herausgestellt.

Tab. 1: In Deutschland vorkommende Metastrongyloidea bei Hund und Katze.

Familie	Spezies	Zwischenwirt	Endwirt	Vorkommen
Angiostrongylidae	*Angiostrongylus vasorum*	Nackt- und Gehäuse-schnecken	Wildkaniden, Hund	endemisch
	Angiostrongylus chabaudi	Nackt- und Gehäuse-schnecken	Wildfeliden, Katze	Einzelfälle (Wildkatzen)
	Aelurostrongylus abstrusus	Nackt- und Gehäuse-schnecken	Wildfeliden, Katze	endemisch
Crenosomatidae	*Crenosoma vulpis*	Nackt- und Gehäuse-schnecken	Wildkaniden, Hund	endemisch
	Troglostrongylus brevior	Nackt- und Gehäuse-schnecken	Wildfeliden, Katze	Einzelfälle (Wildkatzen)
Filaroididae	*Oslerus/ Filaroides osleri*	-	Wildkaniden, Hund	sporadisch
	Oslerus/ Filaroides hirthi/milksi	-	Wildkaniden, Hund	Einzelfälle

2.1.1 Angiostrongylidae

Die Familie der Angiostrongylidae umfasst u. a. die Gattungen *Angiostrongylus, Aelurostrongylus, Gurltia* und *Didelphostrongylus* (Anderson 2000). Hier wird nur auf die in Deutschland vorkommenden Arten eingegangen.

2.1.1.1 Gattung *Angiostrongylus*

Vertreter der Gattung *Angiostrongylus* kommen in Deutschland in Wildtierpopulationen und beim Hund vor (Steeb et al. 2014, Härtwig et al. 2015, Maksimov et al. 2017, Schug et al. 2018). In der Bundesrepublik wurde *A. vasorum* (Abb. 1) bei Rotfüchsen (*Vulpes vulpes*) und Hunden sowie *A. chabaudi* bei Wildkatzen (*Felis silvestris silvestris*) nachgewiesen (Barutzki und Schaper 2003, Barutzki und Schaper 2009, Taubert et al. 2009, Härtwig et al. 2015 , J. Hirzmann persönliche Mitteilung).

Angiostrongylus vasorum

A. vasorum wird beim Hund als der pathogenste Lungenparasit angesehen, da er eine lebensbedrohlichen Erkrankung im Endwirt verursachen kann (Denk et al. 2009, Yamakawa et al. 2013, Rinaldi et al. 2014). Aufgrund der Lokalisation der Adulten in der *Arteria pulmonalis* und der rechten Herzkammer des Endwirtes sowie seiner Erstbeschreibung in der Gegend von Toulouse, Frankreich (Serres, 1854), ist dieser Parasit auch als „französischer Herzwurm" bekannt (Bolt et al. 1994, Morgan et al. 2005, Ferdushy und Hasan 2010). In diversen europäischen Ländern, einschließlich Deutschland, wird von einer Ausbreitung von *A. vasorum*, vor allem in nördlichere Regionen, berichtet (Helm et al. 2010, Helm et al. 2015, Barutzki et al. 2017, Maksimov et al. 2017).

Der Lebenszyklus der hier beschriebenen Lungenwürmer ähnelt sich grundsätzlich stark und wird daher nur an dieser Stelle für *A. vasorum* ausführlich beschrieben (Abb. 1), während bei den anderen Spezies lediglich auf Unterschiede verwiesen wird. *A. vasorum* verfügt über ein relativ breites Wirtsspektrum sowohl im Bezug auf den Zwischenwirt (Guilhon 1965, Guilhon 1969, Rosen et al. 1970, Barcante et al. 2003, Ferdushy et al. 2009, Koch und Willesen 2009, Ferdushy und Hasan 2010) als auch auf den Endwirt (Bolt et al. 1994, Ferdushy und Hasan 2010, Morgan und Shaw 2010). Als potentielle Endwirte wurden diverse Caniden und Canoidea wie beispielsweise Wölfe (*Canis lupus*), Kojoten (*Canis latrans*), kleine Pandas (*Ailurus fulgens*) und Dachse (*Meles meles*) beschrieben (Guilhon 1965, Torres et al. 2001, Bourque et al. 2005, Saeed et al. 2006, Patterson-Kane et al. 2009, Hermosilla et al. 2017). Rotfüchse werden als das Hauptreservoir für *A. vasorum* angesehen (Bolt et al. 1992, Bolt et al. 1994). Adulte *A. vasorum*-Stadien befinden sich im Endwirt in der *Arteria pulmonalis* und der rechten Herzkammer. Dort pflanzen sie sich fort und setzen larvenhaltige Eier ab, die in das Kapillargebiet der Lunge geschwemmt werden. Dort schlüpfen die Erstlarven (L1) und migrieren durch das Lungenparenchym in die Alveolen der Lunge. Durch Auslösen des Hustenreflexes sowie durch die mukoziliäre *clearance* werden die L1-Larven in die oberen Atemwege transportiert und heruntergeschluckt. Nach Passage des Hundedarms gelangen L1-Stadien mit dem Kot in die Umwelt (Abb. 1). Die Präpatenz beträgt durchschnittlich 53 Tage, kann aber erheblich variieren (33 - 76 Tage) (Rosen et al. 1970, Oliveira-Junior et al. 2006, Morgan und Shaw 2010). Mit

dem Hundekot werden die Larven von diversen Schneckenarten aufgenommen (Rosen et al. 1970, Bolt et al. 1994, Barutzki 2013). Sie gelangen entweder passiv oral (Ferdushy et al. 2009) oder aktiv durch Penetration der Fußsohle in die Schnecke (Guilhon 1969, Rosen et al. 1970, Barcante et al. 2003, Bourque et al. 2008). Dort entwickeln sie sich zur L2 und anschließend, in Abhängigkeit von Umweltfaktoren wie Temperatur, vom 10. - 30. Tag *post infectionem* (p. i.) zur infektiösen L3 (Grewal et al. 2002, Barcante et al. 2003, Mozzer et al. 2011, Dias und Dos Santos Lima 2012).

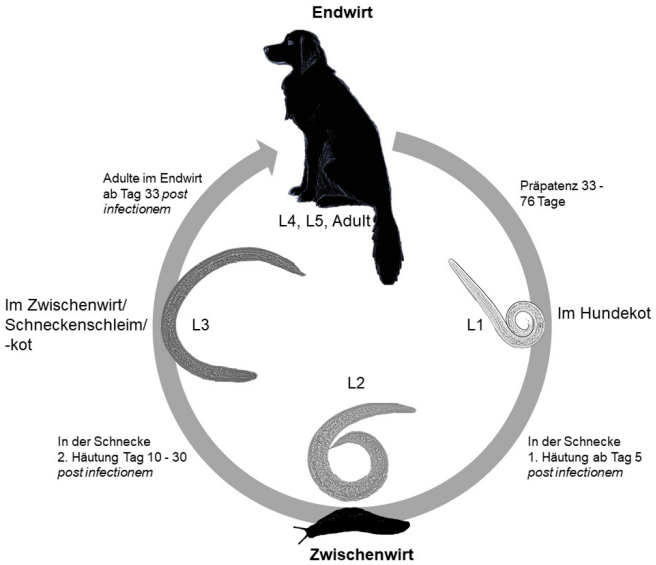

Abb. 1: Lebenszyklus von *Angiostrongylus vasorum*

In Schnecken befindliche L3 werden vom Endwirt oral aufgenommen und während des Verdauungsprozesses frei gesetzt (Rosen et al. 1970, Bolt et al. 1993, Barcante et al. 2003, Conboy et al. 2017). Als alternativer Infektionsweg wird die Aufnahme von Larven aus der Umgebung diskutiert, die zuvor die Schnecke aktiv über Schleim oder Kot verlassen haben (Barcante et al. 2003, Jefferies et al. 2009a, Conboy et al. 2017). Die Larven migrieren durch die Darmwand des Hundes in die Mesenteriallymphknoten, häuten sich zum L4- und L5-Stadium, gelangen in die Blutbahn und mit dem Blutstrom in die Lungenarterie bzw. die rechte Herzkammer, wo sie sich zu Adulten entwickeln (Rosen et al. 1970, Morgan et al. 2005, Koch und Willesen 2009, Barutzki 2013). In der Literatur werden diverse terrestrische Nackt- und Gehäuseschneckenarten als

natürliche Zwischenwirte, sowie aquatischen Schnecken als Auxiliar-Zwischenwirte beschrieben (Rosen et al. 1970, Guilhon und Cens 1973, Bolt et al. 1992, Barcante et al. 2003, Morley 2010). Ein Auxiliar- oder Hilfszwischenwirt ist typischerweise ein Zwischenwirt, der für den betreffenden Parasiten eine geringere Prävalenz und Infektionsintensität aufweist als die Hauptzwischenwirte (Morley 2010).

Die Verbreitung von *A. vasorum* in Ländern mit endemischem Vorkommen weist ein charakteristisches Verteilungsmuster auf, das von isolierten, hyperendemischen Foci (Morgan et al. 2009, Ferdushy und Hasan 2010), die von Gebieten mit einem deutlich geringeren Vorkommen des Parasiten umgeben sind, geprägt ist (Morgan et al. 2009, Ferdushy und Hasan 2010). Epidemiologische Untersuchungen zu *A. vasorum*-Infektionen bei deutschen Rotfüchsen als End- bzw. Reservoirwirte belegen eine starke Verbreitung des Parasiten in dieser Tierart in Deutschland (Härtwig et al. 2015, Schug et al. 2018). Entsprechend waren bis zu 27,3 % der Füchse in Thüringen, Rheinland-Pfalz und Hessen befallen (Schug et al. 2018); in Brandenburg wurde eine Prävalenz von 9 % nachgewiesen (Härtwig et al. 2015). Auch in anderen Ländern Europas wurde die Rolle des Fuchses als Reservoirwirt für *A. vasorum* bestätigt und entsprechend hohe Prävalenzen von 3,4 % bis 80 % (Tab. 2) belegt (Al-Sabi et al. 2014, Eleni et al. 2014b, Garrido-Castañé et al. 2015). Im Gegensatz dazu werden in Hunden i. d. R. deutlich niedrigere Prävalenzen zu *A. vasorum* konstatiert. Diese bewegten sich in den letzten Jahren für Deutschland zwischen 0,9 % und 2,3 % (Taubert et al. 2009, Barutzki et al. 2017, Maksimov et al. 2017). In der Population von Hunden mit respiratorischen Symptomen, Herz-Kreislauf-Erkrankungen, Blutungsneigung oder neurologischen Symptomen betrug die mittlere Prävalenz sogar 7,4 % (Barutzki und Schaper 2009). Kontinuierliche Untersuchungen von Barutzki et al. (2017) über 14 Jahre (2002-2016) wiesen auf ansteigende *A. vasorum*-Prävalenzen in der deutschen Hundepopulation der letzten Jahre hin. Die tatsächliche geographische Ausbreitung dieses Erregers in Deutschland wurde über statistische Analysen in einer aktuellen Studie von Maksimov et al. (2017) belegt. Auch in anderen europäischen Ländern wurden meist niedrige Prävalenzen bei Hunden gefunden (Tab. 3). In Dänemark und England lag die Prävalenz bei 2,2 % bzw. 3,2 %, während in Italien gebietsweise hohe Prävalenzen bis zu 13,2 % nachgewiesen wurden (Taubert et al. 2009, Al-Sabi et al. 2013, Schnyder et al. 2013a, Del Prete et al. 2015). Als Risikofaktoren für den Hund beschrieben Morgan et al. (2010) das Alter (jüngere Hunde sind häufiger betroffen), die Jahreszeit (erhöhtes Risiko zu Beginn des Jahres)

und eine unregelmäßige Entwurmung (Entwurmung 1-12 Wochen vor Kontakt mit Lungenwürmern gilt als Schutzfaktor). Das Geschlecht und die Rasse stellen laut dieser Studie keine Risikofaktoren dar (Morgan et al. 2010). Andere Studien zeigten ein erhöhtes Risiko für solche Hunderassen, die klassischerweise jagdlich genutzt werden (Conboy 2004).

Zur Prävalenz von *A. vasorum* in Zwischenwirten liegen nur wenige epidemiologische Daten aus der EU vor. Diese beziehen sich ausschließlich auf England und Dänemark. Hier wurde eine Schnecken-bezogene, von starken regionalen Schwankungen geprägte Prävalenz von bis zu 29,4 % in Swansea (Süd-Wales, Aziz et al. 2016) und bis zu 26 % in Kopenhagen (Ferdushy et al. 2009) nachgewiesen.

Tab. 2: Prävalenzdaten zu *A. vasorum* bei endemischen europäischen Fuchspopulationen (Erfassung ab 2000)

Land/Gesamtprävalenz		Regionen	Referenzen
Deutschland	14,1 %	Hessen: 8,4 % Rheinland-Pfalz: 27,3 % Thüringen: 19,1 %	Schug et al. 2018
		Brandenburg: 9 %	Härtwig et al. 2015
Dänemark		Kopenhagen: 80 % südliches Jütland: 0%	Al-Sabi et al. 2014
		Kopenhagen: 58 %	Al-Sabi und Kapel 2013
		Nord Seeland: 48,6 %	Saeed et al. 2006
England	18,3 %	Norden: 7,4 % Südosten: 50,8 % Osten: 12,9 %	Taylor et al. 2015
	7,3 %	Norden: 0 % Südosten: 23,2 % Osten: 1,6 % Binnenland: 4,8 % Süden: 6,9 %	Morgan et al. 2008
Italien		Nordwesten: 78,2 %	Magi et al. 2015
		Campagna : 33,3 %	Santoro et al. 2015
		Zentralitalien: 43,5 %	Eleni et al. 2014b
		Toskana: 7 %	Magi et al. 2009
Polen	5,2 %	Augustowska Ur-Wald	Demiaszkiewicz et al. 2014
Ungarn	17,9 %		Tolnai et al. 2015
	5 %		Sréter et al. 2003
Niederlande	4,2 %	Groningen/Limburg	Franssen et al. 2014
Portugal		Mitte, Westen: 7,14 %	Figueiredo et al. 2016
		Dunas de Mira: 16,1 %	Eira et al. 2006
Irland	39,9 %	in allen Bundesländern: 11,1 - 72,7 %	McCarthy et al. 2016
Rumänien	4,2 %	Cluj: 3,0 % Hunedoara: 8,2 % Mures: 10,9 %	Deak et al. 2017b

	Salaj: 4,0 %	
Schweiz	Osten, Nordosten: 47,4 %	Gillis-Germitsch et al. 2017a
Spanien	Pyrenäen: 3,4 %	Garrido-Castañé et al. 2015
	Baskenland: 33,3 %	Gerrikagoitia et al. 2010
	Murcia: 1,8 %	Martínez-Carrasco et al. 2007
	Katalonien: 22,7 %	Mañas 2005

Tab. 3: Prävalenzdaten zu *A. vasorum* bei europäischen endemischen Hundepopulationen (Erfassung ab 2000)

Land/Gesamtprävalenz		Regionen/Zeitraum	Referenzen
Deutschland	2,3 %	Nordrhein-Westfalen: 2,0 % Rheinland-Pfalz: 3,9 % Hessen: 1,8 % Baden-Württemberg: 3,6 % Bayern: 1,4 % Sachsen: 0 %	Maksimov et al. 2017
	0,9 %	2002-2006: 0,1 % 2007-2011: 0,8 % 2012-2016: 1,3 %	Barutzki et al. 2017
		Westen: 2,3 %	Schnyder et al. 2013a
	0,5 %		Barutzki und Schaper 2011
		Bayern: 0,36 %	Schulz et al. 2013
	7,4 %		Barutzki und Schaper 2009
	1,2 %		Taubert et al. 2009
	0,1 %		Barutzki und Schaper 2003
Dänemark	2,2 %		Al-Sabi et al. 2013
	2,2 %		Taubert et al. 2009
England		Süden: 3,2%	Schnyder et al. 2013a
Italien		Campagna: 13,2 %	Del Prete et al. 2015
		Sardinien: 3,4 %	Pipia et al. 2014
		Campagna: 1,04 %	Rinaldi et al. 2014
		Toskana: 2,3 % Ligurien: 3,8 %	Guardone et al. 2013
		Zentralitalien: 2,5 % Süden: 0 %	Di Cesare et al. 2011
Niederlande	0,8 %		van Doorn et al. 2009
Polen	1,3 %		Schnyder et al. 2013b
Portugal	1,3 %		Alho et al. 2016
Schweiz	3.1 %		Lurati et al. 2015

Belgien	4,7 %	Lempereur et al. 2016
Schweden	0,9 %	Grandi et al. 2017
Frankreich	3,2 %	Schnyder et al. 2017
Slowakei	4,4 %	Miterpáková et al. 2015
Ungarn	2,7 %	Schnyder et al. 2015

Lungenwurminfektionen können bei Hunden z. T. schwerwiegende Erkrankungen verursachen, die unterschiedliche Organsysteme betreffen können. So kann die Angiostrongylose mit milden respiratorischen Symptomen einhergehen, zu starker Dyspnoe und Lungenfibrose führen, mit kardiologischen und gastrointestinalen Symptomen behaftet sein oder aber auch subklinisch verlaufen (Brennan et al. 2004, Bourque et al. 2008, Traversa et al. 2008c, Koch und Willesen 2009, Schnyder et al. 2009). Besonders schwere Krankheitsverläufe mit meist tödlichem Ausgang resultieren i. d. R. aus einer infektionsbedingten, verstärkten Blutungsneigung (Traversa et al. 2008c, Denk et al. 2009, Schmitz und Moritz 2009). Weitere Symptome können neurologischer Natur sein oder das Auge betreffen. Diese beruhen meist auf Blutungen (infolge der erhöhten Blutungsneigung) oder auf fehlgewanderten Larven (Patteson et al. 1993, Brennan et al. 2004, Manning 2007, Denk et al. 2009, Koch und Willesen 2009, Barutzki 2013). Klinische Erkrankungen betreffen v. a. junge Hunde im Alter von 1 bis 2 Jahren (Staebler et al. 2005).

Da die Symptomatik der Angiostrongylose häufig wenig spezifisch ist und die L1-Ausscheidung im Kot intermittierend erfolgt (Oliveira-Junior et al. 2006), ist die Diagnosestellung selbst für den erfahrenen oftmals Tierarzt schwer. Der Nachweis von Erstlarven (Abb. 2) im Kot infizierter Tiere wird i. d. R. über das Trichterauswanderverfahren nach Baermann-Wetzel geführt (Cury et al. 2002, Koch und Willesen 2009). Jedoch können mit dieser Methode falsch negative Befunde

erhoben werden aufgrund der intermittierenden Larvenausscheidung, bei einer aus niedriger Wurmbürde resultierenden geringen Larvenausscheidung, bei geringer Vitalität der Larven oder während der langen Prävalenz (Oliveira-Junior et al. 2006, Verzberger-Epshtein et al. 2008, Schnyder et al. 2014). Aus diesen Gründen wird eine Sammelkotprobe von drei aufeinanderfolgenden Tagen empfohlen (Barutzki und Schaper 2009, Taubert et al. 2009, Paradies et al. 2013). Dieselben Einschränkungen betreffen andere koprologische Diagnostikverfahren zum Nachweis von Lungenwurminfektionen (Jefferies et al. 2011).

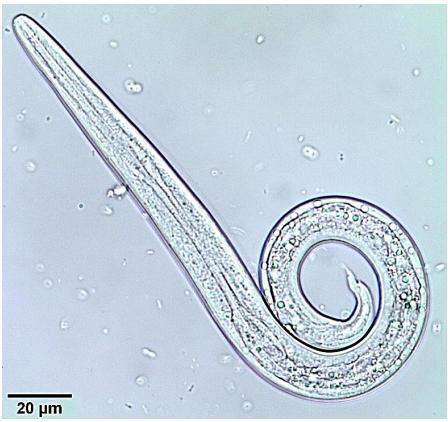

Abb. 2: *Angiostrongylus vasorum*-L1,

Quelle: Lange et al. 2018a

Im Vergleich zum Trichterauswanderverfahren ist der Kotausstrich eine wenig geeignete Methode zum L1-Nachweis aufgrund der geringeren Probenmenge und in Ermangelung einer Anreicherung der Larvenstadien (Traversa und Guglielmini 2008, Humm und Adamantos 2010). Bei der Zink-Sulfat-basierten Flotation ist dagegen oftmals die morphologische Beurteilung der Parasitenstadien infolge der Verformung der Larven erschwert (Conboy 2004, Schnyder et al. 2011a). Dies trifft auch auf einige Lösungen zu, die bei der FLOTAC-Technik eingesetzt werden (Schnyder et al. 2011a). Vergleiche mit den vorher genannten koproskopischen Methoden ergaben eine höhere Sensitivität der FLOTAC-Methode (Schnyder et al. 2011a). Vor allem bei Larven mit eingeschränkter Vitalität nach längeren Transportzeiten bietet diese Methode Vorteile gegenüber dem Trichterauswanderverfahren (Schnyder et al. 2011a).

Die mikroskopische Identifikation der in den Fäzes enthaltenen Lungenwurmlarven orientiert sich an spezifischen, morphometrischen Merkmalen wie Größe und Breite, das Ösophagus/Körper-Verhältnis und die Ausprägung des Hinterendes der Larven (Wetzel 1940, Ash 1970, Guilhon und Cens 1973, Traversa et al. 2010, Di Cesare et al. 2013, Giannelli et al. 2014), die eine Differenzierung von anderen Nematodenlarven ermöglichen (Traversa et al. 2010). Die charakteristische Ausprägung des Hinterendes

der Erstlarven eignet sich zudem zur Unterscheidung der verschiedenen Lungenwurmspezies (Ash 1970).

Für eine frühe Diagnose der Infektion noch während der Präpatenz (ab der 5. Woche *p. i.,* Schnyder et al. 2011b) eignen sich ELISA- und PCR-basierte Verfahren. Erstere weisen zirkulierende *A. vasorum*-Antigene in Hundeseren mit einer Spezifizität von 94 - 100 % und einer Sensitivität von 42,4 - 95,7 % nach (Verzberger-Epshtein et al. 2008, Schnyder et al. 2011b, Schnyder et al. 2014). Allerdings erfolgt der Nachweis des Parasiten mit dieser Methode auch noch eine gewisse Zeit nach dessen Elimination (Schnyder et al. 2011b). Kommerziell ist derzeit nur ein In situ-Test der Firma IDEXX Laboratories (Angio Detect™) erhältlich. Dieser weist ab der 14. Woche *p. i.* eine hohe Sensitivität auf; frühestens feststellbar ist die Infektion ab der 9. Woche *p. i.* (Schnyder et al. 2014). Antikörper-basierte Nachweisverfahren (ELISA) können ebenfalls zur Diagnose der *A. vasorum*-Infektionen bei Hunden eingesetzt werden (Guardone et al. 2013, Liu et al. 2017). Gemäß Schucan et al. (2012) kann über diese Methode die Infektion bereits 13 - 21 Tage *p. i.* (und somit ggf. vor einem Antigen-Nachweis) nachgewiesen werden. Es muss allerdings beachtet werden, dass ein positiver Befund lediglich auf eine erfolgte Infektion hinweist, was nicht mit einer Erkrankung gleichzusetzen ist (Schucan et al. 2012, Schnyder et al. 2013a). Der Nachweis der *A. vasorum*-Infektion über Immunoblotting stellt eine weitere Antikörper-basierte Alternative dar, ist jedoch aufgrund des methodischen Aufwands nur für geringe Probenzahlen geeignet (Cury et al. 2002).

Angiostrongylus chabaudi

A. chabaudi nutzt Wild- und Hauskatzen als Endwirte sowie Schnecken als Zwischenwirte (Biocca 1957, Varcasia et al. 2014, Traversa et al. 2015, Colella et al. 2017). Die Lokalisation der Adulten in der felinen *Arteria pulmonalis* entspricht der Situation bei *A. vasorum,* (Biocca 1957, Varcasia et al. 2014). Die Entwicklung der Larven im Zwischenwirt dauert etwa 6 - 10 Tage (Colella et al. 2017). Weitere Details zu dem Lebenszyklus und der Biologie des Parasiten, wie beispielsweise die Präpatenzdauer, sind noch unbekannt (Traversa et al. 2015, Giannelli et al. 2016, Colella et al. 2017).

A. chabaudi wurde erstmalig in Wildkatzen in Italien beschrieben; aktuell zeigte sich eine Prävalenz von bis zu 85 % (Biocca 1957, Giannelli et al. 2016, Veronesi et al. 2016). Später wurde der Parasit auch in Wildkatzenpopulationen anderer Länder wie Griechenland, Rumänien, Bulgarien oder Deutschland nachgewiesen (Diakou et al. 2016, Gherman et al. 2016, Giannelli et al. 2016, Veronesi et al. 2016 , J. Hirzmann persönliche Mitteilung). Infektionen bei Hauskatzen wurden bisher nur in Italien berichtet (Varcasia et al. 2014, Traversa et al. 2015, Giannelli et al. 2016, Colella et al. 2017). Da ein Großteil der *A. chabaudi*-Nachweise bei bis zu 2 Jahre alten Feliden geführt wurde, wird vermutet, dass jüngere Tiere einem höheren Infektionsrisiko ausgesetzt sind oder sich die Immunität noch bildet (Giannelli et al. 2016).

Sektionen von verunfallten Katzen zufolge löst *A. chabaudi* histopathologische Veränderungen aus, die mit einer Schädigung des Gefäßsystems der Lunge (z. B. hypertrophe Arterienwände) einhergehen und über Ödem- und Thrombenbildung charakterisiert sind (Diakou et al. 2016, Giannelli et al. 2016). Über die Symptomatik der felinen Angiostrongylose ist derzeit nichts bekannt. Aufgrund der phylogenetischen Verwandtschaft der Parasiten und der gleichen Lokalisation der Adulten in den Lungenarterien wird vermutet, dass die Erkrankung der caninen Angiostrongylose ähnelt und tödlich verlaufen kann (Giannelli et al. 2016).

Die Erstlarven im Katzenkot können grundsätzlich morphologisch von anderen Lungenwurmlarven der Katze unterschieden werden und ähneln denen von *A. vasorum* (Giannelli et al. 2016, Colella et al. 2017). Da die morphologische Unterscheidung dieser Stadien jedoch schwierig ist (Deak et al. 2017a) und Mischinfektionen mit anderen Lungenwürmern der Katze vorkommen können (Traversa et al. 2015), sollte die Diagnose über molekularbiologische Methoden wie PCR oder Sequenzierung abgesichert werden (Varcasia et al. 2014, Di Cesare et al. 2015, Giannelli et al. 2016). Derzeit sind keine kommerziell erhältlichen Diagnostika verfügbar. Gemäß Deak et al. (2017) kann *A. chabaudi*-Antigen auch über den Angio Detect™-Test nachgewiesen werden (Deak et al. 2017a).

2.1.1.2 *Aelurostrongylus abstrusus*

Aelurostrongylus abstrusus ist der häufigste Lungenwurm bei Hauskatzen (Pennisi et al. 2015, Traversa et al. 2015). Als Endwirte können neben der Hauskatze auch Wildfeliden fungieren (Scott 1973, Elsheikha et al. 2016). Der Lebenszyklus von

A. abstrusus unterscheidet sich von dem von *A. vasorum* über die signifikante Einbindung paratenischer Wirte, d. h. Katzen infizieren sich vornehmlich über den Verzehr solcher Tiere, die zuvor infizierte Schnecke aufgenommen haben (z. B. Nagetiere, Vögel, Amphibien und Reptilien) (Hobmaier und Hobmaier 1935, Hamilton 1963b, Anderson 2000, Jeżewski et al. 2013). Außerdem befinden sich die adulten Stadien nicht im Gefäßsystem, sondern im Lungenparenchym (v. a. Alveolargänge) und in den Bronchioli der Endwirte (Scott 1973, Elsheikha et al. 2016). Die Entwicklung infektiöser Larven im Zwischenwirt dauert etwa 11 Tage (Giannelli et al. 2014).

Dieser klassische Lungenwurm ist ein weltweit verbreiteter Parasit von Wildfeliden und Hauskatzen (Scott 1973, Barutzki und Schaper 2013). In Deutschland ist *A. abstrusus* endemisch verbreitet und weist schwankende Prävalenzen zwischen 0,5 % und 15,3 % bei Hauskatzen auf (Hiepe et al. 1988, Taubert et al. 2009, Barutzki und Schaper 2011, Barutzki und Schaper 2013). Steeb et al. (2014) wiesen diesen Parasiten bei 24,7 % der Wildkatzen nach. Die Infektion wurde vor allem bei Katzen unter sieben Jahren nachgewiesen (Barutzki und Schaper 2013). Es wird vermutet, dass *A. abstrusus* besonders bei Freigängerkatzen weit verbreitet ist (Barutzki und Schaper 2013). Im Gegensatz zu *A. vasorum* ist das Vorkommen von *A. abstrusus* nicht auf bestimmte Foci in Deutschland beschränkt (Barutzki und Schaper 2013).

Bei der Katze löst *A. abstrusus* respiratorische Symptome mit unterschiedlichem Verlauf aus. Die klinische Ausprägung der Aelurostrongylose kann grundsätzlich von subklinisch bis letal variieren (Traversa und Guglielmini 2008, Traversa et al. 2008c, Barutzki und Schaper 2013). Häufig rufen Infektionen mit *A. abstrusus* allerdings eine milde Symptomatik hervor und können selbstlimitierend sein (Traversa und Guglielmini 2008, Pennisi et al. 2015). Auch Mischinfektionen mit anderen Lungenwürmern wurden beschrieben (Di Cesare et al. 2014b, Traversa et al. 2015).

Kopromikroskopische Nachweisverfahren nutzen die charakteristische Morphologie des Hinterendes der *A. abstrusus*-L1 zur Speziesdiagnose. Dieses wird als eingekerbt und s-förmig beschrieben (Abb. 3) (Scott 1973, Giannelli et al. 2014). *Troglostrongylus brevior*- und *A. chabaudi*-Larven können allerdings morphologisch nur schwer differenziert werden (Brianti et al. 2013, Traversa et al. 2015). Daher sollten kopromikroskpopische Diagnosen mittels der PCR-Technik bestätigt werden (Penagos-Tabares et al. 2018a). Diverse PCR-basierte Nachweissysteme zeigten eine hohe Sensitivität und Spezifität für den *A. abstrusus*-Nachweis (Traversa et al. 2008a) und werden auch für die Analyse von Mischinfektionen genutzt (Annoscia et al. 2014, Traversa et al. 2015). Derzeit sind keine kommerziell erhältlichen, serologischen Tests zum Nachweis von *A. abstrusus*-Infektionen verfügbar (Barutzki und Schaper 2013, Elsheikha et al. 2016). In Forschungsarbeiten gelang der serologische Nachweis von *A. abstrusus*-spezifischen Antikörpern mittels IFAT (Briggs et al. 2013) oder ELISA (Schnyder 2015a, Elsheikha et al. 2016).

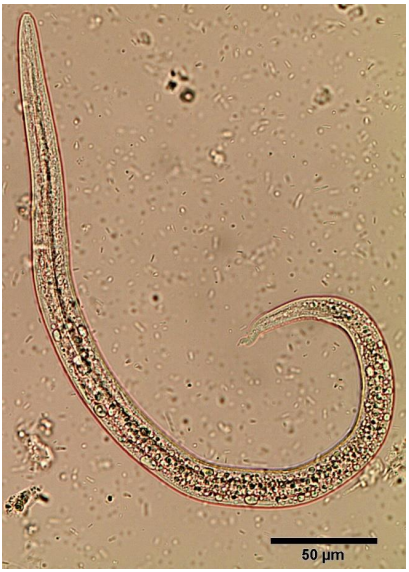

Abb. 3: *Aelurostrongylus abstrusus*-L1

Quelle: Penagos-Tabares et al. 2018b

2.1.2 Crenosomatidae

Innerhalb der Crenosomatidae kommt *C. vulpis* bei deutschen Haushunden und Rotfüchsen sowie *T. brevior* bei Wildkatzen vor (Taubert et al. 2009, Barutzki 2013, Steeb et al. 2014, Schug et al. 2018).

2.1.2.1 *Crenosoma vulpis*

Als Endwirte des Fuchslungenwurms dienen diverse Wildkaniden (Rotfuchs, Polarfuchs, Marderhund, Wolf, Kojote) und der Haushund (Wetzel und Müller 1935, Rausch et al. 1990, Thiess 2001, Bagrade et al. 2009, Bridger et al. 2009, Bružinskaitė-

Schmidhalter et al. 2012). Eine Besonderheit dieses Lungenwurms ist, dass er im Gegensatz zu *A. vasorum* eine Leberwanderung durchführt und in Folge Lebergewebsschäden hervorrufen kann (Stockdale und Hulland 1970, Barutzki 2013). Die Adulten residieren in den Bronchioli und Bronchien; die Präpatenz beträgt etwa 19 Tage (Stockdale und Hulland 1970). Die Entwicklung zur infektiösen L3 im Zwischenwirt dauert etwa 10 - 15 Tage (Colella et al. 2016).

Die Verbreitung von *C. vulpis* ist nahezu weltweit (Rausch et al. 1990, Sréter et al. 2003, Conboy 2004, Davidson et al. 2006, Magi et al. 2009). Die Prävalenzen von *C. vulpis* in der europäischen Fuchspopulation (Tab. 4) schwanken zwischen 1,3 % und 58 % (Davidson et al. 2006, Al-Sabi et al. 2014, McCarthy et al. 2016). In Europa wurden zur caninen Crenosomose vornehmlich Fallberichte veröffentlicht und nur wenige aktuelle epidemiologische Studien durchgeführt (zur Übersicht siehe Tab. 5). Dabei betrug die Prävalenz von *C. vulpis* bei dänischen Haushunden zwischen 0 % und 1,4 % (Taubert et al. 2009, Al-Sabi et al. 2013). Zu Deutschland liegen sowohl einzelne Fallberichte zur Crenosomose beim Hund als auch epidemiologische Studien beim Fuchs und beim Haushund vor (Kriegleder und Barutzki 1988, Taubert et al. 2009, Barutzki 2013, Maksimov et al. 2017, Schug et al. 2018). Die Prävalenz in der deutschen Hundepopulation schwankt von 0,4 % bis 6 % (Barutzki und Schaper 2009, Taubert et al. 2009, Barutzki und Schaper 2011, Maksimov et al. 2017). Das geographische Verteilungsmuster von *C. vulpis* ist im Gegensatz zu *A. vasorum* gleichmäßig (Barutzki 2013, Maksimov et al. 2017).

Tab. 4: Prävalenzdaten zu *Crenosoma vulpis* bei Füchsen in Endemiegebieten Europas (Erfassung ab 2000)

Land/Gesamtprävalenz		Regionen/Zeitraum	Veröffentlichung
Bosnien und Herzegowina	45,7 %		Hodžić et al. 2016
Dänemark	22,9	Kopenhagen, südliches Jütland	Al-Sabi et al. 2014
	19 %	Seeland, Jütland	Al-Sabi und Kapel 2013
	17,4 %		Saeed et al. 2006
England	10,8 %	Norden: 4,3 %	Taylor et al. 2015
		Binnenland: 10,5 %	
		Osten: 4,3 %	
		Südosten: 25 %	
		Süden: 9,7 %	
	2 %		Morgan et al. 2008
Irland	1,3 %		McCarthy et al. 2016
Italien		Süden 8,7 %	Latrofa et al. 2015
		Nordwesten 15,8 %	Magi et al. 2015
		Toskana 14,7 %	Magi et al. 2009
Litauen	53,8 %		Bružinskaitė-Schmidhalter et al. 2012
Niederlande		Groningen-Limburg 16,7 %	Franssen et al. 2014
Norwegen	58 %		Davidson et al. 2006
Portugal		Mitte, Westen 39,3 %	Figueiredo et al. 2016
		Dunas de Mira 3,2 5	Eira et al. 2006
Schweiz	10,5 %		Gillis-Germitsch et al. 2017a
Slowenien	2,8 %		Vergles Rataj et al. 2013
Spanien		Pyrenäen 44,8 %	Garrido-Castañé et al. 2015
		Murcia 0 %	Martínez-Carrasco et al. 2007
		Katalonien 33,9 %	Mañas 2005
Ungarn	24,6 %		Tolnai et al. 2015
	24 %		Sréter et al. 2003

Tab. 5: Prävalenzdaten zu *Crenosoma vulpis* bei Hunden in Endemiegebieten Europas (Erfassung ab 2000)

Land/Gesamtprävalenz		Regionen/Zeitraum	Veröffentlichung
Deutschland	2,2 %	Nordrhein-Westfalen: 1,7 % Rheinland-Pfalz: 1,9 % Hessen: 3,2 % Baden-Württemberg: 2,1 % Bayern: 2,5 % Sachsen: 3,3 %	Maksimov et al. 2017
	0,4 %	2002-2006: 0,2 % 2007-2011: 0,5 % 2012-2016: 0,5 %	Barutzki et al. 2017
		0,4 %	Barutzki und Schaper 2011
		6,0 %	Barutzki und Schaper 2009
	2,4 %		Taubert et al. 2009
	0,6 %		Epe et al. 2004
	0,3 %		Barutzki und Schaper 2003
Dänemark	1,4 %		Taubert et al. 2009
	0 %		Al-Sabi et al. 2013

Infektionen von Hunden mit *C. vulpis* bleiben asymptomatisch oder führen zu milden respiratorischen Symptomen (Stockdale und Hulland 1970, Shaw et al. 1996, Barutzki 2013). Entsprechend zeigen sich Husten, Rhinitis, Leistungsschwäche, Anorexie, Tachypnoe und Dyspnoe (Stockdale und Hulland 1970, Bylin 2010, Barutzki 2013). Bei der *C. vulpis*-Infektion können auch Leberschädigungen auftreten, die aus der Leberwanderung der Larven (Stockdale und Hulland 1970, Bylin 2010) resultieren. Auch chronische Verläufe wurden beschrieben (Conboy 2004).

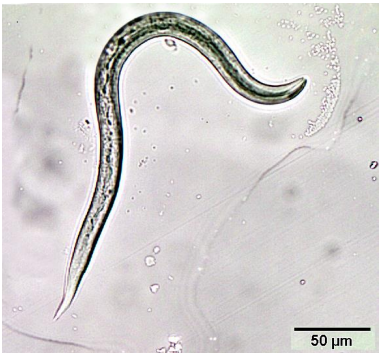

Abb. 4: *Crenosoma vulpis*-L1

Zur morphologischen Diagnose eignet sich das punktförmig gerade zulaufende Hinterende der L1 von *C. vulpis,* das leicht gebogen ist (Abb. 4) (Traversa et al. 2010, Barutzki 2013). Neben koproskopischen Diagnostikmethoden kann auch eine spezifische PCR zum Nachweis von *C. vulpis* verwendet werden (Schug et al. 2018). Derzeit befinden sich keine kommerziellen Testverfahren zum Nachweis von *C. vulpis*-Infektionen auf dem Markt (Traversa et al. 2010).

2.1.2.2 *Troglostrongylus brevior*

Als Endwirte fungieren bei *T. brevior* Wildfeliden und Hauskatzen (Brianti et al. 2014, Steeb et al. 2014). Als Besonderheit von *T. brevior* wird in Einzelfällen beschrieben, dass eine laktogene oder transplazentäre Übertragung der Infektion von dem Muttertier auf die Welpen erfolgen kann (Brianti et al. 2013). In aller Regel erfolgt die Infektion des Endwirts aber über die perorale Aufnahme infizierter Zwischenwirte oder paratenischer Wirte (Anderson 2000). Die Entwicklung bis zur infektiösen L3 im Zwischenwirt dauert etwa 8 - 40 Tage (Gerichter 1949, Giannelli et al. 2014). Adulte *T. brevior*-Stadien residieren in den Bronchien der Endwirte. Die Präpatenz beträgt 28 Tage (Gerichter 1949).

Die Troglostrongylose löst häufig schwere respiratorische Symptome aus, die zum Tod des Endwirts führen können, vor allem bei jungen Katzen (Brianti et al. 2012, Di Cesare et al. 2014b, Cavalera et al. 2018). Entsprechend werden Dyspnoe, Tachypnoe, Husten, Nasenausfluss, Anorexie und Lethargie beobachtet (Brianti et al. 2012, Traversa et al. 2014).

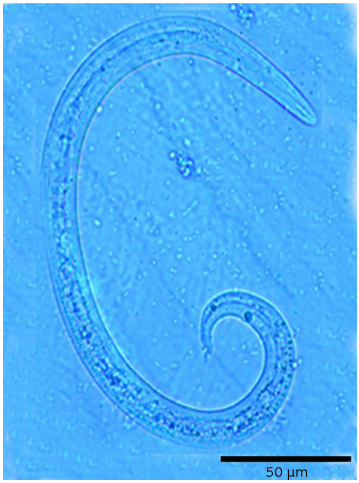

Abb. 5: *Troglostrongylus brevior*-L1

In der koproskopischen Diagnostik sind die Erstlarven unterschiedlicher Lungenwurmarten der Katze nur schwer zu unterscheiden (Brianti et al. 2013, Traversa et al. 2015). Das charakteristische Hinterende von *T. brevior* wird als graduell spitz zulaufend mit einer tiefen Einkerbung beschrieben, welche den Anhang des Hinterendes in einen flacheren ventralen Dorn und einen deutlichen dorsalen Fortsatz unterteilt (Abb. 5) (Brianti et al. 2012, Giannelli et al. 2014). Bisher wurden diverse PCR-Systeme zum spezifischen Nachweis von *T. brevior* beschrieben (Jefferies et al. 2010b, Annoscia et al. 2014, Di Cesare et al. 2015). Allerdings ist derzeit kein serologischer oder molekularbiologischer Test kommerziell erhältlich.

2.2 Gastropoden als Zwischenwirte für Lungenwürmer

Phylogenetisch ist der Parasitismus von Weichtieren weit unter Nematoden verbreitet und hat sich mehrfach und unabhängig voneinander in verschiedenen Vertretern der Nematoda entwickelt (Grewal et al. 2003). Invertebraten als Wirte zu nutzen, scheint benefitär für Nematoden zu sein und diese zeigen eine hohe Plastizität in der Wahl der Weichtier-Wirtsspezies (Grewal et al. 2003). Entwicklungsgeschichtlich wird angenommen, dass die Nutzung von Weichtieren als Wirte der Nutzung von Vertebraten zeitlich vorausgegangen ist (Grewal et al. 2003).

