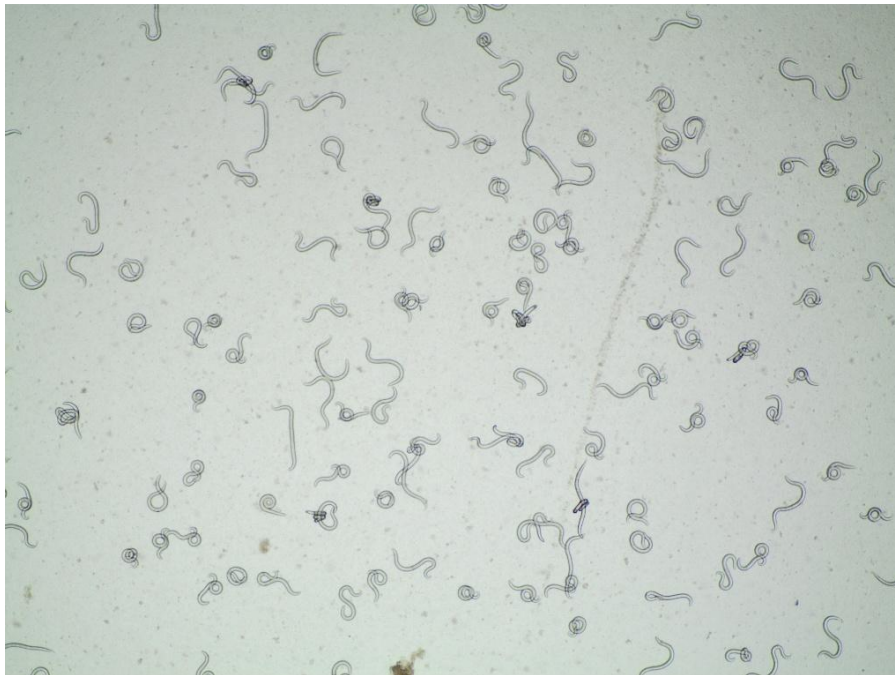


Alena Dusch

**Untersuchungen zu metastrongyloiden Lungenwürmern
im obligatorischen gastropoden Zwischenwirt –
Epidemiologie, Organtropismus und Immunologie**



INAUGURAL-DISSERTATION
zur Erlangung des Grades eines
Dr. med. vet. beim Fachbereich
Veterinärmedizin der Justus-Liebig-
Universität Gießen

Institut für Parasitologie, Fachbereich Veterinärmedizin, Justus-Liebig-
Universität Gießen

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INAUGURAL-DISSERTATION
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Universität Gießen

eingereicht von

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Gießen 2026

Imprimatur

Mit Genehmigung des Fachbereichs Veterinärmedizin
der Justus-Liebig-Universität Gießen

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Tag der Disputation: 09.06.2026

TAGUNGSBEITRÄGE

Daten dieser Dissertation wurden bereits auf den folgenden nationalen und internationalen Tagungen vorgestellt:

- 1. Dusch A**, Segeritz L, Taubert A, Hermosilla C (2023). Re-monitoring of a hyperendemic focus of *Angiostrongylus vasorum* infections in native gastropod populations in Southern Germany. 30th annual meeting of the German Society for Parasitology, 15.-17. März 2023, Gießen.
- 2. Dusch A**, Segeritz L, Taubert A, Hermosilla C (2023). Re-confirmation of a hyperendemic focus of *Angiostrongylus vasorum* infections in gastropod intermediate hosts in Southern Germany. Deutsche Veterinärmedizinische Gesellschaft Parasitologie und Parasitäre Krankheiten, 15.-17. Mai 2023, München.
- 3. Dusch A**, Segeritz L, Taubert A, Hermosilla C (2024). Re-evaluation of a hyperendemic focus of *Angiostrongylus vasorum* infections in gastropod intermediate hosts in Southern Germany. 6.-10. Mai 2024, Modrava, Tschechien.
- 4. Dusch A**, Haas M, Taubert A, Hermosilla C. Die Bedeutung von Gastropoden in der Parasitologie und aktuelle Forschungen. Jahrestagung der Deutschen Malakozoologischen Gesellschaft. 17. Mai 2024, Bad Harzburg.
- 5. Dusch A**, van de Sanden J, Verburg E, Taubert A, Hermosilla C. PET/CT von *Crenosoma striatum*-infizierten *Achatina fulica* (2024). Deutsche Veterinärmedizinische Gesellschaft Parasitologie und Parasitäre Krankheiten, 8-10. Juli 2024, Hannover.
- 6. Dusch A**, van de Sanden J, Verburg E, Conejeros I, Velasquez Z, Taubert A, Hermosilla C. *Achatina fulica* as a possible model organism for gastropod-borne diseases. Joint Parasitology Spring Meeting, 10.-14. März 2025, Würzburg.

ABKÜRZUNGSVERZEICHNIS

¹⁸F-FDG: Fluor-18-Fluordesoxyglukose
aggNET: aggregated neutrophil extracellular traps
CT: Computertomographie
DAMP: damage-associated molecular patterns
diffInEPT: diffuse Invertebrate Extracellular Phagocyte Traps
DNA: Desoxyribonukleinsäure
EET: Eosinophil extracellular traps
ET: Extracellular Traps
FREP: Fibrinogen-related proteins
InEPT: Invertebrate Extracellular Phagocyte Traps
L1: erstes Larvenstadium
L2: zweites Larvenstadium
L3: drittes Larvenstadium
L4: viertes Larvenstadium
L5: fünftes Larvenstadium
LPS: Lipopolysaccharide
MET: Macrophage extracellular traps
MPO: Myeloperoxidase
MRT: Magnetresonanztomographie
NET: Neutrophil extracellular traps
NOX: NADPH-Oxidase
PAD4: Protein-Arginin-Deiminase Typ 4
PCR: Polymerase-Kettenreaktion
PET: Positronen-Emissions-Tomographie
p. i.: post infectionem
PRR: Pattern recognition receptor
REM: Rasterelektronenmikroskop
ROS: Reactive Oxygen Species
SPECT: Single Photon Emission Computed Tomography
sprInEPT: spread Invertebrate Extracellular Phagocyte Traps
TLR: Toll-like receptor

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1. Einleitung

Infektionen mit Lungenwürmern gehören noch immer zu den Parasitosen, die im klinischen Umfeld von praktizierenden Tierärzten stark unterschätzt und vernachlässigt werden (Penagos-Tabares et al. 2018a). Lungenwurminfektionen können zu den verschiedensten Symptomen führen, von asymptomatisch bis zu schwerwiegenden kardiovaskulären und respiratorischen Erkrankungen (Traversa et al. 2015; Colombo et al. 2021).

Zu den Kaniden betreffenden Arten gehören die Nematoden *Angiostrongylus vasorum*, *Crenosoma vulpis* und *Capillaria aerophila* (syn. *Eucoleus aerophilus*). Der pathogenste dieser Lungenwürmer ist *A. vasorum*, auch „Französischer Herzwurm“ genannt. Infektionen mit diesem Lungenwurm können zu respiratorischen, kardiovaskulären, neurologischen, okulären und gastrointestinalen Symptomen führen (Perry et al. 1991; Bolt et al. 1994; Morgan und Shaw 2010; Colombo et al. 2021). Adulte Stadien leben in der Lungenarterie und im rechten Herzen. Infektionen mit *C. vulpis*, entsprechend ihrer Lokalisation in den Bronchien des Endwirts, führen zu Bronchitiden und Husten (Barutzki und Schaper 2009; Choi et al. 2014). *C. aerophila*, der Lungenhaarwurm, infiziert Hunde und Katzen sowie wildlebende Kaniden und Feliden. Infektionen können subklinisch bleiben, aber auch respiratorische Symptome wie Husten, Niesen und Bronchitis auslösen (Majeed et al. 1989; Barrs et al. 2000).

Die häufigste Lungenwurminfektion bei Katzen wird durch *Aelurostrongylus abstrusus* ausgelöst (Pennisi et al. 2015). In jüngster Zeit wurde auch *Troglostrongylus brevior* als neu auftretende Krankheit dokumentiert, die vor allem bei jungen Tieren zu gefährlichen respiratorischen und generalisierten Symptomen führen kann (Cavalera et al. 2018). *Angiostrongylus chabaudi* wurde ebenfalls beschrieben als Parasit, der das kardiovaskuläre System von Feliden infiziert (Varcasia et al. 2014).

Die Ausbreitung von Lungenwurminfektionen nimmt stetig zu, was aus jüngeren Studien hervorgeht (Barutzki und Schaper 2009; Schnyder et al. 2013a; Maksimov et al. 2017). In Europa werden neue hyperendemische Foki beschrieben sowie die Ausbreitung der Lungenwürmer in Regionen, in denen diese vorher nicht vorkamen. Kürzlich wurde auch erstmalig das Vorkommen des zoonotisch relevanten Lungenwurms *Angiostrongylus cantonensis* in Spanien (u. a. Teneriffa, Makaronesien, Valencia) sowie in Italien beschrieben (Segeritz et al. 2021b; Galán-Puchades et al. 2022; Pandian et al. 2025). Diese Lungenwurmart infiziert üblicherweise Ratten, jedoch ist eine *A. cantonensis*-Infektion des Menschen als Fehlwirt möglich. Dort ist der Parasit in der Lage, eine lebensgefährliche eosinophile Meningitis auszulösen (Wang et al. 2010; Martins et al. 2015).

Eine wichtige Rolle in der Übertragung und Epidemiologie von Lungenwürmern spielen die Wildtiere, vor allem Füchse, deren Urbanisation in den letzten Jahren zugenommen hat

(Bružinskaitė-Schmidhalter et al. 2012; Cervantes et al. 2023). Diese bilden ein Parasitenreservoir, wodurch neue parasitäre Infektionen sich auf Haustiere übertragen können. Die Gründe für die zunehmende Verbreitung der Lungenwürmer sind bisher unbekannt. Diskutiert werden der Klimawandel, das vermehrte Reisen von Tierbesitzern zusammen mit ihren Hunden sowie die Einfuhr von Schnecken, die eine wichtige Rolle als Zwischenwirte spielen.

Terrestrische Gastropoden (Schnecken und Nacktschnecken) sind die obligatorischen Zwischenwirte für metastrongyloide Lungenwürmer. Der Endwirt (z. B. Hund, Katze) scheidet im Kot das erste Larvenstadium (L1) der Lungenwürmer aus. Schnecken nehmen diese durch Koprophagie auf. Im Körper der Schnecke entwickeln sich die Larven zum zweiten Larvenstadium (L2) und später zum für Endwirte infektiösen dritten Larvenstadium (L3). Der Endwirt infiziert sich durch die Aufnahme der infizierten Schnecke selbst, durch freie L3 z. B. an Gras oder durch paratenische Wirte (Mäuse, Frösche, Vögel), die diese Schnecken verzehrt haben (Aziz et al. 2016; Valente et al. 2020).

Bisher haben sich die meisten Prävalenzstudien über Lungenwurminfektionen auf die Endwirte konzentriert, in denen sich in den letzten Jahren in mehreren Ländern ein klarer Anstieg der Infektionen verzeichnen ließ. Dazu zählen Deutschland, Dänemark, Italien, das Vereinigte Königreich sowie die Schweiz (Morgan und Shaw 2010; Traversa et al. 2010; Helm et al. 2015; Lemming et al. 2020; Gillis-Germitsch et al. 2020; Tieri et al. 2021; Morgan et al. 2021). Jedoch liegen nur wenige Studien über die Prävalenz von Lungenwürmern im Zwischenwirt Schnecke in Deutschland vor (Lange et al. 2018a; Segeritz et al. 2022).

Ziel dieser Arbeit war es, die Kontinuität eines bereits beschriebenen hyperendemischen „Hotspots“ für Lungenwurminfektionen in Schnecken (Segeritz et al. 2022) zu bestätigen und mögliche Prävalenzschwankungen in den verschiedenen Jahreszeiten festzustellen und daraus das Infektionsrisiko für die Endwirte herzuleiten. Zusätzlich wurde die Schnecke als Zwischenwirt betrachtet: Die Migration der Larven, die Reaktionen des Immunsystems nach der Infektion sowie die langfristige Überlebensfähigkeit der Larven im Modellorganismus *Lissachatina fulica*.

2. Literaturübersicht

2.1 Taxonomie der Lungenwürmer

Die Superfamilie Metastrongyloidea (*Meta-*: nach; *-strongylos*: rund, zylindrisch) umfasst zahlreiche Spezies, von denen viele als Lungenwürmer von veterinärmedizinischer Relevanz sind. Diese Parasiten siedeln sich meist, mit Ausnahme von *A. vasorum*, in der Lunge der Endwirte an, wo es auch zur Reproduktion kommt (Morgan et al. 2008; Traversa und Di Cesare 2013). *Eucoleus aerophilus* (*Capillaria aerophila*) wurde zusätzlich auch als in der Trachea lebend beschrieben; *A. vasorum* hingegen ist im rechten Herz und der Pulmonalarterie lokalisiert (Morgan et al. 2008; Stepanović et al. 2020). Im Gegensatz zu den Familien der Angiostrongylidae und Crenosomatidae, die als heteroxene Lungenwürmer auf Gastropoden (verschiedene Schneckenspezies) als Zwischenwirte angewiesen sind, benötigen die Familien Trichuridae und Filaroididae keinen Zwischenwirt und haben eine direkte Entwicklung (Clayton und Lindsay 1979; Traversa et al. 2011). Die Lungennematoden (Familie Metastrongylidae) der Schweine verfügen ebenso über eine heteroxene Entwicklung, jedoch ist bei diesen Lungenwürmern der Regenwurm als Zwischenwirt eingeschaltet (Schwartz und Alicata 1934; Dunn 1955).

Nematoden der Superfamilie Metastrongyloidea weisen im Vergleich zu anderen Strongyliden mehrere charakteristische morphologische Merkmale auf. Der rhabditoide Ösophagus des ersten Larvenstadiums ist von einfachem Aufbau ohne deutlich differenzierten Präbulbus oder Bulbus (Ferdushy und Hasan 2010). In den ersten drei Larvenstadien der metastrongyloiden Lungenwürmer, die sich im Zwischenwirt befinden, ist der Ösophagus sehr lang, ca. ein Drittel der Körperlänge der Larve. Die Mundkapsel sowie die Lippen sind häufig sehr klein und rudimentär (Skryabin 1992). Die Vulva befindet sich in unmittelbarer Nachbarschaft zum Anus, mit Ausnahme der Familie Crenosomatidae, bei denen sich die Vulva in der Körpermitte befindet (Rosen et al. 1970; Latrofa et al. 2015). Die Bursa copulatrix entspricht dem üblichen Strongyliden-Typ, ist aber arttypisch unterschiedlich reduziert. So lassen sich in der Familie der Filaroididae lediglich noch Papillen nachweisen (Saari et al. 2018). Eine Besonderheit ist die artspezifische Ausprägung des Schwanzes der metastrongyloiden Larven, der eine morphologische Speziesdifferenzierung ermöglicht (Ash 1970; Rosen et al. 1970).

Viele dieser Lungenwurmartarten kommen endemisch in Deutschland vor: *A. vasorum*, *A. chabaudi*, *Ae. abstrusus*, *C. vulpis*, *Crenosoma striatum*, *T. brevior*, *Oslerus osleri*, *Filaroides hirthei* sowie *C. aerophila*. Die zoonotischen Lungenwürmer *A. cantonensis* und *Angiostrongylus costaricensis* wurden bislang nicht in Deutschland beschrieben.

Tab. 1. Taxonomie der Lungenwürmer der Fleischfresser sowie zoonotische Arten

Stamm: Nematoda Ordnung: Strongylida Superfamilie: Metastrongyloidea		
<u>Familie Angiostrongylidae</u> <i>Angiostrongylus vasorum</i> <i>Angiostrongylus chabaudi</i> <i>Angiostrongylus cantonensis*</i> <i>Angiostrongylus costaricensis*</i> <i>Angiostrongylus mackerrasae</i> <i>Angiostrongylus dujardini</i> <i>Aelurostrongylus abstrusus</i> <i>Gurltia paralyzans</i>	<u>Familie Crenosomatidae</u> <i>Crenosoma vulpis</i> <i>Crenosoma striatum</i> <i>Crenosoma vismani</i> <i>Crenosoma melesi</i> <i>Crenosoma goblei</i> <i>Troglostrongylus brevior</i> <i>Troglostrongylus wilsoni</i> <i>Otostrongylus circumlitus</i>	<u>Familie Filaroididae</u> <i>Oslerus osleri</i> <i>Filaroides hirthi</i>

Stamm: Nematoda Ordnung: Trichocephalida
<u>Familie Trichuridae</u> <i>Capillaria aerophila</i>

*zoonotische Arten

2.2 Kaniden betreffende Arten

2.2.1 *Angiostrongylus vasorum*

A. vasorum wurde erstmals 1866 von Baillet in Südfrankreich als *Haemostrongylus vasorum* beschrieben. Dort wurde ein neuartiger Parasit im rechten Ventrikel und Herzohr beschrieben, woraus sich auch der Beinamen „Französischer Herzwurm“ ableitet.

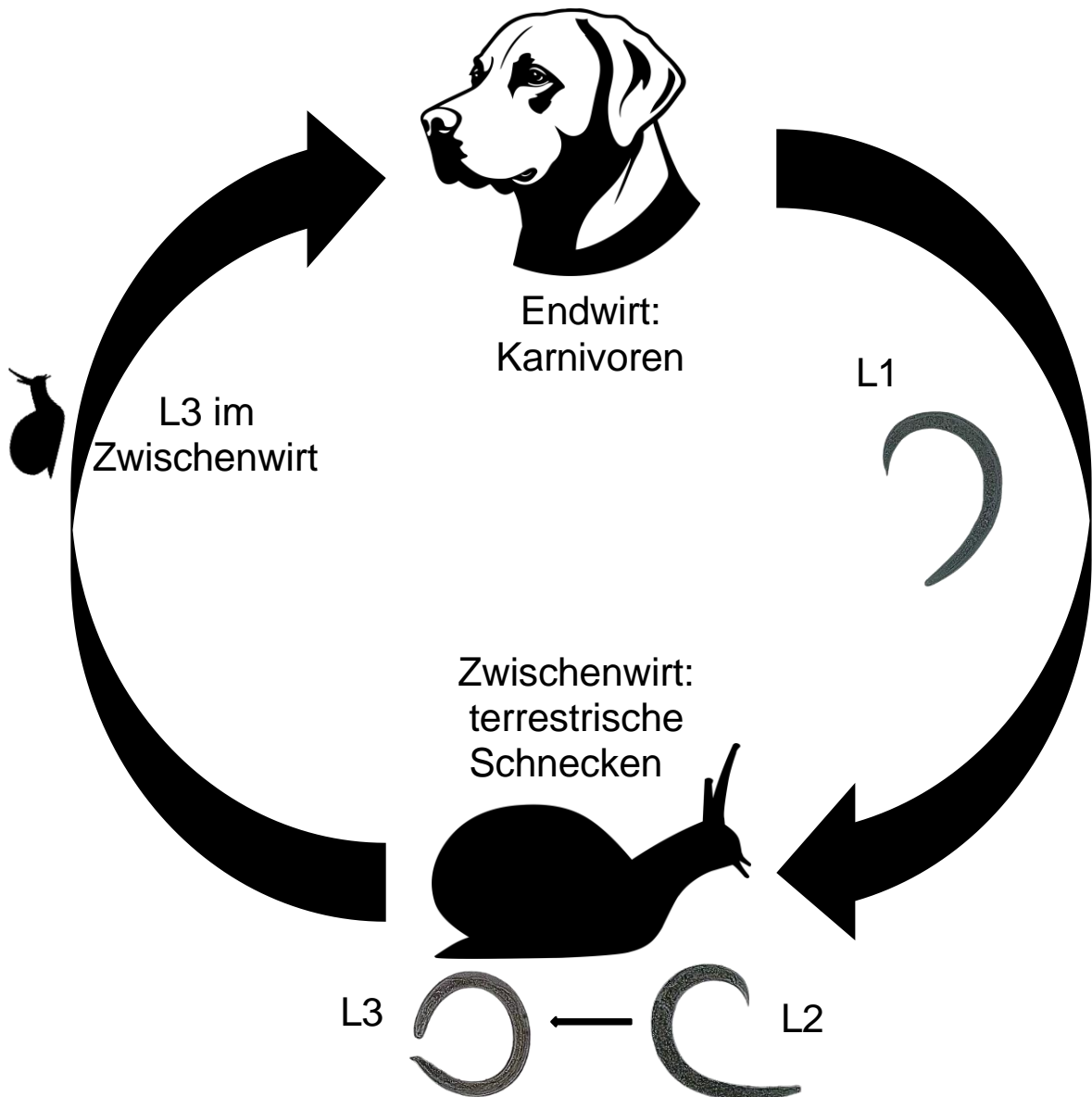


Abb. 1. Lebenszyklus von *A. vasorum*

Der Lebenszyklus von *A. vasorum* ist heteroxen, wobei Gastropoden als obligatorische Zwischenwirte agieren. L1 werden mit dem Kot des Endwirts ausgeschieden. Die Schnecken können sich daraufhin auf verschiedene Arten infizieren: peroral durch Koprophagie, durch das Aufnehmen von Gewebe infizierter Schnecken (Kannibalismus) oder durch aktive Penetration der Kutis der Sohlen durch aus toten infizierten Schnecken ausgeschiedene Larvenstadien (Intermediasis) (Guilhon und Cens 1969; Rosen et al. 1970; Colella et al. 2015; Modrý et al. 2021). Innerhalb von 10-30 Tagen entwickelt sich im Körper der Schnecke erst das zweite (L2), dann das dritte Larvenstadium (L3). Die L3 sind das für den Endwirt infektiöse Larvenstadium. Die Infektion des Endwirts kann auf mehrere Arten geschehen. Eine Möglichkeit ist die Aufnahme der Schnecke durch den Endwirt, entweder bewusst oder durch versehentliche Aufnahme kleiner Schnecken z. B. an überreifem Obst oder Gras. Zudem können sich infektiöse L3 in paratenischen Wirten (z. B. Vögel, Amphibien) befinden, welche durch Endwirte gejagt werden. Zu guter Letzt wird die Aufnahme von freien L3 in der Umwelt, wie etwa durch Wasser oder Gras, diskutiert. Die infektiösen L3 können entweder nach dem Tod der Mollusken freigesetzt werden oder aktiv von lebenden Schnecken über den Schleim abgegeben werden (Conboy et al. 2017; Robbins et al. 2021).

Im Verdauungstrakt des Endwirts angekommen, werden die L3 aktiv, durchbohren die Darmwand und gelangen in die Mesenteriallymphknoten. Dort häuten sie sich in das vierte (L4) und fünfte Larvenstadium (L5). Daraufhin gelangen die Larven in die Blutbahn und werden ins rechte Herz und die Arteria pulmonalis abgeschwemmt. Dort entwickeln sie sich zu den Adultstadien. Die Männchen sind mit 14-18 mm etwas kürzer als die Weibchen (18-24 mm). Die produzierten Eier werden passiv im Blutstrom in die Lunge transportiert. Dort schlüpfen die L1, penetrieren die Lungenalveolen und werden hochgehustet und abgeschluckt. So gelangen die Larven in den Verdauungstrakt, bis sie mit dem Kot ausgeschieden werden. Dadurch schließt sich der Entwicklungszyklus. Die Präpatenz variiert zwischen 38 und 57 Tagen (Morgan et al. 2010; Elsheikha et al. 2014). Die Dauer der Patenz ist ebenfalls variabel und ist aufgrund der intermittierenden Larvenausscheidung schwer zu bestimmen. In empirischen Studien wurde eine Larvenausscheidung über eine Zeitspanne von 31 Wochen bis zu 5 Jahren angegeben (Eckert et al. 2008; Robbins et al. 2021). Daraus lässt sich schließen, dass die Adulti von *A. vasorum* über 5 Jahre im Wirt überleben und Schäden anrichten können.

Bisher wurden verschiedenste terrestrische Nackt- und Gehäuseschnecken als potenzielle Zwischenwirte für *A. vasorum* beschrieben. In experimentellen Versuchen waren auch aquatische Schnecken (z. B. *Biomphalaria glabrata*) als Zwischenwirte geeignet (Barçante et al. 2003). Während viele einheimische Schneckenarten als Zwischenwirte bekannt sind, sind diverse invasive Arten ebenfalls als Zwischenwirte relevant. Dazu gehört die in viele Länder

invasive *Lissachatina fulica* (Afrikanische Riesenachatschnecke) sowie *Arion vulgaris* (Spanische Wegschnecke), *Cepaea nemoralis/hortensis* (Bänderschnecken) und *Cornu aspersum* (Gartenschnecken). Alle diese Schneckenarten wurden bereits als obligate Zwischenwirte für metastrongyloide Lungenwürmer bestätigt (Zmoray et al. 1969; Colella et al. 2016; Penagos-Tabares et al. 2020).

Neben der Übertragung durch Schnecken selbst spielen auch diverse paratenische Wirte eine Rolle in der Übertragung der infektiösen L3 auf die kaniden Endwirte. Nager (Mäuse), Vögel, Reptilien und Amphibien (Frösche, Molche) werden in der Übertragung diskutiert (Bolt et al. 1993; Mozzer und Lima 2015; Colella et al. 2015, 2019).

Verschiedenste Spezies können als Endwirte für *A. vasorum* fungieren und zur Epidemiologie und Ausbreitung der kaninen Angiostrongylose beitragen, wie etwa Haus- und Wildkaniden. Darunter zählen in Europa etwa Wölfe (*Canis lupus*), Rotfüchse (*Vulpes vulpes*) und Goldschakale (*Canis aureus*) (Bolt et al. 1992; Bružinskaitė-Schmidhalter et al. 2012; Szabo et al. 2013; Hermosilla et al. 2017; De Liberato et al. 2017; Gavrilović et al. 2019b; Tieri et al. 2021). Auch außerhalb von Europa ist *A. vasorum* prävalent und infiziert die dort heimischen Kaniden, wie etwa den Brasilianischen Kampfuchs (*Lycalopex vetulus*), den Maikong, auch Krabbenfuchs genannt (*Cerdocyon thous*), den Pampasfuchs (*Pseudalopex gymnocerus*) in Südamerika (Lima et al. 1994; Fiorello et al. 2006; Duarte et al. 2007), den Kojoten (*Canis latrans*) in Nordamerika (Kotwa et al. 2021) und den Afrikanischen Goldwolf (*Canis lupaster*) in Afrika (Mechouck et al. 2024). Doch nicht nur Kaniden werden als Endwirte von *A. vasorum* beschrieben. So werden auch Musteliden wie das Hermelin (*Mustela erminea*), das Zwergwiesel (*M. nivalis*), der Amerikanische Nerz (*Neogale vison*), der Dachs (*Meles meles*), der Fischotter (*Lutra lutra*), und der Europäische Iltis (*M. putorius*) von *A. vasorum* infiziert (Madsen 1999; Simpson 2010; Lemming et al. 2020; Kretschmar; Magi et al. 2009). Zudem wurden Infektionen auch in anderen Tieren beobachtet, darunter der Rote Panda (*Ailurus fulgens fulgens*) und das Erdmännchen (*Suricata suricatta*) (Patterson-Kane et al. 2009; Gillis-Germitsch et al. 2017).

Ursprünglich war *A. vasorum* in Frankreich, Deutschland, Großbritannien und Dänemark verbreitet (Guilhon und Cens 1969; Bolt et al. 1992; Martin et al. 1993; Barutzki et al. 2017). Mittlerweile wurden Endemiegebiete von *A. vasorum* in weiteren Kontinenten beschrieben, darunter Nord- und Südamerika, Afrika und Asien (Kotwa et al. 2021; Uribe et al. 2021; Mechouck et al. 2024). Doch auch in den ursprünglichen Endemiegebieten ist es zu einem Anstieg der Prävalenz gekommen. Aus diesem Grund wurde in den letzten Jahren eine hohe Anzahl an Studien über die Prävalenz von *A. vasorum* in Hunden, aber auch in wildlebenden Kaniden durchgeführt (siehe Tab. 2., 3.).

Tab. 2. Prävalenzdaten zu *Angiostrongylus vasorum*-Infektionen in europäischen Hundepopulationen

Land	Prävalenz	Regionen/Zeitraum	Referenzen
Belgien	4,7 %		(Lempereur et al. 2016)
Deutschland	1,0-3,1 %		(Vrhovec et al. 2022)
	0,9 %	2002-2006: 0,1 % 2007-2011: 0,8 % 2012-2016: 1,3 %	(Barutzki et al. 2017)
	2,3 %	Rheinland-Pfalz: 3,9 % Baden-Württemberg: 3,6 % Nordrhein-Westfalen: 2,0 % Hessen: 1,8 % Bayern: 1,4 % Sachsen: 0 %	(Maksimov et al. 2017)
	0,1 %		(Barutzki und Schaper 2003)
	1,2 %		(Taubert et al. 2009)
	7,4 %		(Barutzki und Schaper 2009)
		Bayern: 0,36 %	(Schulz et al. 2013)
	0,5 %		(Barutzki und Schaper 2011)
		Westdeutschland: 2,3 %	(Schnyder et al. 2013a)
Dänemark	2,2 %		(Taubert et al. 2009)
England	3,2 %		(Schnyder et al. 2013a)
Frankreich	2,0 %		(Schnyder et al. 2017)
Griechenland	3,0 %		(Angelou et al. 2020)
Italien	13,2 %	Campagna	(Prete et al. 2015)
	1,0 %	Campagna	(Rinaldi et al. 2014)
	3,4 %	Sardinien	(Pipia et al. 2014)
	3,8 %	Ligurien	(Guardone et al. 2013)
	2,3 %	Toskana	(Guardone et al. 2013)
	2,5 %	Zentralitalien	(Di Cesare et al. 2011)
	0 %	Süditalien	(Di Cesare et al. 2011)
Niederlande	1,4 %		(Lempereur et al. 2020)
Österreich	1,5 %		(Globokar et al. 2021)
Polen	1,3 %		(Schnyder et al. 2013b)
Portugal	1,1 %		(Leal-Sousa et al. 2025)
Rumänien	2,1 %		(Deak et al. 2019)
Schweden	0,9 %		(Grandi et al. 2017)
Schweiz	3,1 %		(Lurati et al. 2015)

Slowakei	6,2 %		(Miterpáková et al. 2015)
Spanien	1,4 %		(Carretón et al. 2022)
Tschechien	4,7 %		(Hajnalová et al. 2018)
Ungarn	2,7 %		(Schnyder et al. 2015)

A. vasorum weist ein typisches Verteilungsmuster auf: Orte sehr hoher Prävalenz (hyperendemische Foki) befinden sich direkt neben Orten sehr niedriger Prävalenz (hypoendemische Foki) (Colombo et al. 2021; Segeritz et al. 2022). In diesen sogenannten hyperendemischen „Hotspots“, welche in Deutschland vorrangig im Süden des Landes liegen, ist entsprechend die Befallsrate der Zwischenwirte und der Endwirte höher als in übrigen Gebieten. Dennoch lässt sich daraus nicht die generelle Infektionsgefahr in einem Gebiet ableiten, da es selbst in benachbarten Landkreisen zu völlig unterschiedlichen Prävalenzen kommen kann (Colombo et al. 2021). Generell steigt aber die Prävalenz der kaninen Angiostrongylose in Europa, und so stieg die Prävalenz in Hunden in Deutschland von 0,1 % im Jahr 2006 auf 1,3 % im Jahr 2016 (Barutzki et al. 2017).

Wildkaniden, v.a. synanthrope Füchse, spielen eine wichtige Rolle in der Epidemiologie der kaninen Lungenwurminfektionen. In Europa zeigen sich, wie bereits bei Prävalenzdaten von Hunden beschrieben, hyper- und hypoendemische Foki für diese Wildtierart. Dieses Verteilungsmuster lässt sich auch auf die Prävalenz von *A. vasorum* in der Fuchspopulation übertragen (Morgan et al. 2008; Taylor et al. 2015). Die in Europa niedrigste beobachtete Prävalenz befindet sich in Rumänien mit 4,2 % (Deak et al. 2017a), während die höchste Prävalenz in der Schweiz beobachtet wurde (81,8 %) (Gillis-Germitsch et al. 2020). Diese Zahlen liegen, im Vergleich mit den Prävalenzdaten der Hunde, sehr viel höher. Der sylvatische Zyklus der Lungenwürmer, der den Zwischenwirt Schnecke sowie diverse kanide Endwirte beinhaltet, kann entsprechend auf Haustiere überspringen. Einerseits nimmt die Urbanisation von wildlebenden Kaniden zu (v.a. von Füchsen und Kojoten), wodurch *A. vasorum* in Städte hereingetragen werden kann und es schlussendlich zu Infektionen in der Hundepopulation kommen kann (Plumer et al. 2014; Cervantes et al. 2023). Dabei dienen die Wildtiere als natürliche Parasitenreservoir, und eine vorher nicht infizierte Haustierpopulation kann sich dadurch mit *A. vasorum* infizieren. Andererseits können sich Hunde bei Spaziergängen im Wald oder am Waldrand infizieren, wo es zu Überschneidungen zwischen den Lebens- und Aufenthaltsräumen von Fuchs und Hund kommen kann.

Tab. 3. Prävalenzdaten zu *Angiostrongylus vasorum*-Infektionen in europäischen Fuchspopulationen

Land	Prävalenz	Regionen/Zeitraum	Referenzen
Deutschland		Rheinland-Pfalz: 27,3 % Thüringen: 19,1 % Hessen: 8,4 %	(Schug et al. 2018)
		Brandenburg: 9 %	(Härtwig et al. 2015)
Dänemark		Nord Seeland: 48,6 %	(Saeed et al. 2006)
		Kopenhagen: 80 % Südliches Jütland: 0 %	(Al-Sabi et al. 2014)
	6,8 %		(Lemming et al. 2020)
England	7,3 %	Südosten: 23,2 % Süden: 6,9 % Binnenland: 4,8 % Osten: 1,6 % Norden: 0 %	(Morgan et al. 2008)
	18,3 %	Südosten: 50,8 % Osten: 12,9 % Norden: 7,4 %	(Taylor et al. 2015)
Irland	39,9 %		(Mccarthy et al. 2016)
Italien	75 %		(Tieri et al. 2021)
		Toskana: 7 %	(Magi et al. 2009)
		Süden: 33,3 %	(Santoro et al. 2015)
		Zentralitalien: 43,5 %	(Eleni et al. 2014)
		Nordwesten: 78,2 %	(Magi et al. 2015)
Niederlande	4,2 %		(Franssen et al. 2014)
Polen	5,2 %		(Demiaszkiewicz et al. 2014)
Portugal	16,1 %		(Eira et al. 2006)
Rumänien	4,2 %		(Deak et al. 2017a)
Serbien	13,2 %		(Gavrilović et al. 2019a)
Schweiz	21,5-81,8 %		(Gillis-Germitsch et al. 2020)
Slowakei	5,43 %		(Čabanová et al. 2018)
Spanien		Nordosten: 3,4 %	(Garrido-Castañé et al. 2015a)
		Nordwesten: 43,0 %	(Martínez-Rondán et al. 2019)
Ungarn	17,9 %		(Tolnai et al. 2015)

Im Gegensatz zu den Endwirten sind Studien zur Prävalenz von metastrongyloiden Lungenwurminfektionen im Zwischenwirt Schnecke bisher noch rar, obwohl diese ein obligater Bestandteil des Lebenszyklus und der Übertragung der Lungenwürmer auf den Endwirt sind. Genau wie die Prävalenz im Endwirt schwankt auch die Prävalenz in den gastropoden Zwischenwirten sehr stark (Tab. 4). So wurde in Deutschland für *A. vasorum* in den westlichen/zentralen Bundesländern Rheinland-Pfalz und Hessen eine Prävalenz von 4,0 % und 5,1 % nachgewiesen, während in den südlichen Bundesländern Baden-Württemberg 18,0 % und in Bayern 0,5 % der Schnecken mit *A. vasorum* infiziert waren (Lange et al. 2018a; Segeritz et al. 2022). Eine hohe Prävalenz im Zwischenwirt geht auch mit einer erhöhten Infektionswahrscheinlichkeit für den Endwirt einher, und umgekehrt kann durch eine hohe Prävalenz im Endwirt auch eine höhere Anzahl an Zwischenwirten infiziert werden.