Neben den metastrongyliden Lungenwürmern sind auch protostrongylide Lungenwürmer auf Schnecken als Zwischenwirte angewiesen (Anderson 2000, Grewal et al. 2003). *Muellerius capillaris*, *Cystocaulus ocreatus* oder *Neostrongylus linearis* sind beispielsweise typische Lungenwürmer bei kleinen Wiederkäuern, während *Elaphostrongylus* und *Parelaphostrongylus* vor allem in nördlichen Regionen Cervidae befallen (Boev 1975, Rezác et al. 1994, Anderson 2000, Georgiev und Georgiev 2002, Grewal et al. 2003, Jenkins et al. 2006). Bei diesen Protostrongyliden wurden diverse Nacktschneckenspezies als Zwischenwirte beschrieben, unter anderem *Limax-, Arion-* und *Deroceras*-Spezies (Boev 1975, Anderson 2000, Grewal

et al. 2003). In einer epidemiologischen Studie von Georgiev und Georgiev (2002) waren 0 - 23,6 % der Schnecken in Bulgarien mit *M. capillaris* infiziert, 0 – 2,7 % mit *N. linearis*, 0 – 10,8 % mit *C. ocreatus* und 0 – 7,3 % mit *Protostrongylus* sp. Bezüglich *Parelaphostrongylus odocoiley* konnte bei Gastropoden in Kanada eine Prävalenz von 0,6 % bis 5,3 % nachgewiesen werden (Samuel et al. 1985). Eine andere Studie aus Kanada wies in 0,6 % der *Deroceras-laeve* Schnecken die morphologisch nicht unterscheidbaren Protostrongyliden *Parelaphostrongylus andersoni* bzw. *Elaphostrongylus rangiferi* nach (Lankester und Fong 1998).

Als Zwischenwirte der metastrongyliden Lungenwürmer der Karnivoren dienen ebenfalls diverse Vertreter terrestrischer Lungenschnecken (Rosen et al. 1970, Guilhon und Cens 1973, Bolt et al. 1992, Anderson 2000). Als Auxiliar-Zwischenwirte wurden auch aquatische Schnecken beschrieben (Barcante et al. 2003, Morley 2010). Landschnecken gehören zu Primärkonsumenten (Wieser 1978) und ihr koprophages Verhalten ermöglicht die Infektion mit metastrongyliden Lungenwurmlarven aus dem Kot des Hundes (Patel et al. 2014, Schnyder 2015b, Aziz et al. 2016). Nackt- und Gehäuseschnecken gehören zum Stamm der Weichtiere (Mollusken) und zur Klasse der Bauchfüßler (Gastropoda), die in die informelle Gruppe der Lungenschnecken (Pulmonata) einzuordnen ist (Nordsieck 2000). In Untersuchungen an Nacktschnecken konnte gezeigt werden, dass die Entwicklung von *A. vasorum*- und *A. abstrusus*-Larven von klimatischen Bedingungen und Umweltfaktoren abhängt (Hamilton 1963b, Lopez et al. 2005, Ferdushy et al. 2010, Di Cesare et al. 2013, Giannelli et al. 2014). Dabei wurde sowohl der Effekt von Größe bzw. Alter der Zwischenwirte als auch die Auswirkung unterschiedlicher Umgebungstemperaturen auf die Entwicklung des Lungenwurms untersucht. Die Autoren gelangten dabei zu dem Ergebnis, dass mit zunehmender Schneckengröße und mit steigender Umgebungstemperatur die Parasitenbürde steigt und die Entwicklung der Larven in der Schnecke gefördert wird (Hamilton 1963b, Lopez et al. 2005, Ferdushy et al. 2010, Di Cesare et al. 2013, Giannelli et al. 2014). Zusätzlich wurde auch der Einfluss der Lagerung der Larven vor der Infektion bei verschiedenen Umgebungstemperaturen auf die Entwicklung der Larven in den Schnecken untersucht (Ferdushy et al. 2010, Dias und Dos Santos Lima 2012, Conboy et al. 2017). Dabei wurde gezeigt, dass eine Lagerung bei höherer Temperatur eine Verringerung der Infektiosität der Larven zur Folge hat. Der gleiche Effekt wurde für die Dauer der Lagerung konstatiert. Die Ausscheidung lebender Lungenwurmlarven im Schleim von Nacktschnecken konnte

bisher nur bei einer von 43 natürlich infizierten Schnecken in einer epidemiologischen Studie aus England nachgewiesen werden (Jefferies et al. 2009a). Bei experimentellen Infektionen wurde gezeigt, dass L3-Larven von *A. vasorum, C. vulpis, A. abstrusus, T. brevior* und *Troglostrongylus wilsoni* die Schnecken aktiv verlassen können (Barcante et al. 2003, Giannelli et al. 2015b, Conboy et al. 2017) und dass das Ausmaß der Ausscheidung infektiöser L3-Larven abhängig von klimatischen Stimuli wie Licht und Wassertemperatur ist (Heyneman und Lim 1967, Barcante et al. 2003, Giannelli et al. 2015b). Die Autoren erklären diesen Effekt durch eine gesteigerte Muskelkontraktion der durch die Umweltstimuli gestressten Schnecken.

In Europa wurden bisher nur wenige Studien zur Prävalenz metastrongylider Lungenwürmer in der Schneckenpopulation durchgeführt. In Dänemark betrug die mittlere *A. vasorum*-Prävalenz in Schnecken aus dem hochendemischen Gebiet Kopenhagens 9 % (Ferdushy et al. 2009), allerdings lag eine starke, gebietsassoziierte Schwankung der Prävalenzen (0-26 %) vor. In Großbritannien zeigten unterschiedliche Studien zu Zwischenwirten mittlere *A. vasorum*-Prävalenzen von 1,6 %, 6,7 % und 29,4 % auf (Patel et al. 2014, Helm et al. 2015, Aziz et al. 2016). Auch hier variierten die Daten stark in geographischer Abhängigkeit.

In Deutschland sind die Gattungen folgender Nacktschnecken am weitesten verbreitet: *Arion, Deroceras* und *Limax* (Lehmann 2005, Naturhistorische-Gesellschaft 2018). Diesen Nacktschnecken ist allen gemeinsam, dass sie Zwitter sind (Nordsieck 2000). Da diese Schneckengattungen in der vorliegenden Arbeit als Zwischenwirte verwendet wurden, werden ihre Eigenschaften hier kurz näher beschrieben.

Arion lusitanicus

Arion lusitanicus, die spanische Wegschnecke (Synonym *A. vulgaris*), stellt taxonomisch eine unklare Gattung mit zum Teil stark voneinander abweichenden Genvarianten dar (NABU 2018). Sie besitzt den typisch glockenförmigen Umriss der Wegschnecken (Familie Arionidae) beim Zusammenziehen des Körpers mit einem Mantel, der nur das vordere Drittel des Körpers einnimmt (Abb. 6). Das Atemloch (Pneumostom) befindet sich in der vorderen Hälfte auf der rechten Seite. Die Farbe der Individuen ist hoch variabel, es kommen hellbraune, orange, dunkelbraune bis fast schwarze, rötlich gefärbte und hellgelbe Individuen vor. Die Jungtiere sind häufig

Abb. 6: *Arion lusitanicus*
(ein juveniles und ein adultes Exemplar)

gelblich mit jeweils einem dunklen Längsband auf jeder Körperseite (Fechter und Falkner 1990, Nordsieck 2000). Die spanische Wegschnecke kann eine Größe von bis zu 8 cm erreichen und ist in der Regel einjährig (Welter-Schultes 2012). Diese Schneckenart wurde lange als invasiver Neozoon aus Südeuropa betrachtet (Zemanova et al. 2016), Genanalysen lassen jedoch vermuten, dass die Schnecke bereits in Mitteleuropa heimisch war (NABU 2018).

Deroceras reticulatum

Der genetzte Ackerschnegel (*D. reticulatum*) ist als Agrar- und Gartenschädling anzusehen (Nordsieck 2000, Barker 2002). Er gehört zur Familie der Ackerschnegel (Agriolimacidae) und ist durch sein distal liegendes Atemloch gekennzeichnet. Der genetzte Ackerschnegel ist eine kleine, einjährige Nacktschneckenart mit einer Größe von bis zu 6 cm (Nordsieck 2000). Gekennzeichnet ist diese Art durch die meist hellbraune Grundfarbe mit dunklen, netzartigen Flecken (Abb. 7). Im Gegensatz zu *A. lusitanicus* fehlt den Schnegeln die Fähigkeit, sich glockenförmig zusammenzuziehen. Das Pneumostom ist von einem leicht helleren Ring umgeben und der Kiel am Schwanzende ist kurz und abgestumpft, die Sohle ist hell

Abb. 7: *Deroceras reticulatum*
(Quelle unteres Bild:
https://en.wikipedia.org/wiki/Deroceras_reticulatum)

(Frömming 1954). Besonders hervorzuheben ist die Paarungsmethode von *D. reticulatum* (Abb. 7), da der Penis ein Stimulationsorgan besitzt, mit dem sich die Schnecken vor der Kopulation reizen (Nordsieck 2000).

Limax maximus

Der Tigerschnegel (*Limax maximus*) stellt eine der größten Schnegelarten Deutschlands dar und ist vermutlich aus Süd- und Westeuropa nach Mitteleuropa eingewandert (Nordsieck 2000). Wie alle Schnegel besitzt auch *L. maximus* einen Rückenkiel, der sich vom Schwanzende bis etwa Mitte des Rückens entlangzieht (Nordsieck 2000). Grundsätzlich besitzt der Tigerschnegel eine hellgraue Grundfarbe mit dunkler Streifung oder Tupfenzeichnung (Nordsieck 2000). Ein eindeutiges Merkmal ist jedoch seine einfarbig hellgraue Fußsohle (Nordsieck 2000). Wie die Mehrheit der Familie der Limacidae ist *L. maximus* eigentlich ein waldlebender Schnegel, hat sich aber hierzulande als Kulturfolger etabliert und ist in Gärten, Parks, Friedhöfen und Kellern zu finden (Nordsieck 2000). Zur Nahrung gehören vor allem Pilze, Flechten und Algen als auch andere Nacktschneckenarten und deren Gelege. Somit ist *L. maximus* nicht primär als Gartenschädling, sondern als Nützling zu betrachten (Nordsieck 2000, Ørmen et al. 2009, Ørmen et al. 2010). Der Tigerschnegel ist heutzutage nahezu weltweit verbreitet (Nordsieck 2000). Bekannt ist *L. maximus* vor allem durch seine artistisch anmutende Paarungsmethode (Chase 1952, Nordsieck 2000), bei der sich zwei Tigerschnegel aus einer erhöhten Position, wie etwa einem Mauervorsprung oder einem Ast, an einem

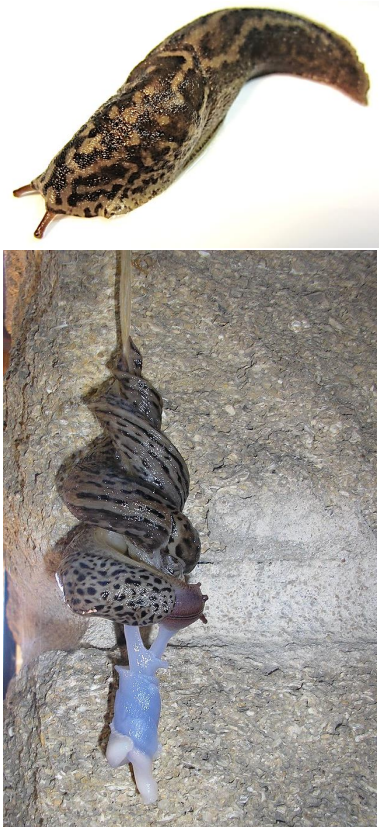

Abb. 8: *Limax maximus*
(Quelle unteres Bild: https://commons.wikimedia.org/wiki/Limax_maximus)

Schleimfaden herablassen. Die Paarung erfolgt dann freihängend und eng umeinander umschlungen (Abb. 8).
Aufgrund der fakultativ karnivoren Lebensweise des Tigerschnegels eignet sich diese Nacktschneckenart besonders für die Erforschung der sogenannten *Intermediesis.* Dieses Phänomen wurde bei Infektionen mit *A. abstrusus* und *T. brevior* bereits beschrieben und bedeutet, dass mit Schneckenschleim ausgeschiedene Larven sowie Larven in toten Schnecken andere Schnecken erfolgreich infizieren können (Colella et al. 2015). Dadurch können Larven aufgrund der erneuten Infektion weiterer Zwischenwirte den schädlichen Umwelteinflüssen entgehen, denen sie ansonsten ausgesetzt wären, wenn sie von Schnecken aktiv oder über deren Tod ausgeschieden würden (Colella et al. 2015).

2.3 Immunreaktionen von Gastropoden

Es wird angenommen, dass Mollusken ausschließlich über ein nicht-adaptives, angeborenes Immunsystem verfügen und sogenannte Hämozyten (Synonym Amöbozyten) die Hauptrolle bei Pathogen-induzierten Abwehrreaktionen übernehmen (Yoshino et al. 2013, Beck und Peatman 2015). Zusätzlich üben Hämozyten zahlreiche weitere Funktionen aus und sind beispielsweise in Wundheilung, Koagulation, Transport von Nährstoffen und anderen Molekülen oder in den intrazellulären Verdau eingebunden (Cheng 1984, Nakayama et al. 1997). Derzeit bekannte Effektormechanismen von Hämozyten umfassen Phagozytose, multizelluläre Einkapselung und zellvermittelte Zytotoxizität (Matricon-Gondran und Letocart 1999, Humphries und Yoshino 2003, Little et al. 2005, Sokolova 2009, Loker 2010).

2.3.1 Einkapselung und Melanisierung

Bei der Einkapselung von großen Pathogenen, wie beispielsweise Lungenwurmlarven, sammeln sich zu Beginn des Prozesses Hämozyten in großer Zahl um den Parasiten an, was als leukozytäre Kapselbildung bezeichnet wird (Sauerländer 1976). Wenige Tage später sind längliche Zellen mit spindelförmigen Kernen sichtbar, die sich konzentrisch in Schichten anordnen; dies entspricht der Phase der fibroblastischen Kapselbildung (Sauerländer 1976). Später flacht die Kapselwand ab und in ihrem Zentrum findet Karyolyse statt, wodurch die Kapselhöhle gebildet wird (Sauerländer 1976). Es wird davon ausgegangen, dass bei Schnecken keine Muskelfasern an der Bildung der Kapsel in Reaktion auf Parasiten beteiligt sind (Sauerländer 1976). In der

späten Phase der Infektion wurde beschrieben, dass sich die Kapsel einseitig abflacht und sich so eine Prädilektionsstelle für den Schlupf der infektiösen L3 bilden könnte (Sauerländer 1976).

L3 von *Angiostrongylus cantonensis* können nach Abschluss der Kapselbildung in diesem Kompartiment auch als Dauerstadium bis zur Infektion des Endwirts verweilen (Cheng und Alicata 1964). Bei Muscheln und Gastropoden folgt auf den Prozess der Einkapselung die Aktivierung der gastropoden Phenoloxidase, was letztendlich in einer Melanisierung mündet (Le Bris et al. 2013, Coaglio et al. 2018), die gelegentlich zur Eliminierung des Parasiten führt (Le Bris et al. 2013). Die Phenoloxidase wird durch externe Komponenten von Mikroorganismen aktiviert, wie beispielsweise durch Lipopolysaccharide (Perazzolo und Barracco 1997). Durch die Hydroxylierung von Monophenolen wird mit Hilfe der Phenoloxidase eine Kaskade ausgelöst, an deren Ende Melanin gebildet wird, welches wiederum zur Erstickung des eingekapselten Parasiten führen kann (Nappi et al. 1992, Söderhäll und Cerenius 1998, Le Clec'h et al. 2016). Während dieses Prozesses werden zytotoxische Substanzen wie reaktive Sauerstoff- und Stickstoffspezies und Chinoide gebildet. Sie stellen Zwischenprodukte des Melanins dar (Coaglio et al. 2018).

Die Einkapselung von Lungenwurmlarven bei Gastropoden hängt von der Umgebungstemperatur und der Schneckenspezies ab (Alicata und Jindrák 1970). Bei mit *A. vasorum* infizierten Achatschnecken (*Achatina fulica*) beginnt ab dem ersten Tag der Infektion eine zelluläre Infiltration um die Larven im Gewebe, verbunden mit Melanindeposition in der Umgebung der Larve (Coaglio et al. 2018). Sauerländer et al. (1976) beschrieben diese leukozytäre, *A. vasorum*-induzierte Kapselbildung in derselben Zwischenwirtsspezies bereits ab zwölf Stunden *p. i.* Bei der Wasserschnecke *Biomphalaria glabrata* findet die zellluläre Infiltration um *Angiostrongylus cantonensis* (zoonotischer Rattenlungenwurm) zwischen 24 und 48 Stunden nach der Infektion statt (Harris und Cheng 1975b, Harris und Cheng 1975a). Bei den Katzenlungenwürmern *A. abstrusus* und *T. brevior* wird das sich bildende Granulom in Gehäuseschnecken (*Helix aspersa*) ab dem neunten Tag *p. i.* als hämozytenreich beschrieben wird (Giannelli et al. 2015b). Bei *A. vasorum*-infizierten *A. fulica* wird ab dem dritten Infektionstag die fibroblastische Kapselbildung beobachtet (Sauerländer 1976). In *B. glabrata* beginnt sich von Tag sechs bis zwölf ein faserreicher Knoten um die *A. vasorum* Larven zu bilden (Kramer et al. 2018). Ab Tag

fünfzehn konnten in *H. aspersa*-Schnecken kleine nekrotische Kapseln um Katzenlungenwurmlarven entdeckt werden (Giannelli et al. 2015b). Insgesamt wurden bei dieser Schneckeninfektion sowohl milde Reaktionen auf die Larven gefunden (Gefäßdilatationen, leichte Zellzahlerhöhung im Gewebe, kleine Granulome) als auch schwerwiegende lokale Reaktionen (nekrotische Granulome) nachgewiesen (Giannelli et al. 2015b). Die schwereren Gewebsreaktionen wurden dabei vornehmlich in Reaktion auf *T. brevior* beobachtet, was die Vermutung nahe legt, dass diese Lungenwurmspezies schädlicher für *H. aspersa* ist und somit schlechter an diese Zwischenwirtsspezies angepasst scheint (Giannelli et al. 2015b). Bei *A. vasorum*-infizierten *A. fulica* zeigten sich ab Tag 18 *p. i.* erstmals abgeflachte Kapseln um die gebildeten L3 (Sauerländer 1976). Auch aus der Kapsel befreite Larven wurden in dieser Untersuchung in einem Lungengefäß der Schnecke festgestellt (Sauerländer 1976). Die Einkapselung erreicht das maximale Ausmaß bei mit *A. vasorum*-infizierten *B. glabrata* ab Tag 20 *p. i.* (Harris und Cheng 1975a, Harris und Cheng 1975b). Eingekapselte Larven überlebten in dieser Wasserschnecke bis zu zwölf Monate (Richards und Merritt 1967). Die Melaninbildung in der Umgebung der *A. vasorum*-Larve nahm bei *A. fulica* ab Tag 13 *p. i.* ab (Coaglio et al. 2018).

2.3.2 Extracellular traps

Das Verständnis der Phagozyten-vermittelten angeborenen Immunabwehr hat sich mit der Entdeckung von sogenannten „*extracellular traps*“ grundlegend verändert (Brinkmann et al. 2004, Cheng und Palaniyar 2013, Silva et al. 2016). Dieser Mechanismus wurde zuerst bei Säugetieren entdeckt (Brinkmann et al. 2004) und wird als nutzbringender Suizid von Phagozyten bezeichnet, der zum Ziel hat, Pathogene extrazelluär zu bekämpfen (Brinkmann und Zychlinsky 2007). Auch für Mollusken, wie Austern, Muscheln und Krabben, konnte gezeigt werden, dass Hämozyten grundsätzlich zur Bildung von „*extracellular traps*“ (ETs) befähigt sind (Poirier et al. 2014, Robb et al. 2014) und dass über ETs Pathogene extrazellulär abgefangen und gegebenenfalls getötet werden können.

Im Verlauf der ETose (zur Übersicht siehe Abb. 9) wird die nukleäre DNA des Phagozyten aus der Zelle ausgeschleust und mit diversen, aus zytoplasmatischen Granula stammenden antimikrobiellen Substanzen sowie mit nukleären Histonen versetzt, die dann auf extrazelluläre Pathogene in konzentrierter Form einwirken

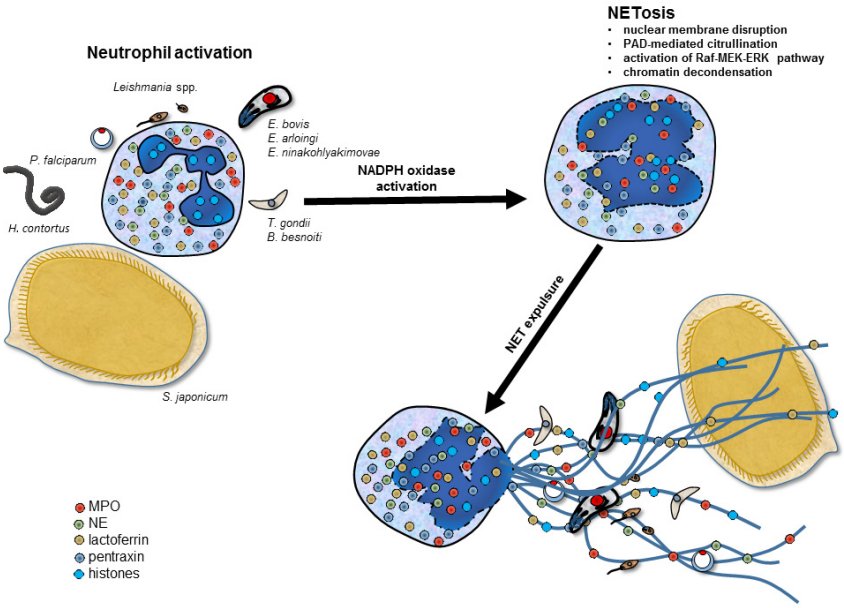

Abb. 9: Schema der NETosis modifiziert nach Hermosilla et al. (2014)

können (Brinkmann et al. 2004). Dabei desintegriert sich nach Stimulation der Phagozyten die Kernmembran, wodurch sich Chromatin mit Proteinen und Peptiden der zytoplasmatischen Granula vermischen kann (Fuchs et al. 2007). Histone (H1, H2A/H2B, H3, H4) werden dabei bei Säugetieren über Aktivitäten von neutrophiler Elastase und Myeloperoxidase degradiert und so die Chromatindekondensierung ermöglicht (Papayannopoulos et al. 2010). Letzteres wird über Hypercitrullinierung spezifischer Histone durch die Peptidylarginin Deaminase Typ 4 erreicht (Wang et al. 2009, Li et al. 2010, Leshner et al. 2012). Für ETose in Säugetieren ist beschrieben, dass dieser Prozess meist in Abhängigkeit von einer NADPH Oxidase-Aktivität abläuft (Abb. 9) (Muñoz-Caro et al. 2014a, Silva et al. 2014, Muñoz-Caro et al. 2015a). Andere benötigte Moleküle sind unter anderem reaktive Sauerstoffspezies (ROS), Defensine, Cathelicidine, Pentraxin, Calprotektin und Laktoferrin (Nathan 2006, Urban et al. 2009, Parker et al. 2012). Bei der ETose handelt es sich um einen hochregulierten, unter Beteiligung diverser antimikrobieller Moleküle ablaufenden Prozess (Fuchs et al. 2007, Papayannopoulos und Zychlinsky 2009, Abi Abdallah und Denkers 2012, Abi Abdallah

et al. 2012, Gray et al. 2013). Die bisher nachgewiesenen, ETose-assoziierten zellulären Signalkaskaden schließen die Beteiligung von *Rapidly Accelerated Fibrosarcoma* (Raf)/ Mitogen-aktivierte Proteine (MAP), über extrazelluläre Signale regulierte (ERK) Kinasen und p38 MAP Kinasen ein (Palic et al. 2007, Muñoz-Caro et al. 2015b). Zusätzlich wird für den erfolgreichen Ablauf der ETose Kalziumeinstrom benötigt (Palic et al. 2007, Muñoz-Caro et al. 2015a, Muñoz-Caro et al. 2015b). Neben der NADPH-Oxidase-abhängigen ETose wurde auch eine NADPH Oxidase-unabhängige Bildung von ETs beschrieben (Nishinaka et al. 2011, Douda et al. 2015, Rochael et al. 2015).

Neben neutrophilen Granulozyten wurde die ETose auch bei anderen, bei Wirbeltieren vorkommenden Phagozytenarten nachgewiesen (von Köckritz-Blickwede et al. 2008, Yousefi et al. 2008, Webster et al. 2010, Denkers und Abi Abdallah 2012), wie z. B. Makrophagen (Aulik et al. 2012, Hellenbrand et al. 2013, Boe et al. 2015), Monozyten (Muñoz-Caro et al. 2014b, Reichel et al. 2015), Mastzellen (von Köckritz-Blickwede et al. 2008, Lin et al. 2011), eosinophilen (Yousefi et al. 2008, Dworski et al. 2011, Muñoz-Caro et al. 2015c) und basophilen Granulozyten (Morshed et al. 2014). Als ETose-auslösende Moleküle wurden u. a. Komplementfaktor 5a (Martinelli et al. 2004, Yousefi et al. 2009), PMA und Zymosan (Brinkmann et al. 2004, Silva et al. 2014, Reichel et al. 2015), LPS (Brinkmann et al. 2004, Pijanowski et al. 2015) und Interleukin 8 (Brinkmann et al. 2004, Gupta et al. 2005) belegt. Kürzlich konnte gezeigt werden, dass ETs nicht nur gegen Bakterien, Viren und Pilze gebildet werden, sondern auch in Reaktion auf Protozoa und Metazoa (Abb. 9) freigesetzt werden (Baker et al. 2008, Guimarães-Costa et al. 2009, Behrendt et al. 2010, Bonne-Annee et al. 2014, Muñoz-Caro et al. 2015c). Auch für vergleichsweise große, extrazellulär lebende Parasiten wie dem Trematoden *Schistosoma japonicum* wurde belegt, dass dieser bzw. seine Stadien ETose auslösen können (Abb. 9) (Chuah et al. 2013). Bezüglich Nematoden wurde gezeigt, dass Larvalstadien von *Haemonchus contortus* (Abb. 9) und von *Strongyloides stercoralis* über ETs immobilisiert und ggf. abgetötet werden können (Bonne-Annee et al. 2014, Muñoz-Caro et al. 2015c).

Die Befähigung zur ETose ist, wie bereits erwähnt, nicht auf Wirbeltiere beschränkt. Kürzlich wurde gezeigt, dass sie auch unter Abwehrzellen der Invertebraten zu finden ist (Poirier et al. 2014, Robb et al. 2014, Lange et al. 2017). Interessanterweise scheinen sogar Acoelomaten, wie die Seeanemone *Actinia equina,* in der Lage sein,

ETs zu bilden (Robb et al. 2014). Dieser Befund legt nahe, dass der Mechanismus der ETosis eine evolutionär gesehen sehr alte Verteidigungsstrategie darstellt, die vor der Entwicklung des Coeloms entstanden sein muss (Robb et al. 2014).

3. Veröffentlichungen

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

Lange M. K., Penagos-Tabares F., Hirzmann J., Failing K., Schaper R., Van Bourgonie Y.R., Backeljau T., Hermosilla C., Taubert, A.

2018, *Vet. Parasitol.* 254: 120-130. doi: 10.1016/j.vetpar.2018.03.011.

Eigener Anteil an der Publikation:

- Projektplanung: 20 %, zusammen mit Betreuern
- Durchführung der Versuche: 80 %, weitestgehend eigenständig
- Auswertung der Experimente: 50 %, zusammen mit Betreuern
- Erstellung des Manuskripts: 80 %, weitestgehend eigenständig

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Research paper

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

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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%).

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

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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 et al., 2004; Saeed et al., 2006; Härtwig et al., 2015; Taylor et al., 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.

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, 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, Table 1). Selection criteria for sampling areas included proximity to sub-urban human settlements with forests and grassland (Table 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.

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 mm^3) 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 of Rowson 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

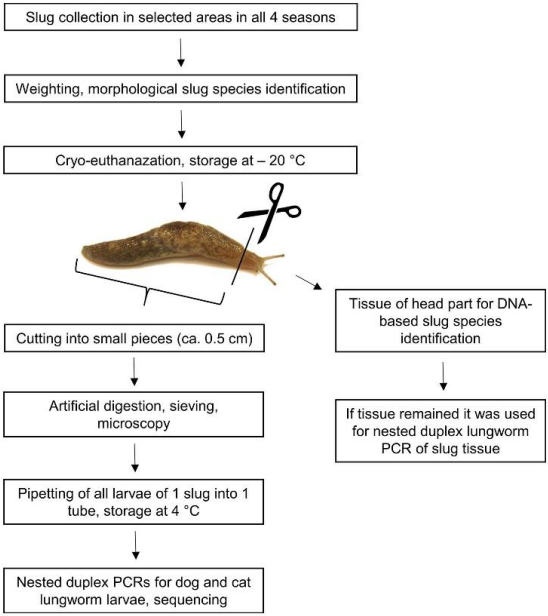

Fig. 1. Processing of samples and overview of analyses conducted.
In total, 2701 slugs were processed and samples were analysed following the here depicted steps. Slugs were cut into small pieces, processed via artificial HCl/ pepsin digestion and samples were stored at 4 °C until further use. Pellets 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.

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

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

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

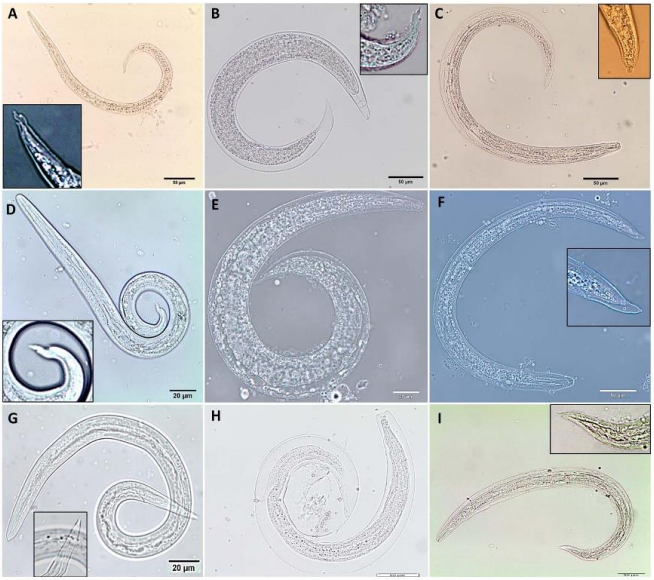

Fig. 2. Morphological characteristics for the identification of larval stages of *Aelurostrongylus abstrusus*, *Angiostrongylus vasorum*, *Crenosoma striatum*.
Key for morphological identification of the three metastrongyloid lungworms *Aelurostrongylus abstrusus* (A–C), *Angiostrongylus vasorum* (D–F) 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.

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. 2A), while tails of L3 stages possess a characteristic rounded terminal knob (Fig. 2C). *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. 2D) and the third stage larvae (Fig. 2F) 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. 2G–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. 2B, 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 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 (*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 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*, 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 $p \leq 0.05$.

3. Results

3.1. Prevalence ofA. 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, Table 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

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.

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.

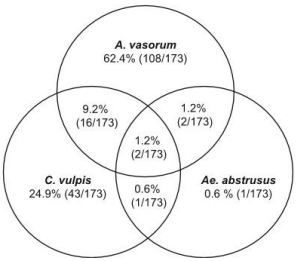

Fig. 3. Mono-, co- and triple infections with*Angiostrongylus vasorum*, *Crenosoma vulpis* and *Aelurostrongylus abstrusus*.
Of the 2701 slugs collected, microscopy revealed 173 lungworm larvae-positive samples. Those were analysed for mono- and co-infections with the different lungworm species via microscopy and PCR.

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.

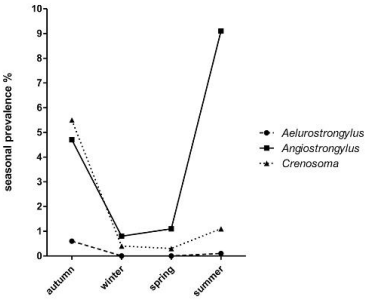

Fig. 4. Seasonal prevalence of*Aelurostrongylus abstrusus*, *Angiostrongylus vasorum* and *Crenosoma vulpis*) in German slugs.
Of the 2701 slugs collected throughout the season in different areas of Germany, 873 slugs were collected in autumn, 254 in winter, 791 in spring and 783 slugs in summer. Here, the data were assigned to the different seasons.

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 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) (Table 2). Broken down by seasons and areas, the overall highest prevalence for *A. vasorum* was found in Otterberg in summer (19.4%) (Table 2). For *C. vulpis* the prevalence varied between 0.3% (Eppstein, Hesse) and 5.9% (Weilburg, Hesse) (Table 2). The highest seasonal prevalence was detected in autumn in Weilburg (Hesse, 10%) (Table 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 (Table 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

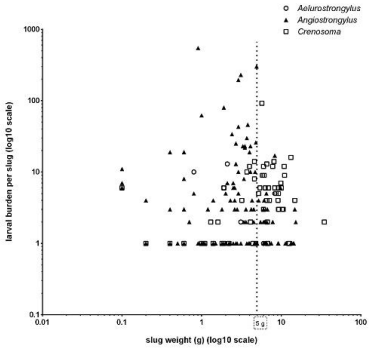

Fig. 5. Correlation of slug weight and*Angiostrongylus vasorum*, *Crenosoma vulpis* and *Aelurostrongylus abstrusus* larval burden.
The larval burden was correlated with the slug weight. X and Y axis are shown as a nonlinear logarithmic scale.

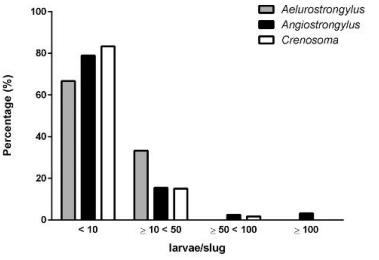

Fig. 6. Larval burden categories for slug*Aelurostrongylus abstrusus*, *Angiostrongylus vasorum* and *Crenosoma vulpis* infections.
Here, the proportion of slugs harbouring < 10, 10–49, 50–100 and > 100 lungworm larvae per specimen is depicted.

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

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.

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 (Table 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* showed a significantly higher

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.

Lungworm species	Intermediate host 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% ($n = 1$)

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).

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, 1953), by observing oesophagus type, tail morphology and parasite size [length and width (Mengert, 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. 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, Table 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 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 (Table 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 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%, Table 2) since larvae in faeces are vulnerable to desiccation (Costa Dias and dos Santos Lima, 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 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; Costa 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 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 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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Ethics approval

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.

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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Appendix A. Supplementary data

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3.2 Regional report on *Angiostrongylus vasorum* in Colombia: Genetic similarity to European lineage.

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Regional report

Regional report on *Angiostrongylus vasorum* in Colombia: Genetic similarity to European lineage

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ABSTRACT

The canine lungworm *Angiostrongylus vasorum* is considered neglected in South America and was only sporadically reported in dogs and wildlife. Gastropods act as obligatory intermediate hosts for this parasitosis. We here analysed *Achatina fulica* (African giant snail) populations from 5 regions of Colombia for *A. vasorum* infections. In total, 609 snails were collected from the departments Antioquia, Valle del Cauca and Putumayo. *Angiostrongylus vasorum*-infected *A. fulica* were found in all departments with a total prevalence of 3.9%. Larvae originating from Putumayo were molecularly characterized and identified as the European lineage of *A. vasorum*. This regional report shows for the first time the presence of *A. vasorum* in intermediate hosts in Colombia and the European genotype in South America.

1. Introduction

The life cycle of *Angiostrongylus vasorum* is obligatory linked to an intermediate host being represented by diverse gastropod species (Patel et al., 2014). Among those is the invasive African giant snail *Achatina fulica* (Sauerländer and Eckert, 1974).

Canine angiostrongylosis represents a challenge to clinicians due to its variety of symptoms (Di Cesare and Traversa, 2014). While this infection is nowadays well-known in Europe, in South America it still represents a neglected disease. So far, *A. vasorum* infection was exclusively reported in Colombian and Brazilian crab-eating foxes (*Cerdocyon thous*) and in Brazilian domestic dogs (Gonçalves, 1961). However, it is under debate whether canid *Angiostrongylus* specimen in South America truly correspond with to *A. vasorum* (constituting different genotypes) or represent with *Angiostrongylus raillieti* a distinct species (Jefferies et al., 2009).

To spread light on this hypothesis, we performed genomic characterization of *A. vasorum* specimens in Colombia.

2. Material and methods

2.1. Study areas and snail collection

In total, 609 *A. fulica* were collected from the Andean region in Andes (n = 238), Ciudad Bolívar (n = 100) and Cañasgordas (n = 100) (all department Antioquia), from the Pacific region in the urban zone of Tuluá, Valle del Cauca (n = 64) and from the Amazonian region Puerto Leguízamo, Putumayo (n = 107).

2.2. Processing of samples

Snails were cryo-euthanized, digested in pepsin solution and examined microscopically (Lange et al., 2017). Larvae were collected by pipetting and larvae-DNA was isolated using a commercial kit (Qiagen DNeasy Blood and Tissue Kit®). A nested PCR of the ribosomal ITS-2 (internal transcribed spacer 2) sequence with the nematode primers NC1/NC2 (Gasser et al., 1993) followed by an *A. vasorum*-specific real-time PCR (Jefferies et al., 2011) was performed.

For sequencing, the NC1/NC2 product was further amplified using the primers NC1 and MetR (5′ CCGCTAAATGATATGCTTA 3′) (Annoscia et al., 2014). The DNA amplicon was purified by PEG

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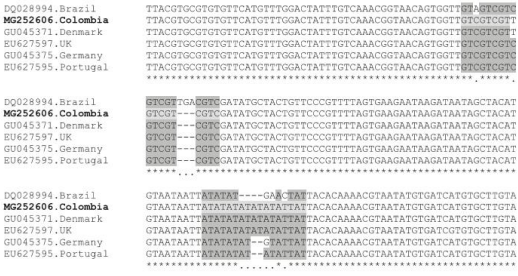

Fig. 1. Alignment of a partial sequence of the ITS-2 region from *A. vasorum* isolates of South America and Europe.
Microsatellites used by Jefferies et al. (2009) served to discriminate between *A. vasorum* genotypes and are highlighted in grey. Sequences from isolates of Europe and Brazil are shown in dark grey and the isolate from Colombia in light grey.

precipitation and sent to a commercial service (LGC Genomics, Berlin, Germany) for direct sequencing using the primer NC1. The electropherograms were verified by eye. Sequences were analysed by BLAST search of the GenBank database, aligned using Clustal Omega© and manually improved (Sequence data available in GenBank under accession number MG252606).

3. Results

Angiostrongylus vasorum-infected snails were found in Andes, Tuluá and Puerto Leguízamo with prevalences of 4.6% (11/238), 6.3% (4/64) and 8.4% (9/107), respectively and a total prevalence of 3.9% (24/609). Larvae were in third stage, showing typical morphology as described previously (Ash, 1970). Larvae from three snails in Puerto Leguízamo were confirmed via *A. vasorum* real-time PCR. Ribosomal ITS-2 region was sequenced. BLAST search provided the identity of *A. vasorum* with 99% (496/497 bp) to a specimen of the European genotype derived from Denmark (accession number GU045375). Comparison to Brazilian sequences revealed a homology (identity) of 94–95% (267–268/283 bp) (accession numbers: DQ028994, DQ028996) with several nucleotide polymorphisms not found in sequences from European isolates (Fig. 1). Unfortunately, samples from other regions did not contain sufficient amplifiable DNA for further sequencing.

4. Discussion

In Colombia, *A. vasorum* was reported in wild crab-eating foxes five decades ago (Gonçalves, 1961). Unfortunately, no genomic characterization was performed on these specimens. Sequence comparisons between European and Brazilian *A. vasorum* specimen revealed two distinct genotypes suggesting that presence of *A. vasorum* in South America results from an ancient evolutionary event that may be due to adaption to different final hosts. The authors predict this evolutionary event to have taken place between 11 and 67 million years ago. However, a new introduction of *A. vasorum* to South America may have occurred at the earliest 10,000 years ago with the introduction of the first dogs (Jefferies et al., 2009; Morgan et al., 2012). In the here presented regional report we analysed the genomic sequence of *A. vasorum* from Colombian intermediate hosts and found a high homology rather to the European lineage than to the South American lineage. Based on these data, it is assumed that the European genotype may indeed circulate in Colombia, most probably in parallel to the ancient South American lineage. This kind of superimposition of historical *A. vasorum* spread onto a more ancient distribution was also described by Jefferies et al. (2010). Areas in which *A. vasorum* was found relate to those predicted to be climatically suitable by Morgan et al. (2009). Since current data represent only a small sample size, further evaluation of the epidemiological situation on *A. vasorum* infections in Colombia is needed. Whether the two different lineages differ in pathogenicity needs to be verified in future.

5. Conclusion

To our knowledge, this study represents the first report on *A. vasorum* in its intermediate host in Colombia and the first report on the European genotype in South America. Since ITS2-based sequencing revealed a high homology to the European genotype, a rather new introduction of *A. vasorum* into Putumayo region may be assumed.

Animal welfare

Not applicable.

Conflict of interest

None.

Funding source

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Ethical statement

Not applicable.

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3.3 Novel approach to study gastropod-mediated innate immune reactions against metastrongyloid parasites.

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ORIGINAL PAPER

Novel approach to study gastropod-mediated innate immune reactions against metastrongyloid parasites

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Abstract
The anthropozoonotic metastrongyloid nematodes *Angiostrongylus cantonensis* and *Angiostrongylus costaricensis*, as well as *Angiostrongylus vasorum*, *Crenosoma vulpis*, *Aelurostrongylus abstrusus* and *Troglostrongylus brevior* are currently considered as emerging gastropod-borne parasites and have gained growing scientific attention in the last years. However, the knowledge on invertebrate immune responses and on how metastrongyloid larvae are attacked by gastropod immune cells is still limited. This work aims to describe an in vitro system to investigate haemocyte-derived innate immune responses of terrestrial gastropods induced by vital axenic metastrongyloid larvae. We also provide protocols on slug/snail management and breeding under standardized climate conditions (circadian cycle, temperature and humidity) for the generation of parasite-free F0 stages which are essential for immune-related investigations. Adult slug species (*Arion lusitanicus*, *Limax maximus*) and giant snails (*Achatina fulica*) were maintained in fully automated climate chambers until mating and production of fertilized eggs. Newly hatched F0 juvenile specimens were kept under parasite-free conditions before experimental use. An improved protocol for gastropod haemolymph collection and haemocyte isolation was established. Giemsa-stained haemolymph preparations showed adequate haemocyte isolation in all three gastropod species. Additionally, a protocol for the production of axenic first and third stage larvae (L1, L3) was established. Haemocyte functionality was tested in haemocyte-nematode-co-cultures. Scanning electron microscopy (SEM) and light microscopy analyses revealed that gastropod-derived haemocytes formed clusters as well as DNA-rich extracellular aggregates catching larvae and decreasing their motility. These data confirm the usefulness of the presented methods to study haemocyte-mediated gastropod immune responses to better understand the complex biology of gastropod-borne diseases.

Keywords Invertebrate immunology · Metastrongyloidea · Haemocytes · *Arion lusitanicus* · *Limax maximus* · *Achatina fulica*

Introduction

Gastropods (snails and slugs) are abundant organisms widely distributed in natural humid habitats worldwide and a source of many kinds of gastropod-borne diseases. Classically, snails and slugs are directly involved as obligate intermediate hosts in the life cycle, dissemination and transmission of several nematode parasitic species infecting humans and domestic animals (Giannelli et al. 2016). Some of these gastropod-borne parasitoses are of increasing importance for human and animal health due to their emergence into previously non-reported geographic areas (Colella et al. 2016; Lv et al. 2009; Traversa and Guglielmini 2008; Traversa et al. 2014). Especially metastrongyloid parasites, such as *Angiostrongylus vasorum*, *A. costaricensis*, *A. cantonensis*, *Aelurostrongylus abstrusus* and *Troglostrongylus brevior*, have gained growing

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attention in basic as well as in applied research areas of human and veterinary medicine (Traversa et al. 2010; Morassutti et al. 2014; Spratt 2015; Hansen et al. 2017; Maksimov et al. 2017). The reasons for the dissemination of metastrongyloid parasites are still unknown. However, it is assumed that shifts in intermediate host populations may be one factor since these parasites are vulnerable to environmental conditions when being released from the final hosts (Dias and Dos Santos Lima 2012; Morgan et al. 2009). Meta-analyses revealed that global warming strongly effects the phenology of molluscs (Root et al. 2003) thereby also affecting their associated parasites (Patz et al. 2000). To date, the knowledge on how gastropods respond immunologically to metastrongyloid infections and how these parasites develop within gastropod species is still very limited (Giannelli et al. 2016; Schnyder 2015).

Gastropods have an innate immune system which strongly resembles that reported in vertebrates in relation to cellular and molecular mechanisms (Patat et al. 2004; Poirier et al. 2014; Robb et al. 2014; Wojda 2016). Invertebrate haemocytes (syn. amoebocytes), which circulate freely within the haemolymph system, are considered as multifunctional phagocytes of the gastropod invertebrate immune system (Beck and Peatman 2015; Fried and Lwaleed 2016; Lange et al. 2017; Loker 2010; Yoshino et al. 2013). Gastropod haemocyte-derived innate immune reactions include phagocytosis, multicellular encapsulation and cell-mediated cytotoxicity as well as the recently described invertebrate extracellular phagocyte trap (InEPT) formation (Lange et al. 2017). Nonetheless, detailed investigations on haemocyte effector mechanisms, as known for vertebrate phagocytes, are still very limited (Lange et al. 2017; Loker 2010; Sokolova 2009). However, research on gastropod-derived innate immune reactions against metastrongyloid parasites and other gastropod-borne parasites (e.g. trematode infections) is fundamental to better understand the molecular, biochemical and signalling pathways involved in these interactions. Improved knowledge on the intricate balance between the gastropod innate immune response and larval-derived infection mechanisms may also provide novel insights into general mechanisms of the evolutionary arms race between host and pathogen. Nowadays, host–pathogen interactions are considered as a crucial area of infectious disease research since new information on host–parasite dynamics will aptly facilitate further development in the field of new drugs, therapies and control strategies (Sen et al. 2016; Yoshino et al. 2013). Especially some of the antimicrobial peptides being produced during innate immune responses appear promising for drug development as it is assumed that they do not induce bacterial resistance (Matsuzaki 2001). Although it is generally accepted that invertebrates only possess an innate immune response (Boehm 2007; Cooper and Alder 2006; Niekerk and Engelbrecht 2015; van Niekerk et al. 2016), recent data suggest that there may also exist some kind of adaptive immune mechanisms (Arala-Chaves and Sequeira 2000; Armitage et al. 2015; Bowden 2017; Coustau et al. 2015; Milutinović and Kurtz 2016; Pham et al. 2007). Invertebrate haemocytes may also play a fundamental role in these adaptive processes (Arala-Chaves and Sequeira 2000; Coustau et al. 2016). For example, gastropod-derived haemocytes were found to be involved in cell proliferation, morphological activation, cellular memory and expression of humoral factors, such as fibrinogen-related proteins (FREPs) and the Down syndrome adhesion molecule (Dscam) (Coustau et al. 2016). Rapid progress is currently made in elucidating molecules involved in the complex invertebrate immunity. However, research on single molecules runs the risk of missing ancient and well-conserved effector mechanisms (for review, see Little et al. (2005)), such as the capacity of invertebrate haemocytes to extrude InEPTs to attack pathogens as previously demonstrated for metazoan parasites in vivo and in vitro (Lange et al. 2017).

Therefore, this work intends to describe detailed in vitro and in vivo systems allowing analyses of gastropod haemocyte-derived innate immune reactions directed against any kind of pathogen of terrestrial molluscs (as an example, we here chose metastrongyloid larvae). Thus, a useful technique for the isolation of adequate haemolymph volumes and haemocyte numbers is presented in addition to a suitable method for generating axenic metastrongyloid larvae and standardized in vitro mollusk breeding conditions. These are essential for basic research on immunobiology of gastropods and to allow future detailed research on gastropod-borne metastrongyloid parasite species, including anthropozoonotic (i.e. *A. cantonensis*, *A. costaricensis*) and domestic/wildlife parasite species (i.e. *A. vasorum*, *A. chabaudi*, *A. mackarrei*, *A. abstrusus*, *T. brevior*, *Crenosoma vulpis*).

Materials and methods

Ethics approval statement

According to the German Animal Welfare Act (Tierschutzgesetz of 25.05.1998—BGBL I S.1105—section 5 paragraph 8a), ethics approval research with invertebrates is only required for experiments where animals of the classes Cephalopoda and Decapoda are used. Thus, an approval of an ethic committee was not necessary for the current studies. Nevertheless, we took every precaution to ensure the animals were under the least amount of pain and stress.

Gastropod maintenance under standardized and parasite-free conditions

Slugs

Terrestrial slugs (*Arion lusitanicus* and *Limax maximus*) (Fig 1a and b) originated from natural populations in the

Federal State of Hesse, Germany. They were then bred and maintained in fully automated climate incubators (model ECP01E®; Snijders Scientific B.V. Tilburg) applying the following conditions: 10 h of dark/10 h of illumination plus 2 h for dawn and dusk each, corresponding to circadian cycles; temperatures ranging from 10 to 16 °C (night/day) and 50% humidity. The specimens used for standardization and establishment of the current model were all F0 generation of the specimens initially isolated from natural occurring slug populations. All slug species were kept in perforated plastic containers (Tupperware®) (boxes 3300–12,000 cm^3; approximately 1 gastropod per 1000 cm^3) supplied with a humidified absorption paper at the bottom, plastic petri dishes (Nunc) for food and a plastic dim house (Tecniplast®) as adequate hiding place to reduce stress (see Fig. 1g). The feedings were performed ad libitum twice a week with lettuce leaves (*Lactuca sativa*), cucumber fruits (*Cucumis sativus*), carrot roots (*Daucus carota* subsp. *sativus*), champignons (*Agaricus campestris*), rabbit pelleted food (VERSELE-LAGA®; CUNIFIT pure) and dry dog food (Purina®, Beneful) (Fig. 1g).

Giant snails

Terrestrial giant African snails (*Achatina fulica*) (Fig. 1c) purchased from a German hatchery (Deine Tierwelt GmbH & Co. KG, Hannover, Germany) were bred and maintained as abovementioned but applying the following conditions: 20 to 25 °C and 60% humidity. For experiment standardization, exclusive specimens of the F0 generation were used. The snails were maintained in plastic containers on terrarium soil (5 cm height, TerraBasis® and TerraCocoshumus® mixed at 1:1 ratio, JBL). The feeding proceedings were performed as described for the slugs; additionally a calcium supplement (ad libitum 21% calcium, Calcina Calcium Citrat®, Canina) was administered (see Fig. 1h).

Generation of parasite-free gastropod F0

Reproduction of the slug species *Ar. lusitanicus* and *L. maximus* as well as the snail *Ac. fulica* occurred under the abovementioned conditions. However, due to special mating behaviour of *L. maximus* [chasing/climbing activities before mating in a free-hanging position (Langlois 1965)], large glass containers (40 cm height) containing branches were used for breeding of this species (Fig. 1d and e). Single eggs or egg clusters of slug/snail species were collected from breeding containers and immediately transferred into small plastic cups with humidified papers and fenestrated lids for aeration. The search for hatching of F0 juvenile slugs/snails was performed weekly (Fig. 1f). Each plastic cup exclusively contained eggs of one species of gastropod which were maintained under the same climatic conditions as adult specimens. Time period (days) from egg deposition to hatching as well as the number of offspring per egg batch were quantified to confirm that the gastropods have appropriate reproductive rates. Freshly hatched F0 slugs/snails were then transferred to plastic containers and counted. Juvenile slugs were observed to feed only on leftovers of empty eggshells during the first day after hatching although food was administered as described above. To confirm the parasite-free status of the slugs a control group of 10 *L. maximus*, 10 *Ar. lusitanicus* and 10 *Ac. fulica* specimens were analyzed by artificial digestion and microscopy as described below.

Isolation of *A. vasorum* first-stage larvae (L1)

The isolation of *A. vasorum* L1 from feces of experimentally infected red foxes (*Vulpes vulpes*) (kindly provided by the Department of Veterinary Disease Biology, University of Copenhagen, Denmark, Danish experimental animal license no. 2010/561-1914) was performed as described by Lange et al. (2017). Briefly, faecal samples were incubated in a funnel partially filled with water, where additionally a sieve (aperture 100 μm) was placed and three layers of gauze (117 threads/cm^2), at room temperature (RT) for 24 h. Owing to positive hydrotactic properties, L1 migrated from the faeces into the water and sedimented. Five-milliliter sediments containing L1 were collected in 15-mL conical tubes (Greiner) by carefully opening the clamp at the bottom of the Baermann funnel apparatus. L1 was pelleted by centrifugation (400 *g*, 10 min, 20 °C). Afterwards, the larvae were separated from the faeces fragments by a 45/72% discontinuous Percoll gradient as described by Graeff-Teixeira et al. (1999). First, an isosmotic (90%) Percoll (IOP) solution was prepared by mixing of 9 parts of Percoll (density 1.128 g/mL, Sigma-Aldrich) with 1 part of 2.5 M sucrose (Carl Roth). Then, 45 and 72% Percoll gradients were prepared with the following: 45% IOP = 3.15 mL IOP + 3.85 mL 0.25 M sucrose and 72% IOP = 3.6 mL IOP + 1.4 mL 0.25 M sucrose. Subsequently, the gradients were prepared in a 15-mL conical tube by overlaying 5 mL 72% IOP with 7 mL 45% IOP. Then, the pellet with larvae was added on top of the gradient. The sample was then centrifuged (400 *g*, 40 min, 20 °C). L1 was then recovered from the boundary layer in between the 45 and 72% gradients.

Isolation of vital *A. vasorum* third-stage larvae (L3) by artificial digestion of experimentally infected slugs

To generate *A. vasorum* L3, *L. maximus* slugs were previously infected orally with vital 100 larvae/slug. First, slugs were placed individually in small plastic containers and not fed for 2 days before infection. The larvae were then resuspended in 200 μL distilled water and carefully deposited on a single dog food pellet with a hollow in the centre (Fig. 2). The slugs were kept in the plastic boxes until the dog food

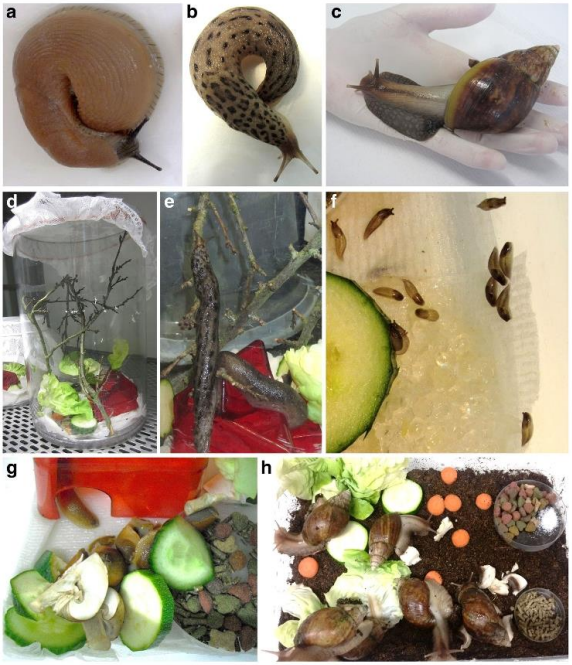

Fig. 1 Terrestrial gastropods maintained under controlled standardized conditions. Adult specimens of (**a**) the Iberian slug (*Arion lusitanicus*), (**b**) the giant garden slug (*Limax maximus*), and (**c**) the giant African snail (*Achatina fulica*). (**d**) Glass container used for reproduction of *L. maximus*. (**e**) *Limax* slugs chasing one another (due to their special mating behaviour) in reproduction container. (**f**) Eggs and freshly hatched F0 specimens of *L. maximus*. (**g**) Slugs were maintained in plastic containers with humidified absorption paper and fed lettuce, cucumber fruits, carrot roots, champignons, rabbit pelleted food and dry dog food. (**h**) Giant snails were maintained in plastic containers on terrarium soil and fed in the same manner as the slugs with addition of a calcium supplement

pellet was entirely eaten to help ensure maximum oral uptake of infective larvae. The larvae recovery rate regarding *L. maximus* was 2.5% mean, ranging from 0.5 to 14% whereas larval recovery from *A. lusitanicus* constituted 2% mean, ranging from 1 to 3.5%.

A. vasorum L3 was isolated 30 days post-infectionem (p.i.) via artificial digestion: the slugs were cut into small pieces and placed in the digestion solution [1 L containing 10 g pepsinogen powder 2000 FIP-U/g (Robert Kind), 8.5 g NaCl (Carl Roth), 30 mL HCl 37% (Carl Roth), distilled water ad 1 l]. The digestion was performed in 50 mL conical tubes (Greiner Bio-One International GmbH) under constant shaking (4 h, 40 °C). Digested samples were sieved first through a 300-μm-pore-size metal sieve (Retsch GmbH) to remove undigested material and then through a 25-μm-pore-size metal sieve (Retsch GmbH). The contents in the last sieve were transferred to 15 mL Falcon tubes and centrifuged (400 ×*g*, 10 min). Pellets were re-suspended and examined microscopically (Leica light microscope at 4× and 20× magnification). Viable *A. vasorum* L3 were carefully collected by pipetting under a microscope (Pasteur pipette, Hirschmann GmbH & Co. KG), washed thrice in sterile PBS in Petri dishes (Greiner Bio-One International GmbH) to remove debris and deposited in 1 mL plastic tubes (Eppendorf).

Preparation of axenic *A. vasorum* first (L1)- and third (L3)-stage larvae

In order to remove any bacterial/fungal contamination and to achieve an axenic status of L1 and L3, the method reported by Barçante et al. (2003) was applied with slight modifications. Briefly, *A. vasorum* larvae were incubated for 10 min in a 10 mL sodium hypochlorite solution (0.5% *v*/*v*) (stock solution containing 12% sodium hypochlorite; Carl Roth)

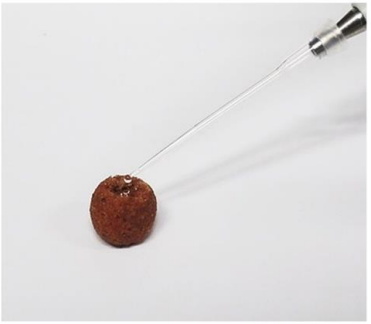

Fig. 2 Oral slug/snail infection. One hundred larvae suspended in 0.2-mL distilled water were deposited on a single dog food pellet

prepared in sterile PBS (Lange et al. 2017). Subsequently, larvae were washed twice (250 ×*g* for 5 min at 20 °C) in sterile PBS supplemented with 3% penicillin (500 U/mL; Sigma-Aldrich) and streptomycin (500 µg/mL; Sigma-Aldrich) followed by two further washings in sterile PBS without antibiotics. To confirm the efficacy of this protocol, axenic larvae (50 larvae per petri dish) were incubated on sterile LB medium (10 g LB, 7.5 g agar, 500 mL distilled water) at 37 °C for 7 days ($n = 5$) and thereafter analyzed for bacterial or fungus contamination. For improved availability, axenic larvae were always prepared 1 day in advance of exposure to gastropod haemocytes in vitro.

Isolation and in vitro culture of gastropod-derived haemocytes and co-culture experiments with vital axenic *A. vasorum* larvae

Adult gastropods (at least 6 months old) were subjected to a 48-h fasting period and cryo-anesthetized (40 min on ice) before haemocyte isolation. Based on a previously published technique for insect (caterpillar)-derived haemocyte isolation (Stoepler et al. 2012), we used a slightly modified serum-free haemocyte collection solution [77% RPMI 1640 medium, 20% anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA and 41 mM citric acid, pH 4.5) and 3% penicillin/streptomycin (Sigma-Aldrich, penicillin 10,000 U/mL, streptomycin 10 mg/mL)] which was freshly prepared under sterile conditions and kept on ice at all times. By using 1-mL syringes (Braun) and capillary needles (30-gauge, Braun), the haemocyte collection solution was injected into each cryo-anesthetized slug at a volume corresponding to 10% of its body weight. Thereafter, slugs were cryo-anesthetized (20 min on ice) again before euthanasia was performed via fast decapitation (Patel et al. 2014). The haemolymph samples were immediately collected from decapitated slugs by careful mechanical pressure and aspiration.

In contrast to the slugs, *Ac. fulica* haemolymph was directly collected from living specimens by aspiration after insertion of a needle with a syringe close to the pneumostome (Cooper 1994). Haemolymph extraction of up to 10% of the snail's body weight was previously described to induce no adverse effects in the animal (Cooper 1998). The extracted snail haemolymph samples were immediately mixed with equal volumes of sterile culture medium [RPMI 1640 medium supplemented with penicillin (500 U/mL; Sigma-Aldrich) and streptomycin (500 µg/mL; Sigma-Aldrich)] (see Fig. 3). The quality of haemocytes was controlled via Giemsa staining (Sigma-Aldrich) of haemolymph smears and based on cell sizes two different types of gastropod haemocytes were found and categorized as type I (small) and II (large) haemocytes according to Accorsi et al. (2013). The haemocytes were washed thrice (250 ×*g*, 5 min, low acceleration) and counted in a Neubauer haemocytometer chamber. Haemocytes were co-cultured with axenic L1 and L3 of *A. vasorum* on poly-$_L$-lysine (Sigma-Aldrich) pre-coated coverslips (Nunc) at a ratio of 200:1 (RT, 30 and 60 min).

Phase-contrast microscopy and scanning electron microscopy (SEM) analyses

Haemocyte-parasite co-cultures were analyzed either by phase contrast microscopy (Olympus IX8® microscope equipped with a digital camera and the Olympus analySIS® software) or by SEM. For SEM analyses, the samples were fixed in 2.5% glutaraldehyde (RT, Merck), which was prepared using 1% osmium tetroxide (Merck), washed in distilled water, dehydrated, critical point dried by CO_2 treatment and sputtered with gold. Thereafter, the samples were examined with a Philips XL30 scanning electron microscope at the Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany.

Results and discussion

Gastropod breeding under standardized climate conditions results in successful generation of parasite-free F0 specimens

In order to maintain terrestrial gastropods as close as possible to their optimal environmental conditions, the animals were kept in fully automated climate chambers, simulating circadian conditions, where successful breeding of all mentioned gastropod species was achieved (Tables 1 and 2). For experiments on innate immune responses, it is mandatory to maintain experimental animals under fully standardized conditions

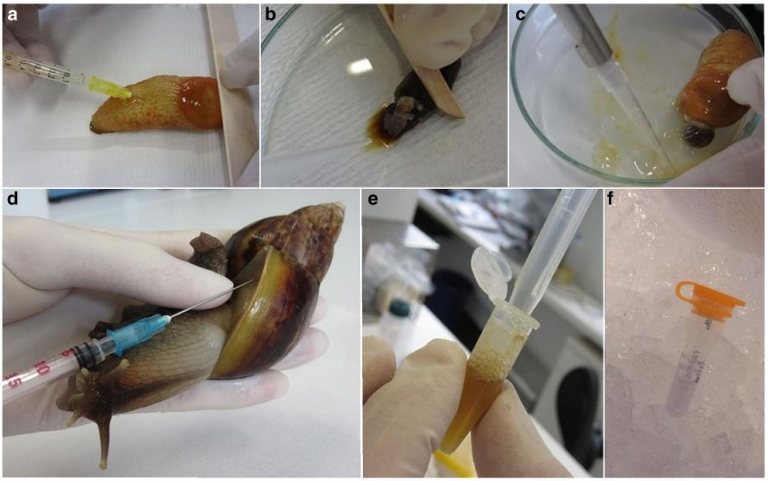

Fig. 3 Haemolymph extraction process. (**a**) Injection of collection solution (~ 10% of the body weigh). (**b**) Haemolymph recovery from *A. lusitanicus* and (**c**) *L. maximus*. (**d**) haemolymph collection via aspiration close to the pneumostome. (**e**) Haemolymph samples from slugs and (**f**) from snails

to avoid interference by other factors, such as diet, climate conditions and stress (Kangassalo et al. 2015; Krams et al. 2015; Wojda et al. 2004). To reduce stress, we administered red dim houses in the slug colonies to be used as shelter and hiding place (this was well accepted by all slug species) (Fig. 1).

The slug and snail mating behaviour, especially for *L. maximus*, were clearly improved under here described conditions and resulted in massive production of fertilized eggs. Newly hatched juvenile parasite-free gastropods of the three species (F0 generation) were efficiently propagated in these climate chambers and successfully used for experimental *A. vasorum* infections or as haemolymph donors. No nematode stages were found in artificially digested F0 generation slugs and snails ($n = 30$) validating their parasite-free status. Diet and climate conditions were, as described above, utterly standardized and automated to guarantee a high reproducibility of the experiments. Under these breeding conditions, climatic factors could easily be controlled and regulated in a way which simulates the in vivo situation, by, for example, adapting the day length to seasonal changes. This allows to evaluate the influence of different climatic scenarios or season-triggered effects on innate immune responses or metastrongyloid infection characteristics in gastropods.

Given that ambient temperature, humidity, circadian cycles and diet can significantly influence the host innate immune response of vertebrates and invertebrates (Kangassalo et al. 2015; Krams et al. 2015; Leicht et al. 2013), these factors should be considered in immunity-related experiments. We recommend and consider these factors vital to maintain gastropod colonies under standardized conditions (i.e. temperature, photoperiod, feeding and humidity) as slight environmental changes can result in the modification of host–pathogen interactions as demonstrated elsewhere (Barber et al. 2016; Mitchell et al. 2005; Seppälä and Jokela 2011). The advantages of controlled climate conditions with circadian cycles also ensure that breeding conditions resemble the in vivo situation. These conditions can then be further manipulated in order to analyze the impact of seasonal variations or climate change on gastropod development or infections. This has previously been suggested to be important for the development of metastrongyloid larvae within terrestrial intermediate hosts (Morgan et al. 2009). Furthermore, the abovementioned investigations can be performed with ease and will contribute to unveil the complex epidemiology of lungworm infections (Maksimov et al. 2017; Morgan et al. 2009).

Table 1 Reproduction of gastropod species in fully automated climate chambers

	Arion lusitanicus	*Limax maximus*	*Achatina fulica*
Mean number of offspring/egg batch ± SD	85 ± 71	109 ± 74	129 ± 103
Maximum number of offspring/egg batch	191	181	202
Minimum number of offspring/egg batch	3	22	56

Axenic status of *A. vasorum* first (L1)- and third (L3) -stage larvae

Analyses on immune responses should generally be performed with axenic larvae in the case of nematode pathogens to avoid reactions due to bacterial or fungal contamination. Due to the lifecycle of metastrongyloids, the isolation of L1 is associated with considerable contamination problems since the larvae have to be separated from the final host faeces. Faeces obviously contain large amounts of bacteria and fungi which themselves function as potent extracellular trap (ET) inducers in mammalian and invertebrate phagocytes (Brinkmann et al. 2004; Ng et al. 2013). In addition, the L3 isolation from dead slugs/snails may also lead to bacterial/protozoal contamination since these intestinal microbes may be set free during the extraction process. Therefore, it is mandatory to generate axenic first- and third-stage larvae to exclude unspecific reactions. The protocol described herein successfully resulted in the generation of axenic metastrongyloid larvae since neither bacterial nor fungal growth occurred after an incubation at 37 °C for 7 days (Fig. 4). In this way, it is possible to declare that immune reactions produced by haemocytes during the experiments were exclusively induced by larval antigens.

Isolation of axenic metastrongyloid larvae from faeces is a pivotal step for in vitro experiments on gastropod immune responses to avoid false interpretation of results (Barçante et al. 2003). The current protocol of L1 purification and sterilisation from carnivore faeces resulted in low levels of debris contamination without reducing the number of viable metastrongyloid larvae. These larvae proved bacteria-free and showed good results in preliminary ET-related experiments. Thus, these larvae may also be used for antigen preparations or other purposes. Furthermore, the development from L1 into L3 of *A. vasorum* was achieved in F0 specimens as demonstrated by viable L3 stages after slug/snail digestion. More importantly, the development time from L1 into infective *A. vasorum* L3 corresponded well to previously reported data (Koch and Willesen 2009).

Haemolymph extraction from slugs and snails

The current haemolymph extraction protocols delivered a rather high volume of haemolymph containing adequate numbers of haemocytes. Thus, Giemsa-stained haemolymph smears revealed the presence of high numbers of intact haemocytes in each gastropod species tested, i.e. in *Ar. lusitanicus*, *L. maximus* and *Ac. fulica* as previously reported (Adamowicz and Bolaczek 2003; Pengsakul et al. 2013) (Fig. 5, Table 3). Type I (small) haemocytes were more abundant (85.2, 94, 96.9%) than type II (large) haemocytes (14.8, 5.9, 3.1%) in the gastropod species *L. maximus*, *Ac. fulica* and *Ar. lusitanicus*, respectively (Table 3). The median collected haemolymph volumes were 112.5 μL in *L. maximus*, 125 μL in *Ar. lusitanicus* and 340 μL in *Ac. fulica*) with total haemocyte counts of 61,250 cells, 27,500 cells and 156,565 cells, respectively. Haemolymph volumes and haemocyte counts varied considerably between individuals of the same gastropod species and also between the different gastropod species (Table 4).

One emphasis of the current study was to develop a rapid and reproducible method for the collection of large volumes of haemolymph for haemocyte isolation. We here provide a novel haemolymph extraction protocol that may potentially be used for the terrestrial intermediate host species: the Iberian slug (*Ar. lusitanicus*), the giant garden slug (*L. maximus*) and the giant African snail (*Ac. fulica*) for a wide range lungworm infections of dogs and cats (i.e. *A. vasorum*, *C. vulpis*, *A. abstrusus*, *T. brevior*) as well as for anthropozoonotic

Table 2 Time period (days) from egg deposition to gastropod juvenile hatching

	Arion lusitanicus	*Limax maximus*	*Achatina fulica*
Batch 1	22	19	30
Batch 2	31.5 ± 5.1	24	31
Batch 3	31.5 ± 7.3	26	
Batch 4	31.0	29.5 ± 7.8	
Batch 5	30.5 ± 4.9		
Mean hatching time	29.5 ± 4.1	24.6 ± 4.4	30.5 ± 0.7
Mean death rate	9.9 ± 7.3%	10.9 ± 15.3%	4.0 ± 2.1%

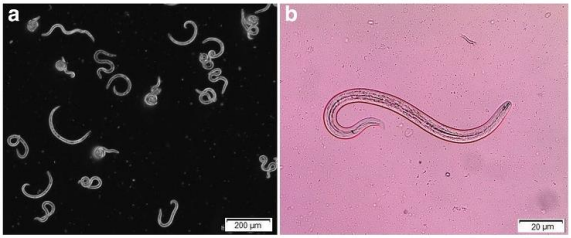

Fig. 4 Axenic culture of *Angiostrongylus vasorum*. (**a**) L1 isolated from feces of experimentally infected foxes. (**b**) L3 isolated from experimentally infected slugs *Limax maximus*

lungworms in tropical/subtropical geographic areas (i.e. *A. cantonensis*, *A. costaricensis*).

The method can easily be transferred to obtain haemocytes from other terrestrial/amphibian or aquatic gastropod species and allows basic research of the snails' immunocompetence against larval stages of other nematodes. The same applies to other gastropod-borne pathogens including important trematode genera affecting public health as well as livestock animals such as *Schistosoma*, *Fasciola*, *Opisthorchis*, *Clonorchis*, *Gastrodiscoides*, *Echinostoma*, *Paragonimus*, *Fasciolopsis*, *Heterophyes* and *Metagonimus* among others.

Since it proved realistic to obtain sufficient haemolymph from giant African snail species achieving a volume of up to 10% of the snail's body weight without sacrificing the donor, estimated volumes of up to 5 mL per snail may be easily extracted from full-grown specimens. Consequently, *Ac. fulica* also appears as a promising and suitable model for more detailed research on invertebrate innate immunity using more cell-consuming techniques, such as fluorescence-activated cell sorting (FACS) analyses or biochemical studies.

Gastropod haemocytes form ETs in response to *A. vasorum* larvae (L1 and L3)

The phase-contrast microscopy showed that haemocytes derived from *Ar. lusitanicus*, *L. maximus* and *Ac. fulica* when exposed to vital *A. vasorum* L1 and L3 larvae formed cellular aggregates and extracellular haemocyte-derived ET-like structures in contact with the larvae or even entangling them so larval movement was decreased (Figs. 6 and 7/Supplementary material Videos 1 and 2). Ultrastructural characterization by SEM analyses confirmed gastropod-derived InEPTs being attached to larval stages (Fig. 7). These extracellular structures were recently analyzed in more detail by Lange et al. (2017). Interestingly, different types of ETs, i.e. spread (*spr*InEPTs) and aggregated InEPTs (*agg*InEPTs) [Figures 2 and 3 in Lange et al. (2017)], are observed in mammalian ETs (Muñoz-Caro et al. 2015b).

Additionally, the phase-contrast microscopy and SEM analyses of haemocyte-larvae co-cultures revealed signs of chemotaxis, the formation of haemocyte aggregates and of intense cellular activity (i.e. vacuolization and fibrillary arrangement). This is in line with observations of Boisseaux et al. (2016) on *Lymnaea stagnalis*-derived haemocytes and on gastropod-derived InEPTs (Lange et al. 2017). Overall, *A. vasorum*-driven InEPTs revealed parasite stage independency, since L1 and L3 induced these reactions. Although several surveys have showed a parasite stage-independent ET formation (Guimaraes-Costa et al. 2009; Hermosilla et al. 2014; Muñoz-Caro et al. 2015a; Silva et al. 2014), differences in efficacy between diverse parasitic stages have been described earlier by Hermosilla et al. (2014). In addition, *A. vasorum*-induced InEPTs also proved to be host species-independent since haemocytes isolated from two different

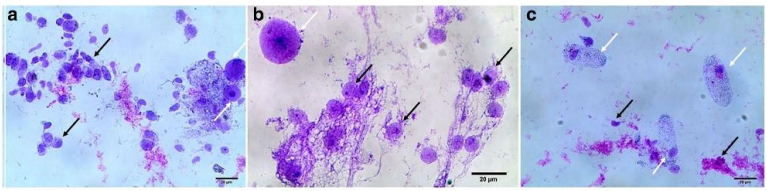

Fig. 5 Haemolymph smears stained by Giemsa. (**a**) Haemocytes of *Arion lusitanicus* (400×), (**b**) haemocytes of *Limax maximus* (400×) and (**c**) haemocytes of *Achatina fulica* (400×). Black arrows indicate type I (small), and white arrows indicate haemocyte type II (large)

Table 3 Proportion of haemocytes type I (small) and type II (large) in gastropod haemolymph samples

	Arion lusitanicus	*Limax maximus*	*Achatina fulica*
Type I cells	559 (96.9%)	104 (85.2%)	112 (94.1%)
Type II cells	18 (3.1%)	18 (14.8%)	7 (5.9%)
Total	577	122	119

slug species and from one snail species performed this effector mechanism, which is in line with previous data on ETosis and InEPTosis (Lange et al. 2017; Muñoz-Caro et al. 2015a).

Intriguingly, many similarities exist between the innate immune system of vertebrates and invertebrates (Coustau et al. 2016; Little et al. 2005; McCormick-Ray and Howard 1991). This applies not only for soluble defense molecules but also for conserved effector mechanisms of professional phagocytes (Hermosilla et al. 2014; Lange et al. 2017; Silva et al. 2016). As such, antimicrobial lectins, peptides, proteases, C-reactive proteins, alpha 2-macroglobulins and histones (H1-like, H5-like) have been reported as effector molecules of the invertebrate immune system (Coates and Decker 2017; Foelix 1996; Goins 2003; Iwanaga and Lee 2005; Little et al. 2005; Liu et al. 2016; Poirier et al. 2014; Van Wettere and Lewbart 2007). Patat et al. (2004) suggest that multifunctional histone proteins are a conserved characteristic of the innate immunity in all organisms possessing histones. In addition, similarities on molecular and structural level have also been assumed for invertebrate and vertebrate effector mechanisms (Arala-Chaves and Sequeira 2000), such as ETosis (Hermosilla et al. 2014; Lange et al. 2017; Poirier et al. 2014; Silva et al. 2016). Consistently, it has been shown that several innate immune signalling pathways and transcription factors are conserved in invertebrates and vertebrates, such as the peptidoglycan recognition protein LC/immune deficiency (PGRP-LC/IMD) pathway, Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) pathways, nuclear factor kappa B (NF-kB) and Toll-like receptors (TLRs) (Coustau et al. 2016; Kang et al. 1998; Pila et al. 2016; Sun et al. 2016; Wojda et al. 2004; Zhang and Coultas 2011). Highly diversified non-self-recognition molecules, such as FREPs and the Dscam (Coustau et al. 2016), are also reported to occur in invertebrates. Haemocytes, as key players of invertebrate immunity (Beck and Peatman 2015), have a pivotal role not only in early innate responses against pathogens by encapsulation, nodulation and melanisation (Tsakas and Marmaras 2010) but also in invertebrate immune memory reactions. As such, haemocytes produce FREPs after their encounter with trematodes (Romero et al. 2011; Zhang et al. 2004). Overall, the invertebrate immune system represents a mosaic of evolutionary conserved processes as well as evolutionarily independent innovative immune mechanisms that require more detailed investigation (Cerenius and Söderhäll 2013; Coustau et al. 2016; Malagoli 2016). In this context, rapid progress has been made in elucidating the molecular mechanisms to be involved in invertebrate innate immunity, particularly in arthropods (Jiravanichpaisal et al. 2006; Milutinović and Kurtz 2016). However, much less data are available on gastropods although these species are well-known for their pivotal role in spreading of anthropozoonotic and veterinary relevant lungworm infections worldwide.

So far, most studies performed on immunological gastropod–parasite interactions have been restricted to the aquatic snail *Biomphalaria glabrata* (Zhang et al. 2007; Zhang and Coultas 2011; Coustau et al. 2015) and related to digenean trematode infections (e.g. schistosomosis, opisthorchiosis, clonorchiosis and fasciolosis), and only few studies have been performed on gastropod-borne nematode infections (van der Knaap and Loker 1990; Ataev et al. 2016; Pila et al. 2016; Lange et al. 2017). Haemocytes obtained from trematode-infected snails have altered morphology, stickiness, spreading behaviour on glass surfaces, and phagocytic activity. The

Table 4 Haemolymph volumes and total cell counts of haemocytes extracted from *Limax maximus*, *Arion lusitanicus* and *Achatina fulica*

Limax. maximus ($n = 12$)	Haemolymph volume (μL)	Haemocyte number
Maximum	250	555,000
Minimum	65	15,000
Median	112.5	61,250
Arion lusitanicus ($n = 18$)	Haemolymph volume (μL)	Haemocyte number
Maximum	600	256,000
Minimum	50	5000
Median	125	27,500
Achatina fulica ($n = 9$)	Haemolymph volume (μL)	Haemocyte number
Maximum	1000	731,200
Minimum	278	156,565
Median	340	396,880

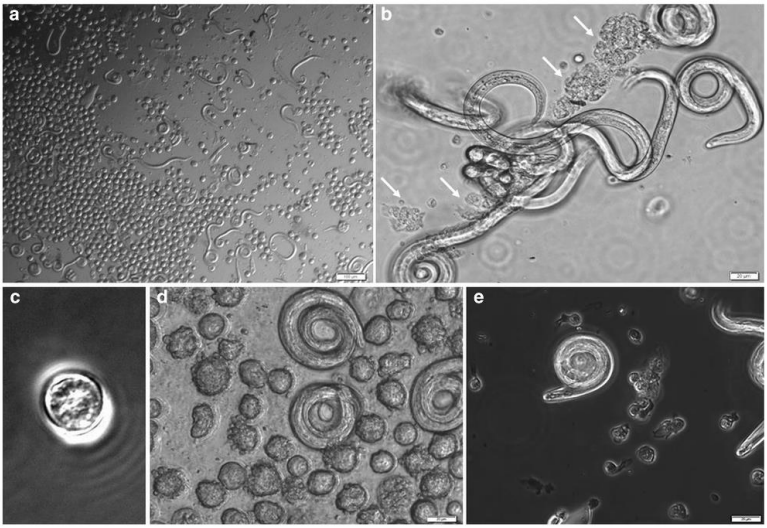

Fig. 6 Co-cultures of gastropod haemocytes and *A. vasorum* larvae. (**a**) *Limax maximus* haemocytes co-cultured with axenic and viable *A. vasorum* L1. (**b**) Cluster of *Arion lusitanicus* haemocytes acting against *A. vasorum* L1s forming aggregated material (arrows, 30 min). (**c**) Non-activated *Limax maximus* haemocyte. (**d**) *Limax maximus* haemocytes reacting against *A. vasorum* L1 showing activated cell surface (arrows, 30 min). (**e**) *Achatina fulica* haemocytes acting against *A. vasorum* L1 at an early time point in the reaction (arrows, 5 min)

nature of the changes depends on both gastropod and trematode species as well as on the developmental stage of the parasite (van der Knaap and Loker 1990). Haemocytes of *B. glabrata* entrapped mother sporocysts of *Schistosoma mansoni* in an in vitro confrontation through extracellular prolongation described as filopodia (van der Knaap and Loker 1990). Given that ETs were not reported until 2004, it would be interesting to investigate if trematode–gastropod interaction can induce similar innate immune mechanisms. Previous data on gastropod-borne trematode infections emphasize that these interactions are regulated by a highly complex molecular crosstalk which involves numerous antigens, immune receptors and anti-effector systems [for details, see Coustau et al. (2015)]. Some of these molecules are highly diversified among gastropods and digenean parasite populations (Adema and Loker 2015; Coustau et al. 2015; Dheilly et al. 2015). These findings could be similar for metastrongyloid infections of humans, domestic animals and wildlife animals. Thus, better understanding of invertebrate-pathogen molecular crosstalk and the identification of key factors capable to impair metastrongyloid development is crucial. Utilizing these novel data raises interesting possibilities for developing new strategies towards blocking/controlling or even disrupting the transmission of gastropod-borne diseases.

Conclusions and future perspectives

The present study describes how gastropods can be bred successfully under standardized conditions and how offspring can be used for immunological analyses. Therefore, emphasis was taken on improved protocols for the collection of sufficient volumes of haemolymph and for the isolation of vital haemocytes which can be used for experiments on haemocyte-mediated innate effector mechanisms. Interactions of gastropod-derived haemocytes with metastrongyloid parasites were also addressed here.

The presented methods will improve basic investigations on molecular immunological interactions between slugs as well as snails and metastrongyloids or other

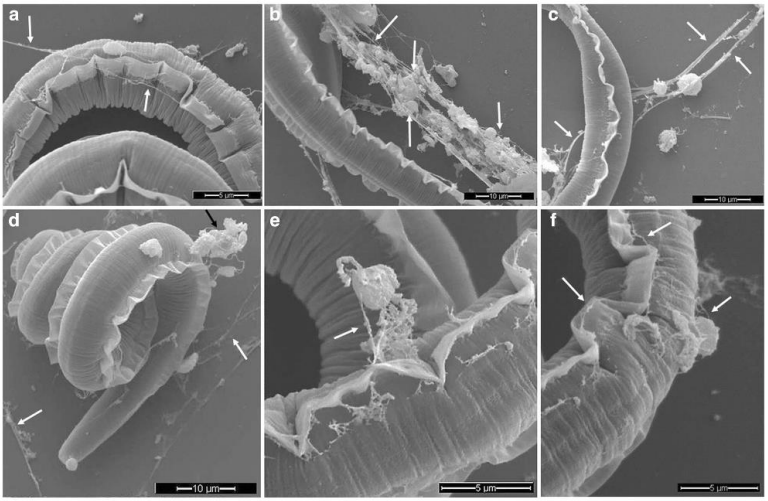

Fig. 7 Scanning electron microscopy analysis of gastropod haemocytes—*A. vasorum*-L1-co-cultures. (**a**) *Limax maximus* haemocytes co-cultured with axenic *A. vasorum* L3, spread ETs (*spr*InEPTs, arrows, 30 min). (**b**) *Limax maximus* haemocytes co-cultured with axenic *A. vasorum* L1, aggregated ETs (*agg*InEPTs, white arrows, 60 min). (**c**) *Arion lusitanicus* haemocytes reacting against *A. vasorum* L1, spread ETs (*spr*InEPTs, arrows, 30 min). (**d**) *Arion lusitanicus* haemocytes reacting against *A. vasorum* L1, aggregated ETs in contact with the larvae's alae (*agg*InEPTs, black arrow) and spread ETs (*spr*InEPTs, white arrows, 30 min). (**e**) *Achatina fulica* haemocytes acting against *A. vasorum* L1, spread ETs (*spr*InEPTs, arrows, 30 min). (**f**) *Achatina fulica* haemocytes acting against *A. vasorum* L1, delicate ET fibres in contact with the alae of the larvae (arrows, 60 min)

nematodes and therefore contribute to more detailed knowledge on invertebrate immunology. Basic research on early innate immune responses against parasites is fundamental in determining which pathways control these interactions. The methods described here could also set the basis for in-depth investigations not only on the pathophysiology and biology of gastropod-borne parasitoses but also on classical immune defense strategies, such as encapsulation, nodulation and melanisation. A solid knowledge on intermediate host–parasite molecular crosstalk and interplay may provide new strategies to disrupt the life cycle of emerging anthropozoonotic parasitic diseases, such as *A. cantonensis* and *A. costaricensis* infections.