Tab. 4. Prävalenzdaten zu metastrongyloiden Lungenwurminfektionen in europäischen Schneckenpopulationen

Land	Region	Prävalenz (%)						Referenz
		<i>Av</i>	<i>Cv</i>	<i>Aa</i>	<i>Tb</i>	<i>Ach</i>	<i>Aca</i>	
Dänemark	Kopenhagen	29,0						(Ferdushy et al. 2009)
Deutschland	Hessen	4,0	3,6	0,3				(Lange et al. 2018a)
	Rheinland-Pfalz	5,1	0,8	0,2				
	Baden-Württemberg	18,0	1,2	2,1	1,52			(Segeritz et al. 2022)
	Bayern	0,5	0	1,0	0			
Österreich	Wien	0,5		1,5	1,0			(Penagos-Tabares et al. 2020)
	Ostösterreich	0,1	0,4	0,2				(Fuehrer et al. 2020)
Griechenland				4,0	2,0	8,0		(Dimzas et al. 2020)
Großbritannien	Swansea	35,2						(Aziz et al. 2016)
	Bristol	0,3						
	Wales	43,0						(Jefferies et al. 2009)
	London	1,6						(Patel et al. 2014)
	Schottland	7,0						(Helm et al. 2015)
Spanien	Teneriffa	10,7		14,3			3,6	(Segeritz et al. 2021b)
	El Hierro	15,0		5,0				

	Teneriffa						60,2	(Martin-Alonso et al. 2015)
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Av: *A. vasorum*, Cv: *C. vulpis*, Aa: *Ae. abstrusus*, Tb: *T. brevior*, Ach: *A. chabaudi*, Aca: *A. cantonensis*

Die Pathogenese von *A. vasorum* im Endwirt ist komplex und beruht auf verschiedenen Faktoren. Zu beachten sind hier mechanische Reizungen der Gefäßendothelien durch die larvalen Wanderstadien und Eier, die Entzündungen besonders in der Lunge verursachen können sowie die erwachsenen Stadien im rechten Herz und der Arteria pulmonalis, die Stoffe sezernieren und das Endothel und umgebendes Gewebe immunologisch reizen.

Klinische Symptome sind oft sehr variabel und nicht zur Diagnosestellung geeignet (Colombo et al. 2021). Verschiedene Formen der kaninen Angiostrongylose wurden beschrieben, darunter perakute, akute, chronische und subklinische Verläufe. Das bekannteste und schwerwiegendste Symptom einer Infektion mit *A. vasorum* ist die erhöhte Blutungsneigung. Häufig stellt sich diese dar in Form von Ekchymosen, Petechien, Einblutungen in die Sklera, Epistaxis, Hämothorax und Schleimhautblutungen (Gallagher et al. 2012; Glaus et al. 2016). Die Ursachen für diese erhöhte Blutungsneigung sind multifaktoriell und noch nicht vollständig geklärt. Diskutiert werden antigeninduzierte disseminierte intravasale Gerinnung (DIC), Beeinflussung des Komplementsystems (v.a. des Lektin-Wegs), Mangel an von-Willebrandt-Faktor auf Endothelien, Thrombozytopenie und Thrombozytopathie sowie die Sekretion von Antikoagulantien durch den Parasiten (Schelling et al. 1986; Caruso und Prestwood 1988; JO'Neill et al. 2010; Hausmann et al. 2016; Tritten et al. 2021). Ein weiterer Faktor ist die durch den Parasiten ausgelöste NETose (Freisetzung von NET) sowie die Hochregulierung von Adhäsionsmolekülen auf Endothelzellen, welche in den Hunden unterschiedlich stark verlaufen und somit die variierenden Schweregrade der kaninen Angiostrongylose erklären können (Grob et al. 2021).

Kardiopulmonale Symptome sind ebenfalls sehr häufig, wie etwa Leistungsintoleranz, Husten, Tachypnoe, Dyspnoe, pulmonale Hypertonie und Rechtsherzinsuffizienz (Martin et al. 1993; Brennan et al. 2004). Zu den gastrointestinalen Symptomen einer kaninen Angiostrongylose gehören u. a. Durchfall, Erbrechen, Gewichtsverlust und Anorexie (Morgan et al. 2010; Colombo et al. 2021; Taulescu et al. 2023). Durch wandernde Larvenstadien sind auch neurologische und okuläre Symptome möglich, wie etwa Ataxie, Einschränkungen der Propriozeption und Blindheit (Chapman et al. 2004).

Über Symptome einer Angiostrongylose in Wildtieren ist bisher wenig bekannt. Während für den Roten Panda bereits Pneumonien berichtet wurden, fehlen derlei Studien für die meisten anderen wildlebenden Endwirte (Patterson-Kane et al. 2009). Die pathologischen Veränderungen in Lunge und Herz bei Wildtieren ähneln denen der Hunde (Schnyder et al.

2010; Eleni et al. 2014; De Liberato et al. 2017), woraus sich grundsätzlich auf eine ähnliche Symptomatik schließen lässt. Aktuelle Studien zeigen, dass Füchse eine weniger schwere Symptomatik mit einer angemesseneren Immunantwort aufweisen (Poli et al. 1991; Webster et al. 2017; Gillis-Germitsch et al. 2021). Aus diesen Gründen wird die Wichtigkeit der Fuchspopulation als Reservoir für *A. vasorum* erneut bestätigt.

Durch die häufig regelmäßigen Entwurmungen und die unspezifischen Symptome werden Infektionen mit Lungenwürmern in den Praxen oft nicht entdeckt. Die üblichen Methoden der Koproskopie, wie das Flotationsverfahren, wie es häufig in Praxen eingesetzt wird, können Lungenwurminfektionen nicht detektieren. Der Goldstandard der Untersuchung auf Lungenwürmer ist das Trichterauswanderungsverfahren nach Baermann-Wetzel, mit dem die motilen und lebendigen L1 nachgewiesen werden. Dazu wird Kot von vorzugsweise drei Tagen gesammelt (da die Ausscheidung der Larven intermittierend verläuft) und auf ein Sieb gegeben. Dieses wird auf einem Trichter platziert, der mit Wasser gefüllt und dessen Ende mit einer Klemme verschlossen wird. Nach 12 Stunden wird die Klemme geöffnet und ein Tropfen unter einem Lichtmikroskop auf die Präsenz von Lungenwurmlarven untersucht. Der Nachteil dieses Verfahrens ist, dass hierfür lebendige, aktiv wandernde Larven im Kot vonnöten sind, also frischer Kotabsatz (Willard et al. 1988; Rode and Jørgensen 1989; Eckert et al. 2008).

Weitere Möglichkeiten schließen serologische (z. B. ELISA) und molekulargenetische Verfahren ein (PCR) (Schnyder et al. 2013a, 2015; Al-Sabi et al. 2014; Massetti et al. 2024). Während die PCR eine hohe Sensitivität und Spezifität hat, so ist das Verfahren aufwändig und kostenintensiv und kann in den wenigsten Praxen durchgeführt werden. Serologische Methoden sind schnell und einfach, können aber oft keine Hinweise auf eine aktuelle Infektion liefern. Beide diagnostische Verfahren lassen weder einen Rückschluss auf die Infektionsstärke noch auf die Larvenausscheidung zu.

Seit einigen Jahren ist auch ein kommerzieller Schnelltest möglich, der parasitenspezifische Antigene von *A. vasorum* im Blut detektiert (IDEXX Angio Detect™). Dieser verfügt über eine hohe Sensitivität (84,6 %) und Spezifität (100 %) und kann bereits eine präpatente Infektion sicher nachweisen (Schnyder et al. 2014).

Die Behandlung von Infektionen mit *A. vasorum* erfolgt kausativ und symptomatisch. Häufig eingesetzte Anthelminthika sind Fenbendazol (25-50 mg/kg KM für 5-21 d), Moxidectin (2,5 mg/kg KM einmalig als Spot-on) und Milbemycinoxim (0,5 mg/kg KM 4x perorale Gabe im Abstand von einer Woche). Um die überschießende Immunantwort abzuschwächen, werden Glucocorticoide gegeben. Falls es zu starken Hämorrhagien kommt, ist eine Bluttransfusion vonnöten.

2.2.2 *Crenosoma vulpis*

Die Art *C. vulpis* (Dujardin, 1845), auch Schachtelhalmförmiger Fuchslungenwurm genannt, ist ein metastrongyloider Lungenwurm, der wildlebende und domestizierte Kaniden befällt. Neben dem Haushund zählen dazu Füchse (*V. vulpes*), Wölfe (*C. lupus*), Goldschakale (*C. aureus*), Marderhunde (*N. procynoides*), Dachse (*M. meles*), Baumarder (*M. martes*) und Steinarder (*M. foina*) (Popiołek et al. 2009; Bružinskaitė-Schmidhalter et al. 2012; Szabo et al. 2013; Al-Sabi et al. 2014; Hermosilla et al. 2017; Deak et al. 2023).

Der Lebenszyklus von *C. vulpis* ähnelt dem von *A. vasorum* stark. Eine Ausnahme davon ist die Leberwanderung von *C. vulpis*-Larven sowie die Lokalisation der Adultstadien. Diese befinden sich nicht in der Blutbahn, sondern im Respirationstrakt (Trachea und Lungenbronchien). Die Präpatenz von *C. vulpis* beträgt 18-21 Tage und die Patenz ca. 10 Monate.

Ein endemisches Vorkommen von *C. vulpis* wurde bisher in Europa und Amerika beschrieben (Nevárez et al. 2005; Fuehrer et al. 2020; Deak et al. 2023). Zudem wurde die Infektion eines Fuchses mit *C. vulpis* in Afrika dokumentiert sowie die Infektion eines Hundes in Korea (Choi et al. 2014; Mechouck et al. 2024).

Die klinischen Symptome einer *C. vulpis*-Infektion sind generell weniger schwerwiegend als bei *A. vasorum*. Entsprechend ihrer Lokalisation in der Lunge führen die Adultstadien zu respiratorischer Symptomatik, wie etwa Husten und Leistungsintoleranz (Choi et al. 2014). Todesfälle bei der kaninen Crenosomose wurden bisher noch nicht beschrieben (Traversa et al. 2010).

Nachgewiesen werden können patente *C. vulpis*-Infektionen ebenfalls mit dem Baermann-Wetzel-Auswanderungsverfahren. Im Gegensatz zu *A. vasorum*-Larven ist der Schwanz der L1 von *C. vulpis* spitz zulaufend, ohne Ausläufer oder Haken (Lange et al. 2018a; Segeritz et al. 2022). Kommerzielle Antigen-Tests sind nicht verfügbar. Zu Forschungszwecken ist eine Differenzierung der *C. vulpis*-Larven per spezifischer PCR möglich (Schug et al. 2018; Segeritz et al. 2022), aber routinemäßig noch nicht in der Diagnostik eingeführt.

Die kausale Therapie einer Infektion mit *C. vulpis* erfolgt mittels Ivermectin (200 µg/kg s.c.) oder Milbemycinoxim (0,5 mg/kg peroral) (Conboy und Adams 1995; Conboy 2004).

Tab. 5. Prävalenzdaten zu *Crenosoma vulpis*-Infektionen in europäischen Fuchspopulationen

Land	Regionen	Prävalenz	Referenz
Bosnien und Herzegowina		45,7 %	(Hodžić et al. 2016)
Dänemark	Kopenhagen, südliches Jütland	22,9 %	(Al-Sabi et al. 2014)
	Seeland, Jütland	19 %	(Al-Sabi und Kapel 2013)
		17,4 %	(Saeed et al. 2006)
Deutschland		32,3 %	(Schug et al. 2018)
England	Südosten	25 %	(Taylor et al. 2015)
	Binnenland	10,5 %	
	Süden	9,7 %	
	Norden	4,3 %	
	Osten	4,3 %	
		2 %	(Morgan et al. 2008)
Irland		1,3 %	(Mccarthy et al. 2016)
Italien	Nordwesten	15,8 %	(Magi et al. 2015)
	Süden	8,7 %	(Latrofa 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)
Polen	Westen	12,8 %	(Matysiak et al. 2025)
Portugal	Mitte, Westen	39,3 %	(Figueiredo et al. 2016)
	Dunas de Mira	3,3 %	(Eira et al. 2006)
Rumänien		32,0 %	(Deak et al. 2020)
Schweiz		3,6-14,9 %	(Gillis-Germitsch et al. 2020)
Serbien		26,5 %	(Gavrilović et al. 2019a)
Slowakei		17,5 %	(Čabanová et al. 2018)
Slowenien		2,8 %	(Vergles Rataj et al. 2013)
Spanien	Pyrenäen	44,8 %	(Garrido-Castañé et al. 2015b)
	Katalonien	33,9 %	(Mañas et al. 2005)
Ungarn		24,6 %	(Tolnai et al. 2015)
		24 %	(Sréter et al. 2003)

Tab. 6. Prävalenzdaten zu *Crenosoma vulpis* in europäischen Hundepopulationen

Land	Regionen	Prävalenz	Referenz
Deutschland		2,2-2,6 %	(Vrhovec et al. 2022)
	Sachsen	3,3 %	(Maksimov et al. 2017)
	Hessen	3,2 %	
	Bayern	2,5 %	
	Baden-Württemberg	2,1 %	
	Rheinland-Pfalz	1,9 %	
	Nordrhein-Westfalen	1,7 %	
		0,4 %	(Barutzki et al. 2017)
		0,4 %	(Barutzki und Schaper 2011)
		6,0 %	(Barutzki und Schaper 2009)
	2,4 %	(Taubert et al. 2009)	
	0,9 %	(Barutzki und Schaper 2003)	
Dänemark		1,4 %	(Taubert et al. 2009)
Finnland		0,2 %	(Rapp et al. 2024)
Österreich		3,0 %	(Globokar et al. 2021)
Slowakei		0,8 %	(Šmigová et al. 2021)
		0,6 %	(Čabanová et al. 2018)
Tschechien		2,0 %	(Hajnalová et al. 2018)

2.3 Metastrongyloide Nematodenarten bei Feliden

2.3.1 *Aelurostrongylus abstrusus*

Ae. abstrusus (Railliet, 1898) ist ein metastrongyloider Nematode, der sowohl domestizierte als auch wildlebende Feliden infiziert. Diese Art gilt als der häufigste Lungenwurm der Katze (Di Cesare et al. 2015; Elsheikha et al. 2016). Neben Hauskatzen (*Felis silvestris catus*) werden auch Luchse (*Lynx lynx*), Europäische Wildkatzen (*Felis silvestris silvestris*), Asiatische Wildkatzen (*Felis silvestris ornata*), Jaguarundis (*Herpailurus yagouaroundi*), Karakals (*Caracal caracal*), Löwen (*Panthera leo*), Geparden (*Acinonyx jubatus*), Servals (*Leptailurus serval*), Sibirische Tiger (*Panthera tigris altaica*), Langschwanzkatzen (*Leopardus wiedii*) sowie Bengalkatzen (*Felis bengalensis euphilurus*) als Endwirte infiziert (West et al. 1977; González et al. 2007; Di Cesare et al. 2016; Moskvina et al. 2018; Stevanović et al. 2019; Kaore et al. 2020; Segeritz et al. 2021a; Quadros et al. 2022; Ré et al. 2022; Haas et al. 2025).

Der Lebenszyklus von *Ae. abstrusus* entspricht größtenteils dem von *A. vasorum*. Bei der Infektion der Endwirte spielen zudem paratenische Wirte, wie Frösche, Reptilien, Mäuse und Vögel, eine große Rolle (Jeżewski et al. 2013; Colella et al. 2019). Nach Aufnahme der L1 durch die Schnecke dauert die Entwicklung zur infektiösen L3 etwa 11 Tage (Giannelli et al. 2014).

Ein Unterschied zu *A. vasorum* ist die Lokalisation der Adultstadien von *Ae. abstrusus*. Diese befinden sich im Respirationstrakt, v.a. den Alveolargängen und den Bronchioli (Elsheikha et al. 2016). Entsprechend dieser Lokalisation führen Infektionen mit *Ae. abstrusus* zu respiratorischen Symptomen, wie etwa Dyspnoe, Husten und erhöhte Atemfrequenz. Die Schwere der klinischen Symptomatik reicht von subklinischen Infektionen bis zu letalem Ausgang der Infektion (Di Cesare et al. 2015; Elsheikha et al. 2016; Lopez-Osorio et al. 2021).

Die Diagnose der felines Aelurostrongylose erfolgt über das Baermann-Wetzel-Auswanderungsverfahren oder über den molekularbiologischen Nachweis durch PCR (Annoscia et al. 2014; Penagos-Tabares et al. 2018a). Kommerzielle serologische Tests sind derzeit nicht verfügbar.

Im Gegensatz zu *A. vasorum* ist die Verteilung von *Ae. abstrusus* geographisch gleichmäßiger, d.h. ohne hypo- und hyperendemische Foki (Schnyder et al. 2021).

Tab. 7. Prävalenzdaten zu *Aelurostrongylus abstrusus*-Infektionen in europäischen Katzenpopulationen

Land	Regionen	Prävalenz	Referenz
Albanien	Tirana	50 %	(Knaus et al. 2011)
Belgien		0,95 %	(Giannelli et al. 2017)
Bulgarien		27,5 %	(Giannelli et al. 2017)
Deutschland	Norden	2,6-6,5 % 12,0 % 1,0 % 0,5 % 5,6 %	(Vrhovec et al. 2022) (Schnyder et al. 2021) (Becker et al. 2012) (Barutzki und Schaper 2011) (Taubert et al. 2009)
Dänemark		8,3 % 13,6 %	(Hansen et al. 2017) (Olsen et al. 2015)
England		1,7 %	(Elsheikha et al. 2019)
Frankreich	Toulouse	1 %	(Henry et al. 2022)
Griechenland		8,47 %	(Giannelli et al. 2017)
Italien	Bari Sassari Messina	5 % 11,6 % 12,94 %	(Giannelli et al. 2017)
Kroatien	Nordwesten Zagreb	22 % 0,38-3,9 %	(Grabarević et al. 1999)
Niederlande		2,6 %	(Robben et al. 2004)
Portugal	Zentrum und Süden Lissabon Norden	1,7 % 11,7 % 0,83 %	(Giannelli et al. 2017)
Rumänien		14,17 %	(Giannelli et al. 2017)
Spanien		5 %	(Giannelli et al. 2017)
Schweiz		0,8 %	(Giannelli et al. 2017)
Ungarn		22,5 %	(Giannelli et al. 2017)
Zypern		2 %	(Diakou et al. 2017)

2.3.2 *Angiostrongylus chabaudi*

A. chabaudi (Biocca, 1957) ist ein metastrongyloider Nematode, der Haus- und Wildkatzen infiziert (Varcasia et al. 2014; Diakou et al. 2016). Die adulten Stadien sind, genau wie bei dem eng verwandten *A. vasorum*, in der Arteria pulmonalis und dem rechten Herz lokalisiert (Giannelli et al. 2016b). Auch für *A. chabaudi* stellen Gastropoden die obligatorischen Zwischenwirte dar, jedoch ist über die Präpatenz und Patenz bisher wenig bekannt (Giannelli et al. 2016b; Diakou et al. 2016).

Zur Symptomatik der felines Angiostrongylose ist bisher noch nichts bekannt. Bei Sektionen infizierter Feliden wurden pathologische Veränderungen an Herz und Lunge beobachtet, woraus sich auf kardiovaskuläre und respiratorische Symptome schließen lässt (Giannelli et al. 2016b; Diakou et al. 2016).

Erstmals berichtet wurde über *A. chabaudi* 1957 in Italien. Berichte liegen mittlerweile vor über die Präsenz des Parasiten in Spanien, Bosnien und Herzegowina, Rumänien, Deutschland und Griechenland (Diakou et al. 2016; Colella et al. 2017; Stevanović et al. 2019; Bisterfeld et al. 2022; García-Livia et al. 2023). Generell wurden *A. chabaudi*-Infektionen hauptsächlich bei jungen Katzen diagnostiziert, weshalb vermutet wird, dass die Immunitätsausbildung noch nicht vollendet ist oder für diese ein höheres Infektionsrisiko besteht (Giannelli et al. 2016b).

Wie bei anderen Lungenwürmern können auch Infektionen mit *A. chabaudi* mithilfe des Baermann-Wetzel-Auswanderungsverfahren nachgewiesen und anschließend mittels Schwanzmorphologie differenziert werden. Alternativ ist auch die molekularbiologische Bestimmung durch PCR möglich. Kommerzielle Diagnostikartikel spezifisch für *A. chabaudi* sind derzeit noch nicht vorhanden, aber eine Infektion kann auch über den Angio Detect™-Test (IDEXX) nachgewiesen werden (Deak et al. 2017b).

2.3.3 *Troglostrongylus brevior*

Die Nematodenart *T. brevior* (Gerichter, 1949) infiziert ebenfalls wildlebende Feliden (z. B. Europäischer Luchs, Wildkatze) und Hauskatzen (Traversa et al. 2015; García-Livia et al. 2023; Haas et al. 2025). Der Lebenszyklus und die Lokalisation der adulten Stadien in den Bronchien und Bronchioli ähneln der von *Ae. abstrusus* stark. Eine Besonderheit im Lebenszyklus von *T. brevior* ist die vertikale Übertragung des Parasiten, wodurch sich Jungtiere bereits pränatal (diaplazentar) oder kurze Zeit postnatal (laktogen) infizieren können (Brianti et al. 2013). Mischinfektionen mit *Ae. abstrusus* wurden ebenfalls beschrieben (Di Cesare et al. 2014).

Besonders relevant sind Infektionen mit *T. brevior* für Jungtiere, wo sie starke klinische Symptome verursachen, darunter Tachypnoe, Dyspnoe, Husten, Anorexie und Lethargie (Di Cesare et al. 2014). Unbehandelt kann eine Infektion in schweren Fällen zum Tod führen (Brianti et al. 2012; Traversa 2014). Besonders in Südeuropa wurde eine hohe Prävalenz von *T. brevior* nachgewiesen, wobei starke Diskrepanzen zwischen wildlebenden Feliden und Hauskatzen vorliegen. So wurde in Italien eine Prävalenz in Wildkatzen von 71,4 % nachgewiesen (Falsone et al. 2014), während die Prävalenz bei Hauskatzen bei 5,1 % lag (Giannelli et al. 2017). Die Rolle der paratenischen Wirte in der Übertragung der felines

Troglostrongylose ist noch nicht vollständig geklärt (Crisi et al. 2018). Durch die hohe Anzahl an streunenden Katzen sowie Wildkatzen in Südeuropa ist es wahrscheinlich, dass *T. brevior*-Infektionen von diesen freilebenden Feliden auf die Hauskatze übertragen werden können.

Die Diagnose einer patenten Troglostrongylose erfolgt, wie bei anderen Lungenwürmern auch, über Nachweis der lebendenden L1 im Baermann-Wetzel-Verfahren. Zur Unterscheidung zu *Ae. abstrusus*-Larven dient die subterminale Lage der Mundöffnung sowie das spitz zulaufende Hinterende mit dorsalem Fortsatz, ventralem Dorn und tiefer Einkerbung (Alić et al. 2015). Eine andere Möglichkeit besteht in der Durchführung einer PCR zum molekularbiologischen Nachweis der Infektion (Di Cesare et al. 2014). Weitere molekularbiologische oder serologische Tests sind derzeit kommerziell nicht erhältlich.

2.4 Andere metastrongyloide Lungenwurmart

2.4.1 *Crenosoma striatum*

C. striatum (Zeder, 1800) gilt als sehr wirtsspezifischer Parasit (Lämmler und Saupe 1968) und infiziert den Europäischen Igel (*Erinaceus europaeus*) (Mirzaei 2015), den Nördlichen Weißbrustigel (*E. roumanicus*) (Kirillov et al. 2022), den Südlichen Weißbrustigel (*E. concolor*) und den Algerischen Igel (*Atelerix algirus*) (Delgado Serra et al. 2024).

Auch hier sind verschiedene Schneckenarten als Zwischenwirte bestätigt (Lämmler und Saupe 1968). Besonders relevant für die Prävalenz und Übertragung des Parasiten ist die Tatsache, dass Schnecken ein Teil der natürlichen Nahrung des Igels darstellen, was eine *C. striatum*-Infektion umso wahrscheinlicher macht (Yalden 1976). Bisher liegen noch keine Daten zur Entwicklungsdauer in der Schnecke vor, aber es ist anzunehmen, dass diese vergleichbar mit den anderen Lungenwürmern (z. B. *C. vulpis*) ist.

Die adulten Nematoden befinden sich in den Bronchien und Bronchioli des Endwirts (Beck 2007; Barradas et al. 2020). Entsprechend dieser Lokalisation führen *C. striatum*-Infektionen zu verminösen Bronchopneumonien, Husten, Dyspnoe und in schweren Fällen zum Tod (Beck 2007; Barradas et al. 2020).

C. striatum-Infektionen bei wildlebenden Igeln weisen eine hohe Prävalenz auf. Aktuelle Studien sprechen von einer Prävalenz von 77,6 % in Deutschland (Schütte et al. 2025) und 59 % in der Schweiz (Lehmann et al. 2024). Der Nachweis kann post mortem erfolgen via Nekropsien (Barradas et al. 2020; Lehmann et al. 2024) oder am lebendigen Tier durch das Baermann-Wetzel-Auswanderungsverfahren (Schütte et al. 2025). Die Behandlung kann

erfolgen durch kommerzielle Antiparasitika, wie etwa Moxidectin in Kombination mit Praziquantel (Adam und Eshar 2024).

2.4.2 Zoonotisch relevante metastronglyoide Lungenwürmer

Die beiden zur Gattung *Angiostrongylus* gehörenden Lungenwürmer *A. cantonensis* und *A. costaricensis* infizieren üblicherweise Ratten, sind aber für ihre zoonotische Relevanz bekannt.

A. costaricensis (Morera, 1971) wurde erstmals in Costa Rica als Abdominalparasit des Menschen beschrieben (Morera und Céspedes 1971). Der Mensch stellt sowohl für *A. costaricensis* als auch für *A. cantonensis* einen Fehlwirt dar. Menschen infizieren sich über den Verzehr von infizierten Schnecken, die in vielen tropischen und subtropischen Ländern regelmäßig als Eiweißquelle konsumiert werden. In den folgenden Jahren wurde über weitere Fälle in verschiedenen Ländern Süd- und Mittelamerikas, aber auch den USA berichtet (Ubelaker und Hall 1979; Malek 1981; Duarte et al. 1991). Als Endwirte von *A. costaricensis* wurden unter anderem die Hausratte (*Rattus rattus*), die Norwegische Ratte (*R. norvegicus*) und die stachlige Baumwollratte (*Sigmodon hispidus*) identifiziert, in denen patente Infektionen mit Larvenausscheidung nachgewiesen wurden (Graeff-Teixeira et al. 1990; Kaminsky 1996). Als obligate Zwischenwirte dienen verschiedene Schneckenspezies, darunter *L. fulica* und weitere Nackt- und Gehäuseschneckenarten (Bonetti und Graeff-Teixeira 1998). Die *A. costaricensis*-Adulti sind in den Mesenterialarterien oder den ileokolischen Arterien lokalisiert. Wie bei anderen *Angiostrongylus*-Arten wurden auch hier verschiedene Schnecken-Spezies als Zwischenwirte identifiziert (Bonetti und Graeff-Teixeira 1998). In Menschen führen Infektionen zum akuten Abdomen mit Palpationsschmerz, Schmerzen in der rechten Beckengrube sowie leichtem Fieber (Loría-Cortés und Lobo-Sanahuja 1980; Graeff-Teixeira et al. 1991b). Während die Symptome und die Eosinophilie Hinweise liefern, ist der Goldstandard für die Diagnose einer Infektion mit *A. costaricensis* bei Menschen die histopathologische Untersuchung (Graeff-Teixeira et al. 1991a; Rodriguez et al. 2008). Die Behandlung erfolgt mittels chirurgischer Intervention, da bislang keine Chemotherapeutika eine Wirkung zeigten und die Läsionen sogar teilweise verschlimmerten (Loría-Cortés und Lobo-Sanahuja 1980; Graeff-Teixeira et al. 1991b).

A. cantonensis gilt in 30 verschiedenen tropischen und subtropischen Ländern in Nord- und Südamerika, Afrika, Asien sowie der Pazifikregion als endemisch (Kim et al. 2002; Wang et al. 2008; Barratt et al. 2016). Infektionen in europäischen Reisenden galten bisher als typische Reiseinfektionen bei Reisen in endemische Länder (Luessi et al. 2009; Morgan et al. 2021). Mittlerweile wurde dieser Parasit aber auch in Süditalien in der Ratten- und Schneckenpopulation nachgewiesen sowie in der Ratten- und Schneckenpopulation in

Makaronesien (Foronda et al. 2010; Martin-Alonso et al. 2015), was für eine Ausbreitung in bisher nicht endemische Gebiete spricht (Segeritz et al. 2021b; Pandian et al. 2025). Zu den Zwischenwirten gehören auch hier verschiedene Nackt- und Gehäuseschnecken, darunter die invasive Art *L. fulica*, die als Neozoon eine Rolle in der zunehmenden Ausbreitung von *A. cantonensis* spielen kann (Thiengo et al. 2010). *A. cantonensis* (Chen, 1935) gilt als der wichtigste Auslöser der eosinophilen Meningitis des Menschen (Wang et al. 2008). Infektionen werden ausgelöst durch den Verzehr unzureichend gekochter Schnecken sowie einer Vielzahl paratenischer Wirte (z. B. Krabben, Garnelen, Frösche, Eidechsen) (Turck et al. 2022). Typische Symptome sind Kopfschmerzen, Fieber, Nackensteifheit, Parästhesie, Übelkeit und Erbrechen (Wang et al. 2008, 2012). Auch okuläre Formen der Infektion sind möglich, aber sehr selten (Feng et al. 2013). Die Diagnose erfolgt durch eine ausführliche Anamnese, eine Untersuchung der zerebrospinalen Flüssigkeit bei Gehirnmanifestation sowie eine optische Untersuchung der Augen bei dem Verdacht einer okulären Manifestation (Sawanyawisuth et al. 2007; Sawanyawisuth und Sawanyawisuth 2008; Feng et al. 2013). Die Behandlung beider Formen erfolgt durch die Gabe von Prednisolon oder anderen Kortikosteroiden (Sawanyawisuth et al. 2007; Sawanyawisuth und Sawanyawisuth 2008; Feng et al. 2013).

2.5 Schnecken als obligatorische Zwischenwirte für metastrongyloide Lungenwürmer

Gastropoden spielen eine Rolle als obligatorische Zwischenwirte für verschiedene Parasiten. Dazu gehören nicht nur die bereits genannten metastrongyloiden Lungenwürmer, sondern auch die protostrongyloiden Lungenwürmer der Wiederkäuer (*Muellerius capillaris*, *Elaphostrongylus* sp., *Cystocaulus ocreatus* u. a.), Trematoden (*Fasciola hepatica*, *Schistosoma mansoni*, *Dicrocoelium dendriticum* u. a.) und Cestoden (*Davainea proglottina*) (Abdou 1958; Zmoray et al. 1969; Manga-González et al. 2001; Řezáč und Palkovič Holasová und Bušta 2013; Faro et al. 2013; Kuchboev et al. 2017; Malatji et al. 2020).

Während für die genannten Trematoden hauptsächlich Wasserschnecken eine Rolle als Zwischenwirte spielen (z. B. *Biomphalaria glabrata*, *Radix natalensis*) (Faro et al. 2013; Malatji et al. 2020), sind für die metastrongyloiden und protostrongyloiden Lungenwürmer eine Reihe von Landschnecken als Zwischenwirte bekannt (Abb. 2). Dazu zählen etwa *Helix pomatia*, *Arion vulgaris*, *Limax maximus*, *Helicodonta* sp., *Deroceras reticulatum*, *Cepea nemoralis* sowie die invasive *Lissachatina fulica*. Einige dieser gastropoden Zwischenwirte werden im Folgenden genauer beschrieben.

2.5.1 *Arion vulgaris*

Arion vulgaris, auch als *Arion lusitanicus* oder Spanische Wegschnecke bezeichnet, ist eine ursprünglich als invasiv bezeichnete Spezies aus der Familie der Arionidae. Diese Nacktschnecke erreicht eine Größe von bis zu 8 cm und ist von hellroter, brauner oder orangener Farbe (Welter-Schultes 2012). In der Regel ist *A. vulgaris* einjährig, die Fortpflanzungszeit liegt im Sommer und die Adulti sterben nach der Eiproduktion ab (Slotsbo 2012). Während diese Schneckenspezies über viele Jahre als invasiv bezeichnet und ihre Herkunft auf der Iberischen Halbinsel vermutet wurde, lassen neue genetische Studien darauf schließen, dass diese Nacktschnecke doch in Mitteleuropa heimisch ist (Zajac et al. 2020). *A. vulgaris* wurde als obligatorischer Zwischenwirt für metastrongyloide und potentiell protostrongyloide Lungenwürmer bereits beschrieben; ob sie eine Rolle im Lebenszyklus anderer Parasiten spielt, ist fraglich (Gerichter 1951; Penagos-Tabares et al. 2020; Segeritz et al. 2022).



Abb. 2. Häufige invertebrate Zwischenwirte für metastrongyloide Lungenwürmer. A: Weinbergschnecke (*Helix pomatia*); B: Afrikanische Riesenschnecke (*Lissachatina fulica*); C: Tigerschneigel (*Limax maximus*); D: Wegschnecke (*Arion* sp.); E: Hain-Bänderschnecke (*Cepaea nemoralis*)

2.5.2 *Limax maximus*

Der Tigerschneigel *L. maximus* zählt mit einer Körperlänge von 10-20 cm zu den größten Schneigelarten Deutschlands (Kerney et al. 1983). Die Schneigel sind üblicherweise von einer beigen Farbe mit einer schwarzen Flecken- oder Streifenzeichnung und einem kurzen Kiel, welcher sich über das hintere Körperdrittel bis zur Schwanzspitze zieht (Wiese 2016). *L. maximus* ist in Deutschland weit verbreitet und gilt als ungefährdet. Dies liegt u. a. an den vielfältigen Lebensräumen, die dieser Schneigel bewohnen kann, wie Wälder, Wiesen, Gebüsch, Gärten und sogar Gebäude (Wiese 2016). Die sehr aktiven und beweglichen Schneigel ernähren sich hauptsächlich von Algen, Pilzen und verfaulten Pflanzen, weshalb

L. maximus als Nützling betrachtet wird (Wiese 2016). Zudem ist *L. maximus* für seinen Kannibalismus bekannt, der grundsätzlich eine Rolle in der Übertragung von Parasiten zwischen den individuellen Schnecken führen kann (Intermediasis), sollte *L. maximus* das Gewebe von infizierten Schnecken aufnehmen (Karlin und Bacon 1961; Colella et al. 2015).

2.5.3 *Deroceras reticulatum*

Bei *D. reticulatum*, auch Genetzte Ackerschnecke genannt, handelt es sich um kleine und aktive Nacktschnecken von hell- bis mittelbrauner Färbung mit einer charakteristischen Flecken- oder Netzzeichnung (Wiese 2016). *D. reticulatum* kommt deutschlandweit häufig vor und gilt als Schädling an Kulturpflanzen (Wiese 2016). Auch diese Nacktschnecke ist in der Lage, verschiedenste Lebensräume zu besiedeln und tritt dementsprechend häufig synanthrop auf, was ihre potentielle Bedeutung als Zwischenwirt für metastrongyloide Lungenwürmer erhöht (Wiese 2016). So ist *D. laeve* als Zwischenwirt für *A. costaricensis* bereits bestätigt worden (Maurer et al. 2002).