Authors' contributions CH, AT and FPT conceived and designed the protocols. FPT and MKL performed haemocyte-related experiments. UG and AS performed the scanning electron microscopy (SEM) analysis. HM contributed with the constant supply of *A. vasorum* larvae. FPT, MKL and CH drafted the work. CH, AT and HM revised the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

The study published does not involve living vertebrates or invertebrates of the classes Cephalopoda or Decapoda. Nevertheless, gastropods involved were treated humanely avoiding unnecessary pain, distress, suffering or lasting harm. All applicable international, national (German Animal Welfare of 25.05.1998—BGBL I S.1105—section 5 paragraph 8a) and/or institutional guidelines for the care and use of animals/invertebrates were followed. The isolation of *A. vasorum* L1 from faeces of experimentally infected red foxes (*Vulpes vulpes*) were kindly provided by the Department of Veterinary Disease Biology, University of Copenhagen, Denmark, Danish experimental animal license no. 2010/561-1914.

According to the German Animal Welfare Act (Tierschutzgesetz of 25.05.1998—BGBL I S.1105—section 5 paragraph 8a), ethics approval

research with invertebrates is only required for experiments where animals of the classes Cephalopoda and Decapoda are used.

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could bear a potential conflict of interest.

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3.4 Gastropod-derived haemocyte extracellular traps entrap metastrongyloid larval stages of *Angiostrongylus vasorum*, *Aelurostrongylus abstrusus* and *Troglostrongylus brevior*.

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Gastropod-derived haemocyte extracellular traps entrap metastrongyloid larval stages of *Angiostrongylus vasorum*, *Aelurostrongylus abstrusus* and *Troglostrongylus brevior*

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Abstract

Background: Phagocyte-derived extracellular traps (ETs) were recently demonstrated mainly in vertebrate hosts as an important effector mechanism against invading parasites. In the present study we aimed to characterize gastropod-derived invertebrate extracellular phagocyte trap (InEPT) formation in response to larval stages of important canine and feline metastrongyloid lungworms. Gastropod haemocytes were isolated from the slug species *Arion lusitanicus* and *Limax maximus*, and the snail *Achatina fulica*, and exposed to larval stages of *Angiostrongylus vasorum*, *Aelurostrongylus abstrusus* and *Troglostrongylus brevior* and investigated for gastropod-derived InEPT formation.

Results: Phase contrast as well as scanning electron microscopy (SEM) analyses of lungworm larvae-exposed haemocytes revealed ET-like structures to be extruded by haemocytes thereby contacting and ensnaring the parasites. Co-localization studies of haemocyte-derived extracellular DNA with histones and myeloperoxidase in larvae-entrapping structures confirmed classical characteristics of ETs. In vivo exposure of slugs to *A. vasorum* larvae resulted in InEPTs being extruded from haemocytes in the slug mucous extrapallial space emphasizing the pivotal role of this effector mechanism against invasive larvae. Functional larval entrapment assays demonstrated that almost half of the haemocyte-exposed larvae were contacted or even immobilized by released InEPTs. Overall, as reported for mammalian-derived ETs, different types of InEPTs were here observed, i.e. aggregated, spread and diffused InEPTs.

Conclusions: To our knowledge, this study represents the first report on metastrongyloid lungworm-triggered ETosis in gastropods thereby providing evidence of early mollusc host innate immune reactions against invading larvae. These findings will contribute to the better understanding on complex parasite-intermediate host interactions since different gastropod species bear different transmitting capacities for metastrongyloid infections.

Keywords: Gastropod-borne diseases, Metastrongyloidea, Extracellular traps, Lungworm, Innate immune response

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Background

Nowadays increasing attention is being paid to gastropod-borne diseases, both in veterinary and human medicine, on academic, pharmaceutical and clinical practice levels [1–5]. In the last decade canine and feline lungworm species such as *Angiostrongylus vasorum*, *Aelurostrongylus abstrusus* and *Troglostrongylus brevior* are emerging in several countries and spreading into previously non-reported areas [5–12]. Especially the former parasite induces a debilitating disease of the cardiorespiratory system [5, 13] but can also cause neurological, ophthalmic and systemic disease with sometimes life threatening coagulopathies in dogs. The knowledge on how lungworm larval development occurs within gastropod species in vivo is still scarce. Consequently, very little is known on early gastropod-mediated innate immune reactions against these parasites and respective research is urgently needed [12].

In contrast to mammalian species which possess both an adaptive and an innate immune system, gastropods exclusively rely on innate immune responses for pathogen inactivation. Typical mammalian professional mononuclear phagocytes, such as polymorphonuclear neutrophils (PMN), monocytes and macrophages, are lacking in molluscs but are replaced by gastropod-specific phagocytes known as haemocytes (syn. amoebocytes), an invertebrate immune cell subtype that freely circulates within the haemolymph system [14]. Haemocytes were reported to be involved in several physiological functions such as wound repair, coagulation, transport of nutrients and other molecules and intracellular digestion [15, 16]. Overall, this cell type is known as the key player of the molluscan innate immune system [17]. The currently known effector mechanisms of haemocytes are phagocytosis, multicellular encapsulation and cell-mediated cytotoxicity. However, the detailed molecular mechanisms of these immunological processes are not well understood, so far [18–22].

Beginning with the landmark study of Brinkmann et al. [23] which introduced with (N) ETosis a new effector mechanism of PMN, the paradigm of how professional phagocytes fight and kill pathogens has profoundly been changed. ETosis represents a novel type of programmed cell death of PMN and other leucocytes in which the nuclear chromatin and granular proteins are expulsed to the extracellular environment forming thin fibre-like extracellular structures bearing the capacity to capture and inactivate invasive pathogens [24–29]. Besides histones/DNA, several antimicrobial granular molecules, such as defensins, cathelicidins, pentraxin, myeloperoxidase (MPO), calprotectin and lactoferrin [30–32], were reported to be contained within ETs. ETs are cast by different types of leukocytes (e.g. PMN, macrophages, mast cells, monocytes, eosinophils) [33–37], in response to different microorganisms such as bacteria [23], fungi [38], viruses [39] and protozoan/metazoan parasites [29, 40, 41]. ETosis has been reported to occur in numerous vertebrate host types, such as humans, cattle, goats, seals, fish or birds [25, 33, 42–45] as effective innate immune defence mechanism. Consistently, also haemocytes were recently reported to release ETs in invertebrate organisms such as crustaceans and bivalves [46–49]. Extracellular traps have, however, not yet been described in gastropods.

The aim of this study was to investigate for the first time gastropod-derived invertebrate extracellular phagocyte traps (InEPTs) as early host innate immune reactions in different gastropod species (*Limax maximus*, *Arion lusitanicus* and *Achatina fulica*) in response to infective metastrongyloid larvae of *Angiostrongylus vasorum*, *Aelurostrongylus abstrusus* and *Troglostrongylus brevior* in vitro and in vivo. We here present first indications of InEPTs being cast by gastropod haemocytes upon larval exposure. This gastropod immune reaction might have an impact on the development of these obligate heteroxenous lungworm parasites in their intermediate hosts.

Methods

Gastropod maintenance

Terrestrial slugs (*A. lusitanicus* and *L. maximus*) and terrestrial giant African snails (*A. fulica*) were bred and maintained in fully-automatized climate incubators (model ECP01E®; Snijders Scientific B.V. Tilburg, the Netherlands) under the following controlled conditions: 50% humidity, 10 h of dark/10 h of illumination corresponding to circadian cycles plus 2 h for dusk and dawn each, temperature ranging from 10 to 16 °C (night/day) regarding the slug species and 20–26 °C for *A. fulica*. The slug species were kept in plastic containers supplied with a humidified absorption paper at the bottom, plastic Petri dishes for food and a plastic dim housing area (Tecniplast®). Gastropod feedings were performed *ad libitum* twice a week with lettuce leaves (*Lactuca sativa*), cucumber fruits (*Cucumis sativus*), carrot roots (*Daucus carota sativus*), champignons (*Agaricus campestris*), rabbit pellet food (VERSELE-LAGA®; CUNIFIT pure) and dry dog food (Purina®, Beneful). *Achatina fulica* were maintained in plastic containers on terrarium soil (5 cm height, TerraBasis® and TerraCocoshumus® mixed at 1:1 ratio, JBL) being supplemented with calcium supplement (*ad libitum* 21% calcium, Calcina Calcium Citrat®, Canina).

Generation of axenic metastrongyloid first- (L1) and third-stage (L3) larvae

Angiostrongylus vasorum first-stage larvae (L1) were obtained from fresh faeces of experimentally infected red

foxes (*Vulpes vulpes*) kindly provided by the Department of Veterinary Disease Biology, University of Copenhagen, Denmark (Danish experimental animal licence no. 2010/561-1914). *Aelurostrongylus abstrusus* and *T. brevior* larvae were recovered from infected cat faeces (kindly donated by S. Rehbein, Merial, Germany). All lungworm larvae were isolated by the classical Baermann funnel technique: 20 g of faeces were placed in a Baermann apparatus equipped with a sieve (aperture 100 µm) and three gauze layers. The funnel was slowly filled with water (20–25 °C) until half of the faecal sample was immersed in water. The apparatus was incubated at room temperature (RT) for 24 h during which the larvae migrated from the faeces into the water owing to positive hydrotaxis and sedimented. By carefully opening the clamp 5 ml sediment were collected in 15 ml conical tubes (Greiner). Then the larvae were concentrated and freed from faecal contamination by Percoll gradients (Merck) as reported elsewhere by Graeff-Teixeira et al. [50].

Angiostrongylus vasorum third-stage larvae (L3) were generated from experimentally infected *L. maximus* slugs at 30 days post-infection (p.i.) *via* artificial digestion. The experimental procedure was the following: slugs were cut in small pieces and digested in digestion solution [1 l contained 10 g pepsinogen powder 2000 FIP-U/g (Robert Kind), 8.5 g NaCl (Carl Roth), 30 ml HCl 37% (Carl Roth), distilled water ad 1 l]. The digestion was performed in 50 ml Falcon tubes (Greiner) under constant shaking (4 h, 40 °C). The digested samples were sieved firstly through a 300 µm-metal sieve (Retsch) to remove undigested material and then through a 25 µm-metal sieve (Retsch). The remnants of the last sieving were transferred to 15 ml Falcon tubes and centrifuged (400× *g*, 10 min). The pellets were resuspended and examined microscopically (Leica light microscope at 4× and 20× magnification). Viable metastrongyloid larvae were carefully collected by pipetting (Pasteur pipette, Hirschmann GmbH & Co. KG).

To remove any bacterial contaminants and to achieve axenic L1 and L3, larvae were incubated for 10 min in 10 ml sodium hypochlorite solution (0.5% *v*/*v*; Carl Roth) prepared with sterile phosphate-buffered saline (PBS) as previously described elsewhere [51]. Additionally, the larvae were washed twice (250× *g*, 5 min, 20 °C) in sterile PBS supplemented with 3% penicillin (500 U/ml; Sigma-Aldrich) and streptomycin (500 µg/ml; Sigma-Aldrich, Darmstadt, Germany). Axenic larvae were prepared two days before InEPT-related experiments in order to conserve a high larval viability.

Slug exposure to *Angiostrongylus vasorum* first-stage larvae (L1) in vivo

In order to evaluate the earliest time point of gastropod-mediated innate immune reactions directed against invading metastrongyloid nematodes, a living juvenile slug (*L. maximus*) of *c*.5 mm of length was exposed to viable axenic L1 of *A. vasorum*. Therefore, the juvenile slug was allocated on a cover slip of 10 mm diameter in a 12-well-plate and confronted to 500 L1 axenic *A. vasorum* larvae diluted in 1 ml sterile PBS. After 10 min of incubation the juvenile slug was cryo-anesthetizied (-20 °C, 5 min), fixed in 2.5% glutaraldehyde (60 min, RT, Merck) and processed for scanning electron microscopy (SEM).

Scanning electron microscopy (SEM)

The fixed samples were post-fixed in 1% osmium tetroxide (Merck, Darmstadt, Germany), washed in distilled water, dehydrated, critical point dried by CO_2-treatment and sputtered with gold. Thereafter, the samples were examined with a Philips XL30 scanning electron microscope at the Institute of Anatomy and Cell biology, Justus Liebig University Giessen, Germany.

Haemolymph extraction and in vitro culture of gastropod haemocytes

Gastropods were subjected to a 48 h fasting period and cryo-anesthetized (40 min on ice) before haemocyte isolation was performed. A modified serum-free haemocyte collection solution [77% RPMI, 20% anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA and 41 mM citric acid, pH 4.5) and 3% penicillin/streptomycin (Sigma-Aldrich, penicillin 10,000 U/ml, streptomycin 10 mg/ml)] according to Stoepler et al. [52] was injected into each cryo-anesthetized gastropod corresponding to 10% of its body weight. Thereafter, slugs were cryo-anesthetized (20 min on ice) again before euthanasia was performed via fast decapitation as described elsewhere [53]. The haemolymph samples were collected by aspiration from decapitated slugs and mixed immediately with 200 µl sterile culture medium composed of penicillin- (500 U/ml; Sigma-Aldrich) and streptomycin- (500 µg/ml; Sigma-Aldrich) supplemented RPMI 1640 medium (Gibco). The haemolymph of *A. fulica* was collected *via* insertion of a needle with syringe close to the pneumostome as described elsewhere [54] and treated the same way as the haemolymph of slugs. The cells were washed thrice (250× *g*, 5 min) and counted in a Neubauer haemocyte chamber. The gastropod haemocytes were co-cultured with axenic L1 of *A. vasorum*, *A. abstrusus* and *T. brevior* as well as with L3 of *A. vasorum* (Table 1) on poly-$_L$-lysine (Sigma-Aldrich) pre-coated coverslips at a ratio of 200:1 (RT, in the dark 30 min). Thereafter, the samples were fixed either in 2.5% glutaraldehyde (RT, Merck) for SEM analysis or in 4% (*w*/*v*) paraformaldehyde for fluorescence microscopic analyses and stored at 4 °C until further used. For negative controls cells were treated the same

Table 1 Overview of conducted nematode/gastropod confrontations

	Limax maximus	*Arion lusitanicus*	*Achatina fulica*
Angiostrongylus vasorum	L1		L1 and L3
Aelurostrongylus abstrusus	L1	L1	L1
Troglostrongylus brevior	L1		
In vitro	×	×	×
In vivo	×		
SEM	×		×
Phase contrast microscopy	×	×	×
Immunofluorescence microscopy		×	×

way as mentioned above. Instead of lungworm larvae only culture medium was added ($n = 5$).

Characteristics to determine ET morphology

According to Muñoz-Caro et al. [29], Schauer et al. [55] and Hakkim et al. [56] ET structures were described referring to their appearance as "diffuse" ETs (*diff*ETs), "spread" ETs (*spr*ETs), "aggregated" ETs (*agg*ETs) and ETs that display an anchor-like effect. "Diffuse" ETs (*diff*ETs) are described as consisting of a globular and compact form with a size of 25–28 nm diameter, whereas "spread" ETs (*spr*ETs) were observed consisting of smooth and elongated web- like structures composed exclusively by thin fibers with a diameter of 15–17 nm. Furthermore, so-called "aggregated" ETs (*agg*ETs), according to Schauer et al. [55] were displayed as large clusters with a "ball of yarn"-like clumpy and massive appearance involving a high number of immune cells. These ET structures appeared with sizes larger than 50 μ m in diameter. A reduction in larval forward-motility due to fine ET structures (*spr*ETs) which are connected to the *agg*ETs entrapping larvae and clearly hampering larval motility was referred to as an anchor-like effect.

Immunofluorescence analyses of InEPTs

For DNA staining the samples were stained by DAPI according to Martinelli et al. [57] and Lippolis et al. [58]. For the detection of nuclear histones and MPO within haemocyte-derived ET structures the following specific monoclonal antibodies were used: anti-histone (H1, H2A/H2B, H3, H4; Merck Milipore, MAB3422, 1:1000) and anti-MPO (Biorbyt; orb11073, 1:1000). The samples were washed twice in PBS and blocked with bovine serum albumin (BSA, 2%, Sigma-Aldrich) and reacted with primary antibody solutions (1 h, RT). After two washings, the samples were incubated for 1 h (RT, in the dark) either in Alexa Fluor-conjugated goat anti-mouse monoclonal antibody solution (mouse clone, 1:1000, Thermo Fisher Scientific, for histone detection) or in goat anti-rabbit antibody solution (1:1000, Thermo Fisher Scientific, for MPO detection). The samples were then washed in PBS, mounted in anti-fading buffer (Mowiol®; Sigma-Aldrich) and examined microscopically (Olympus IX81® phase contrast microscope equipped with a digital camera and the analySIS® software).

InEPT-entrapment assay

Gastropod haemocytes ($n = 3$, 1×10^4) were seeded on poly-$_L$-lysine pre-coated coverslips and exposed to axenic *A. vasorum*-L1 (50 larvae/sample) in 300 μl RPMI medium 1640 (1% penicillin/streptomycin, without phenol red, Sigma-Aldrich, for 30 min at RT). Thereafter, the coverslips were fixed (4% paraformaldehyde) and InEPT-entrapped larvae were counted by using an inverted DMIRB® phase contrast microscope (Leica). Larvae were considered as entrapped when cell aggregates of at least two haemocytes or stretches of different kinds of ETs (*agg*InEPTs, *spr*InEPTs or *diff*InEPTs) were in direct contact with the larvae. The data were expressed as percentage of entrapped L1 relative to the total amount of *A. vasorum* L1.

Haemocytes of three gastropod species (*Limax maximus*, *Arion lusitanicus* and *Achatina fulica*) were confronted to three nematode species (*Angiostrongylus vasorum*, *Aelurostrongylus abstrusus* and *Troglostrongylus brevior*) of different parasitic stages (L1 and L3) in in vitro and in vivo experiments with the use of different visualisation methods (SEM, phase contrast microscopy, immunofluorescence microscopy).

Results

Metastrongyloid lungworm larvae induce gastropod InEPTs in a parasite species- and stage-independent manner

Microscopic analyses revealed that exposure of first- and third-stage larvae of *A. vasorum* to gastropod haemocytes trigger the formation of ET-like structures indicating a stage-independent process (Additional file 1) since both larval stages (L1, L3) were attacked by InEPTs. To account for parasite-specificity we also tested *A. abstrusus*- and *T. brevior*-L1 for their capability to induce InEPTs and indeed both parasite species triggered InEPT formation in exposed haemocytes (see Additional file 1; Additional file 2: Figure S1; Additional file 3: Figure S2). These data clearly provide evidence against a parasite-specificity of metastrongyloid-triggered InEPT formation highlighting the capacity of gastropod haemocytes to equally react to different lungworm parasites therefore the reaction is species-independent.

Overall, we detected typical 'diffuse' InEPTs (*diff*InEPTs) which are reported to be composed of a complex of extracellular decondensed chromatin adorned with antimicrobial histones and proteins and show a rather compact form with sizes of 20–30 μm in diameter [29]. Additionally, 'spread' InEPTs (*spr*InEPTs) were here observed consisting of smooth and extremely elongated web-like extracellular structures (Figs. 1, 2 and 3). Interestingly, in the case of *A. vasorum* larvae InEPTs were consistently observed in close proximity to the alae of the larvae indicating this structure as a possible target of haemocytes (Fig. 3d). Furthermore, the presence of metastrongyloid-triggered 'aggregated' InEPTs (*agg*InEPTs), in accordance with Schauer et al. [55] and Muñoz-Caro et al. [29], were also detected as large clusters of ET-like structures with a clumpy morphology reaching sizes larger than 50 μm in diameter and involving a high number of haemocytes (Fig. 3c). Especially the combination of these structures with *spr*InEPTs appeared strong enough to hamper larvae from movements by entangling them (see Fig. 3 and Additional file 1). Thus, anchor-like effects originating from few haemocytes captured lungworm larvae mainly at one end of the body and were often connected to *agg*InEPTs. Although larvae moved rigorously to escape they seemed entrapped in these InEPT structures (see Fig. 3 and Additional file 1).

Irrespective of the InEPT types, antibody-based experiments revealed that gastropod-derived InEPTs were composed of extracellular chromatin being decorated with histones (H1, H2A/H2B, H3, H4) and MPO-like proteins thereby confirming classical characteristics of ETs (Fig. 4).

Metastrongyloid larvae-driven InEPTosis is (intermediate) host-independent

Lungworm parasites are known to infect a broad panel of gastropod intermediate host species. In order to evaluate whether metastrongyloid-mediated InEPTosis is an intermediate host-specific event or rather represents a general effector mechanism accounting for most slugs and snail species, we also analysed this parasite-triggered InEPT formation in three different gastropod species, i.e. in *A. lusitanicus*, *L. maximus* and *A. fulica* (Table 1). Overall, haemocytes of all three gastropod species cast InEPTs in response to vital metastrongyloid lungworm larvae which argues against a strict intermediate host-specific reaction.

Gastropod InEPTs entrap *Angiostrongylus vasorum* larvae

In order to analyse the efficacy of haemocyte-derived InEPTs to entrap viable *A. vasorum* L1, we established a quantitative parasite-entrapment assay by exposing

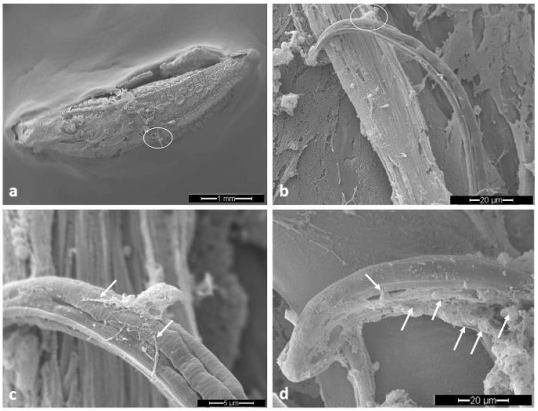

Fig. 1 In vivo InEPT formation in *Limax maximus* as early response against living *Angiostrongylus vasorum* larvae. **a** Scanning electron microscopy overview on the total slug, *circle* shows the area of interest which is magnified in (**b**). **b** *Angiostrongylus vasorum* larvae on the slug surface, *circle* shows area of interest which is magnified in (**c**). **c** Haemocytes in tight contact to lungworm larvae releasing delicate ET-like fibres (*spr*InEPTs, *arrows*). **d** Haemocytes (*arrows*) on the surface of the slug attached to a L1 larvae releasing delicate web-like structures (*diff*InEPTs) which cover the surface of the larvae

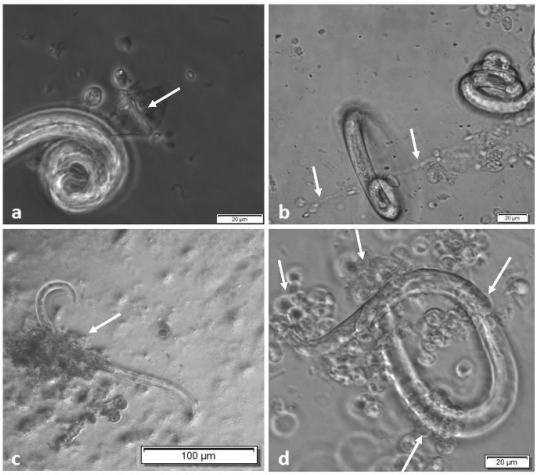

Fig. 2 Early ET formation against metastrongyloid lungworm larvae. **a** *Achatina fulica* haemocytes acting against *A. vasorum* L1, spread ETs (*spr*InEPTs, *arrow*, 30 min). **b** *Achatina fulica* haemocytes acting against *A. vasorum* L1, spread ETs (*spr*InEPTs, arrows, 30 min). **c** *Arion lusitanicus* haemocytes acting against *A. abstrusus* L1, aggregated ETs (*agg*InEPTs, *arrows*, 60 min). **d** *Achatina fulica* haemocytes against *A. vasorum* L1, aggregated ETs (*agg*InEPTs, *arrows*, 60 min)

haemocytes to *A. vasorum*-L1 and thereafter counting larvae that were found either entrapped within extruded InEPT structures or attached by haemocyte agglomerates. Functional parasite-entrapment experiments revealed a proportion of 41% of *A. vasorum* larvae to be in contact and most probably immobilized by InEPT structures (see Fig. 5). These results indicate a rather high efficacy of gastropod InEPT-mediated entrapment considering that almost every second larva was ensnared and most probably hampered from intermediate host invasion.

In vivo slug exposure to *Angiostrongylus vasorum*-L1 results in extrapallial InEPT formation

Given that slug haemocytes are known to be also present in the peripheral extrapallial fluid [17, 59, 60], we here intended to visualize the first contact of invading parasites on the surface of the slug. In vivo SEM evaluation of this earliest host innate immune reaction against *A. vasorum*, clearly demonstrated that extrapallial haemocytes were present in the slug mucus and firmly attached to the cuticle of invading larvae (Fig. 1). As a defence mechanism, some of these haemocytes cast ET-like structures towards the nematode cuticle (Fig. 1). These ET-like extracellular structures were also detected in a more spread, net-like pattern originating from more than one haemocyte on the surface of the larvae. In line with previous descriptions of mammalian ET morphologies [29] we here found both, smooth and elongated extracellular structures composed exclusively of thin fibres (Fig. 1c) and rather web-like spread forms (Fig. 1d). Given that the experiment was performed already 10 min after the first contact between parasite and intermediate host these data clearly confirm that InEPT induction represents a very fast and early effector mechanism that even precedes pathogen invasion into the slug corpus, i.e. in the extrapallial space (mucus) of gastropods.

In the negative controls no cell aggregates were observed in four of the five experiments. Only in one coverslip cells formed occasionally aggregates without typical ET-like fibres.

Discussion

To our knowledge, this study delivers first evidence on the release of ETs as part of the early innate immune response of gastropod haemocytes acting against metastrongyloid lungworm larvae. So far, ETosis was reported

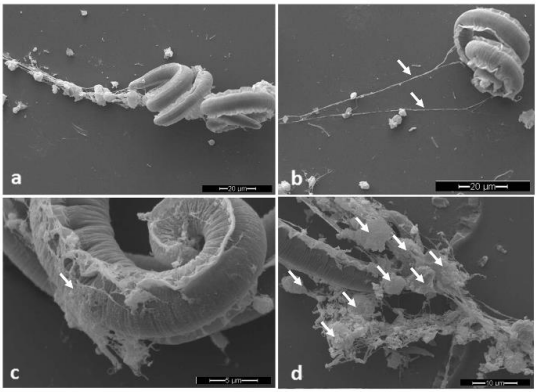

Fig. 3 ETs formed by gastropod haemocytes after the confrontation with *Angiostrongylus vasorum* L1. Scanning electron microscopy analysis revealed InEPTs being formed by haemocytes of *A. fulica* co-cultured with L1 showing different types of InEPTs **a** two larvae entrapped by a chain of InEPTs and haemocytes, **b** spread InEPTs (*spr*InEPTs, *arrows*), **c** diffused InEPTs (*diff*InEPTs, *arrows*), **d** haemocytes (*arrows*) forming aggregated InEPTs (*agg*InEPTs)

only in few other invertebrate hosts, such as oysters (*Crassostrea gigas* [46, 61]), the shore crab *Carcinus meanas* and the blue mussel *Mytilus edulis* [47] and shrimp [62]. ETs mainly consist of chromatin, nuclear histones (H1, H2A/H2B, H3, H4) and granular components, such as NE, MPO, lactoferrin, pentraxin and gelatinase amongst others [24, 26, 40]. In accordance to recent findings on InEPT reactions of the shore crab [47] we here confirmed the typical decoration of externalised chromatin of InEPTs by co-localization with histones (H1, H2A/H2B, H3, H4) and MPO on gastropod-derived InEPTs being cast against lungworm larvae. These results are also in accordance to reports on parasite-triggered ETosis in the mammalian system, which demonstrated histones and MPO as main components of ETs in vitro as well as in vivo [25, 29, 33, 40, 43, 63–65]. Regarding the effect of deoxyribonuclease (DNase) treatment in mammalian ET-formation Behrendt et al. [25] described the disintegration of NETs, highlighting the backbone-nature of chromatin. Moreover, DNase treatment on parasite-triggered ET formation illustrated that entrapped *Besnoitia besnoiti* tachyzoites were neither killed by ETs since their host cell infectivity was entirely restored upon DNase treatment [37, 64]. Until now not much is known on ET-evasion-mechanisms of parasites [40] but Guimarães-Costa et al. [66] mentioned the ability of *Leishmania* parasites to escape NET-mediated killing by producing nucleases. For future investigation it would be furthermore of interest to conduct in vitro assays with known ETosis inhibitors to proof that InEPT formation in gastropods is controlled and deliberate and is not a consequence of parasite-haemocyte damage that allows the nuclear material to escape passively from the host cell.

During the life-cycle of most metastrongyloid nematodes of domestic animals and humans, exogenous metastrongyloid L1 must actively invade terrestrial mollusc intermediate hosts to fulfil further development into infective L3 [2, 67]. By performing this obligate step of the life-cycle, larvae will become potential targets of the mollusc innate immune system. Mollusc haemocytes, which correspond to mammalian professional phagocytes, such as neutrophils, monocytes, macrophages, are known to be actively recruited to the site of infection and additionally to actively migrate into the gastropod mucous extrapallial space [17, 59, 60]. The current in vivo and in vitro data demonstrate that gastropod-derived ETosis is a conserved, ancient and efficient effector mechanism as already demonstrated elsewhere [24, 25, 33, 40, 64, 68]. So far, several reports exist on protozoan-triggered ETosis [25, 42, 43, 63–65, 68–70] whilst only few data are available on metazoan-triggered ETosis. First ever reported metazoan-induced ETosis was reported on trematodes. As such, *Schistosoma japonicum* has recently been identified as potent ET-inducer in vitro and in vivo [41]. Besides the here described lungworms, ET-inducing nematodes species

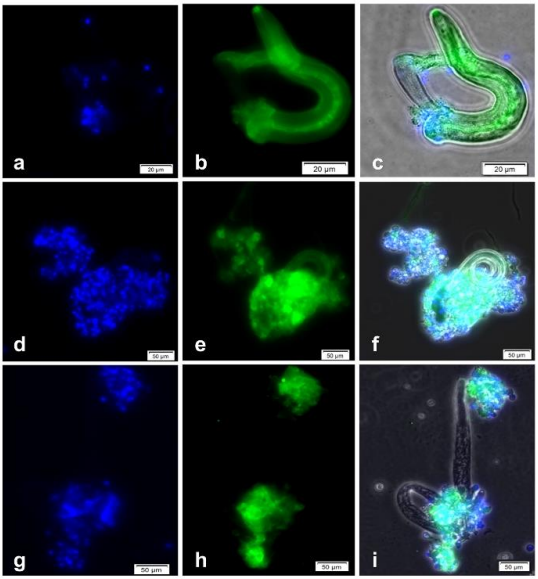

Fig. 4 Co-localisation of DNA, histones (H1, H2A/B, H3, H4) and MPO in InEPT structures. Co-cultures of *A. lusitanicus* haemocytes with *A. abstrusus* (**a**-**c**) and *A. fulica* haemocytes *with A. vasorum* L1 (**d**-**i**) induced the formation of ETs with the classical constituents: DNA (*blue* DAPI, **a**, **d**, **g**), histones (*green*, **b**) and MPO (*green*, **e**, **h**). The merges illustrate the larvae and the constituents of the ETs derived from haemocytes (**c**, **f**, **i**)

include only *Strongyloides stercoralis* [71] and *Haemonchus contortus* [29].

The current data suggest that gastropod-derived InEPT formation is a rather general effector mechanism against metastrongyloid parasites. Thus, gastropod-derived InEPT release occurred irrespective of the parasite species (i.e. *A. vasorum, A. abstrusus*, *T. brevior*), the parasite stage (L1 *vs* L3) and of the haemocyte origin (i.e. *A. lusitanicus*, *L. maximus*, *A. fulica*). In agreement, data on different protozoans, such as *Leishmania* spp. [69] and *Eimeria* spp. [65], also indicated ETosis as a parasite species- and stage-independent defence effector mechanism. In addition, *Eimeria bovis*- and *Cryptosporidium parvum*-triggered ETosis has recently also been reported as host origin-independent innate immune reaction [65]. It is worthwhile to mention, that the efficacy of ET-mediated nematode entrapment differed between different parasite and host species. Thus, in the current work, up to 41% of larval entrapment was observed which is considerably lower compared to *H. contortus* NET-mediated larval entrapment in the mammalian system [29]. This survey represents the first report on InEPTs extruded by haemocytes against lungworms and these data will serve as a base line for further in depth investigation.

As an interesting feature, we observed the in vitro-release of different types of InEPTs, such as *diff*InEPTs, *spr*InEPTs and *agg*InEPTs and anchor-like effects upon contact with larvae of the lungworm species *A. vasorum*, *A. abstrusus* and *T. brevior*. Anchor-like nematode entrapment performed by gastropod haemocytes is consistent to previously reported NETs acting against *Haemonchus contortus* [29]. At least two types of InEPTs (i.e. *diff*InEPTs, *spr*InEPTs) were additionally found to occur in vivo within the mucus of slugs acting against actively invading *A. vasorum* L1. All types of InEPTs promoted efficient

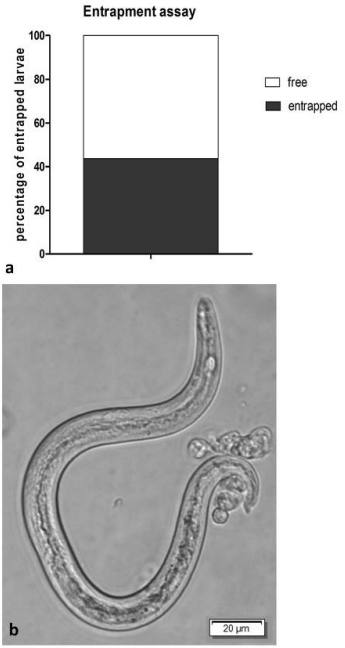

Fig. 5 Entrapment assay of *Angiostrongylus vasorum* L1 exposed to haemocytes of *Achatina fulica*. Co-cultivation of haemocytes of *A. fulica* were seeded on poly-$_L$-lysine pre-coated coverslips and exposed to *A. vasorum* first-stage larvae. Larvae were considered as entrapped when cell aggregates of at least two cells were in contact with the larvae (**b**). The data are expressed as percentage of attacked L1 relative to the total amount of L1 (**a**)

ensnarement of the larvae since almost half of the larvae were found immobilized by these extracellular structures. However, in contrast to other invertebrate ET reactions [48], lethal effects of InEPTs could not be observed even within a prolonged incubation period (4 h). Consequently, the tight immobilization of larvae seems to represent the key mechanism of larval-induced InEPTs. Thus, we postulate that even though InEPT formation themselves do not seem capable of killing nematode larvae they might entrap them and prevent active invasion and further migration through the mollusc body. Moreover, it seems plausible to assume that InEPT-entrapped larvae might become exposed to other recruited haemocytes [24, 40].

Importantly, *A. vasorum*-mediated InEPT induction already occurred within the mucous surface of the gastropod tegument, i.e. even before larval host penetration, thereby representing the earliest possible time point of parasite-intermediate host interactions. Given that generally only few larvae are detected in gastropod intermediate hosts [72], this early attack may thus have a high impact on the dispersal of the disease. So far, it is still unclear how slug haemocytes recognize the larvae in terms of ETosis and which parasite-derived molecules are involved in this process. Nonetheless, the current data on vital metastrongyloid larvae suggest that molecules are most probably present on the cuticle, but it cannot be excluded that also excretory/secretory molecules might be able to act as triggers of InEPT formation. In this context, haemocyte-derived sensing of the larvae might also be a matter of size since Branzk et al. [27] reported on the ability of professional phagocytes to distinguish between small- and large-sized pathogens and to selectively cast ETs in response to large pathogens. Recently, physical properties of particles, such as shape and rigidity, have also been demonstrated to influence on the type of response of phagocytes [73]. These mechanisms may apply for ET induction by large metazoan parasites such as lungworms, *S. japonicum*, *S. stercoralis* and *H. contortus*, especially since phagocytosis is ineffective against these large multicellular parasites. In agreement with Muñoz-Caro et al. [29], the main two functions of gastropod-derived InEPTs acting against large nematode larval stages may be firstly to reduce proper larval invasion and dispersal within the gastropod host and secondly to expose InEPT-entrapped larvae to other immune cells in vivo. A partial or even complete blockage of larval invasion by gastropods could result in diminished parasite burden and thereby significantly influence the intermediate host capacity of certain species. Considering the fact that ETs are mainly composed by antimicrobial histones, peptides and proteins, we also speculate that InEPTs might serve to administer haemocyte-derived anthelmintic compounds to the larval cuticle or in the near vicinity of entrapped parasites. As such, in the mammalian system, eosinophil-derived neurotoxin (EDN) will be more efficient if localized close to the larvae [29]. The release of EDN might restrict the larval motility thereby preventing further development and allowing adhesion of eosinophils to discharge toxic granule contents on the larval surface [74]. As such, eosinophil-derived ETosis was recently demonstrated as a response to the nematode *H. contortus* [29]. However, whether gastropod haemocyte granules also contain EDN-like molecules remains to be ascertained in the future. Nonetheless, our data strongly suggest mollusc InEPTs as

an important effector mechanism of haemocytes acting against metastrongyloid lungworms of dogs and cats.

Conclusions

In summary, we postulate that InEPTosis might diminish the establishment and development of metastrongyloid larvae within the obligate gastropod intermediate host by immobilizing the larvae and hampering them from migration through the tegument or body. Mollusc InEPTs may further facilitate the exposure and attack of entrapped large-sized parasites by other immunocompetent haemocytes. In consequence, lungworm larvae-triggered InEPTosis will also have an impact on the in vivo situation and may therefore influence the intermediate host capacities of different slug/snail species. Comparative studies on zoonotic relevant parasites, such as *A. cantonensis* and *A. costaricensis*, are urgently required to gain more knowledge on gastropod-borne parasitoses. Thus, we call for more mollusc-based investigations in order to better understand the actual spread of gastropod-borne diseases in humans, domestic as well as wild animals.

Additional files

Additional file 1: Metastrongyloid larvae entrapment by gastropod-derived InEPTs. (*MOV* 5119 kb)

Additional file 2: Figure S1. Metastrongyloid larvae of *Troglostrongylus brevior* and *Aelurostrongylus abstrusus* attacked by mollusc haemocytes. Early ET formation against L1 of *Troglostrongylus brevior* confronted to haemocytes of *Limax maximus* analysed with contrast phase microscopy. (DOCX 376 kb)

Additional file 3: Figure S2. Early ET formation against L1 of *Aelurostrongylus abstrusus* confronted to haemocytes of *Limax maximus* analysed with contrast phase microscopy. (DOCX 1533 kb)

Abbreviations

*agg*InEPTs: Aggregated InEPTs; DAPI: 4′,6-diamidino-2-phenylindole; *diff*InEPTs: Diffused InEPTs; DNA: Deoxyribonucleic acid; DNase: Deoxyribonuclease; EDN: Eosinophil-derived neurotoxin; EDTA: Ethylenediaminetetraacetic acid; ET: Extracellular traps; InEPT: Invertebrate extracellular phagocyte traps; L1: First-stage larvae; L3: Third-stage larvae; MPO: Myeloperoxidase; NE: Neutrophil elastase; PBS: Phosphate-buffered saline; PMN: Polymorphonuclear neutrophils; RPMI: Roswell Park Memorial Institute medium; SEM: Scanning electron microscopy; *spr*InEPTs: Spread InEPTs

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Availability of data and materials

All data generated or analysed during this study are included in the article and its Additional files.

Authors' contributions

CH, AT, RS, HM, MKL and FPT conceived and designed the InEPTs experiments. MKL, FPT, TMC and UG performed gastropod InEPT-related experiments. HM contributed with the supply of *A. vasorum* larvae. AT, CH, RS and HM revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval

Not applicable.

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4. Zusätzliche Untersuchungen und Ergebnisse

Während meines viermonatigen Auslandsaufenthaltes in Kolumbien wurde eine epidemiologische Studie zur Ermittlung der Prävalenzen von metastrongyliden Lungenwürmern in Achatschnecken (*Achatina fulica*) durchgeführt. Einige Daten dieser Untersuchungen sind bereits veröffentlicht (siehe 3.2.). Das Manuskript zu den rein epidemiologischen Daten wurde bereits eingereicht und ist hier im Anhang hinterlegt (Penagos-Tabares F., Lange M. K., Vélez J., Gutiérrez J., Hirzmann J., Taubert A., Hermosilla C., Chaparro Gutiérrez J. The invasive giant African snail *Achatina fulica* as natural intermediate host of *Aelurostrongylus abstrusus*, *Angiostrongylus vasorum*, *Troglostrongylus brevior* and *Crenosoma vulpis* in Colombia, 2018, *PLOS Neglected Tropical Diseases*), ist jedoch noch nicht akzeptiert. In Folge werden die entsprechenden Untersuchungen und Daten hier näher besprochen.

Des Weiteren wurde in Zusammenarbeit mit den kolumbianischen Kollegen eine Übersichtsarbeit unter meiner Ko-Autorenschaft veröffentlicht, welches auf den vernachlässigten Status von *A. abstrusus* und *A. vasorum* in Südamerika hinweist. Diese Veröffentlichung ist in Kapitel 6 hinterlegt.

4.1. Epidemiologische Untersuchungen zur Lungenwurmprävalenz in kolumbianischen Achatschnecken

Die Achatschnecke (*Achatina fulica*) wird in Kolumbien als invasiver Wirtschaftsschädling betrachtet und gilt als eine der am meisten verbreiteten invasiven Schneckenspezies im tropischen und subtropischen Raum (Raut und Barker 2002, Vogler et al. 2013). In Kolumbien ist diese Gehäuseschnecke bereits in 26 der 32 Provinzen vorgedrungen (Thiengo et al. 2007, Beltramino et al. 2015). Die Achatschnecke fungiert u. a. als Zwischenwirt diverser Metastrongyliden, die zu Erkrankungen sowohl bei Tieren als auch Menschen führen können (Lowe et al. 2000, Jing et al. 2015). Aufgrund ihrer Größe (7 - 20 cm, Abb. 10), ihrer Lebensspanne (bis zu 9 Jahre) sowie ihrer invasiven Tendenz wird angenommen, dass *A. fulica* in der Verbreitung von metastrongyliden Parasiten eine bedeutende Rolle zukommen könnte (Alicata 1966, Raut und Barker 2002, Graeff-Teixeira 2007, Moreira et al. 2013).