2.5.4 *Lissachatina fulica*

Lissachatina (Achatina) fulica, die Afrikanische Riesenschnecke oder Achatschnecke, stammt ursprünglich aus Ostafrika. Aufgrund ihrer hohen Tenazität und des Handels ist diese Schnecke mittlerweile in vielen Ländern invasiv, darunter Indien, Kolumbien, Brasilien und die Vereinigten Staaten (Rekha Sarma et al. 2015; Penagos-Tabares et al. 2019; Carvalho et al. 2024). Nicht nur gilt diese Schneckenspezies als Schädling, der ganze Ernten vernichten kann, sondern auch als Überträger von Parasiten und anderen Erkrankungen (Rekha Sarma et al. 2015; Thakuri et al. 2019; Abdulla 2024). So sind für *L. fulica* 17 Parasiten beschrieben, in denen die Schnecke als Zwischenwirt fungiert (Gippet et al. 2023). Durch die zunehmende Verbreitung der Schnecke und der durch den Klimawandel bedingten mildereren Temperaturen muss bedacht werden, dass sich auch in der Schnecke befindliche Pathogene verbreiten können.

2.5.5 Methodische Grundlagen zur Haltung von *L. fulica* unter standardisierten Laborbedingungen und Infektionsversuche

L. fulica wurden in automatischen Klimaschränken (ECP01E®, Snijders Scientific B.V. Tilburg, Niederlande) unter standardisierten Laborbedingungen gezüchtet und gehalten. Die Luftfeuchtigkeit betrug dabei 50 % und die Temperatur variierte zwischen 20-26 °C. Zudem wurde ein Lichtzyklus etabliert mit 10 Stunden Licht, 10 Stunden Dunkelheit und jeweils 2

Stunden für Morgen- und Abenddämmerung. Die Schnecken wurden zu zweit oder zu dritt in Plastikboxen mit Luftlöchern gehalten, deren Boden mit einer ca. 5 cm dicken Schicht aus Kokoserde (TropicShop) bedeckt war. Um den pH-Wert anzuheben, wurde zudem Gartenkalk (Rheinkalk GmbH) unter die Erde gemischt. Sepia-Schalen wurden ad libitum zur Verfügung gestellt und dienten als Kalzium-Quelle; als Proteinquelle wurde kommerzielles Hundefutter (Romeo, Aldi) angeboten. Zudem wurde folgendes Gemüse gefüttert: Zucchini (*Cucurbita pepo*), Paprika (*Capsicum annuum*), Karotten (*Daucus carota*), Gurken (*Cucumis sativus*) und Salat (*Lactuca sativa*) (Abb. 3A).

Gastropode Zwischenwirte infizieren sich in der Regel mit metastrongyloiden Lungenwürmern durch die orale Aufnahme der L1 in den Fäzes eines infizierten Endwirts. Um eine natürliche Infektion nachzuahmen, wurden *L. fulica* mit einer festgelegten Dosis an vitalen L1 in Lösung (z. B. Kotwasser, Fruchtsaft) oral mithilfe einer Spritze infiziert. Die Schnecken nahmen diese Flüssigkeit willentlich bei vollem Bewusstsein auf (Abb. 3B).

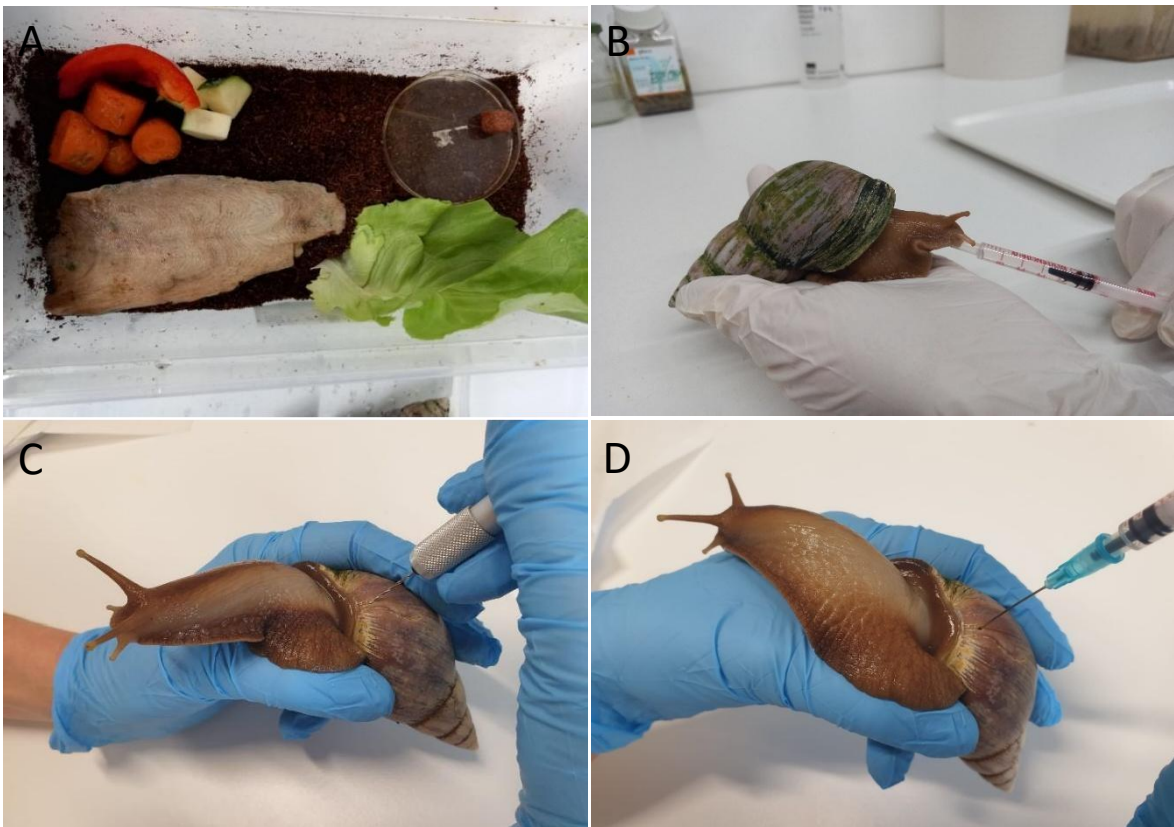


Abb. 3. Haltung von *L. fulica* unter standardisierten Laborbedingungen. A: Haltungsverbehälter; B: Infektion mit vitalen L1 metastrongyloider Lungenwürmer, C: Perforation des Schneckenhauses mittels elektrischen Bohrers (Dremel 3000®), D: Herzpunktion und anschließende Hämolymphextraktion.

Anders als Säuger verfügen Schnecken nur über ein rudimentär entwickeltes Blutgefäßsystem, weshalb die Entnahme von Hämolymphe (vergleichbar mit dem Blut der Säuger) eine Herausforderung darstellt. Ein weiteres Hindernis stellt die Anatomie der Schnecke dar, da diese sich in Sekundenschnelle in ihr Haus zurückziehen kann und jegliche Manipulation am Weichkörper verhindert. Aus diesem Grund entschieden wir uns für das Verfahren der Herzpunktion nach Penetration des Schneckenhauses. Dazu wurde das Gebiet gereinigt und die Punktionsstelle neben dem Pneumostom lokalisiert. Daraufhin wurde ein elektrischer Bohrer (Dremel 3000, Dremel) angesetzt und das Schneckenhaus vorsichtig angebohrt, bis es zur Perforation kam (Abb. 3C). Im Anschluss wurde eine sterile Spritze mit einer 18G-Kanüle eingeführt, das Herz punktiert und die Hämolymphe vorsichtig aspiriert (Abb. 3D). Die Hämolymphe verhält sich wie das Säugetierblut und koaguliert *in vitro* innerhalb kurzer Zeit, weshalb die Verwendung eines Antikoagulans notwendig war. Dieses wurde 1:1 mit der Hämolymphe vermischt, die im Anschluss für weitere Verarbeitung zur Verfügung stand. Ein Vorteil dieser Hämolymp-Extraktionsmethode besteht darin, dass diese sehr schonend für die Schnecke ist und dieser wenig Schmerzen, Leiden oder Schäden zufügt. Während der Herzpunktion ziehen die Schnecken sich weder in ihr Haus zurück noch zeigen sie andere Abwehrreaktionen. Daraus lässt sich schließen, dass die Extraktion selbst keine Schmerzen verursacht. Das Loch, das zur Hämolymp-Extraktion gebohrt wird, remineralisiert sich innerhalb weniger Wochen, sodass keine Verbindung mehr zwischen der Körperhöhle und der Außenwelt vorhanden ist. Zudem überlebt *L. fulica* die Hämolymp-Extraktion, solange nur 1 % der Körpermasse der Schnecke extrahiert wird, was insbesondere im Rahmen des 3R-Prinzips von Bedeutung ist. Aus diesem Grund ist eine geringere Anzahl an Schnecken erforderlich (*Reduce* = Reduzieren) und sie können nach vier Wochen erneut zur Hämolymp-Extraktion herangezogen werden.

2.6 Das gastropode Immunsystem

Im Gegensatz zu Säugetieren verfügen Gastropoden nicht über ein erworbenes Immunsystem, sondern nur über ein angeborenes nicht-adaptives Immunsystem (Pinaud et al. 2016; Pila et al. 2016a). Die Effektorzellen sind die bereits erwähnten Hämozyten (Syn. Amoebozyten), die frei in der Hämolymphe zirkulieren und verschiedene Aufgaben im Immunsystem und Stofftransport übernehmen (Yoshino et al. 2013; Huang et al. 2020; de la Ballina et al. 2022). Darunter fallen Koagulation und Wundheilung, der Transport von Nähr- und Mineralstoffen sowie der intrazelluläre Verdau (Cheng 1984; Nakayama et al. 1997; Machałowski und Jesionowski 2020; Furukawa et al. 2021).

Zudem verfügen gastropode Hämozyten über potente Abwehrmechanismen gegen eindringende Pathogene: *i)* zellvermittelte Zytotoxizität, *ii)* sogenannte Invertebrate **Extracellular Phagocyte Traps (InEPT)**, *iii)* Phagozytose, *iv)* Einkapselung und *v)* Melanisierung (Robb et al. 2014; Lange et al. 2017; Huang et al. 2020; Falkingham und Rae 2021; Al-Khalaifah 2022). Gastropode Hämozyten können durch sogenannte **Pattern Recognition Receptors (PRR)**, wie etwa **Toll-like Rezeptoren (TLR)** invasive Pathogene, inklusive Parasiten, erkennen (Marmaras und Lampropoulou 2009; Elvitigala et al. 2013; Pila et al. 2016b; Eleftherianos et al. 2021). Zudem gibt es Hinweise, dass Hämozyten eine Rolle in der nicht-adaptiven Immunantwort spielen, da Hämozyten die Zellproliferation regulieren und humorale Faktoren freisetzen, wie etwa **Fibrinogen-related proteins (FREPs)**, Lektine, Lysozyme und Peroxidasen (Coustau et al. 2016; Al-Khalaifah 2022). Zudem lassen weitere Studien vermuten, dass gastropode Hämozyten, wie bereits bei Arthropoden gezeigt wurde (Krejčová und Bajgar 2025), eine gewisse „angeborene Gedächtnisfunktion“ besitzen und nach einem zweiten Kontakt mit einem Pathogen stärker und anhaltender reagieren können (Pinaud et al. 2016; de Melo et al. 2020).

2.6.1 Extracellular traps (ET) und InEPT

Die Fähigkeit zur Ausbildung von ET wurde zuerst in menschlichen neutrophilen Phagozyten als sogenannte **neutrophil extracellular traps (NET)** beschrieben (Brinkmann et al. 2004). In den folgenden Jahren wurde diese Fähigkeit in verschiedenen Säugetierspezies nachgewiesen, darunter Rinder, Schafe, Ziegen, Hunde, Seehunde, Delfine und Mäuse (Reichel et al. 2015; Imlau et al. 2019; Worku et al. 2021; Tucker et al. 2021; Grob et al. 2021).

Die ET-Bildung, auch als **ETose** bezeichnet, wurde beschrieben als nützlicher Suizid der Immunzelle, um dadurch Pathogene zu immobilisieren (Brinkmann und Zychlinsky 2007). Als auslösende Faktoren gelten Kontakt mit Bakterien, Viren, Pilzen und Parasiten (Branzk und Papayannopoulos 2013; Schönrich und Raftery 2016; Niedźwiedzka-Rystwej et al. 2019; Zhong et al. 2022). Zu den ETose-auslösenden Parasiten gehören sowohl apikomplexe Protozoa (z. B. *Besnoitia besnoiti*, *Eimeria tenella*, *Toxoplasma gondii*) (Rentería-Solís et al. 2024; Conejeros et al. 2025; Turra et al. 2025) sowie Metazoa (*A. vasorum*, *Dirofilaria immitis*) (Lange et al. 2017; Penagos-Tabares et al. 2018b; Muñoz-Caro et al. 2018). Neben Parasiten wurden auch weitere NET-Stimuli *in vivo* nachgewiesen: IL-8 und **damage associated molecular patterns (DAMP)** (Gupta et al. 2005; Huang et al. 2015).

Im ersten ETose-Aktivierungsschritt nach Erregerkontakt kommt es zur Dekondensation des Heterochromatins im Zellkern. Auslöser hierfür ist die Aktivität der durch Ca^{2+} aktivierten **Protein-Arginin-Deiminase Typ 4 (PAD4)**, wodurch die Histon-Arginine zu Histon-Citrollinen

konvertiert werden (Demaurex et al. 1994; Sørensen und Borregaard 2016). Auf diese Weise lässt die stark positive Ladung der Histone nach, die elektrostatische Bindung zwischen Histonen und der DNA wird schwächer und es kommt zur Dekondensierung des Chromatins. Im nächsten Schritt werden die Membranen des Zellkerns sowie der Granula aufgelöst und die dekondensierte DNA trifft auf die antimikrobiellen Granula-Moleküle. Diese Granula beinhalten u. a. Elastase, Myeloperoxidase, Laktoferrin und Pentraxin, die sich mit der dekondensierten DNA vermischen (Fuchs et al. 2007). Dieser DNA-Enzym-Komplex wird daraufhin aus der Zelle ausgeschleudert, um Pathogene zu immobilisieren. Eine kernlose Zelle bleibt zurück, die zugrunde geht (Fuchs et al. 2007; Sørensen und Borregaard 2016).

Nur ca. 20 % der neutrophilen Granulozyten sind in der Lage dazu, NET zu bilden, wobei die Gründe für dieses Phänomen noch immer unbekannt sind (Phillipson und Kubes 2011). Womöglich existieren verschiedene Arten von Neutrophilen im peripheren Blut, die auf unterschiedliche Weise auf eindringende Pathogene reagieren (Phillipson und Kubes 2011; Sørensen und Borregaard). Doch auch andere Zellen sind in der Lage, ET zu bilden. Eosinophile Granulozyten beispielsweise formen ebenfalls netzartige Strukturen aus DNA und Histonen, die Pilze, Bakterien und Parasiten einfangen und abtöten können (Muñoz Caro et al. 2015; Ueki et al. 2016; Mukherjee et al. 2018; Fukuchi et al. 2021). Im Vergleich zu den NET sind die Stränge dieser eosinophil extracellular traps (EET) dicker und weniger anfällig durch den proteolytischen Abbau durch Leukozyten (Ueki et al. 2013, 2016). Auch basophile Granulozyten können ET bilden, wobei diese im Gegensatz zu Mastzellen unabhängig von der Aktivität der NADPH-Oxidase (NOX) ist (Morshed et al. 2014; Möllerherm et al. 2016). Eine ET-Bildung wurde auch bei den versatilen Monozyten und Makrophagen (MET, macrophage extracellular traps) nachgewiesen (Boe et al. 2015; Doster et al. 2017; Pertiwi et al. 2019).

Trotz ihrer effizienten Abwehr gegenüber potentiellen Krankheitserregern können NET/MET auch nachteilig wirken, indem sie beispielsweise Spermien schädigen und dadurch zu Fruchtbarkeitsstörungen führen können (Zambrano et al. 2016; Moya et al. 2022). Die Beteiligung von NET ist zudem in mehreren Krankheitsbildern beschrieben, darunter Appendizitis sowie Autoimmunerkrankungen, darunter Lupus, rheumatoide Arthritis und Psoriasis (Brinkmann et al. 2004; Simon et al. 2013; He et al. 2018; Boettcher et al. 2020). Zudem wirken NET Thrombose-fördernd, da sie bei Aktivierung in Blutgefäßen zur Aggregation von Thrombozyten und damit zur Verstopfung des Gefäßes führen (Fuchs et al. 2010; Lou et al. 2023).

Auch in den Hämozyten von Wirbellosen konnte die Ausbildung von InEPT beschrieben werden (Robb et al. 2014; Lange et al. 2017). In verschiedenen Schneckenarten (*L. maximus*, *L. fulica*), der Gemeinen Strandkrabbe (*Carcinus maenas*), der Pazifischen Auster (*Crassostrea gigas*) der Kirschessigfliege (*Drosophila suzukii*) sowie weiteren Invertebraten

wurde die Ausbildung von InEPT beschrieben (Robb et al. 2014; Poirier et al. 2014; Lange et al. 2017; Carrau et al. 2021). Diese Tatsache legt nahe, dass die ETose ein evolutionär hochgradig konservierter Mechanismus von hoher Effizienz ist, der viele Tierarten und Millionen von Jahren überdauert hat.

2.6.2 Einkapselung

Die Einkapselung ist ein weiterer zellulärer Effektormechanismus von gastropoden Hämocyten (Sminia et al. 1974; Sauerländer 1976). Dieser Prozess wird ausgelöst durch Kontakt der Schnecke mit verschiedenen Pathogenen und/oder Fremdkörpern, wie etwa Parasiten, darunter die Lungenwürmer *A. vasorum* und *A. cantonensis* sowie Bakterien (Sauerländer 1976; Al-Khalaifah 2022; Wang et al. 2023). Die Einkapselung findet nicht statt in Allografts und Autografts, was bestätigt, dass das nicht-adaptive Immunsystem der Schnecke zwischen verschiedenen Gewebetypen und inertem Material unterscheiden kann (Sminia et al. 1974).

Der Prozess der gastropoden Einkapselung läuft in mehreren Phasen ab und konnte bei experimentell *A. vasorum*-infizierten *L. fulica* näher untersucht werden. Die Abwehrreaktionen gegen *A. vasorum*-L1 begannen mit einer großen Hämocytenansammlung (Aggregation) um die Larven bereits 5 Stunden p. i. (Sauerländer 1976). Mit fortschreitender Zeit und zunehmender Anzahl der Hämocyten um die Larven kam es zur Abflachung der Zellkerne und einer initialen Kapselbildung, in fortschreitendem Stadium zur Karyolyse in den zentralen Hämocyten, woraus sich später die Kapselhöhle bildete (Sauerländer 1976). Dadurch ergab sich ein faserstrukturähnliches Bild, also eine konzentrische Anordnung der einzelnen Hämocyten-schichten zueinander (Sauerländer 1976). Der Aufbau der bindegewebigen Kapsel stellt sich dreischichtig dar: die innere und äußere Schicht bestehen jeweils aus abgeflachten, eng verbundenen Hämocyten, während die mittlere Schicht aus regulär morphologischen Hämocyten aufgebaut ist (Sminia et al. 1974; Sauerländer 1976). Der Aufbau der Kapsel ist in allen Organen der Schnecke gleich (Sauerländer 1976). Während der Entwicklung der *A. vasorum*-L1 zur L3 flachte sich die Kapselwand einseitig ab, was als mögliche Prädilektionsstelle für das Auswandern der Lungenwurm-Larven vermutet wird und als ein Evasionsmechanismus der Einkapselung interpretiert werden kann (Sauerländer 1976).

Als weiterer Schritt nach der Einkapselung schließt sich häufig die Melanisierung an, ein Prozess, der zur Abtötung der eingedrungenen Pathogene führen kann (Le Bris et al. 2013). Dabei kommt es durch β -1,3-Glucane sowie Lipopolysaccharide (LPS) zur Aktivierung der Phenoloxidase, die als Pro-Phenoloxidase inaktiviert in der Hämolymphe vorkommt (Vargas-Albores et al. 1997; Perazzolo und Barracco 1997). Diese oxidiert und polymerisiert schließlich Phenole, wodurch es zur Produktion von Melanin und dessen Anlagerung an Pathogene kommt. Das Melanin selbst als Polymer kann die Pathogene schädigen, indem es Proteasen

und die Nahrungsaufnahme der eingedrungenen Mikroorganismen hemmt (Chen und Chen 1995; Wilson et al. 2008). Zudem wird während dieses Prozesses Stickstoffmonoxid (NO) gebildet, welches ebenfalls eine Beteiligung in der Abwehr gegen Pathogene hat (Nappi et al. 2000; Coaglio et al. 2018).

2.6.3 Organtropismus metastrongyloider Lungenwürmer im gastropoden Zwischenwirt

Bislang gibt es noch wenige Studien zum Verhalten metastrongyloider Lungenwürmer im obligatorischen gastropoden Zwischenwirt, insbesondere was den Organtropismus und die Wanderwege der Larven betrifft. Ein solcher Schneckenorgantropismus ist bereits bei anderen Parasiten (z. B. Trematoden) bekannt. So wurde für einige Trematodenarten beispielsweise ein Tropismus für das Reproduktionssystem beschrieben, wobei es in vielen Fällen zur sogenannten parasitären Kastration der Schnecken kommen kann (Reinhard 1956; Faro et al. 2013). Auch verschiedene *Babesia*-Arten lassen einen Organtropismus für das Reproduktionssystem ihrer Endwirte erkennen, indem diese Einzeller die Ovarien befallen und somit die nächste Zeckengeneration infizieren können (Büscher et al. 1988; Boldbaatar et al. 2008). *Dicrocoelium dendriticum* ist sogar in der Lage, das Verhalten seines Zwischenwirts zu verändern, indem eine Zerkarie in das Unterschlundganglion einer Ameise eindringt (Manga-González et al. 2001). Selbiges ist für *Toxoplasma gondii* bekannt, welches häufig das ZNS befällt und somit das Verhalten von Mäusen dahingehend verändert, dass diese sich für Katzenurin interessieren, was die Wahrscheinlichkeit erhöht, dass die Mäuse gefressen werden (Kannan et al. 2010).

Eine solche Verhaltensveränderung mit Lungenwürmern infizierter Schnecken ist bislang nicht bekannt, wenn auch ein gewisser Organtropismus nachgewiesen werden konnte. So wurde in einer Studie von Sauerländer et al. (1976) ein Tropismus für die Lunge sowie den Fuß der Schnecke berichtet. Eine Untersuchung über die genauen Wanderwege liegt bisher nicht vor. Während ein künstlicher Verdau zu verschiedenen Zeitpunkten einen Einblick in die Wanderwege ermöglicht, besteht auch die Möglichkeit, die Lungenwurmlarven selbst radioaktiv zu markieren und auf diesem Weg durch moderne bildgebende Verfahren die Wanderwege *in vivo* sowie den frühen Organtropismus zu analysieren.

2.7 Bildgebende Verfahren

Das PET (**P**ositronen-**E**missions-**T**omographie)/CT (**C**omputertomographie)-Verfahren wurde im späten 20. Jahrhundert entwickelt und wird heute hauptsächlich in der Onkologie und Nuklearmedizin eingesetzt, wie der Diagnose und dem „Staging“ von Tumoren sowie der Therapiekontrolle (Krause et al. 2013; Meyer et al. 2016). Das PET/CT ergänzt und erweitert die klassischen Verfahren der Nuklearmedizin. Dazu zählt insbesondere die Szintigraphie, bei der Gamma-Emitter-Tracer detektiert werden, um die Funktion von Organen und Geweben zu beurteilen. Auf dieser Basis wurden etwa das PET/MRT (**M**agnetresonanztomographie), das PET/CT sowie das SPECT/CT (**S**ingle **P**hoton **E**mission **C**omputed **T**omography) entwickelt. Wie bei der Szintigraphie verwendet das SPECT/CT die Detektion von Gammastrahlen, die direkt von dem verwendeten Radionuklid (z. B. ^{99m}Tc) ausgehen und von Gammakameras um den Patienten detektiert werden. Das PET/MRT verbindet die funktionellen PET-Informationen mit den hochauflösenden Bildern von Organen und Geweben aus dem MRT-Verfahren. All diese Verfahren unterscheiden sich in der Sensitivität, der räumlichen Auflösung sowie der Tracer-Auswahl. So ist im PET/CT die Darstellung des Knochen- und Lungengewebes von hoher Genauigkeit, während das PET/MRT für die Darstellung des Weichgewebes präferiert wird. Sowohl das SPECT-Verfahren als auch die Szintigraphie verfügen über eine geringere Auflösung, sind aber leichter verfügbar und die Strahlenbelastung ist im Allgemeinen geringer.

In heute eingesetzten modernen Geräten können das PET- sowie das CT-Verfahren gemeinsam durchgeführt werden, indem nacheinander die präzisen Signale des radioaktiven Zerfalls analysiert und diese daraufhin anatomisch durch das CT-Verfahren lokalisiert werden. Dazu wird dem Patienten ein radioaktiver Marker oral oder intravenös verabreicht, der sich innerhalb von 30-60 min im Körper verteilt (Uptake-Zeit). ^{18}F -Fluordesoxyglucose (^{18}F -FDG) ist ein radioaktives Glucose-Analogon, welches als Marker eingesetzt wird (Krause et al. 2013). Anhand dieses Markers wird im Folgenden die Funktionsweise des PET/CT erklärt. Tumoren sind metabolisch hochgradig aktive Zellen, die im Vergleich zu gesunden Körperzellen über einen höheren Glucoseumsatz verfügen (Ancy et al. 2018). ^{18}F -FDG wird über den GLUT-Transporter wie Glucose in die Zelle aufgenommen, und wird durch die Hexokinase zu FDG-6-Phosphat phosphoryliert (Krause et al. 2013; Ancy et al. 2018). Dieses kann im Körper durch die Glucose-6-Phosphat-Isomerase nicht abgebaut werden, sondern reichert sich in metabolisch aktiven Geweben an („metabolic trapping“). Dort zerfällt das instabile Radionuklid und setzt dabei Positronen frei (β^+ -Zerfall), die auf Elektronen treffen. Nach dem Aufeinandertreffen kommt es zur gegenseitigen Auslöschung (Annihilation), wodurch zwei Gamma-Photonen mit je 511 keV entstehen. Diese bewegen sich 180° voneinander weg in unterschiedliche Richtungen und werden von umliegenden Detektoren registriert. Dadurch werden metabolisch hochaktive Strukturen wie Tumoren oder das Gehirn

sichtbar, sowie die Leber, Niere und Harnblase, die an der Ausscheidung des ^{18}F -FDG beteiligt sind. Im Anschluss folgt das CT-Verfahren, welches hochauflösend anatomische Strukturen darstellt. Durch die Fusion beider Verfahren kann die Lage und Größe eines Tumors oder potentieller Metastasen bestimmt werden (Röhrich 2023).

^{18}F -FDG verfügt nur über eine sehr kurze Halbwertszeit (110 min), was im praktischen Umfeld durchaus erwünscht sein kann, da die Strahlenexposition des Patienten gering bleibt. In manchen Situationen ist jedoch die Verwendung radioaktiver Tracer mit längerer Halbwertszeit vonnöten, sollten etwa die Transportwege des Tracers zum Patienten länger sein oder der Tracer weiter verarbeitet werden. ^{64}Cu -Derivate etwa verfügen über eine vergleichsweise sehr lange Halbwertszeit von 12,7 Stunden, was eine Darstellung der metabolischen Aktivität von Geweben über einen längeren Zeitpunkt zulässt (Holland et al. 2009).

PET/CT findet zudem nicht nur in der Onkologie, sondern auch in der Diagnostik verschiedener metabolischer Erkrankungen eine Anwendung, darunter Polymyalgia rheumatica, Alzheimer sowie kongenitale Herzerkrankungen (Meyer et al. 2016; Chiaravalloti et al. 2017; Witte et al. 2023). Auch in der Veterinärmedizin wird das PET/CT-Verfahren bereits experimentell in der Tumordiagnostik verwendet, ist aber noch kein Bestandteil der Routinediagnostik (Kim et al. 2014; Sánchez et al. 2019). In der Forschung wird PET/CT mittlerweile breitflächig eingesetzt. Für die Maus als Tiermodell stehen eigens entwickelte Kleintier-PET/CT-Scanner zur Verfügung (Cheng et al. 2023).

2.8 Zielsetzung der Dissertation

Ziel dieser Dissertation war es, das Wissen um die Epidemiologie der Lungenwürmer im gastropoden Zwischenwirt im Hinblick auf den Jahresverlauf der Infektionen zu erweitern. Zudem sollte die zeitliche Stabilität eines bereits identifizierten hyperendemischen Fokus untersucht werden. Eine weitere Fragestellung beschäftigt sich mit dem Organtropismus der wandernden Lungenwurmstadien im gastropoden Zwischenwirt. Während bereits einige Studien zur Wanderung der Larvenstadien im Endwirt vorliegen, ist das Wissen um die Wanderung der Larven im Zwischenwirt gering. Unser Ziel war es, die Wanderung der Larven im Zwischenwirt zu verschiedenen Zeiten p. i. zu analysieren, potenzielle Wanderwege aufzuzeigen sowie Prädilektionsorgane zu identifizieren. Dazu sollen klassische Verfahren wie die künstliche Verdauung einzelner Organe sowie moderne Methoden wie die radioaktive Markierung der Larven und deren Verfolgung im PET/CT eingesetzt werden. Abschließend wurde das gastropode Immunsystem hinsichtlich seiner Reaktionen auf metastrongyloide Lungenwürmer analysiert, um grundlegende Erkenntnisse über das bisher vernachlässigte

Feld der gastropoden Immunologie zu gewinnen und Einblicke in die Immunabwehrmechanismen gegen metastrongyloide Lungenwürmer zu erhalten.

3. Publikationen

3.1 Re-Evaluation of a Hyperendemic Focus of Metastrongyloid Lungworm Infections in Gastropod Intermediate Hosts in Southern Germany

Alena Dusch, Lisa Segeritz, Judith Schmiedel, Anja Taubert, Carlos Hermosilla



Pathogens 2025, 14, 800. <https://doi.org/10.3390/pathogens14080800>

Eigener Anteil an der Publikation:

- Projektplanung: 20 %, zusammen mit Betreuern
- Durchführung Versuche: 70 %, zusammen mit Ko-Autoren
- Auswertung der Experimente: 80 %, weitgehend eigenständig
- Erstellung des Manuskripts: 80 %, weitgehend eigenständig

Article

Re-Evaluation of a Hyperendemic Focus of Metastrongyloid Lungworm Infections in Gastropod Intermediate Hosts in Southern Germany

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Abstract

The metastrongyloid nematodes *Angiostrongylus vasorum*, *Aelurostrongylus abstrusus*, and *Crenosoma vulpis* can cause severe cardiopulmonary and respiratory symptoms in domestic dogs and cats and free-ranging canids and felids (e.g., foxes, wolves, wild cats, lynxes). Recent data on the prevalence of *A. vasorum* infections in dogs and foxes and on the prevalence of *Ae. abstrusus* and *Troglostrongylus brevior* infections in free-ranging lynxes and wild cats revealed several endemic and hyperendemic foci in Germany. Nonetheless, long-term investigations on the prevalence of metastrongyloid larvae infecting gastropod intermediate hosts are still scarce for Germany. To fill this gap, we conducted an epidemiological survey on native slugs and snails in a selected meadow close to Obrigheim, previously identified as a hyperendemic focus for canine angiostrongylosis. To re-evaluate this location as a ‘hotspot’ of canine angiostrongylosis, terrestrial slugs and snails ($n = 533$) were collected in all seasons, artificially digested, and microscopically and molecularly analyzed for the presence of metastrongyloid lungworm larvae. Here, the prevalence ranged greatly between seasons. In summer, 27.46% (59/215) of gastropods were infected with metastrongyloid larvae. In fall, the prevalence dropped to 10.00% (16/160) and lowest infection rates were observed in both winter (5.65%) and spring (1.47%). In total, *A. vasorum* was detected in 12.01% (64/533), *Crenosoma* sp. in 0.94% (5/533), and *Ae. abstrusus* in 0.38% (2/533) of gastropod samples. Even though total *A. vasorum* infection levels were revealed to be considerably lower than in the prior study, this epidemiological survey in principle reconfirms Obrigheim as a stable hyperendemic focus and thereby as a location with high metastrongyloid infection risk for domestic dogs, cats, and wildlife throughout the year. These results call for continuous epidemiological studies on gastropod populations to better understand metastrongyloid lungworm spread and infection dynamics over the years.

Keywords: gastropod-borne disease; *Angiostrongylus vasorum*; lungworm; morphology; PCR; Germany



Academic Editor: Geoff Hide

Received: 3 July 2025

Revised: 30 July 2025

Accepted: 7 August 2025

Published: 9 August 2025

Citation: Dusch, A.; Segeritz, L.; Schmiedel, J.; Taubert, A.; Hermosilla, C. Re-Evaluation of a Hyperendemic Focus of Metastrongyloid Lungworm Infections in Gastropod Intermediate Hosts in Southern Germany. *Pathogens* **2025**, *14*, 800. <https://doi.org/10.3390/pathogens14080800>

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1. Introduction

Germany hosts multiple metastrongyloid lungworm species that infect different wildlife and domestic animals. Their life cycle is heteroxenous, with gastropods (i.e., snails and slugs) acting as obligatory intermediate hosts. The first-stage larvae (L1) are shed by the definitive host via its feces and are then taken up by the gastropod. In gastropods, the larvae

develop to the third stage (L3). These enter the definitive host either by direct consumption of the gastropods or by accidental oral uptake after gastropod larval shedding [1,2]. As such, metastrongyloid lungworm infections are considered gastropod-borne diseases.

Angiostrongylus vasorum, also known as the French heartworm, is a parasite that lives in the right heart and the pulmonary arteries. It is considered the most pathogenic lungworm species in dogs. Clinical signs range from mild respiratory symptoms like coughing to severe respiratory distress, neurological disorder, hemorrhaging, and in some cases, even death [3–5]. This parasite does not only infect domestic dogs, but also foxes (*Vulpes vulpes*) [6,7], wolves (*Canis lupus*) [8,9], golden jackals (*Canis aureus*) [10,11], raccoon dogs (*Nyctereutes procyonoides*) [12], badgers (*Meles meles*) [13], and red pandas (*Ailurus fulgens*) [14]. Due to the rising urbanization of wild-living animals, especially synanthropic foxes, the life cycle easily shifts to domestic animals, such as dogs [7]. *A. vasorum* infections are occurring globally, being characterized by a patchy distribution of hyper- and hypoen- demic foci in close proximity [3,15]. Recently, a ‘hotspot’ area was identified in 2018 in the city of Obrigheim within the Federal State of Baden-Wuerttemberg in Southern Germany reaching a prevalence of 62.96% in terrestrial gastropods [15].

Crenosoma vulpis is a metastrongyloid lungworm that infects both wildlife (e.g., foxes and wolves) and domestic canids. Adult parasites live in the bronchioles, bronchi, and trachea and lead to respiratory signs, such as acute coughing, dyspnea, bronchitis, and chronic cough [9,16–19]. Another important representative of this genus is *Crenosoma striatum*. Adult *C. striatum* nematodes are located in bronchi and pulmonary tissue of European hedgehogs (*Erinaceus europaeus*) [20]. European hedgehogs are omnivorous and consume terrestrial slugs/semi-slugs/snails on a regular basis, rendering them highly susceptible to this lungworm infection, which leads to respiratory disease, pneumonia, and even death in heavily *C. striatum*-infected animals [21,22].