Abb. 10: *Achatina fulica*
Quelle: Penagos-Tabares et al. 2018b

Bisher liegen aus Kolumbien keinerlei Daten zu Metastrongylideninfektionen in Zwischenwirten vor und die meisten Lungenwurmarten werden in Kolumbien zu vernachlässigten Parasitosen gezählt (Penagos-Tabares et al. 2018a). Zudem wurden bisher weder *T. brevior* noch *C. vulpis* oder *A. cantonensis* in Kolumbien nachgewiesen, obwohl zumindest *C. vulpis* grundsätzlich eine weltweite Verbreitung aufweist (Bihr und Conboy 1999, Matos et al. 2016), *A. cantonensis* weltweit in tropischen und suptropischen Regionen vorkommt (Wang et al. 2008, Cowie 2013, Kim et al. 2014) und beide Spezies bereits in Nachbarländern Kolumbiens endemisch

sind (Pincay et al. 2009, Oyarzún-Cadagán 2013, Dard et al. 2017, Guerino et al. 2017). Um diese epidemiologische Lücke zu schließen, wurden eine epidemiologische Studie durchgeführt und kolumbianische Achatschnecken untersucht. Dazu wurden insgesamt 609 *A. fulica* aus folgenden fünf Regionen Kolumbiens gesammelt (Abb. 11): Cañasgordas, Ciudad Bolívar und Andes (alle aus der Provinz Antioquia), Tuluá (Provinz: Valle del Cauca) und Puerto Leguízamo (Provinz Putumayo). Cañasgordas, Ciudad Bolívar und Andes gehören zum Großraum der Anden. Tuluá liegt im Großraum des pazifischen Küstentieflands und Puerto Leguízamo im Großraum Amazonien.

Abb. 11: Sammelorte von *A. fulica* in Kolumbien
(Quelle: US Dept. of State Geographer. 2017 Google Image Landsat / Copernicus. Data SIO, NOAA, US Navy, NGA, GEBCO. Google Earth Pro® 2017)

Die Achatschnecken wurden mittels künstlicher Verdaumethode, Mikroskopie, PCR und Sequenzierung unter Anwendung der unter 3.1 beschriebenen Methoden analysiert. Dabei wurden folgende Lungenwurmspezies mikroskopisch nachgewiesen und per PCR/Sequenzierung bestätigt: *A. abstrusus*, *T. brevior*, *C. vulpis* und *A. vasorum* (Tab. 6). Des Weiteren wurden *Angiostrongylus*-Larven gefunden, deren Spezies aufgrund degradierter DNA nicht eindeutig bestimmt werden konnte. Von den 609 Proben waren 9,4 % positiv für *A. abstrusus*-Larven und 1,3 % für *T. brevior*-Larven (Tab. 6). In 1,1 % und 3,9 % der Proben wurden *C. vulpis*- und *A. vasorum*-Larven nachgewiesen (Tab. 6).

Die Lungenwurmarten waren insgesamt unterschiedlich in den einzelnen Regionen verbreitet. Die höchste regionale Prävalenz von 53,3 % wurde für *A. abstrusus* in der Region von Putumayo festgestellt, in den anderen Regionen wurde dieser Parasit dagegen nicht gefunden. *T. brevior* kam in Puerto Leguízamo und in Ciudad Bolívar

mit einer regionalen Prävalenz von 6,5 % und 1 % vor. Lediglich in einer Schnecke aus Cañasgordas wurde *C. vulpis* gefunden (1 %) sowie in 5,6 % der Proben aus Putumayo. Für *A. vasorum*-Larven ergaben sich folgende Prävalenzen: Puerto Leguízamo (8,4 %), Tuluá (6,3 %) und Andes (4,6 %) (Tab. 6). Somit wurden nur in Puerto Leguízamo alle untersuchten Lungenwurmspezies (jeweils in der höchsten Prävalenz verglichen mit den anderen Regionen) in *A. fulica* nachgewiesen.

Tab. 6: Prävalenzen metastrongylider Lungenwürmer in kolumbianischen *Achatina fulica*

Region / Spezies	Antioquia			Valle del Cauca	Putumayo	GESAMT
	Andes (*n* = 238)	Cañas-gordas (*n* = 100)	Ciudad Bolívar (*n* = 100)	Tuluá (*n* = 64)	Puerto Leguízamo (*n* = 107)	**(*n* = 609)**
A. abstrusus	-	-	-	-	53,3 % (57)	9,4 % (57)
T. brevior	-	-	1 % (1)	-	6,5 % (7)	1,3 % (8)
C. vulpis	-	1 % (1)	-	-	5,6 % (6)	1,1 % (7)
A. vasorum	4,6 % (11)	-	-	6,3 % (4)	8,4 % (9)	3,9 % (24)
***Angiostrongylus* sp.**	0,4 % (1)	-	-	1,6 % (1)	-	0,3 % (2)
Lungenwurm-befall insgesamt	5 % (12)	1 % (1)	1 % (1)	7,8 % (5)	73,8 % (69)	**16,1 %** (98)

Die Larvenbürde variierte zwischen 1 und 314 Larven pro Schnecke bezüglich *A. abstrusus* und zwischen 1 und 286 Larven für *T. brevior* (Abb. 12, 13). Für *C. vulpis* wurden ähnliche Larvenbürden beobachtet (1 - 208 Larven). Die Larvenbürde von mit *A. vasorum* infizierten Schnecken war hingegen relativ niedrig (1 - 30 Larven pro Schnecke, Abb. 12, 13). In insgesamt 92,3 % der Proben waren weniger als 10 *A. vasorum*-Larven pro Schnecke vorhanden (Abb. 12). Bei den verbleibenden 7,7 % der Proben lagen mehr als 10, aber weniger als 50 Larven vor. Bezüglich der *A. abstrusus*-Infektionen enthielten 43,9 % der Schnecken weniger als 10 Larven pro Tier, während 38,6 % 10 – 49 Larven pro Schnecke aufwiesen. Zusätzlich enthielten 7 % der Proben 50 - 99 Larven und 10,5 % der Proben wiesen 100 oder mehr Larven auf. Bei mit *T. brevior* und *C. vulpis* infizierten Schnecken lag eine ähnliche Verteilung der Larvenbürden vor, wobei die Mehrheit der Proben ebenfalls weniger als 10 Larven enthielt (71,4 %, Abb. 12). 10 – 49 Larven wurden bei 14,3 % der *T. brevior*-positiven

Schnecken gefunden. Im Gegensatz dazu zeigten 14,3 % der *C. vulpis*-positiven Schnecken eine Larvenbürde von 50 – 99 Larven pro Tier. Zusätzlich wurden bei 14,3 % der Proben, die positiv für *C. vulpis* und *T. brevior* getestet wurden, 100 oder mehr Larven nachgewiesen (Abb. 12).

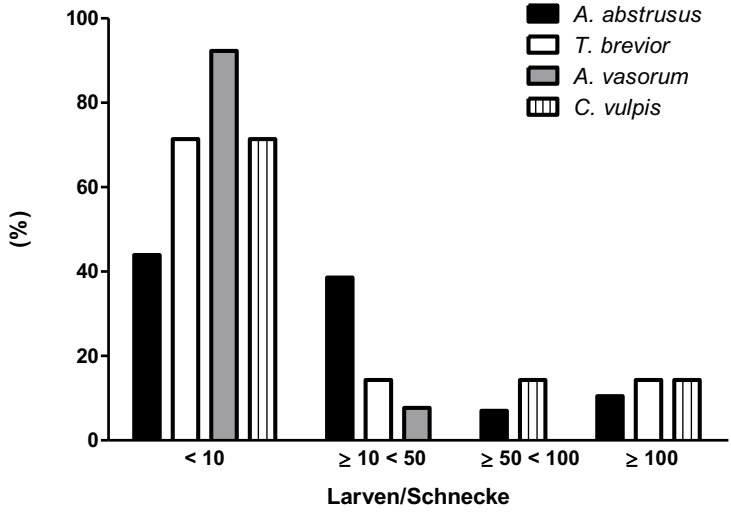

Abb. 12: Larvenbürden von *A. abstrusus, A. vasorum*, *C. vulpis* und *T. brevior* in *A. fulica*

Der Großteil der Lungenwurm-positiven Schnecken (74,7 %) hatte ein Gewicht von mehr als 10 g (Abb. 13).

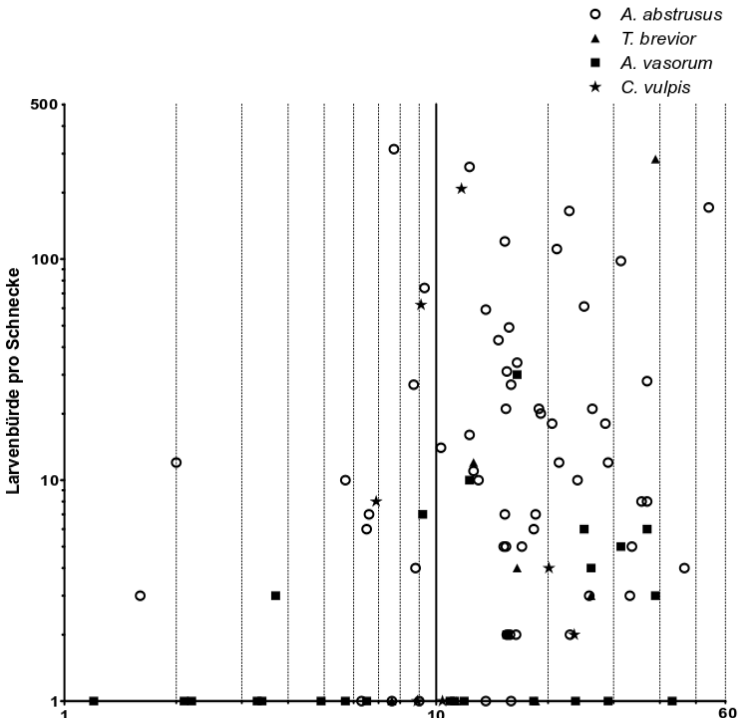

Abb. 13: Larvenbürde von *A. abstrusus*, *A. vasorum*, *C. vulpis* und *T. brevior* in Abhängigkeit vom Schneckengewicht

Ko-Infektionen wurden bei insgesamt 19 % (16/84) aller Lungenwurm-positiver Schnecken gefunden (Abb. 14). Dabei wurde in einer Schnecke *C. vulpis* und *T. brevior* nachgewiesen (1,2 %), in einer weiteren Schnecke *T. brevior* zusammen mit *A. vasorum*. Ko-Infektionen mit *T. brevior* und *A. abstrusus* kamen in 4,8 % aller positiver Proben vor. Der höchste Anteil an Ko-Infektionen (*n* = 7; 8,3 %) bezog sich auf *A. abstrusus* und *A. vasorum*. In drei Schnecken wurden *A. abstrusus*- und *C. vulpis*-Larven nachgewiesen (*n* = 3, 3.6 %, Abb. 14). Ko-Infektionen mit mehr als zwei Lungenwurmspezies wurden nicht festgestellt.

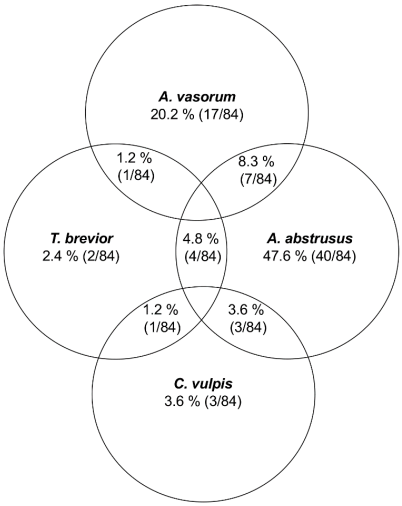

Abb. 14: Mono- und Ko-Infektionen mit *A. abstrusus*, *A. vasorum*, *C. vulpis* und *T. brevior* in kolumbianischen Achatschnecken

Bei 21 der 84 positiven Proben wurde eine DNA-Sequenzierung durchgeführt und die Sequenzen anschließend in einer NCBI-gestützten Homologiesuche mit bereits veröffentlichten Sequenzen verglichen. Dabei wurden die Spezies *A. abstrusus*, *A. vasorum*, *C. vulpis* und *T. brevior* über eine Sequenzidentität von 99 - 100 % bestätigt (Tab. 7). Daneben wurde in einem Fall eine DNA-Sequenz mit einer Identität von nur 82 % für *A. abstrusus* nachgewiesen, was auf eine nah verwandte, aber nicht identische Spezies schließen lassen könnte. Die verschiedenen, *A. abstrusus*-positiven Proben wiesen in der Sequenzierung zwei unterschiedliche Haplotypen auf, die hier mit A und B bezeichnet werden (Tab. 7). Typ A (*n* = 11) stimmte mit den in Europa vorkommenden Haplotypen überein, was anhand der 6 – 9 *Repeats* der GTC-Satelliten bestätigt wurde. Der Haplotyp B (*n* = 3) enthielt dagegen nur 3 *Repeats* der GTC -Satelliten und wurde in dieser Form noch nie veröffentlicht. Dieser Haplotyp könnte somit eine südamerikanische Variante darstellen. Zusätzlich wurde in einer Schnecke, die sowohl Haplotyp A als auch B von *A. abstrusus* enthielt, noch ein dritter Haplotyp (AB) gefunden, der 4 *Repeats* aufwies und somit einen Hybrid der Haplotypen A und B darstellen könnte (Tab. 7).

Tab. 7: Homologiesuche zu Metastrongyliden aus kolumbianischen *A. fulica* (NCBI Nucleotide Blast)

Lungenwurmspezies /Haplotyp	*n* =	Sequenz-abdeckung	Sequenz-Identität	Anzahl GTC microsatellite *Repeats*
Aelurostrongylus abstrusus A	11	99 %	100 %	6-9
Aelurostrongylus abstrusus B	3	99 %	91 %	3
Aelurostrongylus abstrusus AB	1	100 %	91 %	4
Angiostrongylus vasorum	1	99 %	100 %	
Crenosoma vulpis	2	98 %	99 %	
Troglostrongylus brevior	6	100 %	99 %	
Metastrongyloidea sp.	1	98 %	82 %	

Bei der vorliegenden Studie zur Prävalenz von metastrongyliden Lungenwürmern in kolumbianischen Achatschnecken handelt es sich um die erste Studie dieser Art in Kolumbien. Es konnte das Vorkommen von zwei Katzenlungenwürmern (*A. abstrusus* und *T. brevior*) und zwei Hundelungenwürmern (*A. vasorum* und *C. vulpis*) nachgewiesen werden. Dabei handelt es sich bezüglich *T. brevior* und *C. vulpis* um Erstnachweise für Kolumbien. Für *A. abstrusus* wurde ein *Hotspot* in der Amazonas-Region (Putumayo) aufgedeckt. Anhand der Sequenzierungen zeigte sich, dass die vorkommende *A. abstrusus*-Population genetisch divers ist.

Das die hier vorgestellten Daten enthaltene, bei *PLOS Neglected Tropical Diseases* eingereichte Manuskript ist in der Anlage dieser Dissertationsschrift hinterlegt.

5. Diskussion

5.1 Vorkommen von Lungenwurminfektionen in Zwischenwirten in Hessen und Rheinland-Pfalz

Die im Rahmen dieser Dissertation durchgeführte epidemiologische Studie sollte erstmals für Deutschland Prävalenzen zu Lungenwurminfektionen in Zwischenwirten erfassen. Dazu wurden insgesamt 1394 Schnecken in Hessen und 1307 Schnecken in Rheinland-Pfalz zu verschiedenen Jahreszeiten gesammelt. Bei der Sammlung haben wir uns auf Nacktschnecken beschränkt, da diese einerseits in den Untersuchungsgebieten häufiger zu finden waren als Gehäuseschnecken (was vermutlich den höheren Habitatansprüchen der Gehäuseschnecken wie kalziumhaltigen Böden geschuldet ist) und da andererseits bei Nacktschnecken die Gefahr der akzidentiellen Aufnahme durch den Hund (beispielsweise beim Fressen von Gras) höher ist als bei Gehäuseschnecken (Ferdushy und Hasan 2010, Helm et al. 2015, Schnyder 2015b).

Die Daten der epidemiologischen Studie belegten das Vorkommen der Lungenwurmarten *A. vasorum, C. vulpis* und *A. abstrusus* in natürlichen Zwischenwirtspopulationen in Hessen und Rheinland-Pfalz. Dabei lagen die Gesamtprävalenzen für *A. vasorum, C. vulpis* und *A. abstrusus* bei 4,7 %, 2,3 % bzw. 0,2 %. *A. vasorum*-Infektionen in Endwirten weisen im Allgemeinen eine fleckenhafte geographische Verbreitung auf (Morgan et al. 2005, Koch und Willesen 2009, Conboy 2011, Aziz et al. 2016), daher zeigte sich auch in anderen Studien eine z. T. stark variierende Prävalenz zu diesem Parasiten in Gastropodenpopulationen. Entsprechend wurden in mehreren, in Großbritannien durchgeführten Studien regional unterschiedliche *A. vasorum*-Prävalenzen von 1,6 %, 6,7 % und 29,4 % festgestellt (Patel et al. 2014, Helm et al. 2015, Aziz et al. 2016). Die einzige weitere Studie zum Vorkommen von *A. vasorum* in Nacktschnecken bezieht sich auf Dänemark und wies eine Gesamtprävalenz von 9 % nach (Ferdushy et al. 2009). Die relativ hohen, hier ermittelten Prävalenzen von *A. vasorum* in Zwischenwirtspopulationen, insbesondere in den identifizierten *Hotspots* in Hessen und Rheinland-Pfalz (bis zu 19,4% im Sommer), zeigten Überlappungen zu Hunde-bezogenen Daten von Maksimov et al.

(2017) und ließen aktive Übertragungswege in diesen Regionen vermuten. Somit bestätigten die vorliegenden Daten ein derzeit bestehendes Infektionsrisiko für Endwirte in Hessen und Rheinland-Pfalz.

Im Rahmen dieser Dissertation konnte gezeigt werden, dass die Prävalenz von *A. vasorum* und *C. vulpis* in Nacktschnecken signifikant von der Saison beeinflusst wird. Für beide Infektionen bestand grundsätzlich das höchste Infektionsrisiko für Schnecken im Herbst. Jedoch wurden auch im Sommer eine hohe *A. vasorum*-Prävalenz festgestellt, mit regional höchsten Werten von 19,4 % in Otterberg (Rheinland-Pfalz). Die regional höchste Prävalenz von *C. vulpis* wurde dagegen in Weilburg (Hessen) im Herbst nachgewiesen und betrug lediglich 10 %. Für *A. abstrusus* konnten solche Daten nicht ermittelt werden, da die geringe Anzahl positiver Proben eine belastbare statistische Auswertung verhinderte.
Neben der Prävalenz war auch die Larvenbürde von *A. vasorum* signifikant von der Jahreszeit abhängig mit statistisch höherer Wahrscheinlichkeit für höhere Larvenzahlen pro Schnecke im Herbst verglichen mit den Jahreszeiten Winter und Frühling. Keine andere Studie bei Nacktschnecken wurde bisher über ein vollständiges Jahr durchgeführt, daher liegen keine weiteren Aussagen zur Saisonalität der *A. vasorum*-Infektion in Gastropoden vor. Folglich liefert die vorliegende epidemiologische Studie einen wichtigen Beitrag zur Saisonalität von Lungenwurminfektionen. Der Einfluss der Saisonalität wurde bei Lungenwurminfektionen von Hunden und Füchsen in Europa bisher nur in wenigen Studien untersucht (Morgan et al. 2008, Maksimov et al. 2017, Schug et al. 2018). Bei der caninen Angiostrongylose und Crenosomatose liegen z. T. widersprüchliche Aussagen zur Saisonabhängigkeit des Infektionsrisikos für Endwirte vor. Bei Taubert et al. (2009) konnte eine Abhängigkeit von der Jahreszeit in Deutschland nur für *C. vulpis*-Infektionen bestätigt werden. Übereinstimmend konnten auch Morgan et al. (2008) keinen statistischen Zusammenhang zwischen der Jahreszeit und *A. vasorum*-Infektionen belegen. Im Gegensatz dazu zeigten Studien mit einer größeren Anzahl untersuchter Hundekotproben eine saisonale Abhängigkeit für beide Lungenwurminfektionen (Barutzki et al. 2017, Maksimov et al. 2017). Bei Hunden ist dabei die Jahreszeit Winter mit einem höheren Infektionsrisiko verbunden (Morgan und Shaw 2010, Barutzki et al. 2017, Maksimov et al. 2017), während bei Schnecken statistisch gesehen das höchste Infektionsrisiko im Herbst in Deutschland festgestellt wurde (vorliegende Untersuchung). Unter Berücksichtigung der Präpatenz von

A. vasorum-Infektionen würden Hunde, die sich im Herbst über perorale Aufnahme von infizierten Schnecken infizieren, koproskopisch ab Winter auffällig werden. Folglich decken sich die Daten zur Saisonalität von *A. vasorum* in Nacktschnecken mit den beschriebenen jahreszeitlichen Zusammenhängen in der Hundepopulation.

Insgesamt variierten die Lungenwurmprävalenzen bei Schnecken erheblich in Abhängigkeit vom Sammlungsort. Entsprechend schwankten die jährlichen, regionalen Gesamtprävalenzen zu *A. vasorum* zwischen 1,5 % in Rockenhausen und 9,9 % in Otterberg (beide Rheinland-Pfalz). *C. vulpis*-assoziierte regionale Gesamtprävalenzen schwankten zwischen 0,3 % in Eppstein und 6,1 % in Weilburg (beides Hessen). Sowohl für *A. vasorum* als auch für *C. vulpis* waren die Prävalenzunterschiede zwischen den Sammlungsorten statistisch hoch signifikant, was eine regional unterschiedlich starke Verbreitung der Parasiten anzeigt. Der Katzenlungenwurm wurde dagegen in vergleichbar niedrigen Prävalenzen lediglich in den Orten Weilburg (Hessen, 0.5 %) und Otterberg (Rheinland-Pfalz, 0.4 %) nachgewiesen.

Auch die Larvenbürde von *A. vasorum*- und *C. vulpis*-infizierten Schnecken war statistisch hochsignifikant vom Sammlungsort abhängig. Dabei lag die *A. vasorum*-assoziierte Larvenbürde in Weilburg signifikant niedriger als in Otterberg, aber höher als in Eppstein. Die Larvenbürde von *C. vulpis* war signifikant höher in Schnecken aus Weilburg verglichen mit Rockenhausen, aber niedriger als in Eppstein. Gebietsweise Schwankungen in der *A. vasorum*-Prävalenz wurden ebenfalls in anderen epidemiologischen Studien aus England und Dänemark festgestellt. So schwankten die Prävalenzen in Dänemark (Großraum Kopenhagen) zwischen 0 % und 26 % (Ferdushy et al. 2009). Aziz et al. (2016) zeigten, dass die *A. vasorum*-Prävalenzen im Süden von Wales und im Südwesten Großbritanniens im Vorstadtgebiet höher als in ländlichen und innerstädtischen Regionen lagen. Dabei variierten die Prävalenzen stark zwischen 0,3 % und 29,4 %. Auch in der Umgebung Londons (0 % und 1,6 %, Patel et al. 2014) und im Südwesten Schottlands (0,8 % und 4,6 %, Helm et al. 2015) wurden deutliche regionale Schwankungen in der *A. vasorum*-Prävalenz beobachtet. Diese Schwankungen entsprechen dem erwähnten fleckenhaften Verteilungsmuster der *A. vasorum*-Prävalenzen in Endwirten, dessen Ursache noch unbekannt ist (Lahodny und Allen 2013). Mögliche Erklärungsmodelle schließen die regionale Variabilität in der Spezieszusammensetzung und die Dichte der Zwischenwirte, sowie neue *A. vasorum*-Einträge in nicht-endemische Regionen durch natürliche oder

akzidentielle Verbreitung infizierter Zwischenwirte als auch Umweltfaktoren, die die Übertragung des Parasiten beeinflussen, ein (Grimm und Paill 2001, Morgan et al. 2004, Lahodny und Allen 2013). Außerdem wurde diskutiert, dass auch der Umfang und die Infektiosität der jeweiligen Larvenpopulation eine Rolle spielen könnte (Yousif und Lämmler 1975, Bolt et al. 1994, Saeed et al. 2006, Kozłowski 2007).

Die Untersuchungsgebiete der hier durchgeführten epidemiologischen Studie wurden anhand vorliegender Daten zu Lungenwurmprävalenzen im Rotfuchs (Schug et al. 2018) ausgewählt. Dabei war ein Auswahlkriterium, dass die Untersuchungsorte hochendemisch sein sollten mit *A. vasorum*-Prävalenzen von über 75 % in der Fuchspopulation. Passend dazu berichtete ein Landwirt, auf dessen Grundstück Sammlungen durchgeführt wurden, dass sein Hund an Angiostrongylose verstorben sei. Da *C. vulpis*-Infektionen im Rotfuchs weit verbreitet sind (Schug et al. 2018), waren die ausgewählten Regionen zusätzlich endemisch für diesen Parasiten, wenn auch nicht mit vergleichbar hohen Prävalenzen wie im Falle von *A. vasorum*. Die hier gemessenen, deutlich niedrigeren Prävalenzen von *C. vulpis* und *A. abstrusus* in Nacktschnecken könnten daher auch in der Auswahl der Untersuchungsorte begründet sein. Weitere Auswahlkriterien für die Untersuchungsgebiete beinhalteten ansprechende Lebensbedingungen für Füchse (Nähe zu Wäldern) und eine Nähe zu Siedlungen, um das Frequentieren der Sammelstellen durch Hunde zu gewährleisten. Außerdem wurden solche Wiesen als Sammelstellen ausgewählt, die den Schnecken als optimale Futterquellen dienen können und so mit breitblättrigen Grünpflanzen, wie Breitwegerich und Löwenzahn (an denen wir besonders oft Schneckenfraßstellen feststellen konnten) bewachsen waren. Während der Sammlungen wurde insbesondere das koprophage Verhalten der Schnecken, die sich in großen Zahlen auf und in Kothaufen von Hunden befanden, auffällig. Dieses Verhalten bestätigt den allgemein angenommenen Lebenszyklus und ist eine Voraussetzung zum erfolgreichen Ablauf desselben, da so die Infektion der Schnecken mit eventuell im Kot vorhandenen Larven sichergestellt wird.

Um den Lebenszyklus der besprochenen Lungenwurmarten zu vollenden, müssen infektiöse L3 vom Endwirt aufgenommen werden. Bei Füchsen, die als Wildtierreservoir dienen, erfolgt die Infektion vermutlich in erster Linie über die Aufnahme paratenischer Wirte, wie kleiner Nagetiere (Jędrzejewski und Jędrzejewska 1992, Reynolds und Tapper 1995). Auch ein Verzehr von Gastropoden ist denkbar, da

Invertebraten ca. 10 % der Diät eines Rotfuchses ausmachen können (Reynolds und Tapper 1995). Auch die Aufnahme von freien Larven aus der Umwelt, ähnlich wie beim Hund, scheint möglich. Bei Haushunden wurde zwar gelegentlich angezweifelt, dass sie aktiv Schnecken konsumieren (Morgan et al. 2005, Schnyder 2015b), es wird aber dennoch davon ausgegangen, dass dies der Hauptinfektionsweg ist (Hurnikova et al. 2013). In diesem Zusammenhang werden auch verschiedene Möglichkeiten der akzidentiellen Aufnahme von Gastropoden diskutiert, wie beispielsweise beim Spielen mit Stöckchen, an denen Schnecken haften (Schnyder 2015b) oder beim Verzehr von Gras (Barcante et al. 2003), was zum natürlichen Verhaltensrepertoir von Hunden gehört (Bjone et al. 2007). Interessanterweise konnten wir während der Schneckensammlung die Aufnahme von einem mit Schnecken bestückten Apfel durch einen Hund beobachten, dessen Besitzer in einem Gespräch zuvor deutlich verneint hatte, dass sein Hund außerhalb des Hauses Dinge aufnimmt. Neben den genannten Infektionswegen können die für den Hund infektiösen L3 grundsätzlich auch durch Schnecken ausgeschieden werden (Barcante et al. 2003, Colella et al. 2015, Conboy et al. 2017), dies scheint aber vernachlässigbar zu sein. Eigene Untersuchungen bestätigten grundsätzlich die Freisetzung von Lungenwurmlarven in Kot und Schleim von Nacktschnecken, dies erfolgte allerdings auf einem sehr niedrigen Niveau (max. 13 Larven/Schnecke) und nahezu ausschließlich nach zuvorigem Stimulus (Licht, Temperatur, Dehydratation) (M. Lange, unveröffentlichte Daten). Entsprechend könnten sich Hunde auch ohne Aufnahme des Zwischenwirtes durch freie Larven im Schneckenschleim auf dem Hundefutter oder im Gras infizieren (Morgan et al. 2005, Schnyder 2015b). Auch bei Wölfen wird angenommen, dass sie Schnecken als Proteinquelle konsumieren. Dabei sollen gemäß einer Studie in Kroatien ca. 9 % der Diät von Wölfen aus Invertebraten bestehen (Hermosilla et al. 2017).

Da Schnecken sich als Zwischenwirte für mehrere Lungenwurmarten eignen, könnten sie mehrfach infiziert sein und über ihre Aufnahme Ko-Infektionen der Endwirte bewirkt werden. Grundsätzlich wurden Ko-Infektionen von Lungenwürmern bei Hunden und insbesondere bei Füchsen berichtet (Jeffery et al. 2004, Saeed et al. 2006, Barutzki und Schaper 2009, Taubert et al. 2009, Maksimov et al. 2017, Schug et al. 2018). In der vorliegenden Studie wurden neben Zweifachinfektionen (*A. vasorum* + *C. vulpis; C. vulpis* + *A. abstrusus; A. abstrusus* + *A. vasorum*) auch Dreifachinfektionen von einzelnen Schnecken festgestellt (*A. vasorum* + *C. vulpis* + *A. abstrusus*). Beim Verzehr einer solchen, dreifach infizierten Schnecke durch eine Katze oder einen Hund

ist jedoch davon auszugehen, dass sich die Tiere nur mit den jeweils typischen Lungenwürmern infizieren würden. Allerdings existieren Berichte von experimentellen Infektionen von Katzen mit *A. vasorum* sowie ein Fallbericht einer Katze mit einer patenten *A. vasorum*-Infektion (Dias et al. 2008, Bangoura et al. 2013), so dass sich zumindest in der Katze potentiell vier Spezies (*A. vasorum, A. chabaudi, T. brevior, A. abstrusus*) ansiedeln könnten. Epidemiologisch scheinen Katzen für *A. vasorum* aber keine Bedeutung zu haben, da sich nur wenige Adulte und gravide Stadien in den Katzen entwickeln (Dias et al. 2008).

Als Risikofaktor für *A. vasorum*-Infektionen bei Hunden gilt das Vorhandensein von Gebieten mit einem hohen Anteil an Holzgewächsen sowie Laub- und Mischwälder (Maksimov et al. 2017). Typische Areale, in denen Besitzer ihre Hunde ausführen, sind Parks in Städten oder Naherholungsgebiete an Stadträndern. Diese weisen oft Wälder und Gebiete mit Holzgewächsen auf, die ideale Schnecken- und Fuchshabitate darstellen (Maksimov et al. 2017). Als nicht geeignete und damit mit einem negativen Risiko behafteten Areale werden dagegen solche mit hohen Nadelholzbeständen genannt (Maksimov et al. 2017). Bei *C. vulpis* hingegen gelten als Risikofaktoren das Vorhandensein von Moorland und Siedlungen, während als schützende Faktoren die Präsenz von Wasserkörpern und Agrarwirtschaft angesehen wird (Maksimov et al. 2017). Beiden Parasiten ist gemein, dass das Vorkommen von Landwirtschaft negativ mit dem Vorkommen der Parasitosen korreliert. Über die Gründe für diese Annahmen kann nur spekuliert werden. Neben der intensiven Nutzung der Flächen und damit ggf. verbundenen Umgrabungen der Erde und Austrockung des Areals ist anzunehmen, dass in der Landwirtschaft eingesetzte Pestizide Schneckenpopulationen reduzieren und somit den Zyklus unterbrechen könnten. In der Nähe von landwirtschaftlichen Ackerflächen konnten während der Schneckensammlungen vermehrt tote Nacktschnecken auf den Wegen beobachtet werden. Diese lagen meist in einer Lache aus eigenem Schleim, was als eine Reaktion auf mögliche Pestizide interpretiert werden könnte.

Als Risikofaktoren in Bezug auf *A. vasorum*-Prävalenz und -Larvenbürde in Zwischenwirten gelten große Spezies und hohe Körpermassen der Gastropoden (Yousif und Lämmler 1975, Ferdushy et al. 2009, Ferdushy et al. 2010, Patel et al. 2014). Sowohl für *A. vasorum* als auch für *C. vulpis* konnte im Rahmen der hier durchgeführten epidemiologischen Studie ein statistisch signifikanter Zusammenhang

zwischen Schneckenkörpermasse und Prävalenz belegt werden. Dabei stieg das Risiko einer *A. vasorum*-Infektion bei Nacktschnecken um den Faktor 2,8, wenn sich die Körpermasse der Schnecke verzehnfachte. Dieser Effekt war bei *C. vulpis* noch stärker ausgeprägt. Hier war das Risiko um den Faktor 5,8 bei einer Körpermassevergrößerung um den Faktor 10 erhöht. Bezüglich der Larvenbürde war ein signifikanter Effekt der Schneckenmasse nur für Infektionen mit *A. vasorum* nachweisbar, nicht dagegen für *C. vulpis.* Demnach erhöht sich die Larvenbürde in der Nacktschnecke um den Faktor 9,4, wenn die Körpermasse sich verzehnfachen würde. Es wird angenommen, dass über eine längere Lebensspanne von Schnecken (die mit einer Vermehrung der Körpermasse einhergehen kann) die Wahrscheinlichkeit des Konsums von L1-haltigem Hundekot erhöht wird, was eine gesteigerte Larvenbürde nach sich ziehen könnte (Rollo 1983, Ferdushy et al. 2009). Es erscheint ebenso offensichtlich, dass große Schnecken über ein erhöhtes Futtervolumen wahrscheinlicher mit Lungenwurmlarven infiziert werden könnten. Gemäß beider Annahmen wären langlebige oder große Schneckenarten wie *A. lusitanicus* oder *L. maximus* eher infiziert oder mit hohen Larvenbürden behaftet. Passend dazu wurde bezüglich Schneckenspezies-bezogener Risikofaktoren bisher berichtet, dass die vergleichsweise großen Schneckenspezies der Familie Arionidae häufiger infiziert sind als kleinere Schneckenspezies (Patel et al. 2014, Schnyder 2015b, Aziz et al. 2016). Die vorliegenden Daten bestätigten dies bezüglich der *A. vasorum*-Prävalenz bei der großen Art *A. lusitanicus* im Vergleich zu *D. reticulatum*. Für *C. vulpis* ließen sich solche Zusammenhänge jedoch nicht feststellen. Erstaunlicherweise verhielt es sich bezüglich der Larvenbürde umgekehrt und die kleinere Spezies *D. reticulatum* erwies sich als diejenige Schneckenspezies, die mit höheren Larvenbürden zu *A. vasorum* belastet war. Allerdings zeigten sich diese Effekte nicht für *C. vulpis*, denn die höhere Larvenbürde wurde hier auch für *A. lusitanicus* gemessen. Demnach stellt die große Nacktschneckenspezies *A. lusitanicus* für *C. vulpis*-Infektionen den geeigneteren Zwischenwirt dar. Bezogen auf *A. vasorum*-Infektionen ist die Frage des besser geeigneten Zwischenwirtes dagegen nicht eindeutig zu beantworten, da, wie oben benannt, jeweils eine andere Schneckenart die höchste Prävalenz bzw. Larvenbürde aufwies. Bei *D. reticulatum* als Träger einer ggf. hohen *A. vasorum*-assoziierten Larvenbürde ist insbesondere zu bedenken, dass diese kleine Schneckenart vermutlich eher akzidentiell durch den Hund aufgenommen wird als die deutlich größeren *A. lusitanicus.* Warum für die beiden Lungenwürmer hier Unterschiede

festgestellt wurden, ist nicht geklärt. Eine mögliche Erklärung könnte in Schneckenspezies-abhängigen, angeborenen Immunreaktionen liegen (Lange et al. 2017, Lange et al. 2018a). Auch in anderen Studien zur Epidemiologie von *A. vasorum* in Zwischenwirtspopulation waren vor allem Nacktschnecken der Familie Arionidae mit hohen Prävalenzen assoziiiert (Ferdushy et al. 2009, Helm et al. 2015, Aziz et al. 2016). In den Studien aus Großbritannien wurden in Schottland *A. vasorum*-Infektionen nur bei *Arion ater, Arion hortensis,* aber auch bei der Gehäuseschnecke *Helix aspersa* festgestellt (Helm et al. 2015). Im Gegensatz dazu wurden im Südwesten Großbritanniens Infektionen bei *Arion flagellus, Arion rufus* und *Limacus maculatus* (Aziz et al. 2016) und im Südosten bei *Arion distinctus, A. ater, Tandonia sowerbyi* und einer zur Familie der Limacidae gehörenden Schnecke gefunden (Patel et al. 2014). Auch bei der Studie aus Dänemark wurden hauptsächlich Arionidae als Träger von *A. vasorum*-Infektionen nachgewiesen, zusätzlich erwies sich auch *L. maximus* als große Schneckenart als infiziert (Ferdushy et al. 2009). Bei keiner der genannten Untersuchungen wurde - im Gegensatz zur vorliegenden Studie - ein statistisch signifikanter Unterschied zwischen den Spezies festgestellt. Dies könnte in der vergleichsweise niedrigen Anzahl der untersuchten Schnecken begründet sein (180 - 381 Schnecken aus England und Dänemark gegenüber 2701 Schnecken in der vorliegenden Arbeit). Eine andere mögliche Erklärung liegt in der unterschiedlichen Zusammensetzung der jeweiligen Schneckenfauna. So war beispielsweise in allen anderen Studien *D. reticulatum* kaum und stattdessen stets mehrere Spezies der Familie Arionidae präsent (Ferdushy et al. 2009, Patel et al. 2014, Helm et al. 2015, Aziz et al. 2016).

Zur erfolgreichen Bekämpfung von Lungenwürmern von Karnivoren können unterschiedliche Strategien verfolgt werden. Bei Hunden wurde gezeigt, dass regelmäßige Entwurmungen der Erkrankung vorbeugen können (Morgan et al. 2010). Um erkrankte Hunde und Katzen erfolgreich zu behandeln, bedarf es einer verlässlichen Diagnostik. Zudem ist es unerlässlich, dass Tierärzte in Endemiegebieten über Lungenwurmerkrankungen aufgeklärt sind und diese entsprechend als Differentialdiagnose bei Hunden mit respiratorischer oder unspezifischer Symptomatik sowie bei neurologischen Symptomen oder Blutungsneigung in Betracht ziehen (Helm et al. 2010, Maksimov et al. 2017). Auch kann durch Aufklärung von Tierbesitzern in den entsprechenden Regionen das Verbreitungsrisiko dadurch verringert werden, dass die Besitzer die Kothaufen ihrer

Haustiere und damit die unmittelbare Infektionsquelle für Schnecken direkt entfernen. Viele Hundebesitzer suchen mit ihren Tieren immer dieselben Areale auf (Schnyder 2015b) und Schnecken nutzen Hundekot offensichtlich als eine wichtige Futterquelle. Wir konnten bei den Sammlungen oftmals mehrere Schnecken pro Hundehaufen entdecken und in einem besonders offensichtlichen Fall erwies sich eine dieser Schnecken als infiziert mit *C. vulpis.* Wenn ein infizierter Hund wiederholt dieselben "Hundekotwiesen" kontaminiert, steigt entsprechend das Risiko für andere Hunde, die dieselbe Wiese frequentieren. Das Bewusstsein für diese Gefahr scheint bei den meisten Hundebesitzern nicht ausgeprägt zu sein, da nur die wenigsten, die wir während unserer Sammlungen getroffen haben, die Bereitschaft erkennen ließen, auch außerhalb der Stadt die Kothaufen ihrer Haustiere zu entfernen.

Neben der Therapie der Endwirte oder dem Abbruch des Entwicklungszyklus über Entfernung der Fäzes wird auch das Auslegen von Schneckenkorn oder anderen Ködern zur Schneckenbekämpfung diskutiert, um über die Senkung der Schneckenpopulation das Infektionsrisiko für Hunde zu verringern (Giannelli et al. 2015a). Es ist fraglich, wie effizient diese Methode ist, da sie ein Einwandern neuer Populationen nicht verhindert. Zudem sind gesundheitliche Aspekte bei direkter oder indirekter (z. B. über paratenische Wirte) Aufnahme der „vergifteten" Schnecken zu bedenken (Nolte 2012). Außerdem ist zu beachten, dass Schneckengifte auch Gartennützlingen schaden, wie zum Beispiel *L. maximus*, der *A. lusitanicus* attackiert und dessen Eier frisst (Nordsieck 2000). Neben chemischen Verfahren zur Schneckenbekämpfung können auch biologische Ansätze, z. B. über Laufenten (eine Rasse von *Anas platyrhynchos*), verfolgt werden (siehe z. B. http://www.laufis.de/rent/, http://www.schnecken-bekämpfen.de/laufenten-zur-schneckenvernichtung/). Als biologische Maßnahme wird auch ein fakultativ parasitoider Nematode, *Phasmarhabditis hermaphrodita* verwendet, der seinen Wirt tötet und dann in dem Schneckenkadaver heranreift (Mengert 1953, Grewal et al. 2003). Da dieser Nematode allerdings nicht ausschließlich auf Schnecken als Wirte angewiesen ist, scheint dies kein sehr effizienter Weg der Schneckenbekämpfung zu sein. Interessanterweise wird aktuell bei anderen Schnecken-übertragenen Parasiten wie *Schistosoma mansoni* der Einsatz eines resistenten Schneckenstammes untersucht, der ggf. mit Molluskiziden kombiniert werden soll (Coelho et al. 2008). Solche Untersuchungen liegen für die Lungenwürmer der Caniden und Feliden bisher nicht vor. Insgesamt erscheint eine Kombination aus mehreren Bekämpfungsansätzen, die

nicht nur die Schneckenpopulation verringert, sondern auch die Kontamination der Umwelt mit Larven im Hundekot vermeidet und von frühzeitigen Therapiemaßnahmen bei den Hunden begleitet wird, am erfolgversprechendsten zur Vermeidung einer weiteren Ausbreitung dieser Parasitosen (Giannelli et al. 2015a). Als Voraussetzung für eine erfolgreiche Bekämpfung dieser Parasitosen gilt sicherlich die Kenntnis über endemische Gebiete (Giannelli et al. 2015a). Mit den hier vorgelegten epidemiologischen Untersuchungen zu Rheinland-Pfalz und Hessen ist ein erster Schritt getan, um diese Wissenslücke zu füllen. Weitere Studien zur epidemiologischen Situation in anderen Teilen Deutschlands sind notwendig für eine großflächige Risikobeurteilung.

5.2 Untersuchungen zum Vorkommen von Lungenwurminfektionen in kolumbianischen Achatschnecken

5.2.1 Epidemiologische Studie in Kolumbien

Bisher liegen keinerlei Untersuchungen zu Lungenwurminfektionen bei gastropoden Zwischenwirten aus Kolumbien vor. In der hier durchgeführten epidemiologischen Studie zu Achatschnecken aus 5 Regionen Kolumbiens konnten Gesamtprävalenzen von 9,4 %, 1,3 %, 1,1 % und 3,9 % für *A. abstrusus, T. brevior, C. vulpis* bzw. *A. vasorum* festgestellt werden. Dabei handelte es sich bezüglich *T. brevior*- und *C. vulpis*-Infektionen um Erstbeschreibungen für Kolumbien.

Der französische Herzwurm *A. vasorum* wurde in Kolumbien bisher nur koproskopisch bei einzelnen, wildlebenden Krabbenfüchsen (*Cerdocyon thous*) nachgewiesen (Gonçalves 1961, Varela-Arias et al. 2014), nicht jedoch in der Population kolumbianischer Haushunde. Die hier gefundene Prävalenz von *A. vasorum* in kolumbianischen Schnecken ist mit 3,9 % vergleichbar mit der Prävalenz in deutschen Nacktschnecken (4,7 %) und legt somit ein endemisches Vorkommen dieses Parasiten in den Untersuchungsgebieten nahe. Dabei ist insbesondere zu berücksichtigen, dass in Kolumbien, im Gegensatz zur deutschen Studie, nicht gezielt in hochendemischen Gebieten gesucht wurde. Daher könnte – unter der Voraussetzung einer fleckenhaften Verbreitung – die Prävalenz von *A. vasorum* in anderen Gebieten Kolumbiens noch deutlich höher liegen. In Südamerika scheint *A. vasorum* in Brasilien, Argentinien und Bolivien in der Population der Haushunde und Wildkaniden endemisch zu sein

(Gonçalves 1961, Venturini und Boren 1991, Fiorello et al. 2006). Allerdings liegen bisher nur Fallberichte vor, epidemiologische Untersuchungen mit Erfassung der Prävalenzen fehlen derzeit.

Die Prävalenz von *C. vulpis* in kolumbianischen Schnecken lag mit 1,1 % niedrig und ist ggf. vergleichbar mit der entsprechenden Prävalenz in deutschen Schnecken (2,3 %). Da der vorliegende Nachweis eine Erstbeschreibung dieses Parasiten für Kolumbien ist, sollten zukünftig auch die Hundepopulationen auf diesen Parasiten untersucht werden. In anderen Ländern Südamerikas liegen derzeit fast keine Berichte zum Vorkommen von *C. vulpis* vor. Lediglich in Chile wurde dieser Parasit bisher mit einer Prävalenz von 1% in der Haushundepopulation beschrieben (Oyarzún-Cadagán 2013).

Die Aelurostrongylose wurde dagegen bei Hauskatzen in Kolumbien bereits berichtet. Die Existenz dieser Parasitose ist seit 2003 in Kolumbien bekannt und wurde vereinzelt über Fallberichte und epidemiologische Studien dokumentiert (Salamanca et al. 2003, Echeverry et al. 2012, Sánchez-Rojas et al. 2017). Die Prävalenz von *A. abstrusus* in kolumbianischen *A. fulica* lag mit 9,4 % relativ hoch (zum Vergleich lag sie in deutschen Schnecken bei 0,2 %), was für eine bereits erfolgte Endemisierung dieses Parasiten in den genannten Untersuchungsgebieten Kolumbiens spricht und dringend Untersuchungen in den dortigen Katzenpopulationen nahelegt. Insbesonders hervorzuheben ist die regional extrem hohe *A. abstrusus*-Prävalenz bei *A. fulica* von 53,3 % in der Provinz Putumayo im Amazonas Gebiet. Bei einer derart hohen Prävalenz kann von einem echten *Hotspot* für *A. abstrusus*-Infektionen ausgegangen werden und es empfiehlt sich dringend, die dortige Katzenpopulation zu untersuchen. Dieses Gebiet besteht zu großen Teilen aus tropischem Regenwald. Da in dieser Region sechs bedrohte Spezies wilder Feliden der Genera *Leopardus, Puma* und *Panthera* leben (IUCN 2017), stellt die Kenntnis dieser potenziell tödlichen Parasitose einen wichtigen Faktor für den Erfolg von Arterhaltungsprogrammen dar. Interessanterweise fiel bei der Sequenzierung der *A. abstrusus*-Isolate auf, dass zwei unterschiedliche Haplotypen vorlagen, von denen einer zu 100 % identisch mit europäischen Isolaten war, während der andere deutlich davon abwich (91 % identisch). Es könnte sich hier um eine südamerikanische Variante des Katzenlungenwurms handeln. Ein dritter Haplotyp, der zusammen mit den beiden anderen in einer Schnecke gefunden wurde, zeigte eine Ähnlichkeit zu den anderen beiden Haplotypen, was vermuten lässt, dass es sich um ein Hybrid aus der

vermeintlich südamerikanischen und europäischen Variante handeln könnte. Diverse Länder Südamerikas sind ebenfalls von der Aelurostrongylose betroffen, wie Uruguay, Brasilien, Chile, Argentinien, und Bolivien (Penagos-Tabares et al. 2018a). Dabei schwankte die Prävalenz zwischen 1,3 % und 35,3 % in der Hauskatzenpopulation, zwischen 35,7 % und 38,1 % in der Wildfelidenpopulation und zwischen 2 % und 80 % in der invertebraten Zwischenwirtspopulation (Esteves et al. 1961, Fiorello et al. 2006, Oyarzún-Cadagán 2013, Ramos et al. 2013, Cardillo et al. 2014, Kusma et al. 2015, Valente et al. 2017).

Die in kolumbianischen Schnecken gemessenen Larvenbürden, die mehrheitlich weniger als 50 Larven aufwiesen, decken sich mit Daten aus deutschen und dänischen Schnecken (Ferdushy et al. 2009, Lange et al. 2018a). Der Anteil von Schnecken mit hohen Larvenbürden (100 und mehr Larven) betrugen bei kolumbianischen Schnecken 14,3 % für *T. brevior* und *C. vulpis* sowie 10,5 % für *A. abstrusus.* Ähnlich hohe Anteile mit hohen Larvenbürden wurde für *A. vasorum* bei Schnecken in Dänemark beschrieben (14 %), während in Deutschland nur 3,3 % der Proben in diese Kategorie fielen (Ferdushy et al. 2009, Lange et al. 2018a). *A. vasorum* kam in kolumbianischen Schnecken generell nur in geringen Larvenbürden vor. Das Vorkommen eines hohen Prozentsatzes von Proben mit geringen Bürden bei einem gleichzeitig geringen Anteil mit hoher Larvenzahl wird als *Overdispersion* bezeichnet (Anderson und Gordon 1982). Das gleiche Phänomen wurde auch von Ferdushy et al. (2009) für *A. vasorum*-Infektionen von dänischen Schnecken beschrieben und deckt sich mit Angaben zu der nah verwandten Spezies *Angiostrongylus costaricensis* (Laitano et al. 2001). Die *Overdispersion* könnte erklären, warum häufig subklinische *A. abstrusus-, A. vasorum-, C. vulpis*- und *T. brevior*-Infektionen bei Endwirten beobachtet werden (Conboy 2004, Ferdushy et al. 2009, Di Cesare et al. 2014a, Traversa und Di Cesare 2014).
Der Fund von Ko-Infektionen verschiedener Lungenwurmspezies (*A. vasorum* + *A. abstrusus; A. vasorum* + *T. brevior; C. vulpis* + *A. abstrusus; C. vulpis* + *T. brevior; A. abstrusus* + *T. brevior*) in kolumbianischen Schnecken deckte sich mit den beschriebenen Ko-Infektionen in deutschen Schnecken und zeigt, dass Schnecken entweder mit mit Kot ko-infizierter Endwirte oder, wahrscheinlicher, mit Kot verschiedener Endwirtindividuen in Kontakt kommen.

Neben *A. vasorum* wurden in kolumbianischen Schnecken dieser Studie zusätzlich weitere Larven des Genus *Angiostrongylus* gefunden, die eine morphologische Ähnlichkeit mit dem humanpathogenen Rattenlungenwurm *A. cantonensis* aufwiesen. Beweisende Sequenzanalysen dazu stehen jedoch noch aus. Sollte sich in zukünftigen Studien dieser Verdacht erhärten, wäre das der erste Bericht zu diesem gefährlichen, humanpathogenen Parasiten in Kolumbien. Da diese Parasitenspezies bereits in Kolumbiens Nachbarländern Ecuador (Pincay et al. 2009, Dorta-Contreras et al. 2011), Brasilien (Moreira et al. 2013, Guerino et al. 2017) und in der Karibik (Chikweto et al. 2009, Dard et al. 2017) endemisch ist, erscheint eine Ausbreitung nach Kolumbien als durchaus wahrscheinlich. Interessanterweise wurden kürzlich in Kolumbien Fälle humaner Meningoencephalitis mit unbekannter Ätiologie berichtet (Velez-van-Meerbeke et al. 2017), deren Symptome denen einer humanen Angiostrongylose ähneln. Daher könnte es sich hier um unentdeckte Infektionen mit *A. cantonensis* handeln (Valente et al. 2018).

5.2.2. *Genetische Analysen zu kolumbianischen A. vasorum-Isolaten*

Das klinische Bild der caninen Angiostrongylose kann stark variieren (Koch und Willesen 2009). Neben Endwirt-bezogenen Faktoren könnten unterschiedlich virulente Stämme des Parasiten eine Erklärung für die klinische Vielfalt bieten. Tatsächlich wurden unterschiedliche Stämme von *A. vasorum* in verschiedenen Ländern nachgewiesen, die potenziell eine unterschiedliche Pathogenität aufweisen könnten (Jefferies et al. 2009b, Morgan et al. 2012).

Mittels molekularer Phylogeographie kann verfolgt werden, wie historisch alte Ereignisse die heutige Verbreitung von Parasiten beeinflusst haben (Riddle et al. 2008, Thompson et al. 2010, Thomson et al. 2010). Bei Untersuchungen zur geographischen Verbreitung von Endoparasiten sollte grundsätzlich bedacht werden, dass diese nicht nur aufgrund eigener Interaktionen mit der Umwelt, sondern auch durch Einschränkungen, die die entsprechende Wirtsspezies betreffen, beeinflusst werden können (Morgan et al. 2012). Außerdem hängt die Verbreitung eines Parasiten davon ab, wie taxonomisch eng das potenzielle Wirtsspektrum ist (Morgan et al. 2012). Diese Verbreitung wird in Abgrenzung zur geographischen Verbreitung als taxonomische Verbreitung bezeichnet (Morgan et al. 2012). Die Fähigkeit von *A. vasorum,* verschiedene Spezies der Überfamilie Canoidea als Endwirt zu verwenden, spricht auf den ersten Blick für eine hohe taxonomische Verbreitung. Allerdings ist von anderen

Parasiten bekannt, dass eine scheinbar hohe taxonomische Verbreitung in Wirklichkeit auf das Vorliegen diverser, wirtsspezifischer Haplotypen oder sogar kryptischer Spezies zurückzuführen ist (Morgan et al. 2012). Inwieweit dies auch auf *A. vasorum* zutreffen könnte, gilt zukünftig zu klären. In Südamerika bestanden lange Zeit Unsicherheiten bezüglich der Spezieszugehörigkeit dort vorkommender *A. vasorum*-Infektionen in wilden Caniden (Jefferies et al. 2009b). Die in Südamerika 1927 zuerst beschriebene Lungenwurmspezies, die bei einheimischen Krabbenfüchsen (*Cerdocyon thous*) vorkam, wurde *A. raillieti* benannt (Travassos 1927). Auch in südamerikanischen Haushunden konnte dieser Lungenwurm nachgewiesen werden (Dougherty 1946). Mehr als 30 Jahre später wurde *A. vasorum* in denselben Endwirtsspezies in Südamerika beschrieben (Gonçalves 1961). Zusätzlich wurde *A. raillieti* 1971 neu beschrieben als *Angiocaulus raillieti* (Grisi 1971). Derzeit wird nach einer taxonomischen Überarbeitung nur *A. vasorum* als vorkommende Spezies anerkannt (Costa et al. 2003, Morgan et al. 2012). Kürzlich konnte jedoch gezeigt werden, dass tatsächlich so große genetische Unterschiede zwischen *A. vasorum*-Stämmen aus Europa und Brasilien vorliegen, und somit eher die Definition zweier kryptischer Spezies als einer einzigen Spezies zuzutreffen scheint (Jefferies et al. 2009b, Morgan et al. 2012). Die Autoren gehen dabei davon aus, dass es sich bei der Auseinanderentwicklung der beiden kryptischen *Angiostrongylus*-Arten um ein phylogenetisch gesehen altes Ereignis handelt. Die Artentstehung der südamerikanischen Füchse wird auf vor etwa 10 Millionen Jahren geschätzt (Wang et al. 2004). Dies deckt sich in etwa mit dem von Jefferies et al. (2009b) benannten zeitlichen Rahmen, in dem sich die beiden phylogenetischen Äste von *A. vasorum* aus Europa und Südamerika auseinander entwickelt haben könnten. Dieser umfasst eine Zeitspanne vor 11 bis 67 Millionen Jahren (Jefferies et al. 2009b). Die erste Einführung von Haushunden geschah vergleichsweise spät erst vor ca. 10.000 Jahren und wird somit nicht als Ursache für das Vorkommen dieses Lungenwurms in Südamerika angesehen (Jefferies et al. 2009b). Für diese Hypothese spricht auch, dass Füchse des Genus Urocyon (Wang et al. 2004), die als Vorfahren des Krabbenfuchses angesehen werden, mit einem Lungenwurm infiziert waren, der *Angiocaulus gubernaculatus* ähnelt (Faulkner et al. 2001). Von diesem Parasiten wird wiederum angenommen, dass er einen gemeinsamen Vorfahren mit *A. vasorum* teilt (Jefferies et al. 2009b). Die Autoren ziehen weiterhin in Betracht, dass mehrfache Wirtswechsel im Laufe der Evolutionsgeschichte von *A. vasorum* stattgefunden haben könnten, da der

Parasit in der Lage ist, verschiedenste Endwirtspezies zu infizieren (Jefferies et al. 2009b). Natürlich kann nicht ausgeschlossen werden, dass *A. vasorum*-Stämme aus Europa mit der Reise von Haushunden auch neuzeitlich nach Südamerika eingetragen worden sind und nun neben dem südamerikanischen *A. vasorum*-Stämmen zirkulieren (Morgan et al. 2012).

Die oben genannte Hypothese wurde durch eigene Untersuchungen an *A. vasorum*-Larven, die aus kolumbianischen Achatschnecken stammen, bestätigt. Dabei wurden dieselben Mikrosatelliten im IST2-Bereich untersucht wie bei der Studie von Jefferies et al. (2009b). Die hier per Sequenzierung ermittelten Daten zeigten eine deutlich höhere Ähnlichkeit zu europäischen Isolaten als zu südamerikanischen Varianten. So waren die kolumbianischen Isolate identisch mit Isolaten aus Dänemark. Dies bestätigte die von Morgan et al. (2012) formulierte Vermutung, dass in Südamerika auch Stämme aus Europa zirkulieren.

5.3 Untersuchungen zu Abwehrreaktionen von Zwischenwirten auf Lungenwurminfektionen

5.3.1 Etablierung von Techniken zur Schneckenzucht, Hämozytenisolation und Kultur axenischer Larven

Die Reproduzierbarkeit von experimentellen Infektionen wird sowohl von endogenen (z. B. individuellen Immunreaktionen) als auch von exogenen Faktoren, wie Ernährung der Tiere, klimatischen Bedingungen oder Stress beeinflusst (Wojda et al. 2004, Kangassalo et al. 2015, Krams et al. 2015). Daher ist bei der Durchführung von Infektionsexperimenten insbesondere im Zusammenhang mit immunologischen Fragestellungen von besonderer Bedeutung, dass sowohl die Haltungsbedingungen als auch die Fütterung der Versuchstiere möglichst standardisiert ablaufen (Penagos-Tabares et al. 2018b). Dies betrifft die Schneckenhaltung genauso wie jede andere Versuchstierhaltung (Mäuse, Ratten etc.). Um standardisierte Bedingungen zu gewährleisten, mussten im Rahmen dieser Dissertation zunächst Vorarbeiten geleistet und die Zucht, Kultur und Haltung der verwendeten Schneckenspezies unter definierten Bedingungen und Nutzung von Klimaschränken etabliert werden. Zudem wurde eine standardisierte Fütterung eingeführt, um exogene Einflüsse soweit wie möglich auszuschließen. Um Stress zu reduzieren, wurden Häuser, die üblicherweise

bei Labormaushaltungen Verwendung finden, eingesetzt (Penagos-Tabares et al. 2018b). Das etablierte System führte letztendlich zu sehr guten und stabilen Zuchterfolgen für die unterschiedlichen Nackt- und Gehäuseschneckenarten und dient nun als Grundlage für weiterführende Untersuchungen (siehe Kapitel 3.3). Insgesamt stellte sowohl die Reproduktion von *L. maximus* aufgrund der in der Einleitung erwähnten speziellen Paarungstechnik als auch die protandrischen Geschlechtsentwicklung bei den zwittrigen Schnecken (die in Abhängigkeit von der sich ändernden Tagelänge erfolgt) die größte Herausforderung dar (Sokolove und McCrone 1978).

Für Infektionsexperimente wurden Schnecken gleichen Alters und definierten Infektionsstatus benötigt. Dies war über Entnahme der Tiere aus der Natur nicht zu bewerkstelligen, daher musst eine eigene Zucht angelegt werden. In dem hier verwendeten Kultur- und Haltungssystem wurde eine durchschnittliche Zeit vom Legen der Eier bis zum Schlupf der juvenilen Schnecken von 29,5 Tagen für *A. lusitanicus*, 24,6 Tagen für *L. maximus* und 30,5 Tagen für *A. fulica* erreicht. Aus den Gelegen schlüpften im Schnitt 85 juvenile *A. lusitanicus*, 109 *L. maximus* und 129 *A. fulica.* In anderen Studien wurden 50-130 Eier pro Gelege bei *L. maximus* beschrieben mit einer Dauer bis zum Schlupf von in ca. 1 Monat (Sokolove und McCrone 1978, Barker und McGhie 1984). Bei *A. lusitanicus* wurden Gelegegrößen von 5 bis 190 Eiern dokumentiert, mit einer Entwicklungsdauer von 2 Monaten (Kozlowski 2000). Eier von *A. fulica* wurden in anderen Studien in Gelegen zu 100 - 200 Eiern gelegt und reiften z. T. aufgrund von Ovoviviparität in 1 – 25 Tagen heran (Pawson und Chase 1984).

Für die geplanten immunologischen Untersuchungen (s. Kapitel 3.4) wurden sowohl vitale Immunzellen von Schnecken als auch „sterile“ (bakterienfreie) Larven benötigt, um die Reaktionen der Immunzellen nicht über potenzielle Kontaminationen zu verfälschen. Durch intensive Vorversuche konnten geeignete Protokolle zur Erstellung axenischer, vitaler Lungenwurmlarvenkulturen und zur Entnahme von Hämolymphe bei Schnecken etabliert werden (s. Veröffentlichung 3.3). Die Bakterienfreiheit der aus Hundekot isolierten und behandelten Erstlarven wurde bestätigt, da Kulturmedien, auf die die gewonnenen Larven aufgebracht wurden, auch nach 7 Tagen Inkubation bei 37 C weder Bakterien- noch Pilzwuchs aufwiesen.

Eine weitere Herausforderung stellte die Isolation von Immunzellen (Hämozyten) aus der Hämolymphe der Schnecken dar. Da die Hämolymphe von Invertebraten extrem

schnell koaguliert und herkömmliche Antikoagulantien nicht zur Vermeidung dieses Effekts ausreichen (Van Wettere und Lewbart 2007), wurde hier ein spezielles Gemisch verschiedener Antikoagulantien verwendet. Bei den Nacktschneckenspezies *L. maximus* und *A. lusitanicus* konnten auf diese Weise Hämolymphvolumina von durchschnittlich 112,5 µl bzw. 125 µl pro Tier gewonnen werden mit einer durchschnittlichen Zellzahl von 61.250 bzw. 27.500 pro Entnahme. Bei den wesentlich größeren *Achatina*-Schnecken wurden durchschnittlich 340 µl gewonnen mit einer mittleren Zellzahl von 396.880 pro Entnahme. Bei *A. fulica* ist beschrieben, dass bis zu 10 % der Körpermasse an Hämolymphe ohne Risiko für das Weiterleben des Tieres entnommen werden kann (Cooper 1994). Somit können beispielsweise bei einer 200 g schweren Achatschnecke bis zu 2 ml der Hämolymphe entnommen werden ohne ihr Weiterleben zu beeinträchtigen. Entsprechend können über diese Technik zukünftig individuelle Verlaufsuntersuchungen zu infizierten Schnecken durchgeführt werden.

Das hier erarbeitete System der standardisierten Schneckenkultur und -haltung bietet die Möglichkeit, reproduzierbare Infektionsexperimente durchzuführen, bei denen verschiedene klimatische Szenarien naturnah simuliert werden können und so beispielsweise der Einfluss des Klimawechsels untersucht werden kann. Dies wird über die Verwendung von Klimaschränken ermöglicht, bei denen die Parameter Licht, Temperatur und Feuchtigkeit beispielsweise so eingestellt werden können, dass die Bedingungen unterschiedlicher Tages- oder Jahreszeiten (einschließlich gradueller Dämmerung) simuliert werden. Dies ist von Bedeutung, da schon leichte Umweltveränderungen die Wechselwirkungen zwischen Pathogen und Wirt beeinflussen (Mitchell et al. 2005, Seppälä und Jokela 2011, Barber et al. 2016).

5.3.2 Bildung von *Extracellular traps* als Abwehrreaktion auf metastrongylide Lungenwurmlarven

In den letzten Jahren wurde für diverse Immunzelltypen und Wirtarten die Befähigung zur Freisetzung sog. *Extracellular Traps* zur Bekämpfung extrazellulärer Pathogene nachgewiesen. Neben Vertebraten wie Mensch, Rind, Hund, Maus, Ratte, Kaninchen etc. ist dieser Effektormechanismus mittlerweile auch für Invertebrate wie Krabben, Seeanemonen und Muscheln (Poirier et al. 2014, Robb et al. 2014) belegt, was eine weit verbreitete Existenz dieses Mechanismus im ganzen Tierreich nahelegt. Auch Schnecken verfügen über ein nicht-adaptives Immunsystem und ihre als Hämozyten

bezeichneten Immunzellen sind in der Lage, über Phagozytose, multizelluläre Einkapselung und zellvermittelte Zytotoxizität Pathogene zu bekämpfen (Matricon-Gondran und Letocart 1999, Humphries und Yoshino 2003, Little et al. 2005, Sokolova 2009, Loker 2010). Anhand der hier vorgelegten Untersuchung konnte erstmalig gezeigt werden, dass Hämozyten von Schnecken auch zur ETose befähigt sind und dass dieser angeborene Immunmechanismus bei verschiedenen Spezies terrestrischer Schnecken als effiziente Abwehrstrategie gegen Larvalstadien unterschiedlicher Lungenwurmarten eingesetzt wird (siehe Veröffentlichung 3.4). Welche Bedeutung dieser Mechanismus in der *in vivo*-Situation hat, ist derzeit nicht geklärt. Dass er tatsächlich auch *in vivo* stattfindet, konnte hier anhand rasterelektronenmikroskopischer Bebilderung gezeigt werden. Im Gegensatz zu den vorliegenden Daten scheint die ETose im Falle von Wasserschnecken bei der Abwehr von *Trichobilharzia regenti* nur eine untergeordnete Rolle zu spielen (Skala et al. 2018). Interessanterweise könnte dieses Phänomen einen Grund dafür liefern, dass die Süßwasserschnecke *Biomphalaria glabrata* als empfänglicher für *A. vasorum* beschrieben wurde verglichen mit der terrestrische Gehäuseschnecke *A. fulica* (Sauerländer und Eckert 1974).

Bei den vorliegenden Untersuchungen zeigte sich, dass die Hämozyten-vermittelte ETose weder abhängig vom Larvenstadium des Parasiten noch von der Spezies des Lungenwurms war. Eine Unabhängigkeit vom parasitären Stadium als auch von der Parasitenart konnte auch für protozoäre Parasiten im Vertebratensystem belegt werden (Muñoz-Caro et al. 2015b). Diese Analogie zeigt auf, dass ETs als relativ unspezifisch agierende Werkzeuge des angeborenen Immunsystems agieren. Robb et al. (2014) zeigten, dass ETosis unter Invertebraten weit verbreitet ist. In Übereinstimmung stellt die ETose offensichtlich einen unter terrestrischen Nacktschnecken und Gehäuseschnecken weit verbreiteten Effektormechanismus der angeborenen Immunabwehr dar, da ETs in Reaktion auf Lungenwurmlarven sowohl von Hämozyten aus *A. lusitanicus* und *L. maximus* als auch aus *A. fulica* reproduzierbar gebildet wurden.

In Analogie zu Untersuchungen in vertebraten Wirten (Muñoz-Caro et al. 2014b, Silva et al. 2014, Muñoz-Caro et al. 2015b) konnte in der vorliegenden Studie gezeigt werden, dass extrazelluläres Chromatin, Myeloperoxidase-ähnliche Enzyme, Elastase und Histone Bestandteile der ETs von Gastropoden darstellen. Bei ETs von

Wirbeltieren ist eine Reihe weiterer Effektormoleküle von ETs bekannt, wie beispielsweise Pentraxin, reaktive Sauerstoffspezies, Defensine, Cathelicidine, Calprotektin und Laktoferrin (Nathan 2006, Shah et al. 2007, Parker et al. 2012). Im gastropoden Wirt ist allerdings die Nachweisbarkeit solcher Moleküle beschränkt, da klassische Werkzeuge wie schneckenspezifische Antikörper fehlen und lediglich über antikörperbasierte Kreuzreaktionen Nachweise geführt werden könnten. Neben den genannten Molekülen spielen reaktive Sauerstoffspezies (ROS) und intrazelluläres Calcium u. a. eine Schlüsselrolle bei der Entstehung von ETs (Muñoz-Caro et al. 2015a, Rochael et al. 2015). Interessanterweise zeigten Coaglio et al. (2018) kürzlich, dass in der Hämplymphe *A. vasorum*-infizierter *A. fulica* im Vergleich zu nicht-infizierten Exemplaren vermehrte Phenoloxidase-Aktivitäten nachzuweisen waren. Der Phenoloxidase-vermittelte Signalweg ist in Schnecken in Melanisierungsprozesse eingebunden, die mit der Bildung von reaktiven Sauerstoff- (ROS) und Stickstoff-Spezies einhergehen und die als Abwehrreaktionen von Gastropoden genutzt werden (Coaglio et al., 2018). Ob bei den genannten Befunden ein Zusammenhang mit der hier beschriebenen ETose vorliegt, muss zukünftig geklärt werden. In Analogie zum Vertebratensystem konnte außerdem gezeigt werden, dass auch bei Gastropoden morphologisch unterschiedliche Typen von ETs gebildet werden, nämlich *aggregated, diffuse* und *spread* ETs (Muñoz-Caro et al. 2015c).

Im vorliegenden Fall wurde beobachtet, dass Lungenwurmlarven über Hämozyten-vermittelte ETs markiert und immobilisiert wurden. Dies entspricht weitestgehend den Befunden zu ET-vermittelten Abwehrreaktionen bei anderen Nematodenstadien (Bonne-Annee et al. 2014, Muñoz-Caro et al. 2015c, McCoy et al. 2017). Im Gegensatz dazu konnte bei einigen Bakterien- und Pilzarten gezeigt werden, dass diese Pathogene über ETose nicht nur abgefangen, sondern auch abgetötet werden (Segal 2005, Urban et al. 2006). Bei Parasiten konnten letale Effekte dagegen bisher nur bei wenigen Spezies gezeigt werden, beispielsweise bei *Leishmania amazonensis* und *Toxoplasma gondii* (Guimarães-Costa et al. 2009, Abi Abdallah et al. 2012). Dabei ist allerdings zu bedenken, dass es sich bei den hier untersuchten metastrongyliden Lungenwurmlarven um vergleichsweise große Pathogene handelt, die entweder nicht über ETs abzutöten sind oder deren Abtötung mehr Zeit oder die Beteiligung einer größeren Anzahl von Zellen in Anspruch nimmt als die Abtötung deutlich kleinerer Pathogenen wie Bakterien. Die hier durchgeführten *in vitro* Versuche wurden jedoch nur über eine relativ kurze Zeitdauer beobachtet, folglich kann nicht beurteilt werden,

ob bei längerer Einwirkdauer der antimikrobiellen Substanzen die ETs doch letale Effekte entfalten würden. Bei der Abtötung von *Strongyloides stercoralis*-Stadien spielen ETs zusammen mit weiteren Abwehrmechanismen eine wichtige Rolle (Bonne-Annee et al. 2014). Dieser humanpathogene Nematode wurde zwar von ETs immobilisiert, aber nicht getötet, jedoch war dieser Schritt essentiell für die darauf folgende zellvermittelten Abtötung des Parasiten durch Neutrophile und Makrophagen (Bonne-Annee et al. 2014).

Bei Schnecken wird als wichtigster, immunzellvermittelter Mechanismus zur Abtötung großer Parasiten die sog. Einkapselung über Hämozyten beschrieben (Matricon-Gondran und Letocart 1999, Little et al. 2005, Loker 2010). Es ist anzunehmen, dass durch die ET-vermittelte Immobilisierung von Lungenwurmlarven die über Einkapselung induzierte Abtötung dieser Pathogene erleichtert wird, sodass beide Mechanismen ggf. als additiv gesehen werden können. Zudem könnten über ETs weitere Hämozyten zur optimierten Abwehrreaktion angelockt werden, diesbezüglich wurde eine „opsonierende" Wirkung von ETs sowohl bei Wirbeltieren als auch Invertebraten beschrieben (Saitoh et al. 2012, Robb et al. 2014). Grundsätzlich wurden Einkapselungen von Lungenwurmlarven (*A. vasorum, A. cantonensis, A. abstrusus, T. brevior*) im Gewebe von Gehäuseschnecken anhand histologischer Untersuchungen bereits belegt (Sauerländer 1976, Giannelli et al. 2015b). Eine solche Einkapselung muss jedoch nicht zwingend letale Effekte entfalten. Im Falle der nah verwandten Lungenwurmart *A. cantonensis* wird davon ausgegangen, dass zumindest ein Teil der Larven die Einkapselung überlebt (Harris und Cheng 1975b). Ob ähnliches auch für die vorliegenden Infektionssysteme zutrifft, muss Gegenstand zukünftiger Untersuchungen sein.

Für einige Pathogene wurde gezeigt, dass sie Mechanismen entwickelt haben, um den letalen Effekten der ETose zu entgehen. So befreien sich einige Bakterien, wie z. B. *Streptococcus pneumoniae,* über Nukleaseaktivitäten aus dem DNA-haltigen Netzwerk der ETs (Beiter et al. 2006, Buchanan et al. 2006). Auch für *Leishmania infantum* wird beschrieben, dass dieser Protozoe über Aktivitäten einer Nuklease einer Elimination durch ETs entgehen kann (Guimarães-Costa et al. 2014). Bei Viren konnte interessanterweise gezeigt werden, dass sich diese durch eine Stimulation der Interleukin 10-Produktion (IL-10) ETs entziehen können (Saitoh et al. 2012). Dabei wurde das Humane Immundefizienz Virus (HIV) zwar erfolgreich von ETs abgefangen

und in Folge vom Immunsystem eliminiert, jedoch konnte das Virus diesem Prozess entgegenwirken, indem es dendritische Zellen dazu stimulierte IL-10 zu produzieren, welches die ET-Bildung inhibiert (Saitoh et al. 2012). Im Falle von Nematodenlarven liegen bisher keine Befunde zu derartigen Mechanismen vor. Da Nematoden jedoch für ihre Freisetzung von exkretorisch-sekretorischen Antigenen, die ihrerseits immunmodulatorisch auf Leukozyten einwirken, bekannt sind, erscheinen solche Mechanismen grundsätzlich denkbar.

Bei Wirbeltierleukozyten werden Pathogene oftmals über Toll-like-Rezeptoren erkannt und in Folge ROS produziert, die ihrerseits in die Auslösung der ETose eingebunden sind (Saitoh et al. 2012). Auch bei Gastropoden ist bekannt, dass ihre Immunzellen TLRs besitzen (Pila et al. 2016). Folglich könnte die Erkennung von Pathogenen im Rahmen der ETose auch bei diesen Zwischenwirtsspezies über einen analogen Weg erfolgen, dies muss jedoch in zukünftigen Studien untersucht werden.

Neben der Einkapselung sind Hämozyten auch in der Lage, Phagozytose durchzuführen. Aufgrund der Größe der Lungenwurmlarven erscheint offensichtlich, dass dieser Mechanismus nicht eine übergeordnete Rolle bei der Abwehr dieser Pathogene spielen kann. Die phagozytotische Aufnahme von Fremdpartikeln wurde bei Gastropoden bereits belegt. Diesbezüglich wurde interessanterweise gezeigt, dass nach erfolgter Phagozytose die Phagozyten samt Fremdpartikel den Körper der Schnecke an verschiedenen Stellen verlassen können (Tripp 1961, Brown 1967). Welche Areale der Schnecke diese Austrittspforten bei den typisch in Deutschland vorkommenden Nacktschneckenarten darstellen, ist derzeit noch unerforscht. Larven von Lungenwürmern sind zwar zu groß, um von Hämozyten phagozytiert zu werden, bei Bakterien hingegen könnte diese Art der Elimination eine Rolle spielen. Bisher ist noch ungeklärt, ob phagozytierte Pathogene über den Inhalt zytoplasmatischer Granula von Hämozyten zersetzt werden können, ähnlich wie es bei den Phagozyten von Wirbeltieren der Fall ist (Maramorosch und Shope 1975).

Auch wenn bislang angenommen wurde, dass Invertebraten nur eine angeborene Immunantwort besitzen, hinterfragen aktuellere Studien die tatsächliche Abwesenheit eines adaptiven, erworbenen Immunsystems zunehmend. So gibt es erste Hinweise darauf, dass Gastropoden Fibrinogen-verwandte Proteine (*fibrinogen-related proteins*) mit Domänen der Immunglobulin Superfamilie produzieren können. Diese könnten ähnliche Funktionen wie Immunglobuline bei Wirbeltieren wahrnehmen (Zhang et al.

2004, Coustau et al. 2016). Ob Schnecken jedoch tatsächlich über erworbene Immunreaktionen verfügen können, werden zukünftige Untersuchungen zeigen müssen.

6. Zusätzliche Publikationen

Review: *Angiostrongylus vasorum* and *Aelurostrongylus abstrusus*: Neglected and underestimated parasites in South America

Penagos-Tabares F., Lange M. K., Chaparro-Gutiérrez J. J., Taubert A., Hermosilla C.

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Eigener Anteil an der Publikation:

- Literaturrecherche: 30 % zusammen mit Ko-Autoren
- Erstellung des Manuskripts: 30 % zusammen mit Ko-Autoren

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Parasites & Vectors

REVIEW Open Access

Angiostrongylus vasorum and *Aelurostrongylus abstrusus*: Neglected and underestimated parasites in South America

Felipe Penagos-Tabares[1,2*], Malin K. Lange[1], Jenny J. Chaparro-Gutiérrez[2], Anja Taubert[1] and Carlos Hermosilla[1]

Abstract

The gastropod-borne nematodes *Angiostrongylus vasorum* and *Aelurostrongylus abstrusus* are global causes of cardio/pulmonary diseases in dogs and cats. In the last decade, the number of reports on canine and feline lungworms has increased in several areas of Europe and North America. The unspecific clinical signs and prolonged course of these diseases often renders diagnosis challenging. Both infections are considered as emerging and underestimated causes of disease in domestic pets. In South America, little information is available on these diseases, apart from occasional reports proving the principle presence of *A. vasorum* and *A. abstrusus*. Thus, the purpose of this review is to summarize reports on infections in both domestic and wildlife animals in South America and to increase the awareness on gastropod-borne metastrongyloid parasites, which also include important zoonotic species, such as *A. cantonensis* and *A. costaricensis*. This review highlights the usefulness of diagnostic tools, such as the Baermann funnel technique, serology and PCR, and proposes to include these routinely on cases with clinical suspicion for lungworm infections. Future national epidemiological surveys are recommended to be conducted to gain a deeper insight into the actual epidemiological situation of gastropod-borne parasitoses in South America.

Keywords: *Angiostrongylus vasorum*, *Aelurostrongylus abstrusus*, Gastropod-borne diseases, Lungworms, Metastrongyloidea

Background

The gastropod-borne metastrongyloid parasites *Angiostrongylus vasorum* and *Aelurostrongylus abstrusus* are known to affect the cardiopulmonary system of canids and the respiratory tract of felids, respectively [1]. These potentially pathogenic parasites have recently gained attention from the veterinary community due to their spread beyond the borders of known endemic areas, mainly in European countries as well as in North America [1–11]. Although they have been reported in both, domestic and wild canids/felids in different South American countries (see Table 1) [12–16], suggesting an endemic presence of both parasites in these regions, only a few epidemiological surveys haven been conducted on these parasites in the past decades. This indicates a neglected and underestimated status of these parasitoses not only by the Latin American veterinary but also by the parasitology community. Thus, more epidemiological research is required to obtain actual, consistent and detailed data on their epidemiology and actual disease occurrence and on the impact of canine angiostrongylosis and feline aelurostrongylosis on domestic and wild canid/felid populations in South America, as already performed in Europe [2, 3].

It is well known that both canine angiostrongylosis and feline aelurostrongylosis can lead to certain diagnostic challenges due to the intermittent excretion of first-stage larvae (L1), the high variability of clinical signs and the frequently occurring chronic and subtle course of infections [2, 17, 18]. In addition, a reliable definitive diagnosis based on clinical-pathological, serological, molecular or coprological approaches is challenging, since all conventional diagnostic methods may fail due to certain deficiencies and limitations of each diagnostic method [2]. The best diagnostic tool for the detection of *A. vasorum* or *A. abstrusus* first larvae in faeces is still represented by the Baermann funnel migration technique, which is unfortunately rarely utilized in small animal veterinary clinics of

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Table 1 Reports on *Angiostrongylus vasorum* infections in definitive hosts in South America

Definitive host species	Geographical location	No. of cases	Reference
Cerdocyon thous	Gioás and Angra Dos Reis, Rio de Janeiro, Brazil	1	Travassos, 1927 [49]
Cerdocyon thous/Canis familiaris	Brazil	na	Dougherty, 1946 [141]
Cerdocyon thous/ Canis familiaris	Colombia and Rio Grande do Sul, Brazil	1	Gonçalves, 1961 [13]
Canis familiaris	Rio de Janeiro, Brazil	na	Langenegger et al., 1962 [54]
Canis familiaris	Rio de Janeiro, Brazil	na	Grisi, 1971 [50]
Canis familiaris	Paraná, Brazil	na	Giovannoni et al, 1985 [55]
Canis familiaris	Minas Gerais, Brazil	na	dos Santos et al., 1985 [56]
Canis familiaris	Argentina	na	Venturini & Borel, 1991 [58]
Lycalopex (syn. *Dusicyon*) *vetulus*	Minas Gerais, Brazil	4/8	Lima et al., 1994 [12]
Lycalopex gymnocercus	Bolivian Chaco	1/10	Fiorello et al., 2006 [82]
Cerdocyon thous	Minas Gerais, Brazil	3/6	Duarte et al., 2007. [57]
Nasua nasua	Paraná, Brazil	1	Vieira et al., 2008 [52]
Eira barbara	Mato Grosso do Sul, Brazil	1	Vieira et al., 2008 [52]
Cerdocyon thous	Pereira, Colombia	1	Varela-Arias et al., 2014 [59]
Cerdocyon thous	Federal District, Midwestern Brazil	1	Ferreira-Júnior et al., 2017 [142]
Cerdocyon thous	Minas Gerais, Brazil	2	Viera et al., 2017 [143]

Abbreviation: na, not applicable

South America [19] even though it is a cheap and easy diagnostic method [20]. As a consequence, only fragmentary information on the true geographical distribution and actual prevalence of these nematodes is available in South and Central America [19, 21].

Overall, some cases of both canine angiostrongylosis and feline aelurostrongylosis were described in several regions of South America (Fig. 1). Furthermore, some reports exist on a specific intermediate host species, the highly invasive terrestrial African giant snail *Achatina fulica* [22–24]. Between 1988 and 1989 this invasive neozoan snail species, originating from East Africa, was introduced to South America (especially to Brazil) for the commercial heliculture industry (snail farming) for human consumption [25]. Since then, this species rapidly spread throughout several South American countries, including Argentina, Colombia, Ecuador, Paraguay, Peru and Venezuela [26]. More importantly, *A. fulica* is considered as the most harmful invading terrestrial snail species on Earth [27, 28] and therefore might contribute to the spread of the here reviewed lungworms and other closely related anthropozoonotic metastrongyloid parasites, such as *Angiostrongylus costaricensis* and *A. cantonensis* as already reported for the Americas [24, 25].

The geographical expansion of lungworm infections, which was reported for several countries [4, 10, 29, 30] throughout the last decades, may rely on climate changes influencing the intermediate host-parasite relationship. In addition, international travelling activities of domestic dogs/cats throughout South America significantly increased in the last years, posing an enhanced risk of parasite import and transmission to previously non-endemic areas as already reported for Europe [20]. Furthermore, the population dynamics of gastropods are strongly affected by a variety of abiotic factors such as temperature and humidity [31]. Taking into account that terrestrial snails/slugs play a crucial role in the life-cycle of *A. vasorum* and *A. abstrusus* [21], an improvement of environmental conditions for gastropods will promote an increased occurrence of aelurostrongylosis and angiostrongylosis in new geographical areas as postulated elsewhere [24]. In addition, wildlife reservoirs, such as red foxes (*Vulpes vulpes*), crab-eating foxes (*Cerdocyon thous*), coyotes (*Canis latrans*), bush dogs (*Speothos venaticus*) and a wide spectrum of wild felid species in South America, request further consideration for the better understanding of the epidemiology. As such, the spread of foxes from sylvatic into suburban/urban areas is a well-known phenomenon in many geographical areas [32]. This 'fox urbanization' can obviously contribute to the import of infectious parasitic stages to domestic dog habitats as recently demonstrated for central Europe [33]. Besides, also wild felids might become urbanized in South American tropical/subtropical cities with vast forested park areas, thus possibly contributing to the spread of feline aelurostrongylosis.