Aelurostrongylus abstrusus is a cat lungworm that is distributed throughout Europe [15, 23,24]. Similar to domestic cats, wild felid species, such as the Eurasian lynx (*Lynx lynx*) and the wild cat (*Felis silvestris*), are susceptible to infection. Thus, feline aelurostrongylosis may spread to domestic cats and vice versa. Symptoms include respiratory distress, coughing, sneezing, and respiratory discharge [25,26].

Troglostrongylus brevior is another cat lungworm present in Germany. It is less often described, even though its relevance seems to be increasing. Feline troglostrongylosis causes mild to severe respiratory disease, which may lead to death in young cats [27,28]. This parasite was recently found in German Eurasian lynxes [29,30] and wildcats [31], suggesting the chance of lungworm transmission between free-living and domestic cats.

Metastrongyloid lungworm infections are spreading worldwide, expanding from regions in which they were formerly endemic [7]. Prevalence studies on lungworm infections in both free-living and domestic definitive hosts were performed in Germany, Denmark, the United Kingdom, France, and Italy, among others [7,8,18,32]. These studies mostly confirmed a classical patchy distribution of lungworm infections in hyperendemic foci, so-called hotspots [3,15]. Recent data suggest that lungworms, especially *A. vasorum*, seem to spread from ‘hotspots’ into new areas where no lungworm infections were previously recorded [7,33,34]. This dynamic spread calls for further studies on both well-known hyperendemic foci and potential new ‘hotspots’ as recently performed for Colombia in South America [35–37].

Metastrongyloid lungworm infections represent under-recognized diseases in both researchers and practicing veterinarians despite their worldwide prevalence [7,38]. While there are numerous studies on prevalences and clinical manifestations of affected definitive hosts in Europe, studies on the prevalence and development of lungworm larvae in gastropod intermediate hosts are highly neglected.

Aim of the current study was to reconfirm an *A. vasorum* hyperendemic focus in Obrigheim, and to identify environmental and biological factors associated with this particular region. Special attention was paid to potential seasonal changes in prevalence of metastrongyloid infections in gastropod intermediate hosts. Furthermore, we intended to inform local veterinarians and dog owners on transmission patterns of canine angiostrongylosis in Obrigheim.

2. Materials and Methods

2.1. Sample Collection

The sampling area of Obrigheim (Baden-Wuerttemberg, Germany) was chosen according to previous published data of lungworm infections in both definitive [39] and intermediate hosts [15]. Thus, a special meadow, frequented by dog walkers and located close to town, was studied again due to its ideal conditions for intermediate host habitat and potential infection area of domestic as well as wild definitive hosts.

Gastropod collection was performed once in all four seasons (spring, summer, autumn, and winter). Please refer to Supplementary Figure S1 for details about sampling conditions. A total number of 533 gastropods (i.e., snails, slugs) including 11 different gastropod species were collected on the hotspot meadow in 2022 and 2023. Representative images of some identified species are shown in Supplementary Figure S2. Since gastropod activity spikes in the early morning hours, the sampling was performed around sunrise. Depending on the weather conditions, gastropod collection required between 1 and 4 h.

Under current German national law, no permission is needed for gastropod collection. Additionally, in April of 2023, water samples (1 L) were collected, since the meadow was flooded due to heavy rain.

2.2. Gastropod Procession

To achieve cryo-euthanasia, gastropods were frozen at $-20\text{ }^{\circ}\text{C}$ and thereafter preserved at $4\text{ }^{\circ}\text{C}$ until further procession. Each gastropod was weighed and morphologically identified before being submitted to artificial digestion according to Lange et al. [40]. Thereafter, the remnants were sieved, and all 533 samples were individually analyzed by light microscopy (Olympus BH-2).

2.3. Microscopical Analysis

Metastrongyloid lungworm larvae were identified according to their morphometric characteristics, such as larval length and width, esophagus length and type (non-rhabditiform, one third to one half of larval length), as well as tail morphology [41]. By this way, lungworm larvae species can be differentiated from each other as well as from possibly present free-living larvae or gastropod parasitic nematodes [40]. Please refer to Figure 1.

2.4. DNA Extraction from Larvae

DNA extraction was performed on larvae collected from 10 gastropods containing high larval burdens in order to assess the applicability of molecular techniques in species identification. Following microscopic analysis, all the larvae found in one individual snail or slug were pooled, thus creating 10 samples for further processing. The DNA of the pooled larvae was then isolated using two different extraction methods.

The first method used was the DNeasy Blood & Tissue Kit (Qiagen) and was carried out according to the manufacturer's instructions.

The second method involved using the EMAG (bioMérieux) automatic nucleic acid extraction system. A customized protocol for extracting DNA from tissue was employed.

In brief, the larvae were homogenized by bead beating and transferred to a new vessel containing a 1:1 dilution of lysis buffer containing guanidine thiocyanate. After a short centrifugation step, the supernatant was loaded into the EMAG. After extraction, the DNA was eluted in Aqua dest.

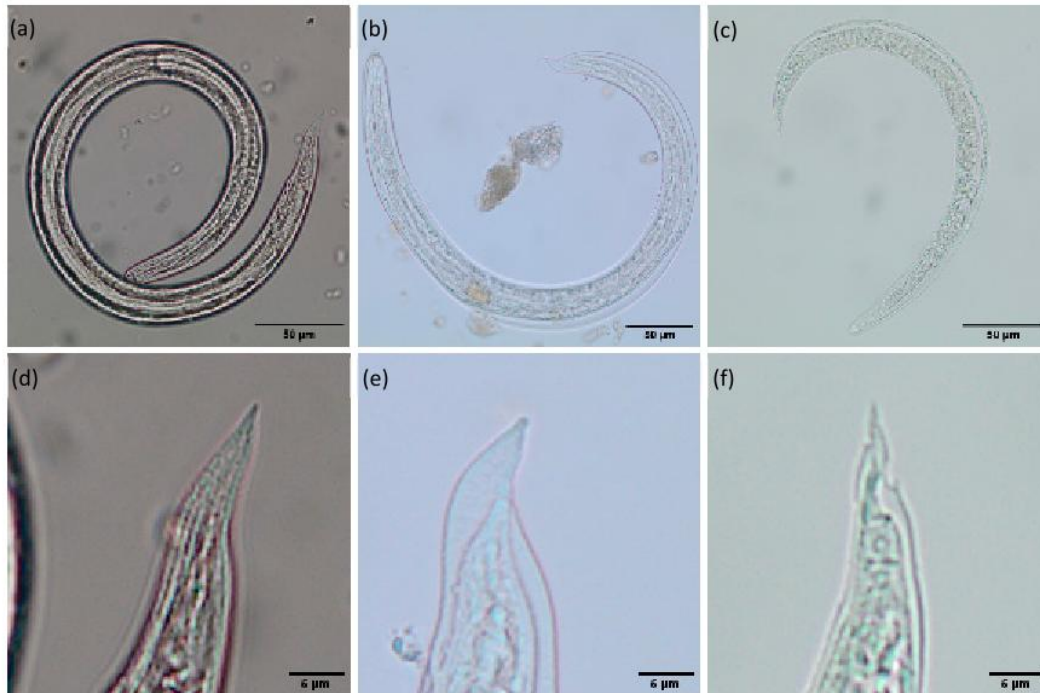


Figure 1. Morphology of metastrongyloid lungworm larvae. (a) *Crenosoma* sp. L3; (b) *Angiostrongylus vasorum* L3; (c) *Angiostrongylus vasorum* L1; (d) *Crenosoma* sp. L3 tail; (e) *Angiostrongylus vasorum* L3 tail; (f) *Angiostrongylus vasorum* L1 tail.

2.5. Molecular Identification of Lungworm Species

PCR was performed on the 10 highly positive samples using the nematode forward primer NC1 5'-ACGCTGGTTCAGGGTTGTT-3' and the reverse primers NC2 5'-TTAGTTTCTTTTCCTCCGCT-3' and MetR 5'-CCGCTAAATGATATGCTTA-3' [15,42,43]. The expected amplicon size was approximately 300 base pairs, targeting the ribosomal RNA (rRNA). Subsequently, 10 samples were sent to LGC Genomics, Berlin, Germany for sequencing. Thereafter, the samples were compared with deposited gene samples in GenBank via BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 10 July 2025)

2.6. Information for Veterinary Practices and Pet Owners

In order to spread awareness among pet owners walking their dogs in or near the hyperendemic focus, an information sign was mounted. The sign contained general information on lungworms, ways of infection, and possible symptoms. A website was created that could simply be accessed by scanning a QR code with a mobile device. The website included a questionnaire for dog owners posing the following questions: age, sex, and breed of the dog, potential stays abroad, anthelmintic status and regimen, diagnoses of lungworms, and related symptoms.

3. Results

In all four seasons, gastropods proved positive for metastrongyloid lungworm larvae with highly variable season-dependent prevalence. A total of 78/533 (14.63%) gastropods

were infected. *A. vasorum* was detected in 12.01% (64/533), *Crenosoma* sp. in 0.94% (5/533), and *Ae. abstrusus* in 0.38% (2/533) of gastropod samples. In summer, 27.46% (59/215) of gastropods were infected with metastrongyloid larvae. In fall, the prevalence dropped to 10.00% (16/160) and lowest infection rates were observed in both winter (5.65%) and spring (1.47%). In total, *A. vasorum* was detected in 12.01% (64/533), *Crenosoma* sp. in 0.94% (5/533), and *Ae. abstrusus* in 0.38% (2/533) of gastropod samples.

PCR was performed on a total of 10 of the infected gastropods in order to check PCR functionality as well as confirm microscopy results. *Crenosoma* sp. was confirmed in four, *A. vasorum* in three, *Ae. abstrusus* in two, and *C. striatum* in a single sample.

Overall, mostly Spanish slugs (*Arion* sp.) were discovered in the meadow (Figure 2). The weight of the gastropods ranged from 0.01 g to 10.11 g and showed an average weight of 4.02 g. In principle, a high prevalence of *A. vasorum* infection was re-confirmed in gastropods.

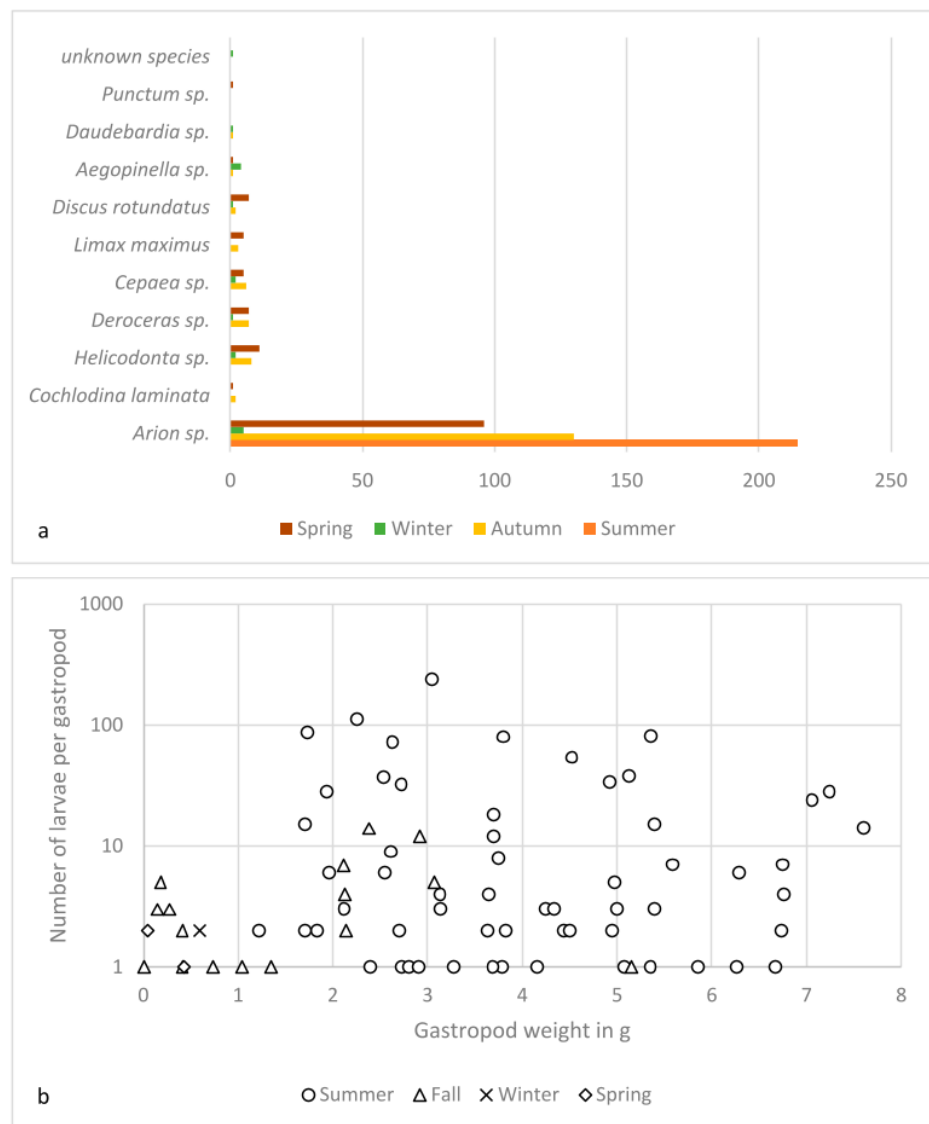


Figure 2. (a) Terrestrial gastropod species collected throughout the seasons; (b) correlation of gastropod weight and larval burden (log 10 scale).

In summer, a total of 27.46% (59/215) of gastropods were infected with metastrongyloid larvae; 22.79% (49/215) of slug samples proved positive for *A. vasorum* larvae (Table 1). Besides *A. vasorum*, *Crenosoma* sp. larvae were also detected (3/215) but at a much lower prevalence (1.40%). In addition, one slug was found positive for *Ae. abstrusus*. The larval burden (number of larvae observed in one individual gastropod) ranged from one to 241 larvae per slug (Figure 2).

Table 1. Metastrongyloid lungworm species prevalences in terrestrial gastropods.

	Spring	Summer	Fall	Winter
<i>A. vasorum</i>	0.74%	22.79%	8.7%	0%
<i>Crenosoma</i> sp.	0%	1.40%	1.2%	0%
<i>Ae. abstrusus</i>	0%	0.47%	0.6%	0%
Metastrongyloidea	0.74%	2.80%	0%	5.56%
Total	1.48%	27.46%	10%	5.56%

In fall, metastrongyloid lungworm species prevalence was considerably lower (Table 1). In total, 10% (16/160) of gastropod samples were positive for metastrongyloid lungworm larvae. *A. vasorum* larvae were diagnosed in 14 slugs, while only 2 slugs were found positive for *Crenosoma* sp. larvae, thus reaching prevalences of 8.7% and 1.2%, respectively. Additionally, *Ae. abstrusus* was observed in a single slug (1/160, 0.6%). In this season, a greater variety of gastropod species was collected (please refer to Figure 2a). While majority of infected gastropods belonged to *Arion* sp., a single leopard slug (*Limax maximus*) was also found positive for metastrongyloid lungworm larvae. The larval burden ranged from 1 to 14 larvae per slug. In addition to the prevalence and the larval burden lower, the average snail weight proved lower when compared to summer with an average gastropod weight of 0.7 g. In this season, a double infection was observed. One *Arion* sp. was infected by both *A. vasorum* and *C. striatum*.

In winter, snow was covering the meadow and gastropods were collected by digging through the snow close to trees and a pile of wood. Given these challenging conditions, a considerably lower number of gastropods ($n = 18$) with a great species diversity were collected. Here, a metastrongyloid lungworm larva was detected only in a single *Deroceras* sp. slug, thus indicating a lungworm prevalence of 5.56%. Unfortunately, the species of this larva could not be identified microscopically. Thus, the larval burden was very low. The average weight of collected gastropods in winter was low as well with 0.6 g.

In spring, a total of 136 gastropods of nine different species were collected at the hotspot meadow. Two of these gastropods were found infected with metastrongyloid lungworm larvae (2/136, 1.48%). Thus, a single *Arion* sp. slug proved infected with metastrongyloid larvae that could not be identified. One *Helicodonta* sp. snail contained a single *A. vasorum* L3. Hence, the larval burden in spring ranged from one to three larvae per gastropod. The gastropods collected in spring were light, with an average weight of 0.45 g. Of note, one gastropod was co-infected with two lungworm species, i.e., *A. vasorum* and *C. striatum*. Triple infections, as well as co-infections with *Ae. abstrusus* and *C. vulpis* were not observed.

The larval burden was highest in summer, with one slug carrying a mean of 77.07 larvae per g bodyweight (BW). The lowest burden was observed in summer as well, with 0.15 larvae per g BW. There was no clear correlation between gastropod BW and larval burden (Figure 2). Most gastropods (56/77) harbored a relatively low larval burden with less than 10 larvae per individual. Very high numbers of larvae (more than 100) were discovered only in two gastropods.

Microscopic species determination in the 10 highly infected gastropods was confirmed by PCR and sequencing (Table 2) according to Segeritz et al. and Ash et al. [15,41]. No differences between the microscopic analysis and PCR was observed.

Table 2. Molecular identification of lungworm larvae species from Obrigheim.

Species	Accession number	Season
<i>A. vasorum</i>	PV917150	Summer
<i>Crenosoma</i> sp.	PV917152	Summer
<i>A. vasorum</i>	PV917158	Summer
<i>Ae. abstrusus</i>	PV917161	Summer
<i>A. vasorum</i>	PV917159	Summer
<i>C. striatum</i>	PV917154	Summer
<i>Crenosoma</i> sp.	PV917156	Summer
<i>Ae. abstrusus</i>	PV917171	Fall
<i>Crenosoma</i> sp.	PV917167	Fall
<i>Crenosoma</i> sp.	PV917170	Fall

The analysis of the water collected in spring revealed the presence of a single metatstrongyloid lungworm larva.

Three veterinary practices around the city of Obrigheim were called and informed about the hyperendemic area but showed little to no interest. The questionnaire linked to the sign mounted on the meadow was filled out by a total of nine pet owners, but none of them reported on a history of lungworm infections in their dogs.

4. Discussion

This study reconfirms Obrigheim as a hyperendemic focus for *A. vasorum* as well as other lungworm species infections in gastropod intermediate hosts as previously reported [15]. The reasons for this meadow being a ‘hotspot’ area may be influenced by various environmental/ecological factors, such as diverse flora (e.g., grassland, trees, fungi, and bushes) and fauna (i.e., mollusks, arthropods, amphibians, reptiles, birds, rodents, and beavers), a stream as water body, and ideal humid climatic conditions throughout the year. Even in very hot and dry summer conditions (i.e., in August 2022), we observed that the meadow still was lush and green. A small stream, called Heiligenbach, runs through the meadow and is surrounded by trees. The stream even has a dam constructed by native beavers. A larger forested area is also located not far from this meadow. All these geographical and ecological conditions also render this meadow ideal for wildlife. As mentioned before, wild canids and wild felids might play a role in the transmission of lungworms to domestic animals [13,31]. While wild cats and Eurasian lynxes are not known to reside nearby, foxes are prevalent all throughout Germany, including Obrigheim. Synanthropic foxes might also defecate on this meadow, thereby infecting gastropods, and the life cycle may spill over to domestic dogs. During sampling, many dog owners were observed walking their dogs on the small path nearby and in the meadow itself.

This study highlights the extreme fluctuating lungworm prevalence in gastropod intermediate hosts. The prevalence of lungworm larvae in gastropods not only varies

drastically in different seasons but also fluctuates between the years. A total gastropod lungworm prevalence of 27.46% was observed in summer. This stands in contrast to the previous study by Segeritz et al. [15], which documented a total prevalence of 17.6% during the summer months. In 2018, the highest prevalence in gastropods was observed in fall with 75.93% [15], thereby contrasting current findings in the same season of 2022, where the total prevalence was only 10%. The higher prevalence in the summer of 2022 may be linked to a different collection time as well as gastropod species collected. Based on the life cycle of *Arion* sp., it is quite logical to find larger and heavier slugs in the summer months. In this season, these slug species tend to reach their maximal length and weight before mating, laying eggs and dying in the late summer months [44–46]. Even though it is possible for multiple slug generations to be formed within a year, a single-generation cycle is more common. Thus, the slugs collected in fall either represented a second and smaller adult generation or the newly hatched juvenile slugs. Of note, most gastropods collected in summer were large *Arion* sp. slugs. Their weight ranged from 0.01 g to 10.11 g with an average weight of 4.02 g. As suggested in previous studies [47,48], there may be a correlation between gastropod maturity and size and even burden of infection. While we were unable to confirm the correlation between gastropod weight and burden of infection, it is quite likely that older gastropods are more prone to being infected, since larger slugs feed on a larger feces volume than smaller slugs, and thus the likelihood of infection may increase. In fall, the gastropods collected and analyzed were generally smaller. The average weight of these gastropods was 0.7 g. The *Arion* sp. slugs collected in October most likely represented, due to their small size, the new generation that hatched later in the year. As such, they did not consume as much feces as their older and larger counterparts. In addition, higher species diversity was observed in fall. This should also be taken into account while comparing the data between the years 2018 and 2022. During winter, *Arion* sp. are known to survive through the juvenile life stages. Other gastropod species, such as the white-lipped snail *Cepaea hortensis*, survive even freezing temperatures by adjusting their metabolic rate [49]. As such, metastrongyloid lungworm larvae are also able to survive the winter inside gastropod intermediate hosts and thereby preserve hyperendemic focus until the gastropods become more active in spring again. As such, the persistence of the life cycle of lungworm infections is possible throughout the seasons and leads to a new cycle in the next year, as reported elsewhere [50,51].

Furthermore, temperature and rainfall play a pivotal role in both survival and multiplication of snails, semi-slugs, and slugs. The summer of 2018 in Germany was extremely hot and dry [52], which influenced both flora and fauna. In these hot and dry climatic conditions, snails and slugs are less active in both reproduction and mobility. These ecological conditions may explain the higher lungworm prevalence in summer of 2022. The lower prevalence in fall of 2022 may also be explained by the higher species diversity. Generally speaking, as mentioned before, *Arion* sp. slugs are described as highly suitable intermediate hosts for metastrongyloid lungworm species. The largest numbers of both collected and infected gastropods were indeed *Arion* sp. This might be linked to multiple factors, such as a potential higher prevalence of those slugs compared to other gastropod species in Obrigheim, or simply by their morphology, which facilitated their visual detection, due to their orange color and size. These invasive slugs are also reported as spreading worldwide. This slug species was reported to be transported passively, for example, in animal fur, clothing, and vehicles [45], and thus to transfer endogenous lungworms, acquiring new habitats. In general, *Arion* sp. were previously described as suitable obligate intermediate hosts for lungworm larvae [2,40]. During field gastropod collections, *Arion* sp. were frequently found feeding on dog feces thereby confirming coprophagic activities [53]. Other

gastropods are less likely to feed on feces, since they prefer other feed, such as a variety of plants, fungi, and seedlings, as well as mycelium [54,55].

Furthermore, the invasive slug *Arion vulgaris* may facilitate parasite spillover from wildlife to domestic animals by increasing environmental contamination with infective larvae. A high abundance of *A. vulgaris* may also cause parasite transmission and facilitate spillback by increasing endemic lungworm infections re-entering wildlife populations. Alternatively, having a range of non-competent or less suitable gastropod hosts present, along with *A. vulgaris*, may have dilution effect to reduce transmission pressure.

In this study, we also found *L. maximus*, *Helicodonta* sp., and *Deroceras* sp. positive for lungworm larvae, even though at low numbers. The slug *L. maximus* is known for cannibalism [56], and the possibility should not be overlooked that this slug species might become infected with metastrongyloid lungworms by feeding on other infected snails or slugs. The number of infected slugs may even increase in this theory when multiple slugs feed on a single infected carcass.

Another important factor to consider in changing prevalences is the behavior of the definitive hosts and pet owners. During cold or rainy periods, they are less likely to be walked in the meadow. In summer months with warm and sunny conditions, pet owners are more likely to go outside for longer walks with their dogs on and/or around this area. Since the prevalence in summer was observed to be higher than in other seasons, the likelihood of both definitive and thus also intermediate host infection is increasing in summer. Not only is it possible for dogs to consume gastropods willingly, but they may also gorge smaller slugs and snails accidentally, for example, while eating grass or feeding on rotten fruits and vegetables. The smallest infected slug we found weighed only 0.01 g. As such, it seems very feasible that a dog or a cat might ingest such tiny gastropods purely by accident. The hunting and consuming of paratenic hosts, such as rodents, reptiles, birds, and frogs, is also an important way of infection, particularly for feline aelurostrongylosis [57,58]. Multiple frogs were observed during the summer sample collection. In addition, while sampling at the meadow, we found dog feces in multiple locations in all seasons. This lack of hygiene by dog owners might additionally contribute to the persistence of this hyperendemic focus in Obrigheim. Since German dog owners travel frequently with their pets, patent lungworm infections may be spilled-over by infected animals into new areas where lungworm infections were not yet prevalent.

Even though we retrieved only a few responses to the questionnaire, it is interesting to notice that none of the pet owners reported on lungworm infections in their dogs. This might be due to multiple reasons. Lungworm infections are not a primary focus in veterinary practices and clinics. Additionally, in order to detect lungworm larvae, a Baermann–Wetzel apparatus needs to be used, since they cannot be detected via flotation in most cases [59]. Furthermore, many lungworm infections are subclinical and with intermittent larval excretion. Moreover, in case of clinical signs like apathy, lethargy, coughing, or gastrointestinal symptoms, these may be unspecific and eventually not be linked to lungworm infections [3,4]. In addition, it is still common practice in Germany to treat dogs with anthelmintics on a regular basis without verifying a potential parasitic infection, for example, once every three months. Commonly used anthelmintic drugs, such as fenbendazole and moxidectin, are effective against multiple species of endoparasites, lungworms included [60,61]. As a result of periodical treatments with endoparasiticides, lungworm infections may be reduced. Nevertheless, it is of utmost importance to raise awareness to potential lungworm infections in both veterinary practitioners and pet owners.

The finding of a single metastrongyloid lungworm larva in the water collected at the meadow indicated that larval survival in water seems in principle possible. It has been described for the zoonotic *Angiostrongylus cantonensis* to actively leave deceased gastropod

intermediate hosts and to survive exogenously as L3 for some weeks [15,39,62]. This L3 survival capacity reveals most likely novel transmission routes for definitive hosts by drinking water from a puddle with a dead but metastrongyloid-infected gastropod.

In this study, PCR was used as an alternative method for metastrongyloid lungworm detection; however, its limitations need to be considered. PCR from digested snail tissue is generally challenging due to various inhibitors, such as mucine and digestive enzymes (endonucleases, proteases) as well as the digestive solution itself [40,63]. Bacteria present in the snail's mucus can also inhibit PCR due to cell wall fragments and nucleases [64]. In addition, low parasite burdens may further complicate the procedure, since the sample can be easily lost during DNA extraction. As such, the PCR technique may not be sensitive enough to detect infections if the parasite burden is too low. Furthermore, no conclusions can be drawn on the parasitic burden, the vitality of the larvae, or the successful development into L3 larval stages.

5. Conclusions

This study reconfirms Obrigheim as a stable hyperendemic focus of Southern Germany for metastrongyloid lungworm infections in gastropods. These epidemiological findings suggest that environmental factors may play a crucial role in the varying prevalence throughout the year. Further research is required to analyze the influences of climatic change and other conditions on the prevalence of lungworm larvae in gastropods and thus the infection risk for definitive hosts.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pathogens14080800/s1>, Figure S1: Sampling conditions throughout the year (a) Summer; (b) Fall; (c) Winter; (d) Spring, flooded meadow. Figure S2: Gastropod species observed in Obrigheim (a) *Limax maximus*; (b) *Helicodonta obvolvata*; (c) *Cepaea* sp.; (d) *Arion* sp. (juvenile).

Author Contributions: Conceptualization, C.H. and A.T.; methodology, C.H. and A.T.; software, A.D. and L.S.; validation, A.D. and L.S.; investigation, A.D. and L.S.; resources, J.S., C.H. and A.T.; data curation, L.S. and A.D.; writing—original draft preparation, A.D.; writing—review and editing, A.D., J.S., C.H., L.S. and A.T.; visualization, A.D. and L.S.; supervision, C.H. and A.T.; project administration, C.H. and A.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained in this article.

Acknowledgments: We are deeply grateful to Christine Henrich (JLU Giessen) for her contribution to the PCR analysis. Furthermore, we thank everyone who helped in the gastropod collection: Honorine Mireille Nguete Ampama, Seyed Sajjad Hasheminasab, Manuel Uribe, Marcel Haas, Charlotte Scriba, Dorin Quernes, Piero Vitolo-Sosa, and Alicia Riedinger.

Conflicts of Interest: The authors declare no conflicts of interest.

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3.2 Organ Tropism of *Angiostrongylus vasorum* Larval Stages in Infected African Giant Snails (*Lissachatina fulica*)

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Pathogens 2024, 13, 946. <https://doi.org/10.3390/pathogens13110946>

Eigener Anteil an der Publikation:

- Projektplanung: 20 %, zusammen mit Betreuern
- Durchführung Versuche: 40 %, zusammen mit Ko-Autoren
- Auswertung der Experimente: 80 %, weitgehend eigenständig
- Erstellung des Manuskripts: 80 %, weitgehend eigenständig

Article

Organ Tropism of *Angiostrongylus vasorum* Larval Stages in Infected African Giant Snails (*Lissachatina fulica*)

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Abstract: *Angiostrongylus vasorum* is a metastrongyloid lungworm causing severe cardiovascular disease in domestic and wild animals. During its heteroxenous life cycle, *A. vasorum* requires obligate gastropod intermediate hosts. Little is known about *A. vasorum* larval organ tropism and development in gastropod intermediate hosts. Thus, the aim of this study was to analyze in vivo development of *A. vasorum* larval stages in experimentally infected African giant snails (*Lissachatina fulica*). Adult *L. fulica* ($n = 26$) were orally infected with *A. vasorum*-L1 and thereafter continuously euthanized. Gastropod organs were artificially digested and microscopically analyzed for the presence of *A. vasorum* larvae. Moreover, paraffin-fixed organs were investigated histologically for snail-borne innate immune response. In the current study, the success of *L. fulica* oral infection was demonstrated, thereby reaching larval recovery rates of up to 49.7%. During snail infection, an organ tropism of *A. vasorum* larvae was detected for the lungs and the foot muscular tissue. Overall, *A. vasorum*-driven gastropod innate immune reactions against larvae varied greatly. In some specimens, larvae were found effectively ensnared by recruited hemocytes, resulting in granuloma formation, whilst in others, hemocyte-mediated reactions were barely observed. Nevertheless, these evidences demand more studies on hemocyte-derived effector mechanisms against *A. vasorum*.

Keywords: *Angiostrongylus vasorum*; *Lissachatina fulica*; innate immunity; hemocytes; organ tropism



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Citation: Dusch, A.; Segeritz, L.; Henrich, M.; Taubert, A.; Hermosilla, C. Organ Tropism of *Angiostrongylus vasorum* Larval Stages in Infected African Giant Snails (*Lissachatina fulica*). *Pathogens* **2024**, *13*, 946. <https://doi.org/10.3390/pathogens13110946>

Academic Editor: Anastasia Diakou

Received: 26 September 2024

Revised: 14 October 2024

Accepted: 24 October 2024

Published: 30 October 2024



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1. Introduction

Angiostrongylus vasorum (Baillet, 1866) is a metastrongyloid lungworm that resides in the cardiovascular system of both domestic and wild canids, including dogs (*Canis familiaris*), wolves (*Canis lupus*) [1,2], golden jackals (*Canis aureus*) [3,4], and red foxes (*Vulpes vulpes*) [5–9], with the latter representing important wildlife reservoirs in Europe. Further definitive hosts include striped skunks (*Mephitis mephitis*), weasels (*Mustela erminea*), ferrets (*Mustela putorius furo*), badgers (*Meles meles*), neotropical otters (*Lontra longicaudis*), raccoon dogs (*Nyctereutes procyonoides*), and red pandas (*Ailurus fulgens*) [10–14]. Due to the rising urbanization of many of these wild animals, the risk of *A. vasorum* transmission to domestic dogs and vice versa is increasing [1,15]. This metastrongyloid parasite can be found on multiple continents and generally shows a patchy epizootiological distribution, with hyper- and hypoendemic foci in close proximity [16,17]. Clinical signs of *A. vasorum* infections in dogs may vary from mild respiratory symptoms to severe cardiopulmonary disorders but also include hemorrhage and neurological, ocular, and gastrointestinal symptoms [13,18–22].

The life cycle of *A. vasorum* is heteroxenous, with terrestrial gastropods acting as obligatory intermediate hosts. Thus, canine angiostrongylosis is considered a gastropod-borne parasitosis with a complex epizootiology, including various slug, semi-slug, and snail species as suitable intermediate hosts [15,23,24]. By feeding on the feces of an *A.*

vasorum-infected definitive host, gastropod intermediate hosts become infected with first-stage larvae (L1). Another possibility is the active penetration of the gastropod epidermis by exogenous L1 [25,26]. While migrating in the mollusc intermediate hosts, *A. vasorum* L1 must molt into second-stage (L2) and third-stage larvae (L3). The canid definitive host will become infected by consuming either an *A. vasorum*-infected intermediate host carrying L3 or by a paratenic host (PH; e.g., amphibians, birds) hosting infectious L3. Alternatively, spontaneous shedding of *A. vasorum* L3 from dead intermediate hosts can occur, which are capable of surviving in the environment for some days or weeks, depending on the climatic condition [27]. As already stated, a wide spectrum of gastropod species may act as intermediate hosts, including the terrestrial African giant snail (*Lissachatina fulica*) (Bowdich, 1822) [28–30].

This gastropod species is considered one of the largest terrestrial snails, and its shell is cone-formed with an average length of 90 mm and contains a minimum of seven whorls in fully grown snails. The shell is medium brown and decorated with darker and lighter stripes, and the tip is usually lighter in color [31,32]. Originally native to East Africa, this terrestrial giant snail is now considered an invasive species (i.e., neozoa) in multiple tropical and subtropical countries, such as Colombia, the United States of America, Brazil, Ecuador, Indonesia, and India [11,33,34]. Furthermore, *L. fulica* has been identified as a natural intermediate host not only for *A. vasorum* but also for *Aelurostrongylus abstrusus*, *Crenosoma vulpis*, and *Troglostrongylus brevior* in the tropics of South America, thereby representing a risk factor for metastrongyloid transmission for both domestic and wild canids/felids [28]. Based on both the invasive nature of *L. fulica* and the emergence of canine angiostrongylosis as a cardiopulmonary disease in various continents, it is of great interest to gain further knowledge on *A. vasorum* L1 migration and organ tropism in *L. fulica* [35], which currently is still not fully understood. Several studies have shown that, after cutaneous or oral gastropod infection, *A. vasorum* L1 must migrate inside the mollusc to find adequate organs for fulfilling further development into L2 and, finally, infective L3 [36–38]. During this larval migration in vivo, *A. vasorum* larvae will be exposed to the gastropod host innate immune system, mainly composed of hemocytes present in the hemolymph, which might not only influence migration routes but also hamper larval development. As opposed to mammals, which own both an innate and an adaptive immune system, gastropods exclusively possess an innate immune system. Hematopoiesis in molluscs is complex and less studied when compared to other metazoan species [39]. Gastropod hematopoiesis leads to the generation of hemocytes (syn. amoebocytes) in hemolymph circulation, tissues, and organs [40,41]. Gastropod hemocytes constitute the main cellular component of hemolymph but, in addition, serve as resident cells at other sites, such as connective and vascular tissues [39,42,43]. Invertebrate hemocytes have multiple functions, such as the transport of molecules, wound repair, coagulation, and defense against invasive pathogens [41]. Hence, hemocytes show diverse effector mechanisms to fight invasive pathogens, such as phagocytosis [44], cell-mediated cytotoxicity, production of reactive oxygen species (ROS), and encapsulation [45]. More recently, also the release of gastropod invertebrate extracellular phagocyte traps (InEPT) against metastrongyloid larvae, including *A. vasorum*, has been reported, which is an effective immune response against invading L1 [40,41,46].

While several histological studies on hemocyte-derived innate immune reactions against nematode larvae exist, much less is known on metastrongyloid lungworm organ tropism [36,47,48], especially on gastropod intermediate hosts being experimentally infected under standardized laboratory conditions, including humidity, light, and temperatures mimicking natural field conditions [36,49]. The aim of this study was, therefore, to gain knowledge on *A. vasorum* larval migration in the gastropod intermediate host *L. fulica*, including organ tropism, with selected histological analyses to tackle hemocyte-mediated innate responses against invasive larvae in vivo.

2. Material and Methods

2.1. Gastropod Maintenance Under Standardized and Parasite-Free Conditions

German-bred African giant snails (*L. fulica*) were kept in a full-automatized climate chamber (ECP01E, Snijders Scientific B.V. Tilburg, the Netherlands) under standardized laboratory conditions with humidity and temperatures mimicking the gastropod's natural habitat by temperatures ranging from 20 to 26 °C and 50% humidity, according to Penagos-Tabares et al. (2018). The light cycle consisted of 10 h of light and 10 h of darkness, in between 2 h for dawn and 2 h for dusk. On the ground of plastic boxes for gastropod maintenance, a layer of coconut soil (Kokosfaser-Humusziegel, TropicShop, Germany) of approximately 5 cm thickness was used. Exclusively parasite-free newly bred *L. fulica* (i.e., F1 and F2 generations) were used for *A. vasorum* infections. These F1/F2 *L. fulica* were fed with lettuce (*Lactuca sativa*), carrots (*Daucus carota*), cucumber (*Cucumis sativus*), zucchini (*Cucurbita pepo*), and commercial dog food (Premium Trockenfutter Romeo, Germany). In order to raise the pH of the soil, garden lime was mixed into the coconut soil. Furthermore, common cuttlefish (*Sepia officinalis*) internal shells (Sepiaschalen, TropicShop, Germany) were offered ad libitum to *L. fulica* as a natural source of calcium.

2.2. Isolation of Vital *Angiostrongylus Vasorum* L1 and Gastropod Oral Infection

Vital first-stage larvae (L1) were isolated from feces of naturally *A. vasorum*-infected dogs (kindly provided by the Institute of Parasitology at the University of Veterinary Medicine Hannover, Hannover, Germany) via the modified Baermann funnel technique, according to Conboy et al. [50]. After 24 h of incubation, 7 mL were obtained from each funnel apparatus, quantified microscopically (Olympus BH-2), and pelleted (200× g, 5 min) to collect vital *A. vasorum*-L1. *L. fulica* ($n = 26$) were orally infected with a dose of 1000 larvae each. In order to be as close as possible to field conditions, the following procedure was used for infection: since gastropods are coprophagic, the remaining fecal solution from the Baermann funnels was used as a suspension medium for the larvae. Thus, 50 mL of diluted canine fecal solution (DCFS) containing 1000 motile larvae were orally fed to the snails via a syringe until complete consumption (please refer to Video 1).

2.3. Gastropod Euthanasia and Organ Isolation

A. vasorum-infected *L. fulica* snails were cryo-euthanized in an ice bath at 4 °C, as reported previously by Lange et al. [51], at different time points post infection (p.i.; please refer to Supplementary Table S1). Following euthanasia, the snails were dissected, and the following 11 organs/tissues were isolated: crop/esophagus, stomach, intestine, hepatopancreas, reproductive tract, albumen gland, foot, kidney, lung, head, and heart. Four gastropods dissected at 5, 14, 29, and 47 days p.i. were used for histological analyses on the different tissues and organs. The organs of these four snails were fixed in 10% neutral-buffered formalin for further histological analyses.

2.4. Artificial Digestion and Identification of Larvae

A total of 22 of the dissected snails were further processed by artificial digestion. Snail single organs and tissues were artificially digested, according to Lange et al. [51]. Afterward, the remains were microscopically analyzed applying 40×, 200×, and/or 400× magnification (Olympus BH-2). To differentiate *A. vasorum* larvae (e.g., L1, L2, L3) from free-living or parasitic larvae of gastropods (genus *Phasmarhaptidis*), the type and length of the esophagus (e.g., non-rhabditiform, 1/2–1/3 of total larvae length), the tail form, and the length and width of the larvae were analyzed [52,53].

2.5. Histology

In total, 11 organs and tissues of four euthanized snails were used for histological analysis. As a first step, the snail's shell was carefully removed. Thereafter, the inner organs were separated and fixed in 10% buffered formalin and embedded in paraffin wax by standard techniques. Tissue/organ sections of 5 µm thickness were cut from

paraffin-embedded specimens and stained following the standard hematoxylin and eosin (HE) protocol for histological examination. HE staining was performed at the Institute of Pathology at the Faculty of Veterinary Medicine, Justus Liebig University Giessen, Giessen, Germany. The tissue slides were analyzed using an optical light microscope (Olympus BH-2) equipped with a digital camera (Olympus SC30) at the Institute of Parasitology of the Justus Liebig University Giessen, Germany.

3. Results

3.1. *Angiostrongylus Vasorum* Larval Development and Organ Tropism in Experimentally Infected *L. fulica* Snails

In order to investigate in detail *A. vasorum* larval organ tropism and development in vivo, single organs of *L. fulica*-infected snails were analyzed for the presence of different larval developmental stages. Overall, all 26 experimentally infected *L. fulica* proved positive for *A. vasorum* larvae. Even though all snails had obtained the same infection dose (1000 L1), different larval recovery rates per animal were observed when summing up all larvae found in one specimen. Thus, the overall larval recovery rates ranged from 1.8% to 49.7% (Table 1).

Table 1. Larval stages of *Angiostrongylus vasorum* in *Lissachatina fulica* tissue.

	OC	SPI	HP	RT	AG	FT	KD	LU	DI	MA	HD	HT	RR
10 min	L1	L1	L1	n.l.d.	n.l.d.	L1	n.l.d.	L1	L1	L1	L1	n.l.d.	10
30 min	L1	L1	L1	n.l.d.	n.l.d.	L1	L1	L1	n.l.d.	L1	L1	n.l.d.	4.8
60 min	L1	L1	L1	n.l.d.	n.l.d.	L1	n.l.d.	L1	n.l.d.	L1	L1	n.l.d.	18.3
90 min	L1	L1	L1	n.l.d.	n.l.d.	L1	n.l.d.	L1	n.l.d.	n.l.d.	L1	n.l.d.	8.8
1 day	L1	L1	L1	n.l.d.	n.l.d.	L1	L1	n.l.d.	L1	L1	L1	n.l.d.	9.6
1 day	L1	L1	L1	n.l.d.	n.l.d.	L1	L1	L1	L1	n.l.d.	n.l.d.	n.l.d.	7.1
4 days	n.l.d.	L1	L1	n.l.d.	n.l.d.	L1	L1	L1	L1	n.l.d.	L1	n.l.d.	4.1
8 days	L1	L1, L2	L1, L2	L2	n.l.d.	n.l.d.	L1, L2	L1, L2	n.l.d.	L1	L1, L2	L1	15.8
11 days	L1	L2	L1	L1	n.l.d.	L1, L2	n.l.d.	L2	n.l.d.	n.l.d.	L1	n.l.d.	2.1
11 days	L2	L2	n.l.d.	n.l.d.	n.l.d.	L1	L2	L2	n.l.d.	n.l.d.	L1, L2	L3	1.8
19 days	L3	L1, L2, L3	L1, L2, L3	n.l.d.	n.l.d.	L1, L2, L3	L1, L2, L3	L2, L3	L2	n.l.d.	n.l.d.	n.l.d.	10.2
20 days	n.l.d.	L2, L3	L1, L2, L3	L2	n.l.d.	L1, L2	L2	L2, L3	n.l.d.	L2	L1, L2	L2, L3	11.4
25 days	n.l.d.	L2, L3	n.l.d.	n.l.d.	n.l.d.	L1, L2, L3	L2, L3	L3	n.l.d.	L3	L1, L2, L3	n.l.d.	2.7
25 days	n.l.d.	L2, L3	L1, L2	n.l.d.	n.l.d.	L1, L2, L3	L3	L3	n.l.d.	L3	n.l.d.	L3	10.9
35 days	n.l.d.	L2, L3	L2, L3	L2, L3	n.l.d.	L2, L3	n.l.d.	L2, L3	n.l.d.	L2, L3	L2	L2, L3	11.6
35 days	n.l.d.	n.l.d.	L2, L3	L2	n.l.d.	L1, L2, L3	L1, L2, L3	L2, L3	L3	L3	L2, L3	L2, L3	13.9
42 days	L3	L3	L2, L3	L3	L3	L2, L3	L3	L3	L3	L3	L3	n.l.d.	40
42 days	n.l.d.	n.l.d.	L2, L3	L3	n.l.d.	L2, L3	L3	L3	L3	L3	L2, L3	L3	10
49 days	L3	L3	L3	L1	n.l.d.	L1, L2, L3	L1, L3	L1, L3	L3	L2, L3	L1	L3	14.2
49 days	n.l.d.	n.l.d.	L1, L2, L3	L3	n.l.d.	L1, L2, L3	L3	L2, L3	L3	L2, L3	L2, L3	n.l.d.	29.6
56 days	L3	L3	L3	n.l.d.	n.l.d.	L1, L2, L3	L3	L3	n.l.d.	L3	L3		26.9
56 days	L2, L3	L3	L3	n.l.d.	n.l.d.	L3	L3	L3	L3	L3	L3	L3	49.7

OC: esophagus and crop, SPI: stomach and proximal intestine, HP: hepatopancreas, RT: reproduction tract, AG: albumen gland, FT: foot, KD: kidney, LU: lung, DI: distal intestine, MA: mantle, HD: head, HT: heart, RR: recovery rate in %. Color coding: number of larvae found in the organs: red > 20, pink: 5–20, light pink: < 5. n.l.d.: no larvae detected.

Referring to the larval development within *L. fulica* snails, L1 stages started to disappear with day 8 p.i. ongoing; in parallel, L2 stages were first detected at 8 days p.i. (Figure 1). The proportion of recovered L2 stages peaked at day 20 p.i. and thereafter declined until the end of the investigation period (56 days p.i.). The first L3 stages were

found at 11 days p.i., and the proportion of L3 in snails increased from this time point onwards; consequently, at day 56 p.i., most of the larval stages had developed into L3 (see Figure 1). Of note is that the speed of larval development varied between individual *L. fulica*. Hence, even after 49 days p.i., some larvae had not developed into the infective L3 stage (please refer to Figure 1 and Table 1). However, when analyzing *L. fulica* long-term infections of two years p.i., vital *A. vasorum* L3 were still found in all four cryo-euthanized snails, even though recovery rates had considerably dropped to 0.1–4.4% after this long period (Table 2).

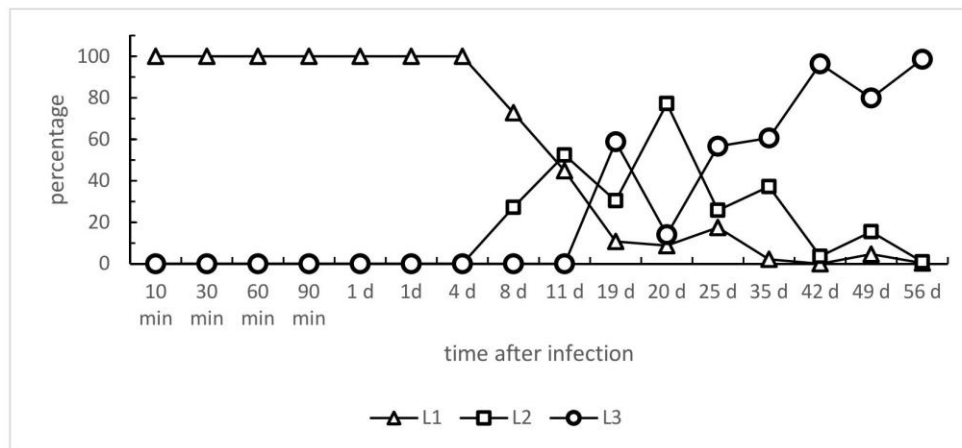


Figure 1. *Angiostrongylus vasorum* larval development in experimentally infected *Lissachatina fulica*.

Table 2. Larval stages of *A. vasorum* in *Lissachatina fulica* tissues after long-term infection (2 years).

	MA, HT	RT	AG	HD	KD	FT	OC	HP	ST	LU	IR
2 y	L3					L3				L3	0.8
2 y					L3	L3					0.2
2 y			L3			L3	L3	L3	L3	L3	4.4
2 y						L3					0.1

MA, HT: mantle and heart, RT: reproduction tract, AG: albumen gland, HD: head, KD: kidney, FT: foot, OC: esophagus and crop, HP: hepatopancreas, ST: stomach, LU: lung, IR: infection rate in %, Color coding: number of larvae found in the organs: red > 20, pink: 5–20, light pink: < 5. n.l.d.: no larvae detected.

Analyses on parasite dispersion in *L. fulica* snails indeed revealed a marked tropism of *A. vasorum* larvae for specific organs and tissues, dependent on the time point of infection. Thus, the overall majority of larvae were found in lung and foot (muscles) tissues, while other organs such as the head, heart, albumen gland, crop-esophagus, intestine, kidney, and reproductive tract contained only a few larval specimens (Table 1). Referring to larval lung tropism, an interesting finding was observed here. The lungs of one *A. vasorum*-infected snail showed macroscopically visible, multiple calcified granulomas, most likely reflecting effective hemocyte-mediated innate immune reactions against invasive *A. vasorum* larvae (Figure 2).

Of note, *A. vasorum* L1 stages rapidly migrated in experimentally infected *L. fulica*, reaching distant organs already within 10 min after oral infection. In addition, the overall L1 migration highly differed between individual snails. As such, at 10 min p.i., 28% and 21% of L1 larvae were still located in the esophagus and stomach, respectively, while 24% were already found in the foot and 13% in the head. At 30 min p.i., most L1 larvae were localized in the foot (50%); 18.75% were found in the head and in the lungs. At 60 min p.i., a larger proportion of L1 was located in the hepatopancreas (36.07%), in the foot (22.4%), and in the head (15.85%). At 90 min p.i., 32%, 25%, and 12% L1 were located in the foot, head, and

hepatopancreas, respectively. In one *A. vasorum*-infected snail, most L1 was detected in the foot (60.42%) at 1 day p.i., while only 19.72% of L1 larvae were located in the foot in another snail harboring 49.30% in the lung instead. Four days p.i., most larvae were observed in the lung (39.02%). Nonetheless, the head (17.07%), foot, and hepatopancreas (both with 12.20%) also contained relatively high proportions. At later time points, e.g., 8 days p.i., a high proportion of L1 was found in the hepatopancreas (41.77%), lung (27.85%), and stomach (17.72%). For the first time, L2 larvae were observed, but without any preference for specific organs. At 11 days p.i., most larvae were located in the lung (36.51%) and foot (17.06%). On this day, the first L3 larval stage was observed, being located in the heart. Of note, two-thirds of the observed larvae were still in the L2 stage. At 19 days p.i., 43.14% and 27.45% of the larvae were located in the lung and hepatopancreas, respectively. It can be noted that in the lung, the vast majority of larvae were L3 stages (40/44), while most larvae in the hepatopancreas were still L2 stages (18/28). At 20 days p.i., more than half of the larvae were found in the lung (55.26%), besides hepatopancreas (21.93%). In line, a high proportion of larvae was observed in the lung (34.09%) and foot (32.18%) at 25 days p.i. Here, the majority of larvae (86/136) had developed into L3 stages. Equally, at 35 days p.i., the lung (46.76%) and foot (29.90%) harbored most of the larvae, with more than half of the larvae (59.76%, 153/256) representing L3 stages. At 42 days p.i., larvae were mainly found in the lung (33.88%), hepatopancreas (17.13%), and stomach (17.13%). L1 larval stages were no longer observed, and almost all larvae were in the L3 stage (97.8%; 489/500). At 49 and 56 days p.i., mainly L3 stages (98.9%) were located in the lung (36.85% and 47.76%, respectively) and foot (45.70% and 25.52%, respectively).

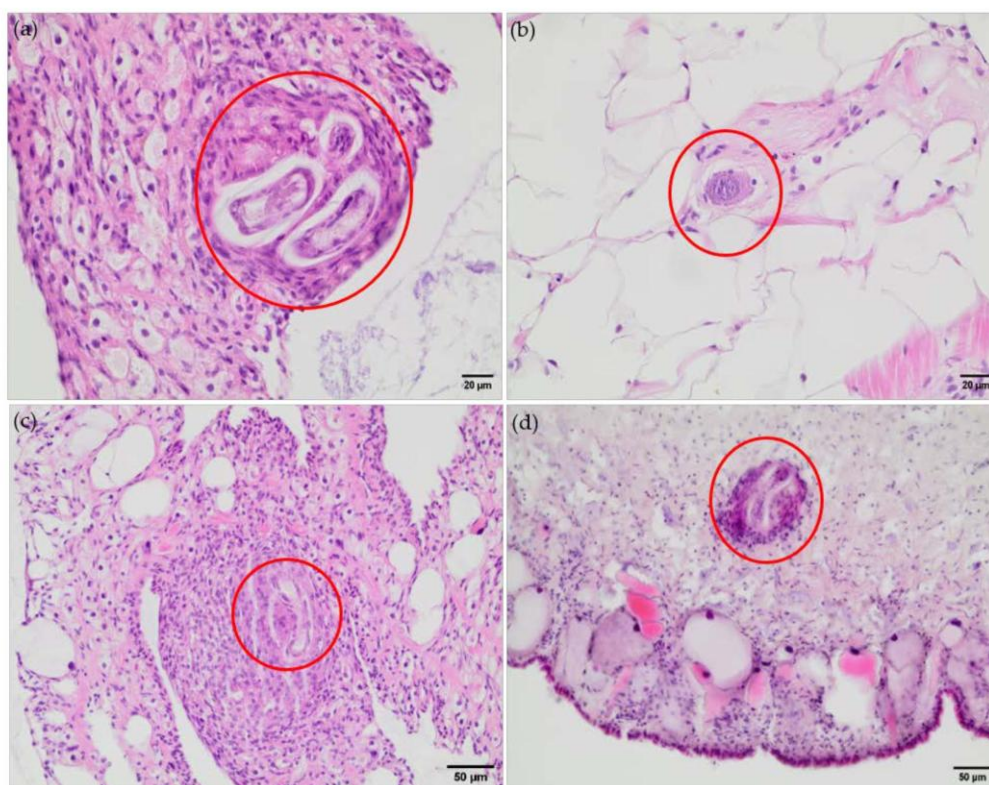


Figure 2. *Angiostrongylus vasorum* larvae in snail tissues (indicated by red circles), HE stained, 40x. (a,b): lung, 29 days p.i.; (c): lung, 5 days p.i.; (d): foot, 47 days p.i.

Considering larval burdens, clear differences were stated for the different organs (Figure 3). In general, the lung showed a high larval burden [up to 511 larvae per gram

(L/g) of organ tissue] and, therefore, was the most infected organ, especially at days 42, 49, and 56 p.i. In contrast to this finding, the reproduction tract and albumin gland showed much lower larval burdens (0.83–1.75 L/g and 2.45 L/g, respectively). Of note, the different organs showed high interindividual variations in terms of larval burden (Table 2). Overall, the larval burden did not affect the larval developmental speed.

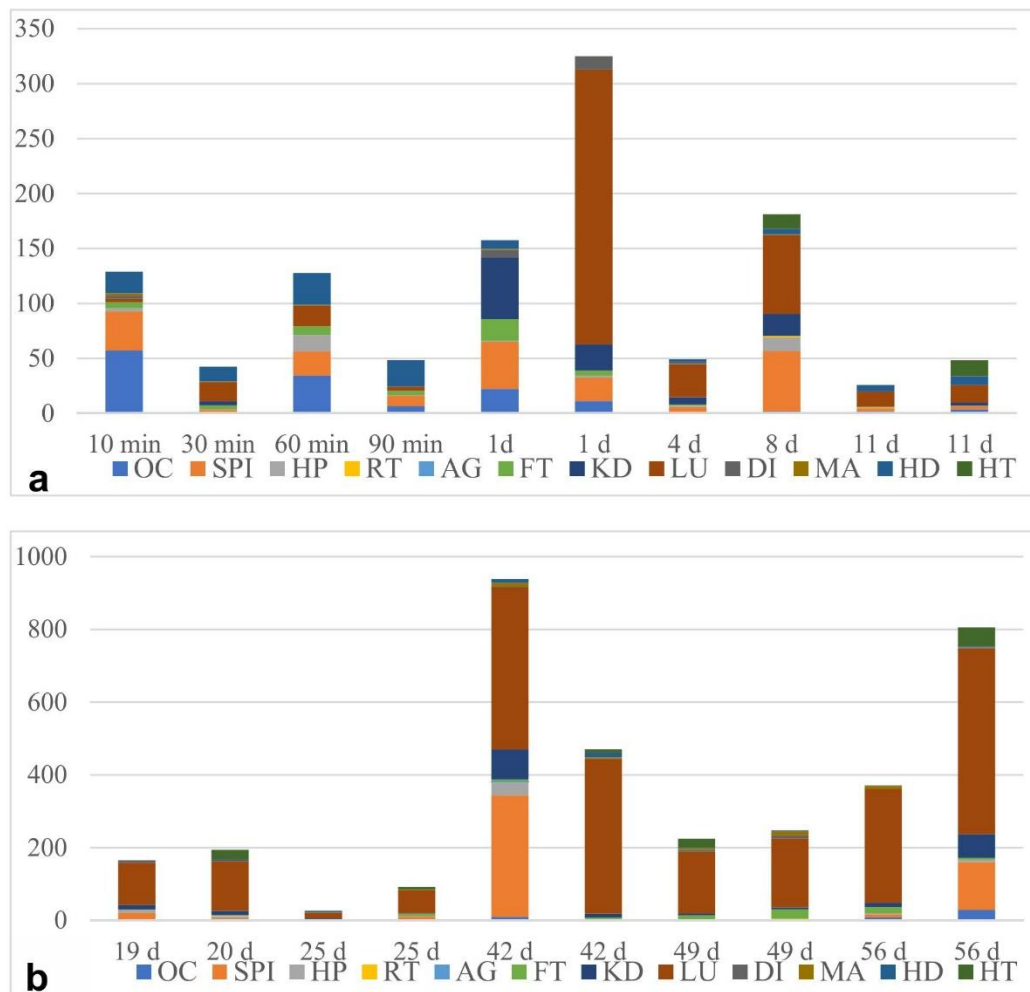


Figure 3. Larval burden of *Angiostrongylus vasorum* in *Lissachatina fulica* tissue in larvae per g organ weight. OC: esophagus and crop, SPI: stomach and proximal intestine, HP: hepatopancreas, RT: reproduction tract, AG: albumen gland, FT: foot, KD: kidney, LU: lung, DI: distal intestine, MA: mantle, HD: head, HT: heart. (a) <14 d p.i.; (b) >14 d p.i.

3.2. Histological Findings Indicate Hemocyte Responses to *Angiostrongylus Vasorum* Larval Stages

The analysis of histological samples confirmed the presence of *A. vasorum*-larvae in snail tissue, e.g., organs like lungs and feet (Figure 2). In the snail's lungs, parasites were found separated by thin hemocyte-derived layers localized in the connective tissue surrounding blood vessels. As an exemplary finding in the connective tissue, we detected a single capsule enclosing three larvae at a time, while other larvae were found separated by thin hemocyte-derived layers (Figure 2). Due to the lack of karyolysis and/or foamy cytoplasm of these gastropod phagocytes, a clear hemocyte activation could not be confirmed for this microenvironment. Moreover, the larvae seemed still intact and showed no signs of

cuticle destruction. In the thin gas exchange surface, no hemocyte accumulation was found in close vicinity to *A. vasorum* larvae, but a thin layer of connective tissue surrounded these pulmonary stages. In the snail foot, many *A. vasorum* larvae were located in the connective tissue. Numerous hemocytes accumulated around these parasitic stages, thereby forming a multicellular capsule. Herein, karyolysis was observed in some of these phagocytes, indicating hemocyte activation, while other hemocytes surrounding the larvae appeared to be flattened and potentially not activated.

As an interesting finding, the macroscopical analysis of the lungs of a long-term infected *L. fulica* snail (euthanized two years p.i.) revealed the presence of multiple calcified granulomas (Figure 4). These pathologic structures were excised, stained, and examined by bright field microscopy. Here, the classical characteristics of granuloma were confirmed histologically, but no larvae were found in any of these pulmonary hemocyte-derived structures, most probably indicating successful larval degradation.

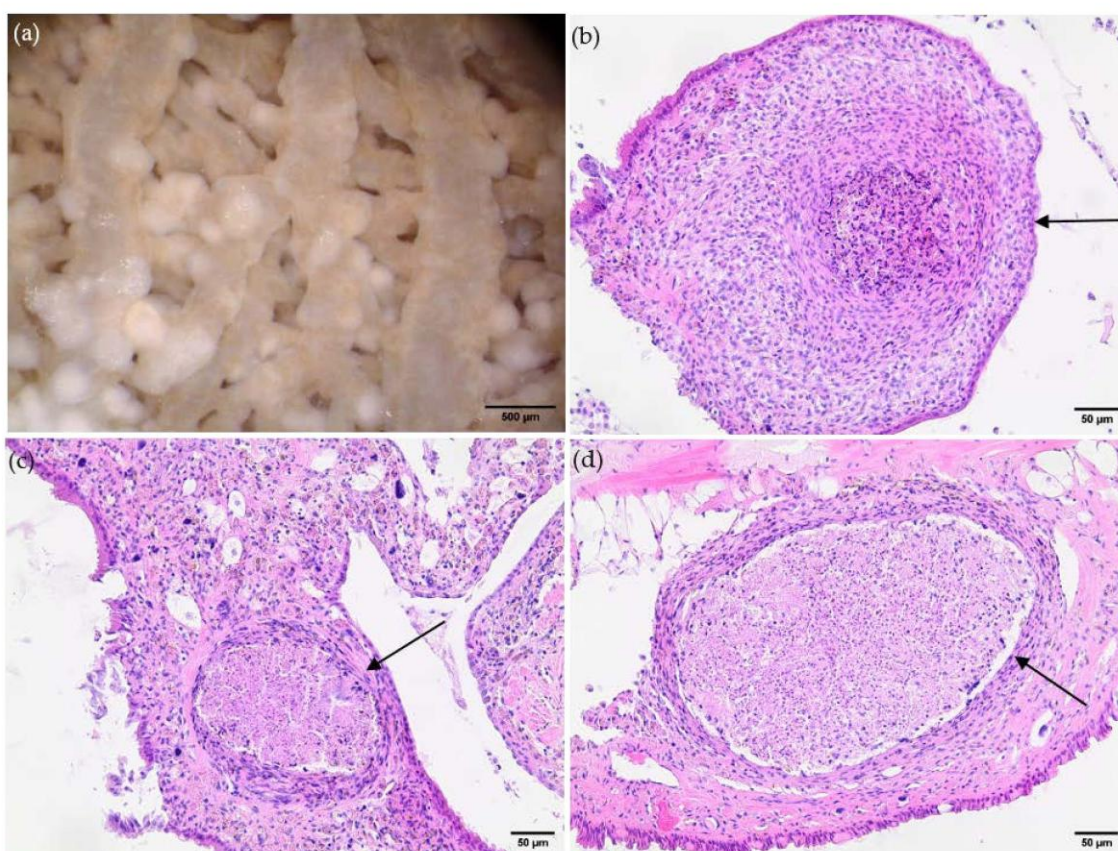


Figure 4. Granuloma-like structures in lung tissue of a long-term infected *Angiostrongylus vasorum*-infected African giant snail (*Lissachatina fulica*). (a): macroscopical illustration of the snail's lung; (b–d): microscopical illustration of granuloma-like structures (indicated by black arrow).

4. Discussion

Detailed investigations on *A. vasorum* larval migration, organ tropism, development, and hemocyte-derived innate immune reactions within gastropod intermediate hosts are still very limited [40,54–56]. The present study confirmed *L. fulica* as a suitable obligate intermediate host for *A. vasorum*, as previously reported in tropical biomes of Colombia [28]. Consequently, all *A. vasorum* developmental larval stages were found in the host, L1, L2, and infective L3. Interestingly, in some experimentally infected *L. fulica*, vital *A. vasorum* L3 was still present two years after infection, indicating long-term larval survival in these

hosts. These long-living stages might be part of not only parasitic survival strategies apart from the canid definitive host but also be relevant for parasite dissemination and transmission. Of note, the neozoan *L. fulica* can live for up to 9 years and thereby migrate over longer distances when compared to short-living terrestrial gastropods [28,29]. As such, *L. fulica* has demonstrated an alarming capacity for rapid spread. In the US State of Florida, three abandoned snails led to massive multiplication and the discovery of more than 18,000 individuals only seven years later, and currently, 15 US states have been colonized by this neozoan species [57]. Even though the mechanisms of *A. vasorum* spreading into non-endemic areas are still under debate [55,56], geographic shifts in mollusc intermediate host populations are assumed as one underlying factor [58,59]. Meta-analyses have revealed that both global warming and anthropogenic pressure strongly affect the phenology of terrestrial gastropods, including slugs, semi-slugs, and snails [16,60].

Referring to experimental infections, a high variation in *A. vasorum* recovery rates was rather obvious in the current study, even though applying the same infection dose (1000 L1/snail). A reason for these deviations might be seen in individual innate immune reactions mediated by hemocytes, as already reported for *Arion* spp. slugs and *L. fulica* snails [30,38,52]. As recently demonstrated in vitro, exposed hemocytes not only firmly adhered to the cuticle of *A. vasorum* L1 and L3 but also reacted by forming invertebrate extracellular phagocyte traps [40,54]. In line, hemocytes circulating within the hemolymph system are considered multifunctional professional phagocytes in gastropods, thereby resembling leukocytes of vertebrates [39,40,61–63]. Hemocytes display diverse effector mechanisms against invasive pathogens, including phagocytosis, encapsulation, cell-mediated cytotoxicity, and release of invertebrate extracellular phagocyte traps (InEPTs) [40,42,54,64]. Some of these hemocyte-derived defense mechanisms were here confirmed in histological analyses of *A. vasorum*-infected *L. fulica* tissues. Hence, accumulation of hemocytes around larvae was found in addition to multicellular larval encapsulation, leading to the formation of large granuloma. Importantly, *A. vasorum* larvae, being surrounded by hemocytes, were detected in several tissues like the foot, distal intestine, lung, and the mantle of snails as early as 10 min p.i. These findings indicated that despite a fast *A. vasorum* larval migration, hemocytes are rapidly recruited to sites of parasite infection [40,54]. Rapid hemocyte recruitment might be a consequence of the open circulatory system of gastropods, with the hemolymph flowing through sinuses and thereby directly bathing organs. Thus, inner organs are surrounded by hemolymph containing circulating hemocytes, which may immediately react against pathogens, including *A. vasorum* larvae [65,66]. Interestingly, percutaneous *A. vasorum* L1 infection of molluscs resulted in InEPTs within gastropod mucous extrapallial space in vivo [30]. Accordingly, gastropod hemocytes spontaneously reacted against vital *A. vasorum* L1 within 30 min by forming InEPTs, resulting in firm entrapment of these motile larvae [54].

In the current study, *A. vasorum* L2 was found as early as 8 days p.i., and the first fully developed L3 stages were present at 11 days p.i. in snail tissues. Consequently, canids might become infected via *L. fulica*-derived L3 as early as 11 days p.i. Compared to a previous report stating a duration of 17 days for *A. vasorum* L3 development [36], the current data indicated a faster development using the same gastropod intermediate host species, which may be due to the climate chamber-based standardized experimental conditions (i.e., a circadian cycle of 10 h of light, 10 h of darkness, 20–26 °C, and 50% humidity) used in the current study. As well documented for other intermediate host species like mosquitoes [67,68], the larval development within intermediate hosts strongly depends on climatic conditions. In line with current observations, the individual developmental rate varied significantly under natural conditions [36]. Hence, the first L3 stages were detected at 11 days p.i., but some L1 stages were still found in foot tissues at 56 days p.i. in a few snail individuals, thereby verifying an asynchronous development of *A. vasorum*-larvae in intermediate hosts. This phenomenon might rely on different biological aspects, such as varying oxygen concentrations and biochemical, cellular, and immunological conditions in different organs or within the same organ, finally resulting in delayed individual larval

development. Alternatively, a discontinued or asynchronous development could represent a kind of larval hypobiosis known from numerous other parasitic nematodes [69,70] to overcome adverse exogenous or even endogenous in vivo conditions.

Regarding organ tropism, clear differences were observed in the early (<1 d) and late (>1 d) stages of infection. Hence, the esophagus and stomach showed a higher larval concentration shortly after an oral infection due to the passive passage of the liquid-containing larvae through the proximal digestive tract. In the hepatopancreas, larval proportions strongly fluctuated, ranging from 0% (11 days p.i.) to 41.77% (8 days p.i.). The physiological functions of the hepatopancreas include resorption and storage of nutrients, as well as mucus and enzyme production [71], thereby representing a potential nutrient source for larval development. In addition, this organ also owns an immune function [71,72], which plays a role in fighting invading pathogens, eventually explaining the varying numbers of larvae in this organ.

Of note, several organs, such as the heart, mantle, and distal intestine, showed a low larval presence throughout the entire infection period. This may be linked to the fact that these inner organs have fewer metabolic activities and nutrient abundance, thus being less favorable for larval development. Conversely, a high larval concentration was observed in the lung in later phases of infection (up to 72.65%) as opposed to earlier time points (max. 18.75%). Here, anatomical restrictions may hamper the larvae from directly migrating to this location, most likely due to the passive flow of the digestive tract, the anatomical location of the lung, and the breathing mechanism [36].

Obviously, the number of *A. vasorum* larvae present in these tissues reflects the organ tropism; however, favorable organ conditions may also be linked to accelerated larval development into L3 stages. As specifically detected on days 20, 25, and 49, the relation between the L2 and L3 stages balanced in favor of the L3 stages in the lung when compared to the foot or hepatopancreas. An accelerated development might be associated with favorable organ-specific metabolic activities or high-rate hemolymph circulation, thereby delivering a more effective nutrient supply to fulfill the needs of *A. vasorum* larvae. The same holds true for gas exchange, including CO₂ and O₂, as previously postulated elsewhere [36]. As reported for other closely related metastrongyloid lungworm species, *A. vasorum* seems to favor hyperoxic conditions (like in the snail lung) since not only the late larval but also adult stages are allocated in the right heart and *Arteria pulmonalis* in the canid definitive host [20,73]. In contrast to lung tissues, very few *A. vasorum* larvae were found in the reproductive tract of hermaphrodite snails, consisting of the uterus, oviduct, vas deferens, penis, vagina, ovotestis and gonopore [36,74], but also of the albumen gland, which secretes the perivitelline fluid being deposited on the eggs of the snail. Both the gland's size and activity are closely linked to the sexual cycle of snails, therefore not representing an ideal place for lungworm larval development. In agreement with current findings, a minor role of the *L. fulica* reproductive tract for larval migration was also described by Sauerländer et al. [36]. However, in contrast to *A. vasorum*, the gastropod reproductive tract may represent a target organ for other parasites, such as trematodes [71,75].