The purpose of this review is to summarize currently available data on the epidemiological situation of *A. vasorum* and *A. abstrusus* infections in dogs, cats and wildlife animals in South America. Given that these parasites indeed occur in several countries of this continent and therefore should be considered as differential

Fig. 1 Reports of natural occurring infections of *Angiostrongylus vasorum* and *Aelurostrongylus abstrusus* in South America

diagnoses in cases of canine cardiopulmonary/feline respiratory disease, the Baermann funnel technique should be included in routine diagnosis. Therefore, important informative aspects, such as the principles of the Baermann funnel technique, larval morphological characteristics and clinical signs are reviewed to encourage veterinarian surgeons and parasitologists to start investigations on these neglected diseases. Finally, novel diagnostic tools, such as serological and molecular approaches, are described briefly to stimulate future large-scale epidemiological surveys on lungworm infections, not only in domestic but also in wildlife animals of South America.

Canine angiostrongylosis in South America

Angiostrongylus vasorum (Baillet, 1866), also known as 'French heartworm', is a parasite of domestic dogs and wild species of the family Canidae, including foxes [34], coyotes [21] and wolves [35, 36] amongst others. Furthermore, this nematode has been reported to occur in other closely related carnivore species, such as mustelids and the red panda (*Ailurus fulgens*) [21]. Experimentally, the Nile rat (*Arvicanthis niloticus*) proved a suitable final host [37]. *Angiostrongylus vasorum* shows a rather patchy geographical distribution worldwide [4, 6, 12, 38–40] and its geographical spread and infection incidence are considered as increasing in the recent years [2, 41]. A broad range of terrestrial snail and slug intermediate host species (e.g. *Arion ater*, *A. rufus*, *A. lusitanicus*, *A.fulica*, *A. distinctus*, *A. hortensis*, *Limax maximus*, *Helix aspersa* and *Tandonia sowerbyi*) [42–45] are infected by L1 either by ingestion while feeding on faeces or by active penetration through the gastropod epidermis [34]. Merely experimentally, the common frog (*Rana temporaria*) and the domestic chicken (*Gallus gallus*) were demonstrated as paratenic hosts for *A. vasorum* [46, 47]. Within the definitive host, adult nematodes mainly reside in the right heart and pulmonary arteries. Adult fertilized females produce eggs which embryonate and hatch within the pulmonary capillaries where L1 penetrate into the alveoli [2], migrate into the trachea, larynx, buccal cavity, are swallowed and finally shed through faeces into the environment.

Canine angiostrongylosis ranges from subclinical cases to severe cardiopulmonary and neurological disorders or coagulopathies besides inducing nonspecific clinical signs [48]. Even fatal infections are frequently reported

[48]. Historically, *Angiostrongylus*-like nematodes in dogs and foxes have been denominated as *A. vasorum* in Europe and as *Haemostrongylus raillieti*, *Angiostrongylus raillieti* or *Angiocaulus raillieti* in Brazil [49–51]. Based on morphological and molecular characteristics, a recent taxonomic revision proposed to amalgamate all these species into one single parasite species, namely *A. vasorum* [41, 51]. However, genetic variations of European and Brazilian *A. vasorum* isolates suggested these isolates as separate cryptic species [41] but further investigations are needed to clarify the final taxonomy. Nonetheless, solving this taxonomic question requires a much larger sample size and the inclusion of additional isolates from different South American countries into genomic analyses, as previously suggested [41].

In Brazil, *A. raillieti* (syn. of *A. vasorum*) was reported in South American coati (*Nasua nasua*) and another *Angiostrongylus* sp. was described in the Tayra (*Eira barbara*). In both wild mammal species, adult parasites were found in the lungs, heart and pulmonary arteries [51, 52]. Based on the specific cardiopulmonary localization and the historical confusion on the nomenclature, we here refer to these reported parasites as *A. vasorum.* Future research on these and other wildlife species is therefore mandatory to elucidate the natural definitive host spectrum of *A. vasorum* in a geographical region to be considered as mega-biodiverse as it is the South America subcontinent [53].

As the first South American report, *A. vasorum* was found in the right ventricle and pulmonary arteries of the crab-eating fox *C. thous* (Linnaeus, 1766) in Rio de Janeiro, Brazil [49]. Thereafter, natural *A. vasorum* infections were reported to occur in domestic dogs in Rio Grande do Sul, Brazil and in the crab-eating fox in Colombia [13]. Later on, more cases of *A. vasorum*-infected crab-eating foxes were described in other Brazilian regions, such as Rio de Janeiro [54], Paraná [55] and Minas Gerais [56, 57]. Additionally, in Minas Gerais *A. vasorum* was found as parasitizing hoary foxes (*Dusicyon vetulus*) (Lund, 1842) (syn. *Pseudoalopex* or *Lycalopex vetulus*) [12]. As also reported for North American free-ranging red foxes (*V. vulpes*) [38], a rather high *A. vasorum* prevalence of up to 50% was detected in Brazilian crab-eating foxes [57] (Table 1). Overall, the sum of these data indicates a broad distribution of *A. vasorum* in wildlife fox populations in South America which will contribute to the parasite propagation and the sylvatic life-cycle maintenance as already reported for Europe [57].

Unfortunately, there also exist ambiguous reports on *A. vasorum* or *Filaroides osleri* canid infections in South America lacking proper morphological diagnosis [58, 59]. One of these reports came from Argentina and included the coprological diagnosis 'lungworm larvae' for two domestic dogs without further characterization [58]. Another report was from Colombia, where a lethal infection of a crab-eating fox was described and diagnosed as lungworm infection based on histopathological findings of the lungs showing parasitic structures which resembled metastrongyloid parasites [59]. Based on the uncertain diagnosis of these two studies, it seems that more detailed research and instructions for veterinarians, pathologists and parasitologists in South America are required. For more detailed data on biology, epidemiology, diagnostic techniques, clinical features as well as anthelminthic treatments the following reviews on canine angiostrongylosis are recommended: Koch & Willesen [21], Helm et al. [44], Schnyder et al. [18] and Traversa & Guglielmini [2].

Feline aelurostrongylosis in South America

Aelurostrongylus abstrusus (Railliet, 1898) infections are distributed worldwide [2, 19]. This parasite represents one of the most important etiological parasitic agents of respiratory alterations in domestic and wild felids [60, 61]. Clinical manifestations of feline aelurostrongylosis range widely from subclinical to a variety of respiratory signs such as dyspnoea, open-mouthed abdominal breathing, coughing, wheezing, sneezing and mucopurulent nasal discharge. Especially in cases of high-dose infections this parasitosis might have a clinical significance [62]. Analogous to *A. vasorum*, *A. abstrusus* has an indirect life-cycle involving a variety of terrestrial gastropods as intermediate hosts (i.e. *A. lusitanicus*, *L. maximus* [45], *A. fulica* [24, 45], *H. aspersa* [63]). Additionally, paratenic hosts such as rodents, frogs, lizards, snakes or birds are known to be involved in parasite life-cycle [60, 64]. In contrast to *A. vasorum*, adult stages of *A. abstrusus* reside in the terminal respiratory bronchioles, alveolar ducts and pulmonary alveoli, where the females produce eggs, which embryonate and hatch within the pulmonary ducts and alveoli [62].

Regarding the presence of *A. abstrusus* infections in South America, there are reports in domestic and wildlife felids from Uruguay [65, 66], Argentina [67, 68], Brazil [15, 69–76], Chile [77–80], Colombia [14, 16, 81] and Bolivia [82] (see Table 2 and Fig. 1). The first report ever on an *A. abstrusus* infection in a cat from South America came from Uruguay in the year 1942 [65]. In 1953, Trein [83] reported 40 cases out of 102 analysed domestic cats which had been submitted to necropsy in Rio Grande do Sul, Brazil. Thereafter, a prevalence of 8.6% was estimated *via* necropsy in cats from Montevideo during the period 1958–1960 [84]. During the 1970s and 1980s, domestic feline aelurostrongylosis was reported in Chile [77–79], Brazil [70] and Argentina [68]. In the 1990s, more reports on feline aelurostrongylosis came from Argentina and Brazil [71, 85, 86].

The report on the highest altitude so far, referred to an incidentally diagnosed *A. abstrusus* infection in a cat

Table 2 Reports on *Aelurostrongylus abstrusus* infections in definitive and intermediate hosts

	Geographical location	Prevalence/no. of cases	Reference
Definitive host species			
Felis catus	Montevideo, Uruguay	1 case	Bacigalupo et al., 1942 [65]
Felis catus	Rio Grande do Sul, Brazil	40/102	Trein, 1953 [83]
Felis catus	Rio de Janeiro, Brazil	na	Langenegger and Lanzieri, 1963 [69]
Felis catus	Montevideo, Uruguay	8.6%	Esteves et al., 1961 [84]
Felis catus	Chile	1 case	Gonzalez & Torres, 1971 [77]
Felis catus	São Paulo, Brazil	na	Campedelli-Filho, 1972 [70]
Felis catus	Valdivia, Chile	na	Torres et al., 1972 [78]
Felis catus	São Paulo, Brazil	8.5%	Fenerich et al., 1975 [144]
Felis catus	Valdivia, Chile	na	Bonilla-Zepeda, 1980 [79]
Felis catus	La Plata, and Buenos Aires Argentina	24.3%, 30.0%	Idiart et al., 1986 [68]
Felis catus	Corrientes, Argentina	30%	Martinez et al., 1990 [85]
Felis catus	Rosario, Argentina	na	Schiaffi et al., 1995 [86]
Felis catus	Santa Maria, Brazil	na	Headley & Conrado, 1997 [71]
Puma yagouaroundi *Leopardus geoffroyi*	Mato Grosso do Sul, Brazil	na	Noronha et al., 2002 [99]
Felis catus	Bogota, Colombia	1 case	Salamanca, 2003 [14]
Felis catus	Uberlândia, Brazil	18%	Mundim et al., 2004 [72]
Felis catus	Santa Maria, Brazil	5.9–25% (mean 18.6%, 1987–1996)	Headley, 2005 [73]
Felis catus	Buenos Aires, Argentina	2.6%	Sommerfelt et al., 2006 [67]
Leopardus pardalis	Bolivian Chaco	5 cases	Fiorello et al., 2006 [82]
Leopardus geoffroyi		3 cases	
F.catus domesticus	Rio de Janeiro, Brazil	1 case	Ferreira et al., 2007 [88]
Felis catus	Quindío, Colombia	0.21% (1/121)	Echeverry et al., 2012 [16]
Felis catus	Cuiaba and Várzea Grande, Matto Grosso, Brazil	1.3%	Ramos et al., 2013 [74]
Felis catus	Montevideo, Uruguay	2/8	Castro et al., 2013 [66]
Felis catus	Río Bueno y La Unión, Provincia del Ranco, Chile	20/200	Oyarzún-Cadagán, 2013 [80]
Felis catus	Rio Grande do Sul, Brazil	29.5%	Ehlers et al., 2013 [87]
Felis catus	Buenos Aires, Argentina	35.3% (6/17)	Cardillo et al., 2014 [91]
Leopardus wiedii, *Leopardus tigrinus*	Natural park De Trê Barras, Três Barras, Brazil	38.1% , 35.7%	Kusma et al., 2015 [15]
Leopardus colocolo	Rio Grande do Sul, Brazil	1 case	Gressler et al., 2016 [75]
Felis catus	Rio Grande do Sul, Brazil	22/2036 (1998–2005)	Pereira et al., 2017 [76]
Felis catus	Caquetá, Colombia	1 case	Sanchez-Rojas et al., 2017 [81]
Intermediate host species			
Achatina fulica	Rio de Janeiro, Goiás, Espirito Santo, Mato grosso, Sergippe and São Paulo, Brazil	5.57% (217/3806)	Thiengo et al, 2008 [90]
Achatina fulica	São Paulo, Brazil	na	Ohlweiler et al., 2010 [145]
Achatina fulica	Puerto Iguazu, Argentina	2%	Valente et al., 2017 [24]
Rumina decollate	Buenos Aires, Argentina	80% (20/25)	Cardillo et al., 2014 [91]

Abbreviations: na, not applicable

from Bogota (Colombia) [14], which is located approximately 2600 meters above sea level (masl), proving the resilience of gastropod intermediate hosts. In 2012, another incidental case of feline aelurostrongylosis diagnosed *via* the Ritchie test came from Quindío (Colombia) during a parasitological survey in domestic cats, this region has an average altitude of 1458 masl, which supports the fact that *A. abstrusus* is adapted to South American mountainous zones [16]. It is worth noting that the Ritchie test only proves positive in cases of highly parasitized and larvae-shedding animals since it is not specific for the detection of L1 in faeces [14]. An epidemiological study on 50 feline necropsies from Uberlândia, Minas Gerais, Brazil, in 2004, revealed an *A. abstrusus* prevalence of 18% [72]. One year later, a retrospective study on *A. abstrusus* infections in domestic cats presented for routine necropsy during 1987–1996 at the Federal University of Santa Maria, Brazil, detected a prevalence of 5.9–25 % [73]. In 2006, 2.6% of stray cats from Buenos Aires, Argentina, were found positive for *A. abstrusus* using the faecal flotation technique [67]. The prevalence of *A. abstrusus* in cats from Porto Alegre, state of Rio Grande do Sul, Brazil remained equal to 29.5% (24/88) during 2008 and 2009 [87].

In a survey in the metropolitan area of Cuiabá, Mato Grosso, Midwestern Brazil, cats revealed a prevalence of *A. abstrusus* of 1.3%, diagnosed *via* necropsy [74]. A recent report came from Chile, where 10% of domestic cats from the cities Rio Bueno and La Union showed *A. abstrusus* infections *via* the Bearmann funnel technique [80]. Recently, *A. abstrusus* was found by necropsy in two out of eight investigated cats in Montevideo, Uruguay [66]. Additionally, a recent retrospective study during 1998–2015 identified 22 cats with *A. abstrusus* infections in Rio Grande do Sul, Brazil [76]. Finally, other case reports on infected domestic cats originated from Rio de Janeiro, Brazil (2007; [88]) and Caquetá, Colombia (2017; [81]).

In addition to domestic felines, *A. abstrusus* infections are also reported in several wildlife species acting as definitive hosts, such as jaguarondis (*Puma yagouaroundi*) and Geoffroy's cat (*Leopardus geoffroyi*), [89], margay (*L. wiedii*), oncilla (*L. tigrinus*) [15] and Colo colo wildcats (*L. colocolo*) [75]. Moreover, infected gastropod intermediate hosts have been reported in Brazil [90] and Argentina [24, 91] (see Table 2). As suitable intermediate host in South America, the terrestrial snail *Rumina decollate* has been reported in addition to the highly invasive African giant snail *A. fulica*. Interestingly, a rather high *A. abstrusus* prevalence was reported in 80% of *R. decollate* [91].

All above mentioned reports show that *A. abstrusus* cycles in both, sylvatic and urban areas. Therefore, it must be considered as differential diagnosis in cases of feline respiratory disease and in the management and conservation programmes on threatened wild felids in various regions of South America. For more details on the biology, epidemiology, pathophysiology, clinic, diagnosis and treatment options of feline aelurostrongylosis we recommend the recently published reviews of Elsheikha et al. [61] and Traversa & Di Cesare [19].

Diagnostic tools for the detection of *A. vasorum* and *A. abstrusus* infections

Coprological diagnostics

All coprological diagnostic methods described here share the limitation that they can be performed no earlier than seven weeks after the infection due to the parasites' prepatency [92].

In 1917, Baermann et al. [93] described a method to detect nematodes present in soil samples which was later on modified for lungworm larvae detection [94, 95]. This method is based on the hydrophilic and thermophilic behaviour of lungworm larvae [96]. Even though it is currently considered as a gold standard for the coprological diagnosis of feline and canine lungworm infections [21, 97], diagnosis may be hampered by the intermittent shedding of the larvae [98], a low viability of larvae [99], the seven week prepatency or scarce larval excretion in low-grade infections [100]. Therefore, the analysis of at least three samples from consecutive days is recommended by some authors [3, 101–103]. Since the Baermann funnel technique is an easy method that does not require specific equipment, it can be carried out in any veterinarian clinic (Fig. 2a). For small-sized samples, a modification of this technique was recently developed by Conboy et al. [104] using 50 ml screw top tubes as shown in Fig. 2b. Following a 12 h incubation, the larvae are here directly sedimented *via* centrifugation [104].

Following the sedimentation step, L1 of metastrongyloid lungworms of domestic carnivores are differentiated microscopically *via* morphological characteristics which are mainly based on size (length, width, body/oesophagus ratio) and distinct tail morphology as reported elsewhere [63, 105–108]. A general morphological characteristic shared by all metastrongyloid lungworm L1 is the non-rhabditiform oesophagus, which forms 1/3–1/2 of the total larval length [4]. Considering the tail morphology, *A. abstrusus* L1 can be identified by its notched S-shaped tail (please see Fig. 3a), which is distinct from *A. vasorum* L1 possessing a sinus wave curve formed tail end with a dorsal spine (see Fig. 3b).

Another coproscopic technique for detection of lungworm larvae is the faecal smear [109], which is limited by a small sample size, includes no concentration step of parasitic stages, has a low sensitivity of 67% [2, 110] and is therefore not recommended as a routine method. The same applies for the zinc sulphate-based flotation method.

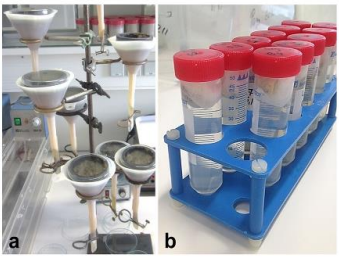

Fig. 2 Illustration of modified Baermann funnel techniques. This technique is considered as the gold standard for the diagnosis of lungworm infections in cat and dog faeces. **a** A household funnel is combined with a plastic tube of 10 cm length and closed by a metal tubing clamp. The funnel is filled with handwarm tap-water. Then a wire mesh screen (9 cm diameter, 0.20–0.25 mm aperture) has to be set in the top of the funnel. **b** Modification by Conboy et al. [104]: instead of a funnel, 50 ml screw-top centrifuge tubes containing warm tap water are used. The faeces need to be placed in a double layer of cheesecloth, placed in the tube and the cap is screwed onto the tube catching a small part of the cheesecloth to keep it in place at the top at the tube. (Pictures taken by Malin K. Lange, Institute of Parasitology, Justus-Liebig-University Giessen)

In one survey, only 8/14 Baermann-positive fecal samples could be detected by this technique [111].

It is very important to emphasize that an accurate and definitive morphological identification of these parasites is a challenging process, which requires well-trained microscopists [2, 19]. So far, there are 181 species in the superfamily Metastrongylidae, many of which with a similar life-cycle and morphology [11]. Other less common parasitic larvae could be detected by the Baermann technique and their morphological and morphometrical key features should be considered since they could be confused with the two species on which this review is focused (see Table 3). In the case of canid samples, it is relevant to consider possible detection of *Crenosoma vulpis* and *Strongyloides stercolaris* [4]. *Strongyloides stercolaris* is spread worldwide [112], while *C. vulpis* is endemic in European and North American red fox (*Vulpes vulpes*) [30, 38] populations and rare in dogs [3]. There is a unique report in South America, specifically in Chile (2013), where 1% (2/200) of the evaluated canine faecal samples resulted positive by the Baermann funnel test [80].

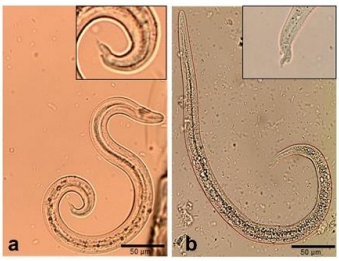

Fig. 3 Morphological characteristics of first-stage larvae of *Angiostrongylus vasorum* and *Aelurostrongylus abstrusus*. **a** The first-stage larvae of *A. vasorum* possess a sinus wave curve formed tail end with a dorsal spine. **b** The first-stage larvae of *A. abstrusus* can be identified by the notched S-shaped tail. (Pictures taken by Malin K. Lange, Institute of Parasitology, Justus-Liebig-University Giessen)

Concerning the microscopical identification of felid coprological samples, it is mandatory to take into account the possible findings of *Troglostrongylus brevior*, *T. subcrenatus* [19, 113], *Oslerus rostratus* [19, 114–116], *Strongyloides stercolaris* [4], *Angiostrongylus chabaudi* [117], and *Angiostrongylus felineus* (recently discovered and described but only with adult stages in *Puma yagouaroundi* from Brazil; this is the reason why its L1 morphological description is lacking) [118]. *Troglostrongylus brevior*, *T. subcrenatus*, *A. chabaudi* and *O. rostratus* have been infrequently reported mainly in wild felids from Europe but not in South America [113, 116, 117]. Additionally, the existence of new metastrongyloid related species in a mega-biodiverse region such as South America could not be rejected and should be contemplated in future studies. Based on the prior observations, it is reasonable to consider the possibility of misdiagnosis in some reports of *A. vasorum* and *A. abstrusus* presented in this review, given that most of these studies were performed by microscopical identification, some of them many years ago, when surely the researchers were not aware of many above mentioned statements and species.

Serological diagnostics

As mentioned before, the gold standard technique for the detection of *A. vasorum* is the Baermann larval migration test [21, 97]. However, this method is constrained by the intermittent shedding of the larvae [98], the seven week prepatency and scarce larval excretion in low-intensity infections [100]. To improve the efficiency and accuracy of *A. vasorum*-related diagnostics, new methods were developed. Thus, enzyme-linked immunosorbent assay (ELISA) tests have been designed to detect circulating *A. vasorum* antigens in serum samples with a specificity ranging between 94–100% and a sensitivity between 42.9–95.7% [99, 100, 119]. However, for some ELISAs non-specific reactions due to antigen-

Table 3 Canid and felid cardiopulmonary nematodes: differential characters of fisrt-stage larvae found by the Baermann funnel technique

Nematode (length × width) (μm)	Morphological keys	Final host	References
Angiostrongylus vasorum (310–400 × 14–16)	A small cup as a cephalic button emerges on the oral extremity	Canids	[1, 2, 4, 146, 147]
	Oesophagus non-rhabditiform, 1/3–1/2 the length of the larva		
	Tip with a dorsal spine and sinus wave curve		
Crenosoma vulpis[a] (240–310 × 13)	Oesophagus non-rhabditiform, 1/3–1/2 the length of the larva	Canids	[4, 111, 148, 149]
	Tail, pointed and straight, without indentations and entirely pointed		
Aulerostrongylus abstrusus (300–415 × 18–19)	Anterior extremity slender, with a short/terminal oral opening leading into a narrow vestibule	Felids	[4, 63, 150, 151]
	Oesophagus non-rhabditiform, 1/3–1/2 the length of the larva		
	Tail S-shaped, with visible dorsal kink, distinct deep dorsal, ventral incisures, a terminal knob-like extremity		
Troglostrongylus brevior[b] (300–357 × 16–19)	Anterior extremity clear and pointed, with a sub-terminal oral opening	Felids	[19, 113, 151–153]
	Oesophagus non-rhabditiform, 1/3–1/2 the length of the larva		
	Tail gradually tapered to dorsal incision, dividing the extremity into two appendices (shallow ventral one, slender dorsal one). S-shaped tail is not obvious, ending straight, gradually tapered		
Troglostrongylus subcrenatus[b] (269–300 × 14–19)	Head pointed, oral opening subterminal (dorsal)	Felids	[19, 113, 153]
	Oesophagus non-rhabditiform, 1/3–1/2 the length of the larva		
	Tail gradually tapered to the extremity with deep dorsal incisure and shallower ventral incisure		
Oslerus rostratus[b] (335–412 × 18–20)	Head rounded, with a central oral opening and a cylindrical buccal capsule	Felids	[1, 4, 151]
	Oesophagus non-rhabditiform, 1/3–1/2 the length of the larva		
	Tail slightly undulated, with a deep ventral notch (ending in minuscule spine) and a shallow dorsal notch		
Angiostrongylus chabaudi[b] (307–420 × 14–16)	Cephalic extremity rounded, with a terminal buccal opening	Felids	[117, 146, 154, 155]
	Oesophagus non-rhabditiform, 1/3–1/2 the length of the larva		
	Caudal extremity with a small dorsal spine and notch, ending in a short sigmoid tail		
Angiostrongylus felineus[c]	?	Felids	[118]
Strongyloides stercoralis (150–390 × 14–23)	Mouth with six lips, mouth-cavity rhabditiform, 1/20–1/21 of the total length of oesophagus	Felids and canids	[1, 4, 156]
	Oesophagus rhabditiform (corpus, isthmus, valvulated bulb), 1/4 of the total length of the larva		
	Pointed and straight tail		

[a]Europe and North America; unique report in South America (1% prevalence in dogs) in Chile [80]
[b]Not reported in South America
[c]Described in 2013 in *Puma yagouaroundi* from Brazil, first-stage larvae have been not described (Viera et al. [118])

based cross-reactivity to other nematode infections were reported [92, 120]. Overall, *A. vasorum* antigen revealed as firstly detectable approximately five weeks after (experimental) infection and appeared to persists for a certain time period after elimination of the parasite [119]. Nevertheless, specific antigen detection may serve as a useful tool for treatment control, as previously proposed [99, 121] since antigen levels significantly decrease after treatment [119]. Thus, an absence of circulating antigens was observed in dogs treated with imidacloprid/moxidectin at 4 or 32 days post-infection (pi) and in dogs treated at 88–92 days pi, circulatory antigens decreased within 13–34 days [119]. Recently, a rapid *in situ* assay (Angio Detect™ Test, IDEXX Laboratories, Westbrook, Maine, USA) was merchandized for the serological detection of circulating *A. vasorum* antigens. This assay showed 100% sensitivity at 14 weeks pi and and with the earliest positive reaction at 9 weeks pi. [99]. When compared to the Baermann funnel assay, the Angio Detect™ Test showed a sensitivity of 97.1% and a specificity of 98.9%

[122]. Thus, this diagnostic test seems to be a useful diagnostic tool in a clinical setting.

Besides antigen detection, serological ELISA tests have been developed to detect specific antibodies raised against the parasite [122, 123]. However, antibody detection in a clinical context depends on the average life span of immunoglobulins and continued antigen stimulation [124]. In the early phase of infection specific antibodies can be detected while antigens are still not detectable [123]. *Angiostrongylus vasorum*-specific antibodies can be detected from 13 to 21 days after infection onwards persisting for up to nine weeks pi [125]. Thus, Cury et al. [97] detected *A. vasorum*-specific antibodies 14–28 days after experimental infection of dogs but humoral responses showed to be highly variable [126]. Serological tests for *A. vasorum*-specific antibody detection based on adult-, excretory/secretory (ES) antigens or L1 antigens showed a sensitivity of up to 85.7% and a specificity of 98.8% during prepatency [125]. However, Schucan et al. [125] found cross-reactions using adult somatic, adult ES antigens and L1 somatic antigen with sera of dogs infected with *C. vulpis*, *Dirofilaria immitis*, *D. repens* and *Eucoleus aerophilus*. When using monoclonal antibody-purified antigens, these cross-reactions were minimalized [125] and specificity was augmented [99, 119, 125, 127].

Angiostrongylus vasorum-specific antibodies can also be detected using the immunoblot (western blot) technique [97]. Although the sensitivity of western blots was higher than above mentioned antibody-ELISA [97], the former technique is only convenient for small sample sizes due to the large effort of this technique.

Both, antigen- and antibody-ELISAs were tested in a field study and compared to the Baermann funnel technique [123]. Thereby, the ELISAs principally confirmed Baermann-positive dogs and additionally detected non-patent infections [123]. As suggested by Schnyder et al. [127], the detection of parasite-specific antigen indicates an actual infection status, while parasite-specific antibodies merely reflect earlier parasite exposure. Consequently, such cases in which both, circulating *A. vasorum* antigens and specific antibodies are detected are assumed as active *A. vasorum* infections while exclusive antibody detection indicates infections that were acquired in the past [119, 125]. Nonetheless, it is important to note that antibody-seropositive dogs may also be free of parasites due to a self-curing process or treatment [125].

Regarding the serological diagnostics for *A. abstrusus*, these techniques are still in development and there are no commercially available serological tests for diagnosis of aelurostrongylosis [61]. Recently, an indirect fluorescent antibody test (IFAT) capable of detecting antibodies against *A. abstrusus* in sera from cats was developed and it showed to be promising in terms of sensitivity and specificity [128]. Furthermore, preliminary results suggest that the detection of antibodies using an ELISA might be a valuable tool for individual diagnosis and also for sero-epidemiological studies on feline aelurostrongylosis [61, 129].

Nonetheless, developing new diagnostic technologies with high sensitivity, specificity, availability and/or efficiency by means of improvement existing assays is necessary [61]. Presently, due to the lack of an optimal commercial serological diagnostic technique the Baermann method is recommended and could be employed for morphological detection of *A. abstrusus* infections which preferably should be confirmed by concurrent PCR. In the same way, larvae obtained from tracheal swabs or bronchoalveolar lavage could confirm the infection *via* PCR [2, 61]

Polymerase chain reaction (PCR)-based diagnostics

Several studies used the PCR technique, mainly based on the second internal transcribed spacer (ITS2) region of ribosomal deoxyribonucleic acid (rDNA), in combination with sequencing of the amplified PCR product to confirm lungworm infections in dogs and wild carnivores [92, 130–135]. Using this molecular technique, different types of samples such as blood, faeces and mucosal smears or even intermediate hosts have successfully been used [132, 135]. However, the sensitivity and reliability of real-time PCR using ITS2 was dependent on the type of sample tested with blood being superior to faeces and pharyngeal or tracheal swabs regarding *A. vasorum* [92, 135, 136]. Houpin et al. [137] described a novel nested PCR- restriction fragment length (PCR-RFLP) [based on *18S* ribosomal ribonucleic acid (rRNA)] for the detection and identification of canine lungworms with a sensitivity of 69.5%. Copro-PCR-based analyses may also be useful in cases of Baermann funnel technique failure due to morphologically altered or less motile *A. vasorum* L1 [121]. However, false negative results in PCR-based analyses were reported depending on quality of the sample and amount of sample used for DNA extraction [92]. Therefore, PCR-based diagnostic techniques for detection of *A. vasorum* are considered less sensitive than ELISA and the Baermann funnel technique [92]. Nevertheless, PCR-based tools developed for the diagnosis of *A. abstrusus* infections showed a specificity of 100% and a sensitivity of ~97% [138]. In addition, an ITS2-based duplex PCR was developed to discriminate between *A. abstrusus* and *T. brevior* (a closely related lungworm parasite species) infections in a single cat [139]. Most recently, a triplex semi-nested PCR for the simultaneous detection of *A. abstrusus*, *T. brevior* and *A. chabaudi* (a rare cardiopulmonary nematode of wild felids) DNA was published [140].

Overall, since these novel molecular diagnostic tools have proven successful and effective for the diagnosis of canine angiostrongylosis and feline aelurostrongylosis thereby partially overcoming limitations of classical diagnostic methods, they may be useful to perform large-scale epidemiological surveys. However, the rather high costs of this molecular technique should also be taken into account, especially in poorer regions of South America.

Conclusions

Considering the wide distribution of canine angiostrongylosis and feline aelurostrongylosis in South America, it is of great interest that small and wildlife practice clinicians consider these infections (and the less common species such as *C. vulpis*, *T. brevior*, *T. subrenatus*, *A. chabaudi*, *A. felineus* and *O. rostratus*) as differential diagnosis in the case of cardiopulmonary disorders. Here, the implementation of routinely applied tools of diagnostics, such as the Baermann funnel technique, parasitological dissection, PCR and/or serology is essential for South America regions since correct diagnosis of infections will significantly contribute to an improved knowledge on the current epidemiological situation of these neglected parasitoses. Phylogenetic studies are also pending to evaluate if *A. vasorum* from South America actually represents a distinct genotype or species. Epidemiological surveys in domestic and wild canid and felids as well as paratenic- and intermediate-hosts with an accurate molecular characterization are required. Additionally, the impact of climatic factors (e.g. altitude, temperature, annual precipitation, relative humidity and biogeographical region) on host-parasite and parasite-intermediate host interactions and the spread of these parasites into non-endemic regions are relevant topics to be considered in future investigations in one of the most biodiverse regions of the planet.

Abbreviations

ELISA: enzyme-linked immunosorbent assay; ITS2: second internal transcribed spacer; L1: first-stage larvae; PCR: polymerase chain reaction; PCR-RFLP: polymerase chain reaction - restriction fragment length polymorphism; rDNA: ribosomal deoxyribonucleic acid; RNA: ribonucleic acid; rRNA: ribosomal ribonucleic acid

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FPT, MKL, JJCC and CH wrote the manuscript. CH and AT revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

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Consent for publication

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Competing interests

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7. Zusammenfassung

Über Gastropoden vermittelte Lungenwurminfektionen spielen zunehmend eine Rolle in deutschen Katzen- und Hundepopulationen. Da zur aktuellen Situation in gastropoden Zwischenwirten von Lungenwurminfektionen bisher keinerlei Daten vorlagen, wurde hier erstmalig eine ganzjährige, epidemiologische Studie in ausgewählten Regionen Deutschlands durchgeführt, die die Prävalenzen von klassischen Lungenwürmern der Karnivoren wie *Angiostrongylus vasorum*, *Crenosoma vulpis* und *Aelurostrongylus abstrusus* in natürlichen Nacktschneckenpopulationen erfassen sollte. Über die Untersuchung von insgesamt 2701 Nacktschnecken aus Hessen und Rheinland-Pfalz konnte gezeigt werden, dass Infektionen mit *A. vasorum*, *C. vulpis* und *A. abstrusus* in unterschiedlichen Häufigkeiten in der Zwischenwirtspopulation nachweisbar sind. Im Falle von *A. vasorum* wurden gebietsweise und jahreszeitlich stark schwankende Prävalenzen (1-19,4 %) gemessen, die dem typischen fleckenhaften geographischen Verteilungsmuster des Parasiten entsprechen. Die Gesamtprävalenz von *A. vasorum* betrug 4,7 %. *C. vulpis* war in 2,3 % der Nacktschnecken zu finden, *A. abstrusus* in 0,2 % der Zwischenwirte. Da zu allen Jahreszeiten Lungenwurm-infizierte Schnecken gefunden wurden, ist davon auszugehen, dass für Hunde, die in Endemiegebieten leben, das ganze Jahr über ein Infektionsrisiko besteht. Dennoch konnte anhand von statistischen Analysen belegt werden, dass das höchste Infektionsrisiko für Hunde bezogen auf *A. vasorum*- und *C. vulpis*-Infektionen im Herbst vorliegt. Bezogen auf die jeweiligen Zwischenwirte belegen die Daten artspezifische Effekte, da *Arion lusitanicus* höhere *A. vasorum*-Prävalenzen als *Deroceras reticulatum* und *D. reticulatum* höhere Larvenbürden als *A. lusitanicus* aufwiesen.

In Südamerika gelten Lungenwurminfektionen von Hunden und Katzen als vernachlässigte Parasitosen und nur wenige epidemiologische Studien existieren zu dieser Thematik. Um diese Lücke zu schließen, wurde im Rahmen dieser Arbeit eine Studie zur Prävalenz von Lungenwurminfektionen in neozoischen Achatschnecken in Kolumbien durchgeführt. Dazu wurden 609 Achatschnecken (*Achatina fulica*) aus fünf Regionen Kolumbiens (Cañasgordas, Ciudad Bolívar, Andes, Tuluá und Puerto Leguízamo) untersucht. Hierbei gelangen Erstnachweise für *C. vulpis*- (Prävalenz 1,1 %) und *Troglostrongylus brevior*- (Prävalenz: 1,3 %) Infektionen für Kolumbien.

Zusätzlich wurden *A. vasorum*-Infektionen bei insgesamt 3,9 % der Achatschnecken festgestellt. Neben einer Gesamtprävalenz von 9,4 % für den Katzenlungenwurm *A. abstrusus* wurde ein *Hotspot* für diesen Parasiten über eine lokal sehr hohe Prävalenz von 53,3% in der Region von Putumayo identifiziert. Über Sequenzanalysen zu *A. vasorum* wurde zudem belegt, dass der europäische Stamm von *A. vasorum* in Südamerika parallel zum südamerikanischen Stamm vorkommt.

Da bisher nur sehr wenig zu Abwehrreaktionen der Zwischenwirte auf Lungenwurminfektionen bekannt ist, wurden zudem Immunreaktionen experimentell infizierter Nacktschneckenspezies untersucht. Dazu wurden zunächst Protokolle sowohl zur standardisierter Haltung und Kultur unterschiedlicher Schneckengenera (*Arion, Deroceras, Limax, Achatina*) als auch zur Gewinnung vitaler Hämozyten als Abwehrzellen von Schnecken und axenischer Lungenwurmlarven etabliert.
Über die *in vitro*-Exposition von Hämozyten unterschiedlicher Gastropodenarten mit Larven unterschiedlicher Lungenwurmspezies konnte hier erstmalig gezeigt werden, dass Hämozyten von Schnecken in der Lage sind, sog. „Extracellular Traps“ (ETs) in Reaktion auf Nematodenlarven zu bilden und über diese die Pathogene zu immobilisieren. Zudem wurde belegt, dass an dieser nicht-adaptiven Immunreaktion zum Vertebratensystem ähnliche Moleküle wie Myeloperoxidase, Elastase und Histone sowie extrazelluläres Chromatin beteiligt sind. In Analogie zum Vertebratensystem konnte außerdem gezeigt werden, dass auch bei Gastropoden morphologisch unterschiedliche Typen von ETs gebildet werden, nämlich *aggregated, diffuse* und *spread* ETs. Durch die Verwendung verschiedener Schnecken- und Lungenwurmarten wurde zudem belegt, dass die ETose unabhängig von der Wirts- und Parasitenart stattfindet. Da unterschiedliche Larvenstadien gleichermaßen ETose auslösten, war dieser Prozess zudem als stadienunabhängig zu bewerten. Die Analyse rasterelektronenmikroskopischer Aufnahmen nach experimentellen Lungenwurminfektionen von Nacktschnecken ermöglichte schließlich den *in vivo*-Nachweis der ETose, da ETs in Reaktion auf eindringende Lungenwurmlarven auf der Körperoberfläche der Gastropoden dargestellt werden konnten. Insgesamt wurde die Hämozyten-vermittelte ETose terrestrischer Gastropoden als ein schnell (bereits 10 Minuten nach der Konfrontation erfolgend) und effizient (fast die Hälfte aller Larven wurde durch ETs gefangen) ablaufender Effektormechanismus identifiziert.

8. Summary

Gastropod-borne lungworm infections of dogs and cats are of increasing concern for veterinarians in Europe. Since data on lungworm infections in German gastropods were lacking, we here conducted an epidemiological survey on gastropod lungworm infections spanning an entire year in selected regions of Germany. The aim was to determine the actual prevalence of classical gastropod-borne canine and feline lungworms, such as *Angiostrongylus vasorum, Crenosoma vulpis* and *Aelurostrongylus* abstrusus. By analysing 2701 slugs from Hesse and Rhineland-Palatine, the presence of *A. vasorum, C. vulpis* and *A. abstrusus* larvae in gastropod intermediate hosts at varying prevalences was proven. The overall prevalence of *A. vasorum, C. vulpis* and *A. abstrusus* in slugs was 4.7 %, 2.3 % and 0.2 %, respectively. Overall, *A. vasorum* prevalences varied strongly (1 % - 19.4 %) in a season- and region-dependent manner thereby corresponding to the well-known patchy geographic distribution of this parasite. Given that slugs proved infected all over the year, dogs are at permanent risk of *A. vasorum*-infections when living in endemic areas. Nevertheless, autumn revealed as the season with the highest infection risk for canine *A. vasorum* and *C. vulpis* infections. Regarding intermediate host susceptability, the current data confirmed species-specific effects, since *Arion lusitanicus* was significantly more often infected with *A. vasorum* than *Deroceras reticulatum* and *D. reticulatum* slugs showed higher larval burdens than *Arion* specimen. Overall, this survey contributed to a better understanding of seasonal fluctuations in lungworm prevalences and revealed slug species-associated risk factors for canine Angiostrongylosis.

In South America, lungworm infections are considered as neglected parasitoses and only few data on both, intermediate and final host infections, are available. To partially fill this gap, an epidemiological survey on lungworm infections in neozoic *Achatina fulica* snails was performed within this work in Colombia. In total, 609 giant African snails (*A. fulica*) were collected in 5 areas of Colombia (Cañasgordas, Ciudad Bolívar, Andes, Tuluá and Puerto Leguízamo) and analysed for canine and feline lungworm infections. We here delivered fist-ever evidence on the presence of *C. vulpis* (prevalence 1.1 %) and *Troglostrongylus brevior* (prevalence 1.3 %) in Colombia. In addition, a total prevalence of 3.9 % and 9.4% was estimated in Colombian *A. fulica* for *A. vasorum* and *A. abstrusus* infections, respectively. For the latter parasite, a local

hotspot with an extraordinary high prevalence of 53.3 % was identified in Putumayo. Sequence analyses on *A. vasorum* isolates furthermore proved an European *A. vasorum* strain to be present in South America along with the South American lineage.

Given that data on innate defense mechanisms of intermediate hosts in reaction to lungworm infections are scarce, haemocyte-related immune reactions against experimental lungworm infections of different slug species were here furthermore analysed. To allow for reproducible *in vitro* experiments, standardised breeding and cultivation of gastropods (*Arion, Deroceras, Limax, Achatina*) and protocols for vital haemocytes (gastopod immune cells) isolation and for axenic lungworm larvae generation were here established.

By confronting gastropod haemocyte with larval lungworm stages *in vitro,* first-ever evidence on gastropod haemocyte-triggered ET formation was given. This defence mechanism led to effective pathogen immobilisation but failed to trigger lethal effects. Moreover, molecules similar to the vertebrate system, such as myeloperoxidase, elastase and histones as well as extracellular chromatin were proven as classical components of this non-adaptive immune mechanism in slugs. In line with findings in the vertebrate system, differend types of ETs, such as *aggregated, diffuse* and *spread* ETs were detected in the gastropod system. Furthermore, gastropod ETosis was proven as host species-independent since several slug species formed ETs in a comparable manner in reaction to lungworm larvae. Given that ETs were equally formed in reaction to three different lungworm species (*A. vasorum, C. vulpis* and *A. abstrusus*) and to two different larval stages (L1, L3), this innate immune mechanism additionally revealed as a parasite species- and parasitic stage-independent process. Finally, scanning electron microscopy-based visualization of ET formation on the body surface of slugs in reaction to skin penetrating lungworm larvae proved the *in vivo* existence of this effector mechanism. Overall, gastropod-derived ETosis revealed as a fast (10 minutes after confrontation with larvae) and efficient effector mechanism of haemocytes (almost half of all larvae used were entrapped by ETs).