As expected, histological analyses of infected snail tissues confirmed that *L. fulica* indeed mounts an innate immune response against *A. vasorum* larvae. Consistently, the strongest hemocyte-mediated reactions were found in foot and lung tissues. In the latter, hemocytes effectively encapsulated *A. vasorum* larvae by pulmonary granuloma formation. So far, the current data do not allow any conclusion on whether encapsulated larvae were killed since their cuticle still seemed intact. Besides granuloma formation, the first in vivo evidence of InEPT extrusion by activated hemocytes was found since some hemocytes showed karyolysis and a foamy cytoplasm, i.e., cellular events being associated with InEPT formation in vitro [40,54]. In contrast to the above findings, *A. vasorum* larvae present in the thin gas exchange surface neither induced hemocyte accumulation nor granuloma formation, indicating niche-specific reactions. However, we can only speculate that this may result from a general lower abundance of hemocytes in gas exchange areas to guarantee undisturbed gas exchange.

A striking evidence of granuloma formation was found in long-term *A. vasorum*-infected *L. fulica*, i.e., as late as two years after infection. Here, a massive accumulation of macroscopically visible granuloma was found in the lungs, unveiling marked snail-derived encapsulation of migrating larvae, as previously reported [30,45]. Histological analyses of these pathological structures confirmed the classical characteristics of granuloma. Surprisingly, neither fragments nor complete *A. vasorum* larvae were found inside these cellular structures. Thus, they may represent long-term remnants of effective hemocyte-mediated innate immune reactions, finally leading to effective killing and dissolution of larvae. Prominent calcification processes in these granulomas may underline this assumption. Another interesting finding in one of these long-term-infected snails was the presence of vital larvae, primarily in the hepatopancreas. It is tempting to hypothesize that the hepatopancreas serves as a safe haven for L3 long-term survival, eventually based on a low abundance of hemocytes in this digestive gland. Undoubtedly, this hypothesis awaits further experimentation.

In conclusion, our findings confirm *L. fulica* as a suitable intermediate host for *A. vasorum* and further demonstrate the prolonged survival of infective L3 stages. Given that *L. fulica* can reach a remarkable age of nine years [72], this long-term survival of *A. vasorum* in its intermediate host may, on the one hand, aid parasite geographic spread to formerly free areas and, on the other hand, pose a considerable long-term risk to susceptible definitive hosts in several regions, endemic for this invasive snail species, such as Colombia, Ecuador, Cuba, the USA, Indonesia, or India [17,28,33,34,76,77]. The preference of *A. vasorum* larvae for certain organs indicates a complex interaction between the parasite and its intermediate host, which warrants further investigation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pathogens13110946/s1>.

Author Contributions: Conceptualization, C.H. and A.T.; methodology, C.H. and A.T.; software, A.D. and L.S.; validation, A.D. and L.S.; investigation, A.D. and L.S.; resources, M.H., C.H. and A.T.; data curation, L.S. and A.D.; writing—original draft preparation, A.D.; writing—review and editing, A.D., M.H., C.H., L.S. and A.T.; visualization, A.D. and L.S.; supervision, C.H. and A.T.; project administration, C.H. and A.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Institute of Parasitology, Justus Liebig University Giessen, Germany.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained in this article.

Acknowledgments: We are grateful to Katharina Raue (TiHo Hannover, Hannover, Germany) for providing feces containing L1 of *A. vasorum*.

Conflicts of Interest: The authors declare no conflicts of interest.

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3.3 Radiolabeling of *Angiostrongylus vasorum* and *Crenosoma striatum* larvae: a novel method using PET/CT to unveil larval migration in the gastropod intermediate host (*Lissachatina fulica*)

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van de Sanden et al. *Parasites & Vectors* (2025) 18:466 <https://doi.org/10.1186/s13071-025-07088-0>

Eigener Anteil an der Publikation:

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Radiolabeling of *Angiostrongylus vasorum*- and *Crenosoma striatum* larvae: a novel method using PET/CT to unveil larval migration in the gastropod intermediate host (*Lissachatina fulica*)

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Abstract

Background Gastropod-borne metastrongyloid lungworm infections are poorly understood despite their importance in both veterinary and human medicine. This study intends to assess the use of nuclear imaging with whole-body positron emission tomography and computed tomography to scan radiolabeled *Angiostrongylus vasorum*- and *Crenosoma striatum* first-stage larvae (L1) while migrating in vivo in the obligate mollusc intermediate hosts. Here, the giant African snail (*Lissachatina fulica*) was used as a novel animal model for lungworm-associated investigations, as this gastropod species is known to act as a natural obligate intermediate host in the tropics for various metastrongyloid lungworms, including the zoonotic-relevant *Angiostrongylus cantonensis*. Radiolabeled *A. vasorum*- and *C. striatum* L1 migration was visualized through nuclear imaging after L1 oral infection or injection.

Methods Live L1 were collected through the standard Baermann funnel technique. After isolation and assessment of larval viability, collected *A. vasorum*- and *C. striatum* L1 were incubated with a radiolabeled glucose analogue, ¹⁸F-fluorodesoxyglucose. Thereafter, radiolabeled L1 were fed orally or injected into adult *L. fulica*, and in vivo scans were performed to visualize larval migration routes at different time points post infection through various gastropod organs/tissues.

Results The most optimal incubation time of larvae and ¹⁸F-fluorodesoxyglucose was 30 min. After washing nonincorporated tracer, metastrongyloid L1 retained on average activity of 0.33 (0.103) KBq per larvae. This was the maximum activity achieved, and even longer incubation times, i.e., 60 and 120 min, did not exceed this value. The in vivo scans showed dispersal from the site of larval injection or feeding. Radiolabeled *A. vasorum*- or *C. striatum* L1 moved rapidly

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from the site of injection or the oral cavity with nonspecific accumulation in one or numerous gastropod organs at 30 min post infection.

Conclusions This study concludes that radiolabeling of metastrongyloid L1 with ^{18}F -fluorodesoxyglucose is achievable up to a level that can be detected in a scan of individual snails. Scanning L1 larvae in *L. fulica* at 60 min still represents larval migration. After 2 h, this study found that the migration is already widespread, and the activity is too low to be narrowed to a specific organ. Detailed in vivo scans of gastropods with not only higher infectious doses but also other radiolabeled tracers and longer observation periods might allow detection of either organ tropism or larval accumulation in certain mollusc tissues/organs for further development into infective stages.

Keywords *Lissachatina fulica*, Radiolabeling, *Angiostrongylus vasorum*, *Crenosoma striatum*, Gastropod-borne diseases

Background

Gastropod-borne parasitic diseases, such as metastrongyloid lungworm infections of humans and animals, are rarely addressed in research. In Europe, multiple species of metastrongyloid lungworm species infect various definitive hosts, both domestic and wild animals. Two highly prevalent lungworms in Europe are *Angiostrongylus vasorum* and the closely related *Crenosoma striatum*. *A. vasorum* infects domestic dogs, wild canids (e.g., wolves, foxes, and jackals), badgers, raccoons, and red pandas [1–5]. All these carnivore species act as definitive hosts (DH) where the adult nematodes reside in the right heart and in the pulmonary artery. Canine angiostrongylosis may lead to a variety of symptoms, ranging from mild to severe ones. Clinical signs include respiratory symptoms, hemorrhages, coagulopathies, neurological and ophthalmic disorders, and gastrointestinal symptoms [5, 6].

Conversely, *C. striatum* is commonly found in the lungs of European hedgehogs (*Erinaceus europaeus*). Respiratory symptoms of crenosomosis in affected hedgehogs include dry cough, bronchitis, hyperthermia, and weight loss [7, 8].

The life cycle of these lungworms is heteroxenous, with terrestrial gastropods (i.e., snails, semi-slugs, and slugs) acting as obligatory intermediate hosts (IH) [9, 10]. Terrestrial gastropods become infected by feeding either on the feces containing first-stage larvae (L1) from an *A. vasorum*-infected DH or from a *C. striatum*-infected European hedgehog. While inside the gastropod IH, the larvae develop into the second-stage larvae (L2) and third-stage larvae (L3), which are then infective for other DH. Therefore, metastrongyloid infections of canids include direct gastropod consumption carrying L3, consumption of paratenic hosts (PH) such as mice, reptiles, and frogs feeding on infected IH, and/or infective L3 in the environment released from either dead IH or PH, for example, in grass or water [11].

The giant African snail *Lissachatina fulica* is one of the largest terrestrial snails in the world. Originally endemic to East Africa, this gastropod species is currently

regarded as one of the most invasive gastropod species worldwide. Stable populations of this mollusc neozoon were found among others in Ecuador, the USA, Brazil, India, and Colombia [12–14]. Moreover, *L. fulica* is known to be an important IH for multiple parasite species, including various lungworm species [13, 15].

There is still little knowledge about the development of metastrongyloid lungworm larvae, organ tropism, and host innate immune reactions against these stages inside the gastropod [16, 17]. To obtain precise data on the parasite's location and distribution speed inside terrestrial gastropods in vivo, imaging techniques can be used. Nuclear imaging with whole-body positron emission tomography and computed tomography (PET/CT) is a well-established technique in the medical field to study various physiological processes within the body, for example, to image and assess tumor sizes and even progression [16]. This noninvasive/minimally invasive technique offers a unique opportunity to observe precise processes in vivo.

For example, ^{18}F -fluorodesoxyglucose (^{18}F -FDG) is a radiolabeled tracer that is being used in PET/CT scanning. It is widely available and routinely employed in clinical practice. With a physical half-life of approximately 109 min, it is classified as a short-lived radionuclide. While clinical protocols typically utilize activity around 1 GBq, a substantially lower activity of 10 MBq was selected for this study to accommodate the smaller body size of snails, consistent with dosing regimens commonly applied in preclinical studies involving mice and rats. As a glucose analogue, ^{18}F -FDG is considered non-toxic at concentrations that do not exceed physiological levels; it is taken up in larger amounts by cells with high metabolic activity, such as in tumors, the brain, the liver, and even parasites [18, 19]. Nematodes, especially, are known to consume and metabolize glucose; as such, the use of a radioactive glucose derivative (^{18}F -FDG) is a suitable approach for tracking larval migration [20, 21].

In our study, we used nuclear imaging to visualize the distribution over time of relevant metastrongyloid parasite species within *L. fulica*, which can be infected with

ease under experimental conditions. The overall aim of this study is to assess the feasibility of radiolabeling *A. vasorum*- and *C. striatum* larvae to visualize their endogenous migration within this large terrestrial gastropod, with the intention of determining their precise route of migration and identification of organ tropism. Understanding the localization of *A. vasorum*/*C. striatum* larvae or other lungworms might lead to not only a better understanding of parasite–*L. fulica* interactions but also to hampering transmission to various mammalian DH, including humans, as is the case for zoonotically relevant *Angiostrongylus cantonensis* and *Angiostrongylus costaricensis*, both species circulating in *L. fulica* populations in the tropics.

Methods

Parasite isolation

Angiostrongylus vasorum- and *C. striatum* L1 were acquired by isolation from naturally infected dogs and European hedgehogs, respectively. Fresh feces were collected and thereafter examined via the Baermann–Wetzel apparatus according to Conboy et al. [22]. The hedgehog feces were kindly provided by the *Egelopvang Papendrecht* (the Netherlands) and the *Wildtierfreunde e.V.* (Germany). The dog feces originated from the Parasitology Diagnostic Unit (PDU) of the Institute of Parasitology, Justus Liebig University Giessen, Giessen, Germany.

Collected larvae were stored in small tubes at 4 °C until used for snail infections. To achieve a higher larval concentration, the solution containing the larvae was centrifuged for 5 min at 800g at room temperature (RT). The supernatant was discarded, and the larvae pellets were resuspended in physiological saline after concentration. Using this method, an infectious dose (ID) of either 1000 or 2000 L1 per snail was created by counting the larvae under a light microscope.

Larval radiolabeling in vitro

¹⁸F-FDG was procured from the Nuclear Department, Faculty of Human Medicine at the Philipps-Universität Marburg, Marburg, Germany. Approximately 1500 *A. vasorum* larvae were divided into five groups: three live study groups (A, B, C) incubated with 1 MBq of ¹⁸F-FDG per well and two negative control groups (DF and LN) (Table 1). Each of the five groups was further divided into six subgroups varying in incubation time (5, 10, 15, 30, 60, and 120 min). Every subgroup was pipetted in an individual well of a 96-well plate (Greiner, Sigma-Aldrich, Germany). For each incubation time point, every well corresponding to a subgroup contained approximately 50 larvae. After incubation, each group was washed three times with phosphate-buffered saline (PBS) by

Table 1 Radioactive uptake in a single *Angiostrongylus vasorum* larva, calculated for live larvae and dead larvae at each time point

Minutes	A + B + C in kBq/larva (SD)	Dead larvae with ¹⁸ F-FDG control in kBq/larva
5	0.08 (0.013)	0.04
10	0.07 (0.016)	0.02
15	0.11 (0.067)	0.04
30	0.33 (0.103)	0.1
60	0.22 (0.084)	0.21
120	0.3 (0.128)	0.04

The number of larvae in a well was divided by the kBq value for each well corresponding to a time point and group. A, B, and C represent three groups of live larvae that were incubated with ¹⁸F-FDG for a specified number of minutes. The result at each time point for A, B, and C was averaged into a single activity per larva. Dead larvae with ¹⁸F-FDG control consist of approximately 50 dead larvae per well per time point that were incubated with ¹⁸F-FDG for a specified number of minutes. As there was only one dead larvae control group, the results are not averaged

SD standard deviation

centrifuging them at 800g (RT) for 5 min and resuspending the specimens in PBS.

The DF and LF groups served as negative controls to validate our results. The DF group consisted of dead larvae that were also incubated with 1 MBq of ¹⁸F-FDG. In contrast, the LF group consisted of living larvae with the same washing steps as the positive groups, but without an ¹⁸F-FDG incubation.

Finally, the larvae were moved to counting tubes and were loaded onto a Perkin Elmer[®] gamma counter. The activity per larva was calculated by dividing the readings from the gamma counter by the total number of larvae that were counted under the microscopes. To determine the activity linked to a reading and to correct for ¹⁸F-FDG decay, two reference samples, vials containing only ¹⁸F-FDG with a known activity of 25 kBq and 50 kBq, were loaded into the gamma counter. The readings from these two samples were used to approximate the amount of activity linked to the readings for each larval sample.

Larval labeling for imaging

The ¹⁸F-FDG used in imaging was obtained as stated above from the Nuclear Department at the Philipps-Universität Marburg (Germany). For the second set of scans, performed in Rotterdam, the ¹⁸F-FDG was produced by Cyclotron Rotterdam B.V., the Netherlands. The larvae were retrieved from the fridge in concentrated form, assessed for viability and ID, and brought to the radioactive workbench. The L1 were combined with ¹⁸F-FDG activity at 30 MBq and incubated at RT for 30 min. The larvae were then washed three times in PBS by centrifuging them for 5 min at 800g on an Eppendorf cup centrifuge and by pipetting off the supernatant and

resuspending it in H₂O. Finally, the activity was measured and noted down as previously described.

Snails

Ten giant African snails (*L. fulica*) were held in a climate chamber (ECP01E, Sniijders Scientific B.V., Tilburg, the Netherlands) at the Institute of Parasitology, JLU Giessen, Giessen, Germany. The light cycle consisted of 10 h of light and 10 h of darkness with 2 h for dawn and dusk, respectively. The snails were fed ad libitum with cucumbers, zucchinis, carrots, green lettuces, and commercial dog food as reported by Dusch et al. (2024). As a natural calcium source, shells of the cuttlefish (*Sepia* spp.) were used here.

Snail infection with either radiolabeled ¹⁸F-FDG *A. vasorum*- or *C. striatum* L1

The snails were carefully cleaned with running water before larval infection and thereafter placed inside a radioactive workbench. The radiolabeled ¹⁸F-FDG L1 of *A. vasorum* suspended in solution were injected near the pneumostome as described by Cooper (1995), placing them close to the gastropod central hemolymph circulation system promoting a quick distribution. After this procedure, the snails were returned to their boxes to wait for 5 min for larval migration. Before PET/CT analysis, the snails were immobilized using an ice bath and placed on the bed for the scanning machine (NanoScan, Mediso).

For the second set of scans, performed in Rotterdam, we decided against parasite injection and opted for voluntary oral infection instead, to be as close as possible to the in vivo situation where larvae (i.e., L1) are ingested by coprophagic gastropods. The snails were fed the radiolabeled ¹⁸F-FDG L1 of *C. striatum* suspended in physiological saline solution with a needleless syringe orally. The rest of the scan protocol was the same as for the metatstrongyloid-injected snails.

PET/CT imaging

PET/CT images (NanoScan, Mediso) were acquired in six with *A. vasorum*-infected *L. fulica* ($n=6$) at 30, 60, and 90 min post infection (p.i.). A seventh non-infected snail ($n=1$) was injected only with ¹⁸F-FDG to serve as a control and scanned afterward in the same way as the infected snails. The CT was obtained using the following parameters: 70 kV, 84 μ As, and one projection. Positron emission tomography (PET) scans were performed using a whole-body window. The first scan was a dynamic scan consisting of ten short scans at a 1-min interval. Images were reconstructed using a reconstruction program and the MLEM algorithm [23].

For the second set of scans, performed in Rotterdam, PET/CT images (VECTor/CT, MILabs, the Netherlands) were acquired in three with *C. striatum*-infected *L. fulica* ($n=3$). All three of the snails were scanned at 5 min p.i., and two of these snails at 2 h p.i. as well. The CT was obtained using the following parameters: 55 kV, 0.17 μ A, and one projection. PET scans were performed using the ultrahigh-definition mouse–rat collimator, scanning four time-windows of 5 min. Images were reconstructed using the MILabs[®] reconstruction program using the SROSEM method with the following parameters: voxel size 0.8 mm, 50 iterations, and 16 subsets. For segmentation and post analysis, Imalytics[®] preclinical software (Gremse-IT, Germany) was used.

Statistics

For statistical analyses, RStudio [2024.09.0 Build 375(R-4.4.2)] was used. The data were separated into three groups, and a linear regression was performed for the time points 5–30 min and the time points 30–120 min to determine if there was a relation between minutes and uptake per larva. Finally, the averages for the different groups were calculated at each time point. All data are displayed as follows: mean (standard deviation [SD]). Significant values of $P < 0.05$ were considered.

Results

Larval incubation

Incubation of live *A. vasorum* L1 with ¹⁸F-FDG resulted in specific uptake in the larvae, which increased between 5 and 30 min after incubation. The ¹⁸F-FDG uptake per larva increased from 0.08 (SD=0.0126) kBq to 0.33 (SD=0.103) kBq per larva (Table 1). A simple linear regression model demonstrated that the uptake time explained a significant amount of the increase in activity per larva for the time points 5–30 min ($t_{(5)}=5.519$, $P < 0.001$). In contrast, in the linear regression model for time points 30–120 min, respectively, uptake time was not a predictor for activity per larvae ($t_{(5)}=-0.12$, $P=0.909$). After 30 min of exposure, for the time points 30, 60, and 120 min combined and averaged, the activity leveled out at 0.282 (SD=0.080) kBq per larva.

PET/CT scans

PET/CT images revealed hotspots immediately after injection, primarily in the snail's mass dorsally from the pneumostome. These indicated limited dispersal in 30 min. Conversely, 60 and 90 min after injection, the scans using *A. vasorum* L1 displayed a more widespread larval distribution throughout the gastropod's body, which is indicative of advanced larval migration (Fig. 1). The activity, however, was in the lower digestive tract and the albumen gland. Two snails showed a distinct

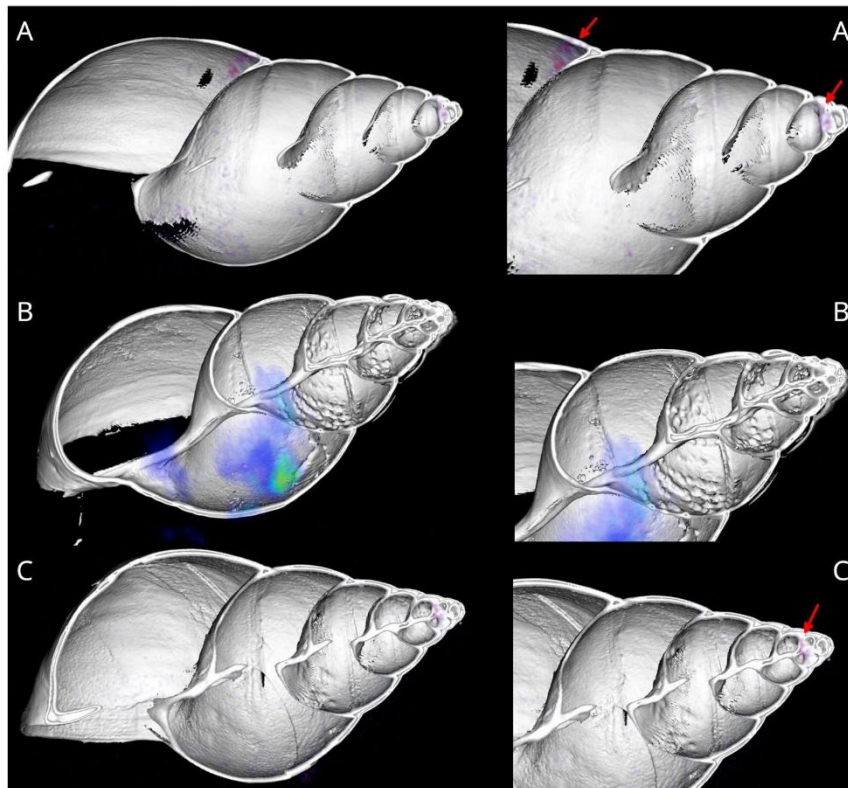


Fig. 1 PET/CT image of two *Lissachatina fulica* snails (A, C) and one control (B) scanned 60 min after injecting ^{18}F -FDG-radiolabeled *Angiostrongylus vasorum* larvae. A, top; red arrows: activity in the digestive tract and the digestive gland. B, middle: control. C, bottom: digestive gland visible

distribution that was not found in the control ^{18}F -FDG-injected snail, a focal point in the top part of the digestive gland (Fig. 1).

In the orally infected snails, *C. striatum* larvae uptake was observed primarily in the snail's digestive tract. These indicated larval dispersal from the esophagus,

the crop, and, in one subject, into the stomach (Fig. 2). After 2 h of larval post-feeding, the scan displayed a more widespread distribution throughout the gastropod's body, which is indicative of advanced larval migration. The ^{18}F -FDG activity, however, was too low to narrow this uptake to a specific inner organ. As such,

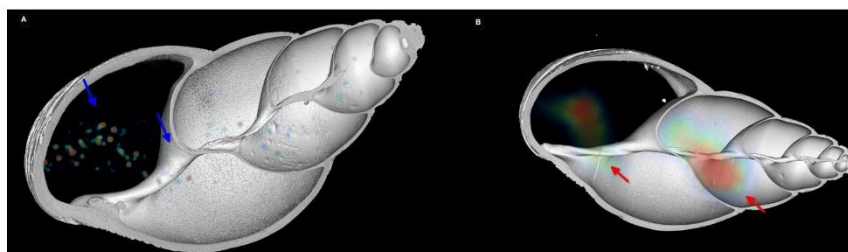


Fig. 2 PET/CT image of two *Lissachatina fulica* snails scanned 10 min after orally feeding ^{18}F -FDG-radiolabeled *Crenosoma striatum* larvae. A, Snail, top-view uptake in digestive tract up to the stomach, blue arrows. B, snail 2, top-view uptake in the digestive tract, red arrows

precise scans could only be performed up to 2 h after injection.

Discussion

This study set out to test whether it was possible to incubate metastrongyloid lungworm larvae with ^{18}F -FDG and to scan them thereafter in vivo in giant African snails (*L. fulica*), which are known to act as suitable obligate IH of various metastrongyloid lungworm species in the tropics of Colombia [13] and Brazil [24].

We decided to use two different lungworm species owing to their differences in localization in the IH. As such, the larvae are exposed to different stressors, which may result in different reactions to both the experimental conditions as well as the organ tropism of the larvae.

The first findings of this work reveal that live metastrongyloid L1 of *A. vasorum* and *C. striatum* can successfully be exposed to the ^{18}F -FDG tracer and after ingestion achieving sufficient radioactivity levels for effective PET/CT scanning. The uptake of ^{18}F -FDG in *A. vasorum* L1 larvae reached approximately 0.3 KBq after 30 min and did not increase any more. This could indicate that the larvae were satiated or that the maximum amount of ^{18}F -FDG was ingested. However, the various replicates showed a difference in the average activity per larva. A possible explanation for this could be individual variance in the isolated larvae, for example, due to larval size and/or metabolic activities or individual larval stress.

The scans performed after injection showed larval activity arising from the injection spot as well as from the lower part of the gastrointestinal tract in four individuals ($n=4$). These results seem to be consistent with other research, which found that the injection method used resulted in most parasites remaining close to their insertion point into the snail, thereby causing a higher number of larvae to be collected from these regions [25–27].

The remaining scans that were performed every 30 min after injection demonstrated a widespread pattern that could not be linked to one specific organ. It is therefore likely that the larvae migrated toward different organs, thereby leaving the gastrointestinal tract. A study by Sauerländer et al. (1976), who used infection doses of 5000 and 20,000 larvae per snail, found that, within 2 h, a large quantity of *A. vasorum*- and *A. cantonensis* larvae had moved toward the lung and foot of the snails [15, 25, 28–30]. Surprisingly, we did not find such a specific signal coming from either organ on the scan after the follow-up period of 2 h. This might be explained by the large volume of both organs and the possibility that the signal was diluted by the lower infection dose the snails received in our study. A distinct signal was however observed at the top of the shell in the digestive gland for two of the snails in the “larval-injected” scans.

In an attempt to mimic a natural infection route and to achieve more accumulation of parasites, the second snail scans occurred after an oral infection route for these parasites. Two different metastrongyloid lungworm species (*A. vasorum* and *C. striatum*) were used to show possible species disparities in migration. In these “larval-oral” scans, the digestive tract of animals showed a large amount of uptake right after initiation of PET/CT scans. Nonetheless, with subsequent scans, the amount of uptake visible in the digestive tract decreased and after 2 h was completely absent. Interestingly, there was no spot visible in the digestive gland.

The most important limitation of radiolabeling lies in the fact that even in the DF control group, a small amount of ^{18}F -FDG uptake is observed, which suggests that our washing protocol was not sufficient to completely wash away all the remaining ^{18}F -FDG. Because of the small sample size ($n=7$), caution must be applied, and, in further studies, the effect of this phenomenon should be minimized by increasing the number of replicates and by improving larval washings. This observation was also present in the first scans performed after larval injection. A large amount of activity was observed from the injection site; however, contamination with the remaining ^{18}F -FDG, which could not be completely washed out of the injection solution, could be a contributing factor to the activity present in this region. Finally, the scans showed some overlap with the control scan, which also unveiled a lot of activity coming from the digestive tract and albumen gland (Fig. 1).

Considering the half-life of ^{18}F -FDG (109.8 min), the signal is halved approximately every 2 h. This by itself could have contributed to the diluted signal found in all infected scans. For a follow-up, the usage of longer half-life radionuclides or a higher initial activity could be more precise in following the larvae during migration to allow scanning over a longer time period. A tracer that is used for nuclear cell imaging, like ^{111}In -oxine, could be an option as it is used for immune-cell tracking [31].

This study set out to establish whether it was possible to radiolabel metastrongyloid lungworm larvae and track larval migration in vivo within gastropods via a PET scan. The most obvious finding to emerge is that it was feasible to label the parasitic larvae with ^{18}F -FDG after 30 min of incubation and that these parasitic stages could be visualized in an early state of their endogenous gastropod migration with a PET/CT scan.

Conclusions

In this study, we were able to show that ^{18}F -FDG-radiolabeling of vital L1 of two endemically occurring metastrongyloid species in Europe, that is, *A. vasorum* and *C. striatum*, can be achieved to track migration

routes within gastropods with minimally invasive (injection of larvae) and with noninvasive (oral uptake of larvae) methods. Despite the low number of snails used here, the study certainly adds knowledge to better our understanding of metastrongyloid parasite migration and localization within mollusc IH. The scans could not pick up a signal after 2 h p.i. that was clear enough to narrow the activity to organs or migration paths. However, this initial work should be repeated and tested on a larger sample of gastropods, with higher infectious doses and with other radiolabeled tracers. Thus, different radionuclides as well as other lungworm species, including zoonotic ones (*A. cantonensis* and *A. costaricensis*) can also be considered in the future since *L. fulica* is known to be a suitable IH in the tropics. The same holds true for species of veterinary relevance in felids and canids such as *Troglostrongylus brevior*, *Aelurostrongylus abstrusus*, and *Crenosoma vulpis*.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13071-025-07088-0>.

Supplementary Material 1. Fig. S1 pictures of first stage larvae of *Crenosoma striatum* and *Angiostrongylus vasorum*

Supplementary Material 2. Table S1 raw readings

Acknowledgements

The authors would like to thank Sandra van Tiel and Marjolein Verhoeven (Erasmus Medical Centre, AMIE Core Facility, Rotterdam, the Netherlands) for their technical support with the PET/CT scanning; Experimenteel diercentrum and the rest of the animal support staff for taking care of and helping with the snail rearing and handling; Egelopvang Papendrecht and Wildtierfreunde e.V. for providing hedgehog feces from *Crenosoma striatum*-infected hedgehogs.

Author contributions

FV, CH, AT, MB, AD, JS, and KW conceived and designed the parasite experiments and scans. KW and FV performed the first set of scans and the parasite incubation. JS and AD performed the second set of scans. JS and AD analyzed the data and scans. FV, CH, MB, AD, and JS revised the manuscript. All authors read and approved the final manuscript.

Funding

The authors did not receive any external funding for this study.

Data availability

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

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 11 June 2025 Accepted: 30 September 2025

Published online: 18 November 2025

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4. Diskussion

Infektionen von Haustieren mit metastrongyloiden Lungenwürmern zählen noch immer zu den vernachlässigten Parasitosen, obwohl diese zu mitunter schweren Erkrankungen und zum Tod des Tieres führen können (Schnyder et al. 2010; Di Cesare et al. 2014; Colombo et al. 2021). Während zur Prävalenz der Lungenwürmer in den Endwirten mehrere Studien durchgeführt wurden, liegen bislang wenige Daten zur Epizootiologie der Lungenwürmer im obligatorischen Zwischenwirt Schnecke vor. In diesem Zusammenhang stellt sich die Frage, ob es in den Schnecken ebenso eine spezifische Prävalenzverteilung wie bei mit *A. vasorum* infizierten Hunden gibt, in der stabile hypo- und hyperendemische Foki oft in unmittelbarer Nachbarschaft liegen.

Die hier durchgeführte Studie hatte den Zweck, einen bekannten hyperendemischen Fokus zu re-evaluieren und Änderungen in der Prävalenz über die Jahre sowie Unterschiede zwischen den Jahreszeiten zu analysieren. Ein weiteres Ziel war es, mögliche Unterschiede in der Entwicklung der Lungenwurmlarven in den verschiedenen Schneckenspezies aufzuzeigen, da die individuelle Biologie der Spezies die Entwicklung der Larven zur infektiösen L3 beeinflussen kann. Dazu wurden 533 Landschnecken in allen vier Jahreszeiten gesammelt, künstlich verdaut und im Anschluss mikroskopisch analysiert. Die Prävalenz variierte stark zwischen den einzelnen Jahreszeiten. Im Sommer waren 27,46 % (59/215) der Schnecken mit metastrongyloiden Lungenwurmlarven infiziert, während im Herbst die Prävalenz auf 10,00 % (16/160) sank. Die niedrigsten Infektionsraten lagen im Winter (5,56 %) und Frühling (1,47 %) vor. Auch die einzelnen Lungenwurm-Spezies wiesen unterschiedliche Prävalenzen auf. So wurde *A. vasorum* in 12,01 %, *Crenosoma* sp. in 0,94 % (5/533) und *Ae. abstrusus* in 0,38 % (2/533) der Schnecken gefunden.

Während wir im Vergleich zur früheren Studie von Segeritz und al. (2021) eine niedrigere Gesamtprävalenz aufweisen konnten, wurde dennoch Obrigheim als stabiler hyperendemischer Fokus bestätigt. Zusätzlich konnten wir erneut verschiedene Schneckenspezies (*Arion* sp., *Helicodonta* sp., *Deroceras* sp., *Limax maximus*) als obligatorische natürliche Zwischenwirte bestätigen. Aus der Tatsache, dass wir in jeder Jahreszeit mit metastrongyloiden Lungenwürmern infizierte Schnecken gefunden haben, ergibt sich ein ganzjähriges Infektionsrisiko für potenzielle Endwirte. Dennoch variiert das Infektionsrisiko zwischen den Jahreszeiten stark durch die schwankende Prävalenz der Lungenwurmlarven in den gastropoden Zwischenwirten. Wir konnten mit 27,46 % die höchste Gesamtprävalenz von Lungenwürmern in Gastropoden im Sommer feststellen, während die Gesamtprävalenz im Herbst nur 10,00 % betrug. Diese Ergebnisse stehen im direkten

Gegensatz zu der Prävalenzstudie von Segeritz et al. (2022), in der die höchste Prävalenz im Herbst mit 75,93 % beobachtet wurde, während die Gesamtprävalenz im Sommer mit 17,6 % vergleichsweise niedrig war. Diese Prävalenzschwankungen zwischen den Jahren 2018 und 2022 lassen sich beispielsweise durch Unterschiede in Temperatur und Niederschlag erklären, welche eine wichtige Rolle in der Populationsdynamik der Schnecken spielen. So nimmt unter heißen und trockenen Bedingungen die Mobilität und Fortpflanzungsrate von Schnecken ab, was sich schlussendlich in der Prävalenz der Lungenwurmlarven spiegeln kann (Zeny et al. 2019; Manara et al. 2025). Der Sommer 2018 galt als ungewöhnlich heiß und trocken, was direkt die Schnecken in ihrem Verhalten und ihrer Überlebensfähigkeit beeinflusst (Rousi et al. 2023).

Dieses Phänomen lässt sich zudem durch den Zeitpunkt des Schneckensammelns erklären. Basierend auf dem Lebenszyklus von *Arion*-Nacktschnecken sind die Exemplare im Sommer oft größer und älter und erreichen ihr maximales Gewicht. Zudem paaren sie sich und sterben gegen Ende des Sommers ab (Yousif und Lämmler 1975; Davies und Davies 1987; Slotsbo 2012). So reichte das Gewicht der Schnecken von 0,01 g bis 10,11 g, wobei das Durchschnittsgewicht bei 4,02 g lag. Aus diesem Grund sind die großen und schweren Schnecken im Sommer in der Lage, eine größere Menge Kot über einen längeren Zeitraum als ihre kleineren Artgenossen aufzunehmen, wodurch sich die Infektionswahrscheinlichkeit dieser Nacktschneckenart erhöht. Frühere Studien beweisen zudem eine Korrelation zwischen Infektionsstärke und Größe der Schnecke, was in unserer Studie nicht der Fall war (Wallace und Rosen 1969; Yousif und Lämmler 1975).