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10. Anhang

Manuskript der epidemiologischen Studie in Kolumbien

The invasive giant African snail *Achatina fulica* as natural intermediate host of *Aelurostrongylus abstrusus, Angiostrongylus vasorum, Troglostrongylus brevior,* and *Crenosoma vulpis* in Colombia

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Abstract

Background

Several metastrongyloid lungworms have never been reported in Colombia, so they are potentially neglected pathogens. There are cases on *Aelurostrongylus abstrusus* and considering that six different wild felids exist in Colombia, whether lungworms are an important cause of disease in their populations would be of great interest to their preservation. The canine lungworm *Angiostrongylus vasorum* has been reported few times in Colombian wildlife. *Crenosoma vulpis*, which targets canids, and *Troglostrongylus brevior,* which infects felids, were not described previously in the Colombian territory. Zoonotic metastrongyloids, such as *A. costaricensis* and *A. cantonensis*, may cause severe gastrointestinal or neurological diseases, respectively. Research on the epidemiology of metastrongyloid parasites in Colombia and South America requires to evaluate the role of snails/slugs as intermediate hosts in these parasite life cycles. Therefore, this study assessed the prevalence of metastrongyloid larvae in populations of the giant African snail *Achatina fulica* from different regions of Colombia.

Methodology/Principal Findings

In total, 609 giant African snails were collected from 6 Colombian municipalities [Tuluá (Valle del Cauca), Andes, Ciudad Bolívar, Cañasgordas (Antioquia) and Puerto Leguízamo (Putumayo)]. The snails were cryo-euthanized and artificially digested in pepsin-HCL solution. The sediments were examined microscopically for the presence of metastrongyloid larvae. Based on morphological characteristics 53.3 % of the snails were infected with *A. abstrusus* larvae, 8.4% with *A. vasorum* larvae, 6.5% with *T. brevior* larvae and 5.6% with *C. vulpis* larvae in Puerto Leguízamo (Putumayo). Snails of Andes and Tulúa were positive for *A. vasorum*

larvae with a prevalence of 4.6 and 6.3 %, respectively. Confirmation of collected metastrongyloid larvae species was performed with PCR and sequencing analyses.

Conclusions/Significance

This epidemiological survey confirms for first time the presence of *A. abstrusus, T. brevior, C. vulpis* and *A. vasorum* in the invasive giant African snail in different geographic areas of Colombia.

Author Summary

Several lungworm species are potentially neglected and underestimated pathogens in Colombia. *Aelurostrongylus abstrusus* and *Troglostrongylus brevior* affect domestic cats as well as felid wildlife. *Angiostrongylus vasorum* and *Crenosoma vulpis* targets the cardiopulmonary system of domestic and wildlife canids. *Angiostrongylus. costaricensis* and *Angiostrongylus cantonensis*, may cause severe gastrointestinal or neurological diseases in humans, respectively. Snails/slugs are involved in the life cycles of these parasites. Thus, we assessed the prevalence of metastrongyloid larvae in 609 specimens of the giant African snail *Achatina fulica* from 6 Colombian municipalities. In Puerto Leguízamo, 53.3 % of the snails were infected with *A. abstrusus* larvae, 8.4% with *A. vasorum* larvae, 6.5% with *T. brevior* larvae and 5.6% with *C. vulpis* larvae. Snails of Andes and Tulúa were positive for *A. vasorum* larvae with a prevalence of 4.6 and 6.3 %, respectively. This epidemiological study confirms for first time the presence of *A. abstrusus, T. brevior, C. vulpis* and *A. vasorum* in the invasive giant African snail in different geographic areas of Colombia.

Keywords: Lungworm, Metastrongyloidae, Gastropod-borne diseases, *Achatina fulica, Aelurostrongylus abstrusus, Angiostrongylus vasorum*, *Troglostrongylus brevior, Crenosoma vulpis*.

Introduction

The giant African giant snail *Achatina fulica* is originally from the east coast of Africa (Prasad et al. 2004). Nowadays, it is considered as one of the most widely extended and invasive snail species in terrestrial ecosystems of tropical and subtropical countries (Raut und Barker 2002, Vogler et al. 2013). As in other South American countries, its presence in Colombia has been reported in 26 of the 32 departments in the country (Thiengo et al. 2007, Beltramino et al. 2015), with the departments of Meta, Valle del Cauca, Putumayo and Caquetá facing a critical ecological threat (De La Ossa-Lacayo et al. 2012). Besides the ecological, agricultural and economic threats associated with this neozoan gastropod, it acts as intermediate host of many metastrongyloid species which can cause disease in animals and humans (Lowe et al. 2000, Jing et al. 2015). Due to its long life span (up to 4.5 years) it is more likely to become infected as demonstrated for other gastropod species (Lange et al., 2017), therefore it constitutes an intermediate host of vital relevance in the epidemiology of metastrongyloid parasites and contributes to their global dissemination (Alicata 1966, Graeff-Teixeira 2007, Lv et al. 2009, Moreira et al. 2013).

In the last decade parasites, such as the canine cardio-pulmonary nematode *Angiostrongylus vasorum* and the feline lungworm *Aelurostrongylus abstrusus,* have gained the attention of the veterinary scientific community (Traversa und Guglielmini 2008, Traversa et al. 2010, Penagos-Tabares et al. 2018a). This is due to their detention in domestic and wildlife animals around many countries and their spread into previously non-endemic geographical areas (Taubert et al. 2009, Traversa et al. 2010, Kistler et al. 2014, Giannelli et al. 2017). Symptoms of canine *A. vasorum* infections vary from asymptomatic subclinical cases to severe cardiopulmonary disorders and coagulopathies which can be fatal (Di Cesare und Traversa 2014). Cases of *A. vasorum* have been reported in Europe (Taubert et al. 2009, Traversa et al. 2010),

Africa (Bwangamoi 1972) as well as North and South America (Lima et al. 1994, Bourque et al. 2005, Kistler et al. 2014). It is considered as one of the most pathogenic species of any cardiopulmonary nematodes in canids (Martin et al. 1993, Taubert et al. 2009), including dogs, wolves (*Canis lupus*) (Eleni et al. 2014a), coyotes (*Canis latrans*) (Bourque et al. 2005), jackals (*Canis aureus*) (Takács et al. 2013), several foxes species (Lima et al. 1994, Fiorello et al. 2006, Duarte et al. 2007, Morgan et al. 2008) as well as other carnivores (Simpson et al. 2016, Gillis-Germitsch et al. 2017b). Several intermediate gastropod hosts have been reported, such as *Arion lusitanicus* (Majoros et al. 2010), *Arion distinctus*, *Tandonia sowerbyi* (Patel et al. 2014), *Deroceras reticulatum* (Lange et al. 2018a), *Arion ater*, *Arion ater rufus*, *Limax maximus* (Ferdushy et al. 2009) and experimentally infected *A. fulica* (Sauerländer und Eckert 1974).

Aelurostrongylus abstrusus is distributed worldwide (Scott 1973, Elsheikha et al. 2016, Penagos-Tabares et al. 2018a) and represents one of the most important lung parasites in felids (Scott 1973, Elsheikha et al. 2016). Clinical manifestations of feline aelurostrongylosis are typical of most other respiratory diseases, and include: dyspnoea, open-mouthed abdominal breathing, coughing, wheezing, sneezing and mucopurulent nasal discharge (Traversa et al. 2008b). In addition to domestic cats, *A. abstrusus* infections are also reported in several felid wildlife species which act as definitive hosts (West et al. 1977, Noronha et al. 2002, González et al. 2007). Different species of gastropods have been reported as intermediate hosts for this nematode, including *Agriolimax agrestis, A. columbianus, Helminthoglypta californiensis, H. nickliniana, Helicella spp*. (Hobmaier und Hobmaier 1935), *Helix aspersa* (Hamilton 1963b), *Mesodonthyroidus, Triodopsisalbolabris, Biomphalaria glabrata*, *Cernuella*

virgate (Lopez et al. 2005), *Achatina fulica* (Ohlweiler et al. 2010), *Rumina decollate* (Cardillo et al. 2014) and *Arion lusitanicus* (Lange et al. 2018a).

Additionally, *Troglostrongylus brevior* is a feline-infecting lungworm, which induces varied symptoms, in range from subclinical cases to severe life-threatening conditions being featured by dyspnoea, mucoid-purulent nasal discharge, sneezing, and anorexia (Mehlhorn 2016). It was firstly documented six decades ago from the respiratory tract of *Felis silvestris lybica* and *F. chausin* from Palestine (Gerichter 1949), more recently it was found in cats from Spain (Jefferies et al. 2010b), Greece (Diakou et al. 2015), Italy, Bulgaria (Giannelli et al. 2017), Cyprus (Diakou et al. 2017) and Romania (Deak et al. 2017a) as well as in *Lynx lynx* from Bosnia and Herzegovina (Alić et al. 2015). Its life cycle and its morphology are very similar to that of *A. abstrusus,* reasons why in the past confusion has been reported (Traversa und Di Cesare 2013). So far and under our current knowledge, the only intermediate host species reported of *T. brevior* is *Helix aspersa* by experimental infection (Giannelli et al. 2014). The scientific information on *T. brevior* (e.g., biology, epidemiology, pathogenesis and immunology) is still in its infancy (Otranto et al. 2013).

Crenosoma vulpis is a worldwide-distributed metastrongyloid, known as the fox lungworm, that disturbs the bronchi, bronchioles and trachea of wild and domestic canids (Bihr und Conboy 1999, Matos et al. 2016). Canine crenosomosis is generally characterized by bronchitis with a dry, unproductive cough that can be induced by tracheal palpation (Cobb und Fisher 1992). High parasite burdens could induce a chronic and productive cough with mucoid or mucopurulent expulsion from the airways (Conboy 2009). This helminth is endemic in European and North American red fox

(*Vulpe vulpes*) populations (Zeh et al. 1977, Sréter et al. 2003, Jeffery et al. 2004, Nevárez et al. 2005, Saeed et al. 2006, Schug et al. 2018). Its first report in dogs in was in 1992 in the UK (Cobb und Fisher 1992). Nowadays, *C. vulpis* has been already reported from dogs in other European countries, such as Ireland (Reilly et al. 2000), Switzerland (Unterer et al. 2002), Germany (Taubert et al. 2009, Maksimov et al. 2017) or Italy (Rinaldi et al. 2007). In South America *C. vulpis* was recently report, specifically in Chile, where 1% (2/200) of dogs evaluated resulted positive by Baermann funnel test (Oyarzún-Cadagán 2013). Some gastropod species reported to be involved in the life cycle of *C. vulpis* are the slugs *Deroceras agrestis*, *Arion circumscriptus* (Malek 2018), *Arion lusitanicus* and *Deroceras reticulatum* (Lange et al. 2018a) as well as the snails *Helix pomatia, Cepea hortensis* (Malek 2018), *Helix aspersa* (Colella et al. 2016).

Besides, parasites of the superfamily Metastrongyloidea (Nematoda) include also potentially life-threatening pathogens of humans (Spratt 2015). The considered zoonotic species are *Angiostrongylus cantonensis* and *Angiostrongylus. costaricensis. Angiostrongylus cantonensis*, commonly known as rat lungworm, triggers in humans a severe neuropathological syndrome characterized by eosinophilic meningoencephalitis (Wang et al. 2008). *Angiostrongylus costaricensis* is characterized for its intestinal tropism, where classical signs of human infection consist of an acute abdomen syndrome characterized by enteritis (mainly ileitis), eosinophilic infiltration, vascular alterations, and granulomas due to the localisation of the adults in the mesenteric arteries (Graeff-Teixeira et al. 1987). The spectrum of described definitive hosts includes different species of rodents (Malek 1981, Romero-Alegría et al. 2014). In Colombia, *A. cantonensis* has not been described yet, but at least 10 human infections by *A. costaricensis* have been reported in the regions of Vaupés, Putumayo, Tolima, Cauca, Valle del Cauca (Herrán und Pérez 1986, Rodríguez 2000). Neural angiostrongylosis,

as well as abdominal angiostrongylosis are infrequently diagnosed due to poor knowledge of clinicians and is usually well tolerated (high subclinical presentation) (Romero-Alegría et al. 2014).

The here mentioned parasitoses are considered neglected and underestimated in Colombia and South America, therefore further research evaluating their epidemiological status in the region is urgently needed. Thus, the aim of the present study is to determine the epidemiological status of metastrongyloid parasites in *A. fulica* populations from the Andean, Pacific and Amazonian Colombian biogeographic regions.

Material and Methods

Study areas and snail collection

In total, 609 *A. fulica* snails were collected between February and October of 2016 from the Andean region in the municipalities of Andes (5° 39′ 20″ N, 75° 52′ 49″ W) (*n* = 238), Ciudad Bolívar (5° 50′ 58″ N, 76° 1′ 13″ W) (*n* = 100), and Cañagordas (6° 44′ 59″ N, 76° 1′ 33″ W) (*n* = 100), which are located in the Department of Antioquia. Additionally, *A. fulica* specimens from the Pacific region were collected in the town of Tuluá, Valle del Cauca (4° 5′ 5″ N, 76° 11′ 55″ W) (*n* = 64), and from the Amazon sylvatic area of Puerto Leguízamo, Putumayo (0° 11′ 38″ S, 74° 46′ 50″ W) (*n* = 107) (Fig 1). The snails were cryo-euthanized and shipped to the parasitology laboratory of the Veterinary Medicine School, at the University of Antioquia in Medellín. This study was approved in the order N° 101 on February 2, 2016 by the Ethics committee for animal experimentation of the University of Antioquia, Medellín, Colombia. The giant African snails is considered as highly invasive gastropod species of Colombia and is controlled by national authorities. The giant African snails do not belong to specially protected fauna regulated by the Act on Nature Conservation and Landscape Management of Colombia.

Fig 1. Sampling locations of giant African snails *Achatina fulica* specimens in Colombia. Cañasgordas, Ciudad Bolívar and Andes (Department of Antioquia) are located in the Andean natural region. Tuluá (Department Valle del Cauca) is situated Pacific natural region and Puerto Leguízamo (Department of Putumayo) in the Amazon natural region. Landsat Image Centre: 3°42′10.81″ N 73°07′56.23″W. Eye altitude: 3151,69 Km. Data from: US Dept. of State Geographer. 2017 Google Image Landsat / Copernicus. Data SIO, NOAA, US Navy, NGA, GEBCO. Source: Google Earth Pro® 2017.

Processing of samples

The snails were weighted and subsequently cut into small pieces and immersed in a digestion solution (10 g pepsinogen powder 2000 FIP-U/g (Robert Kind), 8.5 g NaCl (Carl Roth), 30 ml HCl 37% (Carl Roth) and distilled water to complete 1 L of solution). The digestion was performed overnight at 40 °C in 50 ml Falcon tubes (Greiner) under constant shaking. The digested samples were initially sieved through a 300 µm-metal sieve (Retsch) to remove any undigested material and further passed through a 25 µm-metal sieve (Retsch). The remnants of the last sieving were transferred to 15 ml Falcon tubes and centrifuged at 400 *g* for 10 min. The pellets were re-suspended and examined microscopically with a Leica light microscope at 4x, 20x and 40x magnification. Metastrongyloid larvae were counted and carefully collected by pipetting (Pasteur pipette, Hirschmann GmbH & Co. KG). In cases of high larval burden (more than 50 larvae per snail) only 10% of larval burden were viewed at higher magnifications.

Morphological identification of metastrongyloid larvae

Larval stages of metastrongyloids were identified by means of body measurement (lengths/width) and the form (non-rhabditiform) and ratio of oesophagus to body lengths (1:3 – 1:2) (Traversa et al. 2010) according to Lange et al., 2018 (Lange et al. 2018a). To distinguish the different metastrongyloid larval stages the distinct tail morphology of each species was

examined. The lungworm species of *A. abstrusus, T. brevior, A. vasorum* and *C. vulpis* were identified by their typical larval tail morphology as described elsewhere (Wetzel 1940, Ash 1970, Guilhon und Cens 1973, Di Cesare et al. 2013, Giannelli et al. 2014, Lange et al. 2018a, Penagos-Tabares et al. 2018a).

Confirmation of metastrongyloid larvae via PCR and sequencing

The previously described morphological tail characteristics allowed to distinguish some larvae at genus but not species level. Therefore, these larvae underwent additionally PCR analyses to confirm microscopical findings. After digestion with proteinase K, DNA was isolated according to the protocol of a commercial kit (Qiagen DNeasy Blood and Tissue Kit®), with the final elution volume of 50 µL. To enhance the sensibility of the qPCR a conventional PCR with the universal nematode specific primers NC1/NC2 (Gasser et al. 1993) was performed prior to the specific PCR analyses for indivisual species. In the case of *A. abstrusus* and *T. brevior* a nested duplex three step PCR with melt analysis was carried out, amplifying the ITS-2 region of 220 bp (*A. abstrusus*) and 370 bp (*T. brevior*). This PCR was conducted using the forward primer 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), 0.5 µL of each primer, 13.5 mL sterile water and 1 µL template. As positive control DNA derived from one first stage larvae of each cat-lungworm species, kindly provided by Boehringer Ingelheim, were used. The cycling protocol was 95 °C ×10′, 35 cycles of denaturation 95 °C ×20″, annealing 59 °C ×20″, and extension 72 °C × 30″. For the lungworm species *A. vasorum* and *C. vulpis* a nested two-step duplex real time PCR was performed amplifying a portion of the ITS-2 (internal transcribed spacer 2) gene (180 base pairs) as also reported in Jefferies et al. 2011 (Jefferies et al. 2011). To account for inhibitory effects deriving from snail tissue (Jefferies et al. 2009a, Patel et al. 2014) samples were diluted 10 fold with sterile water and tested again with the above mentioned PCR analyses. Sequencing was performed according to Lange et al. 2018 (Lange et al. 2018a). For

sequencing templates of the above mentioned NC1/NC2 conventional PCR were used if sufficient amplifiable DNA amounts were present. Hence, 21 samples were chosen for further sequencing with use of cloning vectors. Purified products were sent to a commercial service (LGC Genomics, Berlin, Germany) for further sequencing and the electropherograms were verified by eye with the software Chromas Lite (version 2.01). Sequences were analysed by BLAST search of the GenBank database. The graphics were designed in GraphPadPrism (version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.).

Results

In the 609 processed samples several metastrongyloid larvae species were identified by their morphology belonging to the following genera: *Aelurostronglyus* sp., *Troglostrongylus* sp., *Crenosoma* sp. and *Angiostrongylus* spp. Futher molecular analysis with PCR allowed the identification of each lungworm species and overall prevalence for snails from each Colombian region is shown in Table 1 and Fig 2. The distinction between different *Angiostrongylus* species was difficult by means of microscopy. Several of the *Angiostrongylus* spp. positive samples contained larvae which resembled *A. vasorum* (*n* = 24) and some showed characteristics of *A. cantonensis* larvae (*n* = 5). However, not all samples could be confirmed by PCR. For the lungworm larvae of *A. abstrusus*, *T. brevior*, *A. vasorum* and *C. vulpis* morphological identification was confirmed by PCR and sequencing. Molecular biological analyses revealed total prevalences of 9.4%, 1.3%, 3.9% and 1.1%, respectively. The overall prevalence of larvae with a morphology resembling *A. cantonensis,* here named *Angiostrongylus* sp. was 0.8%, varying from 0.4% in Andés to 1.6% in Tulúa. The prevalence of *A. abstrusus* in Putumayo was 53.3%. This parasite species could not be detected in any of the other regions of Colombia. By contrast, *T. brevior* was found in two regions, Puerto Leguízamo and Ciudad Bolívar, with a local prevalence of 6.5% and 1.0%, respectively. *C. vulpis* was found in only one snail from Cañasgordas and in 5.6% of the samples derived from Putumayo. A similar

prevalence of 5% was found for *A. vasorum* in snails from Putumayo and Andes (prevalence 4.6%) and from Tulúa. See Table 1

Table 1. Prevalence of metastrongyloid lungworm larvae in *Achatina fulica* from 5 geographic regions Colombia.

Region / Lungworm species	Antioquia			Valle del Cauca	Putumayo	Total (*n* = 609)
	Andes (*n* = 238)	Cañas-gordas (*n* = 100)	Ciudad Bolívar (*n* = 100)	Tuluá (*n* = 64)	Puerto Leguízamo (*n* = 107)	
Aelurostrongylus abstrusus	-	-	-	-	53.3% (57)	9.4% (57)
Troglostrongylus brevior	-	-	1.0% (1)	-	6.5% (7)	1.3% (8)
Crenosoma vulpis	-	1.0% (1)	-	-	5.6% (6)	1.1% (7)
Angiostrongylus vasorum	4.6% (11)	-	-	6.3% (4)	8.4% (9)	3.9% (24)
***Angiostrongylus* sp.**	0.4% (1)	-	-	1.6% (1)	-	0.3% (2)
Total	5.0% (12)	1.0% (1)	1.0% (1)	7.8% (5)	73.8% (69)	**16.1%** (98)

Fig 2. Larval burden categories for gastropod *Aelurostrongylus abstrusus*, *Angiostrongylus vasorum*, *Crenosoma vulpis* and *Troglostrongylus brevior* infections. Snails were collected in 5 different biogeographic areas of Colombia, artificially digested and analysed microscopically for lungworm infections. Here, the proportion of slugs harbouring <10, 10-49, 50-100 and >100 lungworm larvae per specimen is depicted.

Larval burden ranged from 1 to 314 larvae per snail for *A. abstrusus* and from 1 to 286 larvae per snail for *T. brevior* (Figs 2 and 3). A similar range of larval burden was observed for *C. vulpis*, ranging from 1 to 208 larvae (Figs 2 and 3). By contrast, larval burden of *A. vasorum* was low (1 to 30 larvae per snail, with only 6 out of 21 snails containing more than 1 larvae (Figs 2 and 3). Thus, 92.3% of the larvae of *A. vasorum* belonged to the category of less than

10 larvae per snail and remaining 7.7% to the category of 10 to 49 larvae (Fig 2). Regarding *A. abstrusus,* 43.9% of the samples belonged this first category (less than 10 larvae per snail), whereas 38.6% were allocated to the category of 10 to 49 larvae. 7.0% of the samples contained from 50 to 100 larvae (Fig 2). So, 10.5% of *A. abstrusus*-positive samples contained 100 or more larvae (Fig 2). *T. brevior* and *C. vulpis* showed a similar distribution with the majority of samples containing a larval burden of less than 10 larvae for both species (71.4%, Fig 2). Moreover, 14.3% of snails containing *T. brevior* larvae belonged to the category of 10 to 49 larvae, but (Fig 2), whereas no *C. vulpis* larvae belonged to this category (Fig 2). In the category of 50 up to 99 larvae, *C. vulpis* was found with a percentage of 14.3%, whereas no *T. brevior* larvae belonged to this section (Fig 2). In the last category of more than 100 larvae *T. brevior* and *C. vulpis* both appeared with a percentage of 14.3% each (Fig 2).

Fig 3. Correlation of slug weight and *Aelurostrongylus abstrusus*, *Angiostrongylus vasorum*, *Crenosoma vulpis* and *Troglostrongylus brevior* larval burden. Snails were collected in 5 different biogeographic areas of Colombia, artificially digested and analysed microscopically for lungworm infections. Here, the larval burden was correlated with the snail weight.

Co-infections were detected by means of microscopy and PCR in 19.0% (16/84) of all lungworm larvae positive snails (Fig 4). One co-infection was detected consisting of *C. vulpis* and *T. brevior* larvae (1.2%) and other co-infections consisted of *T. brevior* and *A. abstrusus*

(*n* = 4, 4.8%, Fig 4). *T. brevior* was also found in one co-infection with *A. vasorum* (*n* = 1, 1.2%, Fig 4). Most of the co-infections were involving *A. abstrusus* and *A. vasorum* (*n* = 7, 8.3%, Fig 4). *A. abstrusus* was also found in co-infections with *C. vulpis* (*n* = 3, 3.6%, Fig 4). Co-infections consisting of more than two species were not detected.

Fig 4. Mono- and co-infections with *Aelurostrongylus abstrusus*, *Angiostrongylus vasorum*, *Crenosoma vulpis* and *Troglostrongylus brevior.* Snails were collected in 5 different biogeographic areas of Colombia, artificially digested and analysed microscopically for lungworm infections. Here, the fraction of lungworm-positive snails was analysed for mono- and co-infections with the different lungworm species.

Sufficient amplifiable DNA for PCR analysis was present in 29 of the 84 positively identified snails by microscopy, and 21 of those 29 were randomly selected for sequencing. Specimen of *A. abstrusus, A. vasorum, C. vulpis* and *T. brevior* could be confirmed via sequencing with a query coverage from 98% to 100% and identity of 99-100 %, (Table 2). Besides these four lungworms another metastrongyloid was detected for which no match in GenBank was found. This showed a genetic similarity to *A. abstrusus* with a query cover of 98% and 82% identity, (Table 2). *Aelurostrongylus abstrusus* was found with two haplotypes here named A and B (Table 2). Type A (*n* = 11) was identical to the European strain, containing 6-9 times repetition in the GTC satellite. In contrast to that, type B (*n* = 3), containing 3 times GTC repetition in the microsatellite, has never been reported before and could represent a South American variant (Table 2 and S1). The sequence of type AB found in one snail, which contains 4 times GTC microsatellite repetition, indicates that type A and type B may have hybridised and was found in a type A, type B and type AB co-infection (Table 2 and S1).

Table 2. NCBI Nucleotide Blast of metastrongyloids from Colombian giant African snails (*A. fulica*)

Lungworm species/strain	*n* =	Query cover	Identity	GTC microsatellite repetitions
Aelurostrongylus abstrusus A	11	99%	100%	6-9x
Aelurostrongylus abstrusus B	3	99%	91%	3x
Aelurostrongylus abstrusus AB	1	100%	91%	4x
Angiostrongylus vasorum	1	99%	100%	
Crenosoma vulpis	2	98%	99%	
Troglostrongylus brevior	6	100%	99%	
Metastrongyloidea sp.	1	98%	82%*	

*compared to *A. abstrusus*

Discussion

This survey was aimed to find the prevalences of metastrongyloid lungworm species in giant African snail populations from 5 regions of Colombia and adding novel data on the epidemiology of lungworms. An unknown metastrongyloid species was found and further phylogenetic analysis is needed for proper classification.

The existence of *A. abstrusus* in the definitive host population, namely domestic cats of Colombia, is known since 2003 and was reported only scarcely ever since (Salamanca et al. 2003, Echeverry et al. 2012, Sánchez-Rojas et al. 2017). Furthermore, *A. vasorum* was reported in two Colombian crab-eating foxes (*C. thous*) in 1961 and 2014 (Gonçalves 1961, Varela-Arias et al. 2014), but up to now not yet in the Colombian domestic dog population. Only few data exist on the prevalence of *A. vasorum* in intermediate hosts in other countries and these data vary considerately (1.6 – 43% slug prevalence) depending on the sampling areas (Ferdushy et al. 2009, Jefferies et al. 2009a, Patel et al. 2014, Aziz et al. 2016, Lange

et al. 2018a). These reports correspond well to the observed rather low *A. vasorum* prevalence reported in this survey. Since *A. vasorum* is known to display a patchy distribution pattern with hyperendemic foci being in close proximity to areas of low prevalence (Morgan et al. 2005, Ferdushy und Hasan 2010, Aziz et al. 2016, Maksimov et al. 2017, Schug et al. 2018), further extended epidemiological surveys in Colombia are required to detect hotspots of this canine angiostrongylosis. Surprisingly, recent results of the sequencing of *A. vasorum*-positive samples from Colombia (Lange et al. 2018b) revealed identity with the European strain and not with the South American strain (Jefferies et al. 2009b). As for the other two lungworm species, *C. vulpis* and *T. brevior,* no reports in Colombia existed prior to our study. The majority of snails carried a rather low larval burden (less than 50 larvae) regarding all four investigated lungworm species (Fig 2). These findings have been described before for *A. vasorum* in natural slug populations (Ferdushy et al. 2009, Lange et al. 2018a). Those snails carrying a high larval burden (more than 50 larvae) can be considered most dangerous for the definitive host population since a higher infection dose leads to severer clinical manifestations as described by elsewhere (Schnyder et al. 2010, Philbey et al. 2014). With reference to *A. vasorum,* observed low prevalences in the majority of gastropods are in line with previous reports (Ferdushy et al. 2009, Lange et al. 2018a). Similar findings were also reported for closely related species *A. costaricensis* (Laitano et al. 2001) where 82% of the slugs were weakly infected. The percentage of snails harbouring more than 100 larvae have been reported in Denmark and in Germany with 14% (Ferdushy et al. 2009) and 3.3%, respectively. In the here conducted survey, larval burdens of 100 or more larvae per snail were observed in 14.3% regarding *C. vulpis* and *T. brevior* and 10.5% regarding *A. abstrusus* which is in line with other studies. The observation that the majority of gastropods only contain low metastrongyloid larval burden, whereas only a minority of specimen harbours high larval burdens was described as overdispersion (Anderson und Gordon 1982) and was also reported for *A. vasorum* by Ferdushy et al. (Ferdushy et al. 2009) and for the closely related species *Angiostrongylus costaricensis* (Laitano et al. 2001). This overdispersion may lead to subclinical infections which are frequently observed in the definite hosts regarding *A. abstrusus, A. vasorum, C. vulpis* and

T. brevior (Conboy 2004, Ferdushy et al. 2009, Di Cesare und Traversa 2014, Lange et al. 2018a) speculated that low larval burdens in gastropods may also result from an intact intermediate host innate immune system, since the effective formation of so-called invertebrate extracellular phagocyte traps (InEPTs) in response to metastrongyloid lungworm larvae by gastropod haemocytes was recently shown (Lange et al. 2017). This questions requires, however, further in depth investigation on how gastropods defend themselves against invading metastrongyloid parasites (Penagos-Tabares et al. 2018b). The maximal metastrongyloid larval burden found in this survey was 314 *A. abstrusus* larvae (Fig 2), which is slightly lower than the 392 *A. vasorum* burden found in a Danish slug (Ferdushy et al. 2009) and much lower than the 546 *A. vasorum* larvae found in a German slug (Lange et al. 2018a). The majority of lungworm positive snails were associated with a higher weight (over 10 g) and probably therefore of an older age. Larger and older snails/slugs being predisposed to *A. cantonensis* and *A. vasorum* infections has also been reported by others (Yousif und Lämmler 1975, Barcante et al. 2003, Ferdushy et al. 2010, Chen et al. 2011, Lange et al. 2018a). Co-infections in the definitive hosts are frequently reported for the two cat lungworm species (Jefferies et al. 2010b, Diakou et al. 2015, Traversa et al. 2015, Crisi et al. 2017) and the two dog lungworm species (Jeffery et al. 2004, Saeed et al. 2006, Barutzki und Schaper 2009, Taubert et al. 2009, Maksimov et al. 2017, Schug et al. 2018). Conversely, in the intermediate host, reports of metastrongyloid co-infections are rather scarce. To our best knowledge, there was only one report of multiple simultaneous infections of *A. vasorum, C. vulpis* and *A. abstrusus* in slugs in Germany (Lange et al. 2018a). The here conducted survey reports for the first time on gastropod mixed infections of *T. brevior* and *A. abstrusus*, *T. brevior* and *C: vulpis,* as well as *T. brevior* and *A. vasorum* (Fig 4), although these co-infections were only occasionally detected.

Regarding the feline lungworm *A. abstrusus,* the here detected prevalences indicate Putumayo as a hotspot for this parasite. Large areas of tropical rainforest of Colombia form the natural

habitat of six wild felid species: ocelots (*Leopardus pardalis*) (Paviolo et al. 2015), oncillas (*Leopardus tigrinus*) (Payan und de Oliveira 2016), margay wildcats (*Leopardus wiedii*) (de Oliveira et al. 2015), cougars (*Puma concolor*) (Nielsen et al. 2015), Jaguarundies (*Puma* (Synonym: *Herpailurus*) *yagouaroundi*) (Caso et al. 2015) and Jaguars (*Panthera onca*) (Quigley et al. 2017), whose populations are decreasing and are in greater or lesser degree of threat (Payán und L. 2006, González-Maya et al. 2010, Caso et al. 2015, de Oliveira et al. 2015, Nielsen et al. 2015, Paviolo et al. 2015, Boron et al. 2016, Payan und de Oliveira 2016, Quigley et al. 2017, Boron et al. 2018). All above mentioned wild felid species could be affected by high prevalences of *A. abstrusus*, which may be a relevant threat factor if the high pathogenicity and mortality associated to aelurostrongylosis are considered (Scott 1973). Thus, new knowledge on the epidemiological status of lungworm species in wild cats' populations from South American is needed to strengthen and consolidate more successful conservation programmes (Penagos-Tabares et al. 2018a). Additionally, the two different *A. abstrusus* haplotypes here detected indicate that there is most probably more genetic variation in this species in Colombia. Since haplotype AB was only found in one snail which was also co-infected with the haplotypes A and B we hypothesized that type A and B had hybridised, forming haplotype AB (Tab. 2, Suppl. file 1). Nevertheless, it is also possible that the three here found variants may represent three cryptic species and no hybridizing haplotypes. Thus, further molecular analyses regarding this matter are mandatory and to be considered in future *Aelurostrongylus*-related investigations not only in Colombia but also in other South American countries.

Besides *A. vasorum,* other lungworm larvae of the genus *Angiostrongylus* which resembled *A. cantonensis* could be detected in two regions of Colombia (Tab. 1) via microscopy. This parasite has never been reported in this country before, but in Colombia's neighbouring countries Ecuador (Pincay et al. 2009, Dorta-Contreras et al. 2011), Brazil (Moreira et al. 2013, Guerino et al. 2017) and the Caribbean islands (Chikweto et al. 2009, Dard et al. 2017). In the

last decades, several cases of human meningoencephalitis of unknown aetiology and unidentified have been reported in Colombia (Velez-van-Meerbeke et al. 2017), which might correspond to unreported cases of human angiostrongyliasis could have gone unreported or unrecognized (Valente et al. 2018). Further specific investigations on this zoonosis are necessary to confirm whether or not it is already circulating in definitive and intermediated hosts as well as in exposed human populations, in order to take measurements to inform the Colombian public health institutions and protect society of this life-threatening parasitosis.

Although other metastrongyloid nematodes of wildlife were not detected in the snails, it cannot be excluded that they may be overseen in co-infections in some samples due to possible DNA degradation. Since the specific morphological diagnostic of metastrongyloid lungworm larvae is difficult (Ash 1970, Traversa et al. 2008b, Lange et al. 2018a) and depending on the larval stage and the condition in which the larvae is at the time point of analysis we here combined it with molecular biological techniques to secure correct identification.

Conclusion

To our current best knowledge, this is the first large-scale epidemiological survey confirming by molecular analysis the presence of *A. abstrusus, A. vasorum*, *T. brevior* and *C. vulpis* infections in intermediate hosts in Colombia. Moreover, *T. brevior* and *C. vulpis* findings represent the first report of these parasites in general in this country. Regarding *A. abstrusus,* a hotspot could be detected in the Amazon region, specifically in Putumayo. On the basis of morphological identification the anthropozoonotic metastrongyloid *A. cantonensis* was first detected in this country. More epidemiological research on all these parasites in natural populations of paratenic and intermediate hosts in other geographic areas, as well as data regarding prevalences in humans, domestic and wild definite hosts are required in Colombia as well as in other countries of South, Central America and North America, in order to increase

the knowledge of the impact, dynamics and environmental factors associated with these neglected parasitoses.

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Supporting information 1. Alignment of a partial sequence of the ITS-2 region from *Aelurostrongylus abstrusus* isolates detected in Colombia. The GTC microsatellite (highlighted in grey with black letters) served to discriminate between the different *A. abstrusus* haplotypes. Type A is present in Europe and Colombia, whereas haplotypes B and AB only were detected in Colombia, so far.

```
Haplotype A    1           -
Haplotype A    1
Haplotype A    1
Haplotype A    1
Haplotype A    1
Haplotype A    1 ------------------------------------------------------------
Haplotype A    1
Haplotype A    1
Haplotype A    1
Haplotype A    1
Haplotype A    1
Haplotype AB   1
Haplotype B    1                                          T      T
Haplotype B    1                                          T      T
Haplotype B    1                                          T      T


Haplotype A   60           --------
Haplotype A   61             GTCGTCGTCGTCGTCGTCGTCGTCGTC     G
Haplotype A   61             GTCGTCGTCGTCGTCGTCGTCGTCGTC
Haplotype A   61             GTCGTCGTCGTCGTCGTCGTCGTCGTC
Haplotype A   61             GTCGTCGTCGTCGTCGTCGTCGTC---
Haplotype A    1 ------      GTCGTCGTCGTCGTCGTCGTCGTC---
Haplotype A   61             GTCGTCGTCGTCGTCGTC---------
Haplotype A   61             GTCGTCGTCGTCGTCGTCGTC------
Haplotype A   61             GTCGTCGTCGTCGTCGTCGTC------
Haplotype A   61             GTCGTCGTCGTCGTCGTCGTC------
Haplotype A   61             GTCGTCGTCGTCGTCGTCGTC------
Haplotype AB  61             GTCGTCGTCGTC----------------
Haplotype B   61             GTCGTCGTC-------------------
Haplotype B   61             GTCGTCGTC-------------------
Haplotype B   61             GTCGTCGTC-------------------


Haplotype A  112              -                   C
Haplotype A  121              -
Haplotype A  121              -
Haplotype A  121              -
Haplotype A  118              -
Haplotype A   52              -
Haplotype A  112              -
Haplotype A  115              G
Haplotype A  115              -
Haplotype A  115              -
Haplotype A  115              -                                      G
Haplotype AB 106              -
Haplotype B  103              -   C
Haplotype B  103              -   C
Haplotype B  103              -   C


Haplotype A  171         A
Haplotype A  180
Haplotype A  180
Haplotype A  180
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Haplotype A  177      T
Haplotype A  111
Haplotype A  171
Haplotype A  175
Haplotype A  174
Haplotype A  174
Haplotype A  174
Haplotype AB 165
Haplotype B  162       ---                  C    T   T
Haplotype B  162       ---                  C    T   T
Haplotype B  162       ---                  C    T   T



Haplotype A  231
Haplotype A  240
Haplotype A  240
Haplotype A  240
Haplotype A  237
Haplotype A  171
Haplotype A  231                            C
Haplotype A  235
Haplotype A  234
Haplotype A  234
Haplotype A  234
Haplotype AB 225                    C   A     ---
Haplotype B  219                    C   A     ---
Haplotype B  219                    C   A     ---
Haplotype B  219                    C   A     ---


Haplotype A  291
Haplotype A  300
Haplotype A  300
Haplotype A  300
Haplotype A  297
Haplotype A  231
Haplotype A  291
Haplotype A  295
Haplotype A  294
Haplotype A  294
Haplotype A  294           C
Haplotype AB 282                                    T
Haplotype B  276                                    T
Haplotype B  276
Haplotype B  276                                    T


Haplotype A  351         C                                      C
Haplotype A  360
Haplotype A  360
Haplotype A  360
Haplotype A  357
Haplotype A  291
Haplotype A  351
Haplotype A  355         C                                      C
Haplotype A  354
Haplotype A  354
Haplotype A  354
Haplotype AB 342         C                          ---
Haplotype B  336         C                          ---
Haplotype B  336
Haplotype B  336         C
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Haplotype A  411
Haplotype A  420                      ------------------------------
Haplotype A  420
Haplotype A  420                                 T
Haplotype A  417
Haplotype A  351
Haplotype A  411
Haplotype A  415
Haplotype A  414
Haplotype A  414
Haplotype A  414
Haplotype AB 399                    T
Haplotype B  393
Haplotype B  396
Haplotype B  396
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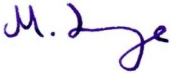
11. Erklärung

Ich habe die vorgelegte Dissertation selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.



Malin Katharina Lange

EIGENANTEILSERKLÄRUNG

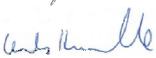
Hiermit erkläre ich, dass ich die in dieser Dissertation mir zugeschriebenen Anteile an den im Folgenden aufgelisteten Publikationen selbst und ohne unerlaubte Hilfe angefertigt habe und alle in Anspruch genommenen Quellen und Hilfsmittel in der Dissertation angegeben habe. Die Mitautoren haben sich einverstanden erklärt, dass die unten stehenden Publikationen in dieser Dissertation verwendet werden.

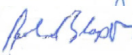
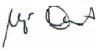
1. Prevalence of *Angiostrongylus vasorum*, *Aelurostrongylus abstrusus* and *Crenosoma vulpis* larvae in native slug populations in Germany

Einverständnis der Mitautoren:

F. Penagos-Tabares 
Y.R. Van Bourgonie 

J. Hirzmann 
T. Backeljau 

K. Failing 
C. Hermosilla 

R. Schaper 
A. Taubert 

2. *Angiostrongylus vasorum* and *Aelurostrongylus abstrusus*: Neglected and underestimated parasites in South America

Einverständnis der Mitautoren:

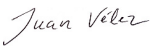
F. Penagos-Tabares 
A. Taubert 

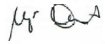
J.J. Chaparro-Gutiérrez 
C. Hermosilla 

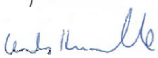
3. Regional report on *Angiostrongylus vasorum* in Colombia: Genetic similarity to European lineage

Einverständnis der Mitautoren:

F. Penagos-Tabares  J.J. Chaparro-Gutiérrez 

J. Vélez  D. Piedrahita 

J. Gutiérrez  A. Taubert 

J. Hirzmann  C. Hermosilla 

4. Novel approach to study gastropod-mediated innate immune reactions against metastrongyloid parasites

Einverständnis der Mitautoren:

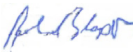
F. Penagos-Tabares  H. Mejer 

A. Seipp  A. Taubert 

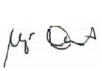
U. Gärtner  C. Hermosilla 

5. Gastropod-derived haemocyte extracellular traps entrap metastrongyloid larval stages of *Angiostrongylus vasorum*, *Aelurostrongylus abstrusus* and *Troglostrongylus brevior*

Einverständnis der Mitautoren:

F. Penagos-Tabares  R. Schaper 

T. Muñoz-Caro  C. Hermosilla 

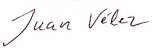
U. Gärtner  A. Taubert 

H. Mejer 

6. The invasive giant African snail *Achatina fulica* as natural intermediate host of *Aelurostrongylus abstrusus, Angiostrongylus vasorum, Troglostrongylus brevior* and *Crenosoma vulpis* in Colombia.

Einverständnis der Mitautoren:

F. Penagos-Tabares  J.J. Chaparro-Gutiérrez 

J. Vélez  J. Gutiérrez 

A. Taubert  J. Hirzmann 

C. Hermosilla 

12. Danksagung

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