Im Herbst wiesen die Schnecken im Allgemeinen eine geringere Größe auf, ihr Durchschnittsgewicht betrug nur 0,7 g. Aufgrund der geringen Größe handelt es sich bei diesen Schnecken vermutlich um die Jungtiere der im Sommer beobachteten Schnecken, die erst vor kurzer Zeit geschlüpft waren. Durch ihre geringe Größe sind diese Schnecken nicht in der Lage, so viel Kot aufzunehmen wie die ältere Generation und entsprechend war sowohl die Infektionsstärke der einzelnen Schnecke geringer als auch die Prävalenz in der allgemeinen Schneckenpopulation. Zu beachten ist auch die größere Artenvielfalt, die im Herbst beobachtet wurde. Während die meisten *Arion* sp. zum Ende des Sommers beziehungsweise im Herbst absterben, können andere Schneckenspezies überwintern. Dazu zählen beispielsweise die Garten-Bänderschnecke *Cepaea hortensis* sowie die Gefleckte Weinbergschnecke *Helix aspersa*, die durch Anpassung ihres Stoffwechsels Minusgrade überstehen können (Ansart et al. 2002; Bech et al. 2020). Auf diese Weise sind metastrongyloide Lungenwürmer in der Lage, den Winter zu überstehen und im Frühjahr neue Endwirte zu infizieren. Durch diese Fähigkeit zur Überwinterung bleibt der Lebenszyklus der Lungenwürmer, wie bereits berichtet, über alle Jahreszeiten hinweg bestehen (Kafle et al. 2018; Anettová et al. 2023). Wie bereits erwähnt, herrschte im Herbst eine größere Artenvielfalt, was sich in der Lungenwurmprävalenz

widerspiegeln kann. Nacktschnecken der Gattung *Arion* gelten als koprophag, was wir bei der Feldarbeit durch die Beobachtung mehrerer Exemplare, die an Hundekot fraßen, bestätigen konnten (2023). Andere Gastropodenspezies ernähren sich eher seltener von Kot und präferieren Pilze, Myzele und verschiedene Pflanzen als Nahrungsquelle (Beeston und Beeston 1919; Kozłowski und Kozłowska 2004). Der Grund für diese größere Artenvielfalt im Herbst lässt sich nicht nur durch den bereits erwähnten Lebenszyklus von *Arion* sp. erklären, sondern auch durch die Morphologie dieser Nacktschnecke. Im Vergleich zu anderen Gastropodenspezies sind diese groß und von einer orangenen Farbe, was dazu führen kann, dass sie bei der Probensammlung leichter entdeckt wurden als andere Schneckenarten. *Arion* sp. wurden bereits als Zwischenwirte für metastrongyloide Lungenwürmer bestätigt (Aziz et al. 2016; Lange et al. 2018a; Segeritz et al. 2022). Zudem liegen Berichte vor, dass die potenziell invasiven *Arion vulgaris* sich weltweit ausbreiten. Diese Ausbreitung kann einerseits durch das aktive Wandern der Schnecken geschehen, andererseits aber auch passiv in Tierfellen, Kleidung, Fahrzeugen und Werkzeug (Davies und Davies 1987). Auf diese Weise können durch die Ausbreitung des Zwischenwirts auch die Lungenwürmer mitverschleppt werden und neue Endemiegebiete erschließen sich.

Doch auch mit dem Endwirt kann ein Parasit verschleppt werden, z. B. indem ein infizierter Hund mit seinem Besitzer reist, wie es zunehmend üblich ist, und dort die heimische Schneckenpopulation infiziert. Ein weiterer Aspekt in der Populationsdynamik der Lungenwürmer ist die Übertragung von Parasiten von Wildtieren auf Haustiere durch die Schnecke sowie das Zurückfließen („Spillback“) der Infektion von der Haustierpopulation auf die Wildtierpopulation durch die erhöhte Anzahl infizierter Zwischenwirte. Zudem kann das Vorhandensein anderer, weniger als Zwischenwirte geeigneter Gastropodenspezies einen sogenannten Verdünnungseffekt bewirken, wodurch mit der Zeit die Prävalenz der Lungenwürmer sowohl in den Zwischenwirten als auch in den Endwirten sinkt.

Bisher wurden in dieser Studie nur *Arion* sp. als Zwischenwirte besprochen, aber wir konnten auch andere Spezies als natürliche Zwischenwirte identifizieren, wie *L. maximus*, *Helicodonta* sp. und *Deroceras* sp. Der Tigerschneegel *L. maximus* ist für seinen Kannibalismus bekannt (Karlin und Bacon 1961). Auf diese Weise kann diese Schnecke sich durch die Aufnahme von mit Lungenwurmlarven infiziertem Schneckengewebe potenziell selbst infizieren. Denkbar ist in diesem Zusammenhang auch die Zunahme der infizierten Individuen, wenn sich mehrere kannibalistische Schnecken von einer infizierten Schnecke ernähren. Besonders bei der Infektion von Katzen mit Lungenwürmern ist die Rolle der paratenischen Wirte (z. B. Maus, Ratte, Amphibien und Reptilien) zu beachten (Lankester und Anderson 1966; Niebuhr et al.

2019). So wurden während der Probensammlung im Sommer mehrere Frösche beobachtet sowie einige Löcher im Boden entdeckt, die auf die Präsenz von Mäusen schließen lässt.

Um zu analysieren, warum sich in Obrigheim ein hyperendemischer Fokus für Lungenwurminfektionen in gastropoden Zwischenwirten befindet, wurden die klimatischen und geografischen Begebenheiten in der Nähe des Hotspots untersucht. Bemerkenswert war hier der Zustand der Obrigheimer Wiese. Im heißen August 2022, in dem das Grasland im Umland vertrocknet war, war das Gras auf der Wiese grün und feucht und bot ideale Bedingungen für das Überleben der Schnecken und damit auch für die Fortsetzung des Lebenszyklus der metastrongyloiden Lungenwürmer. Durch die Wiese fließt zudem ein kleiner Bach namens Heiligenbach, der von Bäumen umgeben ist. Diverse Flora führt zu einer größeren Artenvielfalt gastropoder Zwischenwirte, während die beobachtete Fauna (Frösche, Nagetiere, Vögel und Reptilien) eine Rolle als paratenische Wirte spielen kann. Zusätzlich haben sich am Heiligenbach Biber angesiedelt und einen Damm gebaut, wodurch entsprechend ein neues Feuchtbiotop entstand. In unmittelbarer Umgebung der Wiese befindet sich außerdem ein größeres Waldgebiet, in dem sich die in Deutschland weit verbreiteten Füchse und womöglich andere Endwirte von Lungenwürmern befinden können. Füchse sind für ihre steigende Urbanisierung bekannt, was zur Übertragung diverser Erkrankungen führen kann. Besonders in dieser waldnahen Wiese können sich die Lebensräume von Fuchs und Hund überschneiden, wodurch dieser sylvatische Zyklus vom Fuchs auf Haustiere überspringen kann.

Während der Probenentnahme wurden mehrere Hundebesitzer beobachtet, die ihre Hunde auf der Obrigheimer Wiese ausführten. Das Verhalten dieser Hundebesitzer und ihrer Haustiere ist ein weiterer wichtiger Faktor, der die Prävalenz und Übertragung der Lungenwürmer auf Zwischen- und Endwirte beeinflusst. Hunde, die nicht angeleint sind, haben eine höhere Wahrscheinlichkeit, unkontrolliert Schnecken aufzunehmen, als ein angeleinter Hund. Zudem können Hunde oder Katzen sich nicht nur durch die willentliche Aufnahme von Schnecken infizieren, sondern auch akzidentiell. In Anbetracht dessen, dass die kleinste mit Lungenwürmern infizierte Schnecke nur 0,01 g wog, kann eine versehentliche Aufnahme leicht mit etwas Gras, verfaulten Früchten oder sogar mit Wasser erfolgen. Im Frühjahr 2023 war die Wiese überschwemmt und wir konnten mehrere tote Gastropoden beobachten, die sich in Pfützen auf der Wiese befanden. In den dort genommenen Wasserproben befand sich eine einzelne metastrongyloide Larve, woraus sich schließen lässt, dass das Überleben der Larven im Wasser zumindest für kurze Zeit möglich ist. So wurde für den zoonotischen *A. cantonensis* bereits beschrieben, dass dieser die Zwischenwirte aktiv verlassen und mehrere Wochen als infektiöse L3 in der Umwelt überleben kann (Rivory et al. 2023).

Während der Probenentnahme konnten wir in allen Jahreszeiten Hundekot an verschiedenen Stellen der Wiese beobachten. Durch diese mangelnde Hygiene kann Obrigheim als hyperendemischer Fokus aufrechterhalten werden. Da insgesamt nur neun Hundebesitzer auf den Fragebogen geantwortet haben, ist eine repräsentative Aussage über die Lungenwurmprävalenz bei Hunden in Obrigheim nicht möglich. Dennoch berichtete keiner der Tierbesitzer über eine diagnostizierte Lungenwurminfektion in seinem Haustier. Dies könnte mehrere Gründe haben. Einerseits ist es in Deutschland noch immer üblich, Hunde regelmäßig zu entwurmen, ohne den Kot vorher auf potenzielle parasitäre Infektionen untersucht zu haben. In der Praxis üblicherweise verwendete Anthelminthika wie Moxidectin und Fenbendazol wirken gegen ein breites Spektrum an Parasiten, darunter auch Lungenwürmer (J. Nolan und B. Lok 2012; Helm und Morgan 2017). So können einerseits Lungenwurminfektionen im Endwirt vorherrschen, aber nie diagnostiziert werden, andererseits aber auch der Übertragungszyklus der Lungenwürmer durch die Behandlung des Endwirts unterbrochen werden. Ein weiterer Aspekt ist die Tatsache, dass Lungenwurminfektionen oft asymptomatisch verlaufen oder nur unspezifische Symptome wie Apathie, Lethargie, Husten, diffuse gastrointestinale oder neurologische Symptome verursachen (Bolt et al. 1994; Colombo et al. 2021). So stehen Parasitosen im Allgemeinen und Lungenwurminfektionen im Speziellen nicht im Vordergrund in der praktischen Veterinärmedizin und werden als potenzielle Differentialdiagnose diffuser Symptomatik oftmals nicht in Betracht gezogen. Während in der Praxis gelegentlich das Flotationsverfahren durchgeführt wird, können auf diese Weise keine Larven von Lungenwürmern nachgewiesen werden (Morgan 2011). Dazu ist das Trichterauswanderungsverfahren nach Baermann-Wetzel vonnöten, welches nur selten von praktizierenden Tierärzten durchgeführt wird und keinen Teil der Routinediagnostik darstellt.

Schließlich wurden in dieser Studie mittels PCR die Lungenwurmart in den Zwischenwirten charakterisiert. Während dieses molekularbiologische Verfahren einige Vorteile bietet, wie etwa die genaue Bestimmung der Spezies, so müssen doch auch die Einschränkungen beachtet werden. So kann weder die Larvenvitalität noch das Larvenstadium oder die Befallsintensität festgestellt werden. Zudem befinden sich im Schneckenschleim Muzin sowie verschiedene Enzyme (Proteasen, Endonukleasen), welche die DNA der Lungenwürmer angreifen und so als natürliche Hemmstoffe die PCR inhibieren können (Lange et al. 2018a; Adema 2025). Zu guter Letzt sind die in den Schnecken vorhandenen Bakterien ebenso in der Lage, durch ihre Zellwandbestandteile sowie Nukleasen die PCR zu inhibieren (Wilson 1997).

Abschließend ist festzuhalten, dass trotz der niedrigeren Prävalenz im Jahr 2022 die Wiese in Obrigheim als stabiler hyperendemischer Fokus für *A. vasorum* bestätigt werden konnte. Daraus ergibt sich ein ganzjährig erhöhtes Risiko für domestizierte und wildlebende Endwirte,

sich mit Lungenwürmern zu infizieren. In weiteren Studien sollte die Populationsdynamik der einzelnen Schneckenspezies über die Jahreszeiten analysiert werden sowie die Prävalenz der Laven in den Schnecken in den einzelnen Jahreszeiten. Zudem sollte das Bewusstsein der praktizierenden Tierärzte für Lungenwurminfektionen geschärft werden.

Bislang liegen wenige Untersuchungen zur metastrongyloiden Larvenmigration, deren Entwicklungs- und Überlebensdauer in gastropoden Zwischenwirten vor. Zur Entwicklungszeit der L1 zur infektiösen L3, deren Wanderung in der Schnecke sowie dem Organotropismus ist entsprechend wenig bekannt, obwohl diese Kenntnisse die Epidemiologie dieser Parasiten beeinflussen können. Daher wurde in der hier vorliegenden Studie der Organotropismus der L1 des metastrongyloiden Lungenwurms *A. vasorum* im natürlichen Zwischenwirt *L. fulica* näher untersucht. Dazu wurden erwachsene Afrikanische Riesenschnecken ($n = 26$) oral mit vitalen *A. vasorum*-L1 infiziert, um den natürlichen Infektionsweg *in vivo* nachzuahmen. Nach unterschiedlichen Zeitpunkten p. i. wurden die Schnecken euthanasiert und die individuellen Organe einzeln künstlich verdaut. Im Anschluss wurden die Organe auf die Präsenz, Anzahl und das Stadium der Larven näher untersucht. Dabei wurden bis zu 49,7 % der Larven nach dem künstlichen Verdau in verschiedenen Stadien und unterschiedlichen Schneckenorganen wiedergefunden. Zusätzlich wurden auch histologische Schnitte der isolierten Organe angefertigt, um Reaktionen des angeborenen Immunsystems von *L. fulica* auf die eindringenden Larven näher zu analysieren.

In dieser Studie konnten wir erneut *L. fulica* als obligaten Zwischenwirt für *A. vasorum* bestätigen, da nach dem künstlichen Verdau alle Entwicklungsstadien (L1, L2 und die für Endwirte infektiöse L3) gefunden wurden. Die Afrikanische Riesenschnecke gilt als eine der invasivsten Spezies, was sich an ihrer alarmierenden Verbreitungsgeschwindigkeit äußert. Ursprünglich war diese Schnecke in Ostafrika nativ, wurde aber mittlerweile in vielen subtropischen und tropischen Ländern als invasive Spezies dokumentiert (Thiengo et al. 2010; Rekha Sarma et al. 2015; Goldyn et al. 2016; Lange et al. 2018b). Ein Beispiel für die alarmierend schnelle Ausbreitung ist im amerikanischen Bundestaat Florida gut dokumentiert. Dort wurden nur drei Exemplare im Jahr 1966 ausgesetzt, die sich in kurzer Zeit so stark vermehrten, dass nur sieben Jahre später eine Population von 18.000 Individuen entdeckt wurde. Mittlerweile wurden *L. fulica*-Riesenschnecken in 14 weiteren Bundesstaaten der USA entdeckt. Die Ursache für diese starke Invasivität lässt sich in der Biologie von *L. fulica* finden. Nicht nur ist die Schnecke in der Lage dazu, lange Strecken zu wandern und niedrige Temperaturen zu überstehen, sondern auch ihre Fähigkeit zur Autobefruchtung führt zu einer explosionsartigen Ausbreitung (Escobar et al. 2011; Dickens et al. 2018). Zwar wurde bisher keine vertikale Übertragung der Lungenwurminfektion auf eine neue Schneckengeneration festgestellt, wie es beispielsweise für Protozoen der Fall ist, aber die Übertragung durch

Kannibalismus, wie er bei anderen Landschnecken vorkommt, ist grundsätzlich vorstellbar (Ozgo und Bogucki 2006). Ein weiterer wichtiger epidemiologischer Aspekt ist die lange Lebenserwartung von *L. fulica*, die bis zu 9 Jahre betragen kann. In unserer Studie wurden noch 2 Jahre nach der experimentellen Infektion der Schnecke lebende L3 von *A. vasorum* gefunden, woraus man auf eine lange Überlebensfähigkeit der Larven in diesem Zwischenwirt schließen kann. Diese langanhaltende Überlebensfähigkeit spielt eine wichtige Rolle in der Überlebens- und Verbreitungsstrategie des Parasiten und stellt ein dauerhaftes Infektionsrisiko für den Endwirt dar (Lange et al. 2018b; Penagos-Tabares et al. 2019).

Weiterhin ist noch nicht vollständig geklärt, wie *A. vasorum* sich innerhalb eines Gebietes verbreitet, in dem der Parasit ursprünglich nicht endemisch vorkommt (Schnyder 2015; Giannelli et al. 2016a). Wie bereits erwähnt, könnte die vorhandene Gastropodenpopulation in diesen nicht-endemischen Gebieten eine Rolle spielen (Lange et al. 2018b). Diverse Schneckenspezies wurden als adäquate Zwischenwirte für *A. vasorum* identifiziert, darunter auch die invasive *L. fulica* (Sauerländer 1976; Coaglio et al. 2018; Lange et al. 2018b). Auf globaler Ebene jedoch tragen auch Umweltveränderungen zur Veränderung der Schneckenpopulationen bei: So wurden durch Meta-Analysen globale Erderwärmung sowie anthropogener Druck als bedeutender Faktor für die Beeinflussung der terrestrischen Nackt- und Gehäuseschneckenpopulation bestimmt (Patz et al. 2000; Maksimov et al. 2017). Durch die immer fortschreitende Erderwärmung und die immer milderen Winter wird in den zukünftigen Jahren das Risiko bestehen, dass *L. fulica* als invasive Spezies bald auch in Mitteleuropa heimisch wird und mitunter gefährliche Parasiten einschleppt, darunter die zoonotischen Lungenwürmer *A. cantonensis* und *A. costaricensis*.

Gastropoden verfügen über ein sogenanntes nicht-adaptives Immunsystem bestehend aus Hämolymphe sowie zirkulierenden Hämozyten, welches auf eindringende Parasitenlarven oder andere Pathogene reagiert. Die immunologischen Reaktionen auf die genannten eindringenden Pathogene ist abhängig von multiplen Faktoren, darunter Fütterung, Umgebung, klimatische Bedingungen sowie dem individuellen Gesundheits- und Immunstatus des Organismus (Wojda et al. 2004; Rouse und Sehwat 2010). Aus diesen Gründen wurden die verwendeten *L. fulica*-Riesenschnecken unter standardisierten Laborbedingungen in Klimaschränken (ECP01E, Snijders Scientific B.V. Tilburg, Niederlande) gehalten. Obwohl in dieser Studie alle Schnecken mit derselben Infektionsdosis von 1000 L1 pro Schnecke infiziert wurden, zeigten sich große Unterschiede in der Wiederfindungsrate der Larven. Ein Grund dafür lässt sich im angeborenen Immunsystem der Schnecke finden. Die Hämozyten, die frei in der Hämolymphe zirkulieren, dienen als Effektorzellen und attackieren Pathogene, darunter auch metastrongyloide Lungenwürmer, durch verschiedene Mechanismen, wie die Formation von InEPT, wie kürzlich *in vitro* und *in vivo* bewiesen wurde (Lange et al. 2017; Penagos-

Tabares et al. 2018b). Diese multifunktionalen Zellen übernehmen die Rolle von Phagozyten und ähneln somit den Leukozyten von Vertebraten (Robb et al. 2014; Poirier et al. 2014; Pila et al. 2016a; Lange et al. 2017). Hämozyten zeigen verschiedene Effektormechanismen gegen eindringende Pathogene, darunter Phagozytose, Einkapselung, zellvermittelte Zytotoxizität und das Ausbilden der bereits erwähnten InEPT (Sokolova 2009; Loker 2010; Lange et al. 2017; Penagos-Tabares et al. 2018b).

Einige dieser Verteidigungsmechanismen konnten wir in dieser Studie in den histologischen Schnitten von mit *A. vasorum* infizierten *L. fulica* nachweisen. Hämozyten lagerten sich um die Larven in multiplen konzentrischen Schichten an und formten auf diese Weise große Kapseln. In einer Studie von Sauerländer et al. (1974) wurden diese Gebilde bereits 10 min p. i. in verschiedenen Geweben gefunden, wie etwa dem Fuß, der Lunge sowie dem Mantel der Schnecke. Daraus lässt sich schließen, dass trotz der schnellen Wanderung der Larven Hämozyten sofort nach Kontakt mit dem Erreger aktiviert werden und sich am Ansiedlungsort der Larve einfinden (Lange et al. 2017; Penagos-Tabares et al. 2018b). Diese schnelle Immunantwort lässt sich mit der Anatomie der Schnecke erklären. Sie verfügt über ein offenes Kreislaufsystem mit einem Herzen und einigen wenigen Blutgefäßen, in dem die Hämolymphe durch sogenannte Lakunen fließt und die Organe umspült. Dadurch sind die einzelnen Organe immer von Hämolymphe und somit Hämozyten umgeben, die nach Erregerkontakt sofort reagieren und in die Organe einwandern können (Sommerville 1973; Smith 1985). Interessanterweise führte eine perkutane Infektion von Mollusken mit *A. vasorum*-L1 *in vivo* zur Ausbildung von InEPT im extrapallialen Schleimraum, wodurch die Larven innerhalb von 30 min immobilisiert wurden (Penagos-Tabares et al. 2018b). Dies bestätigt die außerordentliche Kapazität der Hämozyten zur Wanderung durch das Schneckengewebe und zur Freisetzung von InEPT gegen vitale *A. vasorum*-L1 noch auf der Körperoberfläche der Schnecke.

In unserer Studie wurden die L2 von *A. vasorum* bereits 8 Tage p. i. aufgefunden, während die ersten L3 nach 11 Tagen p. i. vorlagen. Daraus lässt sich folgern, dass ein Infektionsrisiko für Hunde bereits 11 Tage nach erfolgter Schneckeninfektion besteht. Im Vergleich zu einer vorigen Studie, in der eine Entwicklungsdauer von 17 Tagen für die Entwicklung zur infektiösen *A. vasorum*-L3 berichtet wurde, lässt sich hier in derselben Schneckenspezies klar eine schnellere Entwicklungsdauer zeigen. Ein Grund dafür könnte in der standardisierten *in-vitro*-Halteung der Schnecken im Klimaschrank liegen (20-26 °C, 50 % Luftfeuchtigkeit, zirkadianer Rhythmus mit 10 h Licht, 10 h Dunkelheit und 4 Stunden für Morgen- und Abenddämmerung). So wurde für Moskitos, die Zwischenwirte für den Malariaerreger *Plasmodium falciparum* sind, bereits bewiesen, dass die Geschwindigkeit der Sporozitenentwicklung stark von externen klimatischen Bedingungen abhängig ist (Beier 1998; Waite et al. 2019). Doch auch unter den

gleichen standardisierten Laborbedingungen variierte die Entwicklungsrate und -geschwindigkeit in den einzelnen Schnecken stark (Sauerländer und Eckert 1974). In unserer Studie wurden die ersten infektiösen L3 zwar bereits nach 11 Tagen p. i. gefunden, aber selbst 56 Tage p. i. wurden vereinzelte L1-Stadien im Fuß einiger Individuen entdeckt. Daraus lässt sich auf eine asynchrone Entwicklung von *A. vasorum* im Zwischenwirt *L. fulica* schließen. Der Grund dafür lässt sich in der Biologie der individuellen Schnecke finden. So herrschen in unterschiedlichen Organen desselben Tiers oder sogar in einem anderen Bereich desselben Organs unterschiedliche biochemische, zelluläre und immunologische Bedingungen sowie unterschiedliche Sauerstoffkonzentrationen. Durch diese wechselnden physiologischen Bedingungen kommt es möglicherweise zur unterschiedlichen Entwicklungsdauer der Larven. Eine weitere Erklärung lässt sich in der potenziellen Fähigkeit der Larven zur Hypobiose finden, wie sie bei anderen parasitischen Nematodenspezies bereits beschrieben wurde (Blitz und Gibbs 1972; Laabs et al. 2012). Dadurch können Parasiten widrigen Lebensbedingungen trotzen und ihre Infektiosität über einen längeren Zeitraum sicherstellen.

In Bezug auf den Organtropismus von *A. vasorum* wurden deutliche Unterschiede zwischen den frühen (<1 Tag) und späten (>1 Tag) Stadien der Infektion beobachtet. Durch die orale Infektion wiesen in den sehr frühen Stadien vor allem die Speiseröhre und der Magen eine hohe Anzahl an Larven auf. Diese hohe Larvendichte lässt sich nicht durch aktive Wanderung, sondern durch einen passiven Transport mit der Flüssigkeit, in der sich die Larven zur Infektion befanden, erklären. Um den Tropismus der Larven für die anderen Organe zu analysieren und interpretieren, ist es wichtig, die jeweilige Anatomie, Physiologie und Funktion zu beachten. Bestimmte Organe, wie der distale Darm, der Mantel und das Herz wiesen in jeglichen Infektionsstadien eine sehr niedrige Larvenpräsenz auf. Der Grund dafür könnte in der niedrigen Stoffwechselaktivität zu finden sein, wodurch adverse Überlebensbedingungen für die Larven durch Nährstoffmangel entstehen. Eine besonders hohe Anzahl an Larven wurde in den späten Phasen der Infektion in der Lunge beobachtet (bis zu 72,65 %), was im direkten Kontrast zu früheren Zeitpunkten steht (max. 18,75 %). Eine Ursache für die späte Ansiedlung in der Lunge ist die Behinderung der Wanderung durch die anatomische Lage der Lunge (dorsal im Tier), der Atmungsmechanismus sowie der passive Fluss des Verdauungstrakts (Sauerländer und Eckert 1974). Ein Grund für die bevorzugte Wanderung in die Lunge in späteren Phasen liegt in der Präferenz von *A. vasorum* für hyperoxische Bedingungen, wie sie der Schneckenlunge herrschen; denn auch im Endwirt befinden sich die späteren Larvenstadien sowie die Adulti im rechten Herz und der *A. pulmonaris* (Rosen et al. 1970; Traversa et al. 2013). In anderen Organen schwankte der Larvenanteil stark, wie etwa dem Hepatopankreas. In dieser Verdauungsdrüse, zu deren Aufgaben die Resorption und Speicherung von Nährstoffen sowie die Produktion von Schleim und Enzymen gehört, reichte die Prävalenz von 0 % (11 Tage p. i.) bis 41,77 % (8 Tage p. i.). Aus diesen Aufgaben ergibt

sich ein potenziell positives Umfeld für die Entwicklung der Larven durch ein ausreichendes Nährstoffangebot. Die sehr variable Anzahl an *A. vasorum*-Larven in diesem immunkompetenten Organ lässt sich theoretisch durch mögliche Abwehrreaktionen des hepatopankreatischen Gewebes erklären, welches eine tragende Rolle in der Bekämpfung verschiedener eindringender Pathogene spielt (Sorensen und Minchella 2001; Raut und Barker 2002). Anders als in der Lunge der Schnecke wurden durchweg nur sehr wenige Larven in den Organen des Reproduktionstrakts gefunden. Dieser besteht in den zwitterigen *L. fulica* aus Uterus, Eileiter, Vagina, Samenleiter, Ovotestis, Eileiter und Gonopore (Ghose 1963; Sauerländer und Eckert 1974). Diese Ergebnisse stimmen mit denen von Sauerländer et al. (1974) überein, der dem Fortpflanzungstrakt eine untergeordnete Rolle im Organtropismus der Lungenwurmlarven zuschrieb. Dies steht im direkten Kontrast zum Organtropismus der Entwicklungsstadien einiger Trematoden, bei denen sich eine Vorliebe für den Fortpflanzungstrakt in ihrem Zwischenwirt feststellen ließ und sogar zur parasitären Kastration führen konnte (Sorensen und Minchella 2001; Faro et al. 2013). Zusätzlich als Bestandteil des Reproduktionstrakts zu erwähnen ist die Albumindrüse, welche Perivitellinflüssigkeit für die Eiproduktion der Schnecke absondert. Das Interessante an diesem Organ ist seine Kopplung an den Sexualzyklus der Schnecke. Die Größe und die Aktivität der Drüse schwankt stark im Laufe des Reproduktionszyklus, weshalb diese keinen idealen Ort für die Entwicklung der Lungenwurmlarven darstellt.

Während wir bereits postuliert haben, dass die Präsenz und hohe Anzahl von Larven in einem Organ auf günstige Bedingungen für die larvale Entwicklung zurückzuführen sind, können wir auch annehmen, dass diese günstigen Bedingungen in den Organen auch die Entwicklungsgeschwindigkeit der Larven maßgeblich beeinflussen können. An den Tagen 20, 25 und 49 wurde festgestellt, dass das Verhältnis zwischen L2 und L3 in der Lunge im Vergleich zum Fuß oder zum Hepatopankreas zugunsten des L3-Stadiums ausgeglichen war. Wie bereits erwähnt, kann es durch die hyperoxischen Bedingungen in der Lunge beziehungsweise durch das vorteilhafte Verhältnis von O₂ und CO₂ zu einer beschleunigten Entwicklung kommen. Ebenfalls zu beachten ist die Tatsache, dass auch der Fuß der Schnecke die Möglichkeit zum Gasaustausch besitzt und aus diesem Grund positive Auswirkungen auf die Entwicklung der Larven haben kann (Fossati et al. 2024).

In dieser Studie konnten wir durch die histologische Analyse des Gewebes infizierter Schnecken Reaktionen des angeborenen Immunsystems von *L. fulica* auf die Larven von *A. vasorum* bestätigen. Entsprechend des starken Befalls von Fuß und Lunge wurden die stärksten Reaktionen in diesen Geweben festgestellt. Im Lungengewebe kapselten die Hämozyten die *A. vasorum*-Larven durch die Bildung von Granulom-ähnlichen Strukturen wirksam ein. In bisherigen Studien wurde die Einkapselung als wirksame Reaktion des

Immunsystems beschrieben, um Parasiten, darunter auch metastrongyloide Lungenwurmlarven, vom nicht infizierten Gewebe abzukapseln, zu isolieren und in manchen Fällen zu töten (Sauerländer 1976; Loker et al. 1982; Falkingham und Rae 2021). Bislang lassen die vorliegenden Daten keine Schlussfolgerung darüber zu, ob die eingekapselten Larven abgetötet wurden, da ihre Kutikula noch intakt zu sein schien. Zu bedenken ist hier, dass diese Analysen nur kurze Zeit nach der Infektion der Schnecke durchgeführt wurden. In zukünftigen Studien sollte analysiert werden, ob die Einkapselung über einen längeren Zeitraum zur Desintegration der Lungenwurmlarven führen kann.

Die Einkapselung ist jedoch nicht die einzige Reaktion des angeborenen Immunsystems, die wir in dieser Studie beobachten konnten. Einige aktivierte Hämozyten wiesen eine Karyolyse sowie ein schaumiges Zytoplasma auf, was *in vitro* mit der Bildung von InEPT assoziiert wird (Lange et al. 2017; Penagos-Tabares et al. 2018b). Immunreaktionen konnten jedoch nicht in allen Teilen der Lunge festgestellt werden. So ließen sich an der dünnen Gasaustauschfläche keine zellulären Reaktionen um die eingedrungenen Larven feststellen. Histologisch ist diese Gasaustauschfläche mit den Alveolen in Säugern zu vergleichen, in denen das Epithel nur flach und einschichtig ist, um den Gasaustausch nicht zu behindern. Aus diesem Grund vermuten wir, dass es an dieser Stelle durch eine geringe Hämozytenzahl nicht zu einer Kapsel- oder Granulombildung kommt, um den Gasaustausch nicht zu beeinträchtigen.

Auch zwei Jahre nach der Infektion wurde eine massive Granulombildung im Lungengewebe mit *A. vasorum* infizierter *L. fulica* beobachtet. Diese waren makroskopisch sichtbar, etwa stecknadelkopfgroß und bei Palpation von sehr fester Struktur. Daraus lässt sich schließen, dass es sich bei diesen Strukturen um Granulome durch Einkapselung von eingedrungenen Lungenwürmern handelt, wie bereits berichtet (Sauerländer 1976; Loker et al. 1982). Nach histologischer Analyse dieser pathologischen Strukturen konnte hier der klassische Aufbau von Granulomen bestätigt werden. Interessanterweise wurden in den Granulomen weder vollständige Larven von *A. vasorum* noch Fragmente davon gefunden. Zudem wurde eine ausgeprägte Verkalkung dieser zellulären Strukturen beobachtet. Diese Verkalkung und das Fehlen der Larven deutet darauf hin, dass es sich hierbei um eine effiziente Abwehrreaktion des angeborenen Immunsystems der Schnecke handelt, um die eingedrungenen Lungenwurmlarven über die Zeit zu immobilisieren, isolieren, abzutöten und zu verdauen. Interessanterweise wurde in einer weiteren langfristig infizierten Schnecke vor allem im Hepatopankreas das Vorhandensein vitaler und potenziell infektiöser L3 entdeckt, die nicht eingekapselt waren. Dieses Organ könnte ein Refugium für diese Larven darstellen, da nicht viele Hämozyten in dieser Verdauungsdrüse vorhanden sind. Dort könnten diese Larven mehrere Jahre überleben und ihre Infektiosität beibehalten. Auch diese Hypothese sollte in weiterführenden Studien genauer überprüft werden.

Zusammenfassend können wir bestätigen, dass *L. fulica* ein geeigneter Zwischenwirt für *A. vasorum* ist, da alle Entwicklungsstufen in der Schnecke gefunden wurden. Zusätzlich konnten wir die lange Überlebensfähigkeit der infektiösen L3 im Zwischenwirt bestätigen. Angesichts der Tatsache, dass *L. fulica* das bemerkenswerte Alter von 9 Jahren erreichen kann, kann diese invasive Schnecke zur geographischen Ausbreitung von *A. vasorum* beitragen. Durch die Größe der Schnecke ist diese in der Lage, eine größere Menge exogene *A. vasorum*-L1 im Kot zu konsumieren und entsprechend eine höhere Anzahl infektiöser L3 auf den Endwirt zu übertragen. Die Präferenz der *A. vasorum*-Larven für bestimmte Organe deutet möglicherweise auf eine komplexe metabolische Wechselwirkung zwischen dem Parasiten und seinem Zwischenwirt hin, die weitere Untersuchungen rechtfertigt. Die hier durchgeführte Studie ist als Grundlage für die Durchführung weiterer Studien zu betrachten. Insbesondere die langfristige Überlebensfähigkeit der *A. vasorum*-Larven sollte eindringlich studiert werden, besonders in Betracht dessen, dass *L. fulica* die zoonotischen Lungenwürmer *A. cantonensis* und *A. costaricensis* übertragen und diese womöglich in Gebiete einschleppen kann, in denen sie bislang nicht endemisch vorkommen.

Um eine detaillierte *in vivo*-Migrationsanalyse von lebenden Lungenwurmlarven in Schnecken zu dokumentieren, wurden in unserer PET/CT-Studie L1 von *A. vasorum* und *C. striatum* durch den radioaktiven Tracer ^{18}F -FDG markiert und anschließend *L. fulica* injiziert oder oral gefüttert. Im Anschluss wurden die infizierten Schnecken zu verschiedenen Zeitpunkten p. i. mittels des PET/CT-Verfahrens gescannt und die *in vivo*-Verteilung der Lungenwurmlarven im Körper der Schnecke analysiert. Für vergleichende Zwecke wurden hierzu Larven von zwei Lungenwurmfamilien zur Infektion von Schnecken verwendet, nämlich der Familie Crenosomatidae und Angiostrongylidae. Diese Lungenwürmer sind im Endwirt in unterschiedlichen Organen lokalisiert: den Bronchien und der Trachea (*C. striatum*) oder dem rechten Herzen und der A. pulmonalis (*A. vasorum*). Durch diese verschiedenen Siedlungsorte sind *C. striatum* und *A. vasorum* unterschiedlichen Stressoren ausgesetzt, woraus sich Unterschiede im Organotropismus sowie der Reaktion auf die Experimentbedingungen ergeben können. Ausgesucht wurden diese Lungenwürmer auch aufgrund ihrer veterinärmedizinischen Relevanz. Infektionen mit *A. vasorum* führen zu einer Vielzahl an Symptomen, darunter neurologische, gastrointestinale, okuläre sowie kardiovaskuläre Erkrankungen. Besonders zu beachten ist in diesem Zusammenhang auch die schwere Hämorrhagie, die in manchen Fällen zum Tod des Tieres führen kann. Wie bereits erwähnt, kommt es zur vermehrten geographischen Ausbreitung dieses Lungenwurms, wodurch sich seine klinische Relevanz erhöht. Auch *C. striatum* ist ein klinisch relevanter Lungenwurm durch seine hohe Prävalenz in den Endwirten. Dies lässt sich durch den hohen Infektionsdrucks des Endwirts (z. B. Europäischer Igel) erklären, zu dessen Ernährung Schnecken gehören, die in vielen Fällen mit

infektiösen *C. striatum*-L3 infiziert sein können, was aber im Rahmen dieser Arbeit nicht untersucht wurde.

Wir konnten in dieser Studie erfolgreich beweisen, dass die L1 beider erwähnter Lungenwurmspezies mit radioaktivem ^{18}F -FDG inkubiert und später *in vivo* im natürlichen Zwischenwirt *L. fulica* mittels des PET/CT-Verfahrens während ihrer Migration dargestellt werden konnten. Nach der Präparation verfügten die L1 über ausreichende radioaktive Level für ein erfolgreiches Scannen im PET/CT.

A. vasorum-L1 verfügten durch die Aufnahme von ^{18}F -FDG nach 30 min über eine Aktivität von 0,3 KBq. Dieser Wert stieg nach längerer Inkubationszeit nicht, woraus sich schließen lässt, dass die Larven gesättigt waren oder die maximale Aufnahmedosis von ^{18}F -FDG bereits erreicht wurde. In den Replikaten jedoch fanden sich Unterschiede in der durchschnittlichen Aktivität pro Larve, was sich durch individuelle biologische Unterschiede in den einzelnen Larven erklären lässt, wie etwa unterschiedliche Größe, Immunstatus, metabolische Aktivität und Stress.

Da sich unter natürlichen Bedingungen die Schnecken in der Regel oral durch Koprophagie infizieren, haben wir in einem Teil des Experiments die Schnecken oral mit den Larven infiziert, um den natürlichen Infektionsweg nachzuahmen. Dabei nahmen alle Schnecken die Flüssigkeit mit den radioaktiv markierten Larven freiwillig auf, da diese wässrige Lösung (verdünnter Hundekot) den koprophagen Schnecken schmeckt und als Nahrung dient. Unmittelbar nach der Infektion zeigte sich in den ersten Scans ein starkes Signal im Gastrointestinaltrakt der Schnecken, welches jedoch mit der Zeit in den weiteren Scans abnahm und nach zwei Stunden p. i. vollkommen verschwunden war.

In einem weiteren Versuch wurden die radioaktiv markierten Larven in das Gewebe dorsal des Pneumostoms von *L. fulica* injiziert. In diesen Scans ließ sich die Aktivität der Larven in allen Individuen am Injektionsort sowie in vier Individuen ($n = 4$) auch im Gastrointestinaltrakt verfolgen. Diese Ergebnisse stimmen mit denen anderer Studien überein, in denen die Larven ebenfalls in der Nähe der Injektionsstelle gefunden wurden (Sauerländer et al. 1976). Im weiteren Verlauf unserer Studie wurden die infizierten Schnecken alle 30 min p. i. gescannt. Dadurch wurde jeweils eine weitreichende Migration der Larven dargestellt, die aber keinem bestimmten Organ zugeordnet werden konnte.

In vergangenen Studien wurde bereits ein Organotropismus der wandernden Lungenwurmlarven nachgewiesen, woraus sich schließen lässt, dass die Larven hier ebenso zu bestimmten Organen wanderten (Sauerländer 1976; Dusch et al. 2024). Sauerländer et al. (1976) infizierte *L. fulica* mit *A. vasorum* und *A. cantonensis*, die Infektionsdosis variierte von 5000 bis 20.000 Larven pro Schnecke. Als häufiges Ziel der Larvenmigration ergab sich bereits

zwei Stunden p. i. der Fuß sowie die Lunge der Schnecke. In der Studie von Dusch et al. (2024) wurde 90 min p. i. eine Prädilektionsstelle für die Migration von *A. vasorum*-L1 zum Fuß und zum Kopf (nach oraler Infektion) festgestellt. In der aktuellen Studie konnten wir während unseres PET/CT-Scans jedoch kein derart spezifisches Signal aus dem Fuß oder der Lunge feststellen. Ein Grund für dieses Phänomen kann einerseits durch die geringe Infektionsdosis von 1000-2000 Larven pro Schnecke erklärt werden, was zur Abschwächung des Signals in den Organen geführt hat. Eine andere Erklärung lässt sich im großen Volumen der beiden Organe finden. Ein Organtropismus konnte jedoch in zwei der mit *A. vasorum* infizierten Schnecken beobachtet werden. So wurde ein deutliches Signal im oberen Teil der Schnecke in der Verdauungsdrüse beobachtet.

Die Interpretation dieser Ergebnisse sollte jedoch vor dem Hintergrund bestimmter Einschränkungen erfolgen. Besonders zu beachten ist auch die Tatsache, dass selbst in der negativen Kontrollgruppe, die rein aus toten Larven bestand, eine geringe Aufnahme von ¹⁸F-FDG erfolgt ist. Daraus lässt sich schließen, dass die Waschvorgänge nicht ausreichend sind, um das ganze verbleibende ¹⁸F-FDG vollständig auszuwaschen. Weiterhin zu beachten ist die geringe Probandenzahl ($n = 7$). Auch zeigte sich im Bereich der Injektionsstelle eine erhöhte Aktivität, die womöglich auf eine Kontamination mit dem verbleibenden ¹⁸F-FDG zurückzuführen ist. Ein weiterer wichtiger limitierender Faktor lässt sich in dem verwendeten radioaktiven Tracer ¹⁸F-FDG finden, dessen Halbwertszeit nur 109,8 min beträgt, was präzise Scans nur wenige Stunden p. i. ermöglicht. Dies spiegelt sich wider in dem abschwächenden Signal, welches in allen Scans festgestellt wurde.

Wie bereits erwähnt dient diese Studie als Pionierarbeit für weitere Arbeiten dieser Art. So ist in weiterführenden Studien eine Verbesserung der Methodik unabdingbar. In diesem Zusammenhang sind vor allem die Waschvorgänge zu nennen, die gründlicher und umfangreicher durchgeführt werden müssen, um mögliche Kontaminationen mit ¹⁸F-FDG zu vermeiden. Um bessere und statistisch relevante Vergleiche zwischen den individuellen Schnecken ziehen zu können, ist außerdem eine höhere Probandenanzahl unabdingbar. Zudem wäre die Verwendung eines anderen radioaktiven Tracers mit einer längeren Halbwertszeit, wie etwa ¹¹¹-Indium-Oxid, eine gute Möglichkeit, um larvale Migration über einen längeren Zeitraum hinweg zu analysieren. Während in dieser Studie nur zwei Lungenwurmspezies verwendet wurden, sollten diese Scans auch mit weiteren Lungenwürmern durchgeführt werden, etwa den Katzenlungenwürmern *T. brevior* und *Ae. abstrusus* sowie den zoonotisch relevanten Lungenwürmern *A. cantonensis* und *A. costaricensis*.

Während dieser PET/CT-Studie konnte festgestellt werden, dass die wandernden Lungenwurmlarven in der Tat über einen Organtropismus verfügen, was sich unter anderem

mit den in den jeweiligen Organen verschiedenen metabolischen Bedingungen sowie Immunreaktionen erklären lässt (Sauerländer et al. 1976; Dusch et al. 2024). Doch noch immer ist wenig bekannt über das gastropode nicht-adaptive Immunsystem und dessen Abwehrreaktionen auf eindringende Pathogene, wie etwa metastrongyloide Lungenwürmer. Dabei stellen diese Schnecken den obligaten Zwischenwirt in einer Reihe von Parasitosen dar. Das gastropode Immunsystem dient dabei als erste Hürde, die die Lungenwurmlarven in ihrem Lebenszyklus überwinden müssen, woraus sich ein direkter Einfluss des Immunsystems in der Übertragung der Parasitosen ergibt. Aus diesem Grund untersuchten wir in einer weiteren Studie die *in vitro*-Reaktionen des angeborenen Immunsystems von *L. fulica* auf vitale *A. vasorum*-L1 sowie auf lösliches *A. vasorum*-Antigen (*AvAg*). Die benötigte Hämolymphe wurde durch Herzpunktion bei *L. fulica* erhalten und sofort mit einem bereits für Mollusken adaptierten Antikoagulanzpuffer vermischt (Lange et al., 2017; Penagos-Tabares et al., 2018b). Die so erhaltenen Hämozyten wurden den erwähnten lebenden *A. vasorum*-L1 oder dem *AvAg* ausgesetzt und auf Produktion von ROS (**R**eactive **O**xygen **S**pecies) und InEPT sowie allgemeine Aktivierung untersucht. Zur Testung dieser Immunreaktionen wurden folgende Verfahren verwendet: *i*) Durchflusszytometrie, *ii*) Rasterelektronenmikroskopie (REM) sowie *iii*) 3D-Holotomographie-Mikroskopie lebender Zellen (Lopez et al. 2022).

Im Rahmen dieser immunologischen Studie konnten wir in Hämozyten, die *AvAg* ausgesetzt wurden, eine zeitabhängige ROS-Produktion nachweisen. Dies ist ein evolutionär gut konservierter, im Tierreich weitverbreiteter Effektormechanismus von Leukozyten gegen eindringende Pathogene wie Bakterien, Viren, Fungi und Parasiten. Für die Hämozyten anderer wirbelloser Spezies wurde die ROS-Produktion bereits als potenter Abwehrmechanismus beschrieben, darunter die Fruchtfliege *Drosophila* sp., Austern oder andere Muscheln und die Süßwasserschnecke *Biomphalaria glabrata* (Hahn et al. 2000; Donaghy et al. 2012; Myers et al. 2018; de la Ballina et al. 2022). Die Hämozyten von *L. fulica* reagieren auch auf parasitäre Infektionen, wie wir in diesem Experiment beweisen konnten. Zudem wurde die ROS-Produktion von *L. fulica*-Hämozyten als allgemeiner und versatiler Abwehrmechanismus gegen eindringende Pathogene und Fremdkörper bestätigt, wie es auch für Säugetiere bereits nachgewiesen wurde (Torres et al. 2006; Moghadam et al. 2021). Auch Parasiten werden durch ROS angegriffen und so wird die Überlebenswahrscheinlichkeit des Parasiten im Wirt gesenkt (Pawłowska et al. 2023). Das sogenannte zelluläre „oxidative burst“ spielt ebenso eine Rolle in der Verteidigungsstrategie des angeborenen Immunsystems der Schnecke und verteidigt so den Organismus gegen Pathogene wie Bakterien und Parasiten (Hahn et al. 2001; Russo und Madec 2011). Während bereits Studien über die ROS-Produktion von *L. fulica* vorliegen, sind diese Daten dennoch begrenzt (Malagoli et al. 2023). Auch die Ausbildung sogenannter „**E**xtracellular **T**raps“ (ET) gilt als Abwehrmechanismus des angeborenen Immunsystems von Säugetieren und Wirbellosen gegen verschiedene

eindringende Pathogene. So liegen auch Berichte vor, dass die Hämozyten der Gastropoden auf eindringende Helminthenstadien mit der Ausbildung der sogenannten InEPT (Invertebrate Extracellular Phagocyte Traps) reagieren (Lange et al. 2017; Penagos-Tabares et al. 2018b).

In dieser Versuchsreihe konnten wir die Aktivierung der Hämozyten durch verschiedene Stimuli, das heißt AvAg, *A. vasorum*-L1 sowie LPS auslösen. Diese Aktivierung stellte sich durch eine morphologische Veränderung der Hämozyten dar. Zuerst raute sich die üblicherweise glatte Hämozytenoberfläche auf und es kam zur Bildung der sogenannten Pseudopodien durch Dehnung der Hämozytenmembran. Durch die 3D-Holotomographie konnten wir während der spontanen Aktivierung außerdem eine verstärkte Zelladhäsion an die Matrix sowie eine Aggregation der Hämozyten untereinander beobachten. Besonders zu beachten ist hier die Tatsache, dass es vor diesem Aktivierungsprozess zu keiner spezifischen Stimulation der Hämozyten durch Pathogene gekommen ist. Dieses Phänomen kann unterschiedliche Ursachen haben. Obwohl *L. fulica* im Labor unter standardisierten Bedingungen in Klimaschränken gehalten werden, ist es dennoch möglich, dass sie mit diversen Mikroorganismen in Kontakt kommen, die etwa durch das Futter oder die Erde in die Schneckenhaltung eingeschleppt wurden. Auch durch die Hämolymphentnahme durch Herzpunktion kann es zu einer bakteriellen Kontamination der Hämolymphe kommen, da in diesem Verfahren ein steriles Arbeiten nicht möglich war. Zwar befinden sich in dem hinzugefügten Hämolymphe- oder Antikoagulanzpuffer Antibiotika, doch können die Bakterien beispielsweise resistent sein oder die Konzentration der Antibiotika ist nicht ausreichend, um Bakterien effizient abzutöten, bevor es zur Hämozytenaktivierung kommt. Zu beachten ist auch die Tatsache, dass die Hämolymphe der Wirbellosen über ein natürliches Mikrobiom verfügt, wodurch es sich bei den potentiellen bakteriellen Kontaminationen auch um die natürliche Bakterienflora handeln kann (Lokmer und Wegner 2015; Carruthers et al. 2025). Zudem kann es durch mechanische Reize zur Aktivierung der Hämozyten gekommen sein, wie etwa dem Aufziehen in der Spritze, dem Vermischen der Hämolymphe mit dem Antikoagulanz sowie dem Kontakt der Hämozyten mit der Matrix. Dennoch halten wir dieses Verfahren für die geeignetste Methode der Hämozytenisolierung, da sich nur ein Teil der Hämozyten aktivierte. Außerdem stellte dieses Verfahren das Überleben der verwendeten Schnecken sicher und es kam zu wenig Schmerzen, Leiden und Schäden während der Extraktion. Dies spiegelte sich im Verhalten der Schnecken wider, die sich während der Prozedur nicht in ihr Häuschen zurückzogen und aktiv und neugierig blieben. Schließlich kann es sich bei dieser spontanen Aktivierung auch um eine basale Patrouillier- bzw. Surveillance-Aktivität der Hämozyten handeln, wie sie für andere Immunzellen bekannt ist.

Weiterhin unbekannt ist, ob diese durch *A. vasorum* induzierten InEPT-ähnlichen Strukturen der Schnecke dieselben immunologischen Eigenschaften wie ET der Säuger besitzen, wie

etwa die entzündungshemmenden Eigenschaften von *aggNET* (aggregated NET) (Schauer et al. 2014; Knopf et al. 2019), was in zukünftigen Studien noch untersucht werden sollte. Zudem konnten hier als Reaktion auf die Larven sowie das Antigen von *A. vasorum* möglicherweise die Ausbildung verschiedener Arten von InEPT nachgewiesen werden, darunter *sprInEPT* (spread InEPT) und *diffInEPT* (diffuse InEPT). Die REM-Analyse ermöglichte eine klare und detaillierte Darstellung der Aktivierung der Hämozyten in Form der Ausbildung von InEPT. Dabei wurden lange und extrem feine netzähnliche Strukturen dargestellt, was für das Vorhandensein von InEPT spricht. Die Darstellung dieser Strukturen ist mit der konventionellen Lichtmikroskopie nicht möglich, was das Potential der REM in der Darstellung filigraner Strukturen in der immunologischen Forschung unterstreicht.

Zusammenfassend lässt sich sagen, dass wir durch die hier verwendeten Verfahren in der Lage waren, die Reaktionen des gastropoden Immunsystems auf lösliches AvAg sowie der L1 von *A. vasorum* im Detail zu untersuchen. Besonders bemerkenswert ist hier das Verfahren der 3D-holotomographischen Mikroskopie, die die Untersuchung von Hämozyten in nahezu physiologischen Bedingungen erlaubt. Dadurch werden die Zellen nicht vorzeitig und künstlich durch diverse Inkubations- und Färbetechniken aktiviert, wie es bereits in Studien über die ET-Produktion bei Säugern berichtet wurde (Zhou et al. 2019; Conejeros et al. 2019; Grob et al. 2021; Espinosa et al. 2023).

Dennoch sollten die Limitationen der vorliegenden Studie beachtet werden. So liefern die REM-Analysen lediglich Informationen über den ultrastrukturellen Aufbau der möglichen InEPT beziehungsweise der aktivierten Hämozyten, aber die molekulare Zusammensetzung dieser Strukturen bleibt verborgen. Zudem ist die genaue Wirkung der InEPT auf die *A. vasorum*-Larven weiterhin unbekannt; insbesondere in Hinsicht auf eine potenziell abtötende Wirkung auf die Larven. Eine übermäßige Bildung von InEPT kann potenziell aber auch das Gewebe der Gastropoden schädigen, wie es bereits von ET bei Menschen berichtet wurde (Czaikoski et al. 2016; Rasmussen et al. 2021). Eine weitere Limitation wird durch das Verfahren der Hämozytenisolation verursacht. So ist für Leukozyten der Säuger berichtet, dass das Fehlen physiologischer pH- und Sauerstoffkonzentrationen das Verhalten dieser immunkompetenten Zellen beeinflussen kann. Das Aufrechterhalten dieser physiologischen Bedingungen ist bei der derzeit verwendeten Methode der Herzpunktion nicht möglich. Eine Verbesserung dieses Verfahrens ist essentiell, um möglichst physiologische Resultate in Zukunft zu erzielen. Zudem sollte eine höhere Hämozytenzahl erzielt werden, da die bisherigen niedrigen Zellzahlen weitreichende Analysen zu molekularen Mechanismen, Signalkaskaden, Phagozytose und ROS-Bildung bei *L. fulica* oder andere Schneckenarten erschweren.

Insgesamt erweitern unsere Untersuchungen das Verständnis der Interaktion zwischen dem gastropoden Zwischenwirt und metastrongyloiden Lungenwürmern. In dieser Arbeit konnten wir Unterschiede im jahreszeitlichen Verlauf der Prävalenz metastrongyloider Lungenwurmlarven im gastropoden Zwischenwirt aufzeigen. In weiteren Studien wurde der Organtropismus wandernder Lungenwurmstadien im gastropoden Zwischenwirt dargestellt sowie die Möglichkeit der radioaktiven Markierung und der Verfolgung der Wanderung von *A. vasorum* und *C. striatum in vivo*. Schließlich konnten wir auch verschiedene Immunabwehrmechanismen gastropoder Hämozyten auf *A. vasorum*-L1 und AvAg dokumentieren.

5. Zusammenfassung

Metastrongyloide Lungenwurminfektionen gehören zu den in der Praxis vernachlässigten Parasitosen, obwohl sie eine Vielzahl an Symptomen verursachen und zum Tod des Tieres führen können. Besonders *A. vasorum*, ein Parasit, der zu schweren kardiovaskulären Erkrankungen führen kann, breitet sich in den letzten Jahren geografisch aus und die Endemiegebiete nehmen zu. Außerdem wurde kürzlich über die Präsenz des zoonotischen Lungenwurms *A. cantonensis* auf dem europäischen Festland berichtet. Aus diesen Gründen sollte das Bewusstsein für diese Parasitosen geschärft werden.

Aktuelle Studien über das Vorkommen metastrongyloider Lungenwurminfektionen in Endwirten legen nahe, dass in Deutschland mehrere hyperendemische Foki, sogenannte „Hotspots“ existieren und entsprechend das Infektionsrisiko für Endwirte in diesen Gebieten erhöht ist. Jedoch liegen bisher wenige Studien über die Prävalenz der Lungenwürmer in den gastropoden Zwischenwirten vor. Weiterhin unbekannt ist zudem die Populationsdynamik der Lungenwürmer in den Schnecken über eine Spanne von mehreren Jahren sowie den Jahreszeiten in einem Jahr. Aus diesem Grund wurde ein bestehender Hotspot in Obrigheim (Baden-Württemberg) vier Jahre nach Erstuntersuchung erneut analysiert, um diese Wissenslücken zu überbrücken. Eine Gesamtzahl von 533 Schnecken wurde in allen vier Jahreszeiten gesammelt, künstlich verdaut und auf die Präsenz von metastrongyloiden Lungenwürmern untersucht. Die Prävalenz variierte stark zwischen den Jahreszeiten. Die höchste Prävalenz wurde im Sommer mit 27,46 % (59/215) festgestellt. Im Herbst sank die Prävalenz auf 10,00 % (16/160) und erreichte im Winter mit 5,65 % und Frühling mit 1,47 % ihren niedrigsten Wert. Auch die Prävalenzen der verschiedenen Lungenwürmer unterschieden sich. *A. vasorum* wurde in 12,01 % (64/533), *Crenosoma* sp. in 0,94 % (5/533) und *Ae. abstrusus* in 0,38 % (2/533) der Schnecken identifiziert. Während diese Ergebnisse deutlich unter jenen des Jahres 2018 liegen, konnte der hyperendemische Fokus in Obrigheim dennoch bestätigt sowie die Populationsdynamik der Lungenwürmer in den Schnecken im Jahreszeitenverlauf dargestellt werden.

Über das Verhalten der Lungenwurmlarven im obligatorischen gastropoden Zwischenwirt ist bisher wenig bekannt. Aus diesem Grund wurde die Migration dieser Larven im Modellorganismus *L. fulica* näher untersucht. Dazu wurden 26 erwachsene *L. fulica* mit *A. vasorum*-L1 infiziert, zu unterschiedlichen Zeitpunkten euthanasiert, die Organe separiert und im Anschluss künstlich verdaut. Diese wurden auf die Präsenz und das Stadium der Lungenwurmlarven untersucht. Im Rahmen dieser Studie konnte *L. fulica* als Zwischenwirt von *A. vasorum* erneut bestätigt werden. Bis zu 49,7 % der Larven konnten in der Schnecke wieder

aufgefunden werden. Der Infektionserfolg variierte drastisch zwischen den einzelnen Schnecken, wobei sich Prädilektionsstellen für die Ansiedlung der Lungenwurmlarven feststellen ließen. So konnte ein Organtropismus für die Lunge und den Fuß der Schnecke festgestellt werden, während der Reproduktionstrakt und die Albumindrüse selten ein Ziel für die wandernden Larven waren. In zusätzlich angefertigten histologischen Schnitten infizierter Organe ließ sich eine Reaktion der gastropoden Hämozyten auf die eingedrungenen Lungenwurmlarven in Form einer Einkapselung nachweisen.

Besonders über die Migration kurze Zeit nach der Infektion sowie die Wege, die die Lungenwurmlarven im Körper der Schnecke nehmen, ist bisher wenig bekannt. Aus diesem Grund wurden lebende *A. vasorum*- und *C. striatum*-L1 aus Kot isoliert und mithilfe des radioaktiven Tracers ^{18}F -FDG markiert. Die ideale Inkubationszeit betrug 30 min, danach nahmen die Larven kein ^{18}F -FDG mehr auf. Die durchschnittliche Aktivität pro Larve betrug dabei 0,33 (0,103) KBq. Im Anschluss wurden die radioaktiv markierten Larven *L. fulica* injiziert oder oral gefüttert. PET/CT-Scans wurden zu unterschiedlichen Zeiten nach der Infektion durchgeführt, um die frühen Wanderstrecken sowie den Organtropismus der Larven darzustellen. Die Larven migrierten von der Stelle der Infektion zu weiteren, *in vivo* nicht näher zu bestimmenden Organen.

Noch immer ist wenig bekannt über die Reaktionen des gastropoden Immunsystems auf Infektionen mit metastrongyloiden Lungenwürmern. In dieser Studie beabsichtigten wir, die Hämozyten von *L. fulica*, die als Effektorzellen des angeborenen Immunsystems dienen, *A. vasorum*-L1 sowie löslichem Antigen von *A. vasorum* (AvAg) auszusetzen. Die Hämozyten wurden durch Herzpunktion aus der Hämolymphe isoliert und mit einem Antikoagulant vermisch. Im Anschluss wurden die Zellen via 3D-holotomographischer Mikroskopie, Durchflusszytometrie sowie REM auf ihre Reaktionen auf die L1 oder das AvAg untersucht. So wurde in der Durchflusszytometrie eine zeitabhängige Produktion von ROS nachgewiesen. Durch das REM-Verfahren und die 3D-holotomographische Mikroskopie wurde die Aktivierung der Hämozyten dargestellt, was sich zeigte durch die Ausbildung von Pseudopodien, Filopodien und die Produktion von Vesikeln. Zudem ließen sich im REM-Verfahren die mögliche Bildung von InEPT nachweisen.

Die vorliegende Arbeit erweitert den Kenntnisstand zu der Epidemiologie sowie zum Organtropismus metastrongyloider Lungenwürmer im gastropoden Zwischenwirt. Zusätzlich konnte das Verständnis der Reaktionen des gastropoden Immunsystems auf eindringende metastrongyloide Lungenwürmer verbessert werden. Gleichzeitig unterstreichen die Ergebnisse die Notwendigkeit weiterer Untersuchungen, insbesondere im Hinblick auf potenzielle Unterschiede verschiedener Lungenwurmspezies in ihrer Interaktion mit dem gastropoden Zwischenwirt.

6. Summary

Metastrongyloid lungworm infections belong to the most neglected parasitoses in veterinary practice, even though they can lead to a variety of symptoms and may even be fatal. Especially *A. vasorum*, a parasite that leads to severe cardiovascular disease, is spreading geographically and the endemic places are increasing. In addition, the presence of the zoonotic lungworm *A. cantonensis* was recently recorded on the European mainland. For this reason, the awareness for these parasitoses needs to be raised.

Recent studies about the occurrence of metastrongyloid lungworm infections in definitive hosts suggest that there are multiple hyperendemic foci, so-called “hotspots” in Germany, in which the infection risk for definitive hosts is elevated. So far, there are only few studies on the prevalence of lungworm infections in gastropod intermediate hosts. The population dynamic of lungworms in intermediate hosts over the span of multiple years as well in the seasons of the same year remains unknown. For this reason, a previously known “hotspot” was re-evaluated four years after the first examination. A total number of 533 gastropods were collected in all four seasons within this “lungworm hotspot”, artificially digested and analyzed for the presence of metastrongyloid lungworm larvae. The prevalence was found to vary greatly between the seasons. The highest prevalence was discovered in summer with 27.46 % (59/215) of infected gastropods. In fall, the prevalence dropped to 10.00 % (16/160) and reached the lowest level in winter with 5.65 % and spring with 1.47 %. The prevalence of the various lungworm species also differed. *A. vasorum* was discovered in 12.01 % (64/533), *Crenosoma* sp. in 0.94 % (5/533) and *Ae abstrusus* in 0.38 % (2/533) of analyzed gastropods. While these results are considerably lower than the ones observed in 2018, the hyperendemic focus in Obrigheim was once again confirmed and the population dynamic over the seasons was as well illustrated.

So far, there is little knowledge on the behaviour of the lungworm larvae in the obligatory gastropod intermediate host. For this reason, the migration of these larvae in the model organism *L. fulica* was investigated. For this, 26 adult *L. fulica* were infected with *A. vasorum* L1 and euthanized at different time points after infection. The organs were separated, artificially digested and analysed for the presence and the stadium of the lungworm larvae. In this study, *L. fulica* was proven once again to be a suitable intermediate host for *A. vasorum*. The larval recovery rate reached up to 49.7 %. The infection success ranged greatly between the individual snails, and predilection sites were identified. An organ tropism for the lung as well as the foot of the snail was here observed, while the reproduction tract and the albumin gland were rarely found as migration targets for endogenous larvae. In addition, histological sections of infected gastropod organs showed an encapsulation response by gastropod haemocytes.

There is so far little knowledge especially on the migration shortly post infection (p. i) as well as the paths taken by the lungworms in the snail's body. For this reason, vital *A. vasorum* and *C. striatum* L1 were isolated from faeces and marked with the radioactive tracer ^{18}F -FDG. The ideal incubation period was found to be 30 min, after that, the larvae were not capable of taking up any more ^{18}F -FDG. The average radioactivity per larva was 0.33 % (0.103) KBq. Afterwards, the radioactively labeled larvae were either injected or orally fed to *L. fulica*. PET/CT scans were performed at different time points p. i. in order to illustrate the early migration routes as well as the larval organ tropism. The larvae migrated from the site of the infection to further organs that could not be identified *in vivo*.

Little is known on the reaction capacities of the gastropod innate immune system against metastrongyloid lungworm infections. In this study, we aimed to expose *L. fulica*-derived haemocytes, which are the effector cells of the gastropod innate immune system, to *A. vasorum* L1 as well as soluble *A. vasorum* antigen (AvAg). The haemocytes were isolated from *L. fulica* haemolymph via cardiac puncture and mixed with an anticoagulant buffer. Afterwards, the cells were analyzed via live-cell 3D-holotomographic microscopy, flow cytometry as well as scanning electron microscopy for their reactions to *A. vasorum* L1 or AvAg. A time-dependent ROS production was proven via flow cytometry analysis. Haemocyte activation, which was demonstrated by the formation of pseudopodia, filopodia and the production of vesicles, was illustrated with scanning electron microscopy as well as live cell 3D-holotomographic microscopy. Additionally, SEM illustrated most likely the production of InEPT.

This study expands our understanding on epidemiology as well as organ tropism of metastrongyloid lungworms in gastropod intermediate hosts. In addition, our understanding of the gastropod immune system's response to invading metastrongyloid lungworms has been enhanced. At the same time, the results underline the need for further research, especially regarding potential differences among various lungworm species in their interaction with the gastropod intermediate host.

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8. Anhang

Das Manuskript zu den Immunreaktionen der Hämozyten von *L. fulica* auf verschiedene Stimuli wurde bereits eingereicht (MDPI Animals), jedoch noch nicht akzeptiert. Es ist in den folgenden Seiten hinterlegt (Alena Dusch, Ivan Conejeros, Zahady D. Velasquez, Carlos Hermosilla, Anja Taubert. Haemocyte-derived innate immune reactions of the Giant African snail (*Lissachatina fulica*) against the lungworm *Angiostrongylus vasorum*).

Des Weiteren wurde eine Arbeit unter meiner Ko-Autorenschaft veröffentlicht, welche die Parasitenflora des Eurasischen Luchses sowie des Zwischenwirts Schnecke analysierte. Diese Veröffentlichung ist ebenfalls im Anhang hinterlegt.

8.1 Haemocyte-derived innate immune reactions of the Giant African snail (*Lissachatina fulica*) against the lungworm *Angiostrongylus vasorum*

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Abstract

The metastrongyloid parasite *Angiostrongylus vasorum* is currently considered an emerging gastropod-borne lungworm species of canids and has gained growing scientific attention in the last years. Nonetheless, knowledge of gastropod innate immune reactions and how *A. vasorum* larvae are attacked by gastropod-derived haemocytes is still very sparse. The current study aims to investigate gastropod haemocyte innate immune reactions in response to *A. vasorum* L1 larvae and antigen (AvAg) *in vitro*. Gastropod haemocytes were isolated from the Giant African snail *Lissachatina fulica* via cardiac puncture, confronted with AvAg or *A. vasorum* L1, and thereafter assessed for cell activation status, ROS production and extrusion of invertebrate extracellular phagocyte traps (InEPT). Haemocyte-parasite interactions were assessed via flow cytometry, live cell 3D-holotomographic microscopy as well as scanning electron microscopy (SEM). Overall AvAg stimulation activated haemocytes and induced a time-dependent increase in haemocyte ROS production. Moreover, SEM analyses illustrated the formation of 'InEPT-like' structures in response to *A. vasorum* L1. These novel findings on neglected invertebrate innate immune responses will help to better understand the complex mollusc-metastrongyloid parasite interactions, which contribute to the epizootiology of not only canine angiostrongylosis but also of closely related metastrongyloid nematodes of public and veterinary health concern.

Keywords: *Lissachatina fulica*, *Angiostrongylus vasorum*, gastropod-borne diseases, haemocytes, reactive oxygen species

1. Introduction

Gastropod-borne diseases, albeit being neglected, play an important role in both veterinary and human medicine [1]. As such, aquatic-, semi-aquatic- and terrestrial gastropods play a pivotal role as obligatory intermediate hosts in the life cycles of numerous helminths, including all parasitic trematodes [2–4] and some metastrongyloid nematode species [5,6]. Metastrongyloid lungworms of

canids and felids, including the highly pathogenic species *Angiostrongylus vasorum*, are of great veterinary concern worldwide and nowadays emerging into previously non-endemic geographic areas [7]. Clinical signs of *A. vasorum* infections in dogs and wild canids vary from mild respiratory signs to severe cardiopulmonary disorders, haemorrhaging, in addition to neurological, ocular, and gastrointestinal symptoms [8–11]. The heteroxenous life cycle of *A. vasorum* is complex since it includes numerous wild canids (e.g., wolves, jackals, coyotes, foxes) and domestic dogs as definitive hosts and a broad spectrum of gastropods serving as intermediate hosts (i. e., slugs, semi-slugs, snails) besides paratenic hosts [12–16]. By feeding on the faeces of an *A. vasorum*-infected definitive host, gastropod intermediate hosts become infected with first-stage larvae (L1). Alternatively, exogenous L1 may penetrate the gastropod's epidermis for infection [17,18]. While migrating in the gastropod, *A. vasorum* L1 molt into second-stage (L2) and third-stage larvae (L3), the latter of which represents the infective stage for final hosts. Besides *A. vasorum*, zoonotic *Angiostrongylus cantonensis* and *Angiostrongylus costaricensis* have re-emerged in recent years as relevant gastropod-borne human infections, causing severe symptoms like eosinophilic meningitis and abdominal angiostrongyliasis [19,20]. Of note, the invasive terrestrial Giant African snail (*Lissachatina fulica*) is a well-known intermediate host of both *A. vasorum* and *A. cantonensis* in the tropics of South America [1,21,22] and therefore of public health concern. Originally native to East Africa, these giant snails are currently recognized as invasive neozoa in multiple tropical and subtropical countries, including Argentina, Brazil, Colombia, Cuba, Ecuador, India, and the United States of America [23–28]. This invasive and coprophagic giant snail may also carry non-native pathogens, such as bacteria, viruses, fungi and parasites, and thus indirectly affect human and animal health. Additionally, *L. fulica* impacts local agriculture by feeding on a large variety of plants and destroying whole harvests [28–30]. Besides the snail's negative impact on humans, animals, agriculture and ecosystems, it may also be useful as a model organism for gastropod-borne diseases, as recently reported [31–33].

In contrast to innate and adaptive branches of the immune system in mammals and birds, invertebrate gastropods exclusively possess an innate immune system, which plays a crucial role in the defense against invasive pathogens, including lungworms [31,34]. Notably, the gastropod innate immune system resembles that reported in vertebrates on the level of cellular and molecular mechanisms [35–37]. Gastropods have a cardiopulmonary system and vascularization, containing haemolymph with haemocytes (syn. amoebocytes). Haemocytes are the most abundant cells in the haemolymph, representing the first line of defense and acting as multifunctional professional phagocytes. As such, circulating and tissue-resident haemocytes own various effector mechanisms like phagocytosis, encapsulation, cell-mediated cytotoxicity, reactive oxygen species (ROS) production, in addition to secretion of microbiocidal and immunomodulatory proteins and enzymes, such as lysozyme, peroxidases, histones and lectins, among others [38,39]. Haemocyte nitric oxide (NO) synthesis represents another critical effector mechanism, since NO not only acts as signaling molecule but also possesses direct antimicrobial properties [40]. Recent studies also reported on the

formation of invertebrate extracellular phagocyte traps (InEPT), which are released from activated gastropod haemocytes in response to larval stages of parasitic nematodes like *A. vasorum*, *Aelurostrongylus abstrusus* and *Troglostrongylus brevior* [34,40]. Resembling neutrophil extracellular traps (NETs) in mammals, InEPT release is considered a conserved innate effector mechanism and cell death process directed against large-sized pathogens [31]. Even though it is generally accepted that invertebrates exclusively possess an innate immune system [42,43], some findings also indicate the presence of specialized adaptive immune reactions in these organisms [44–48]. Consistently, invertebrate haemocytes may play a pivotal role in these adaptive immune responses, as suggested elsewhere [44,49]. Hence, gastropod haemocytes regulate cell proliferation, express and release humoral factors [e.g. fibrinogen-related proteins (FREPs), lectins, lysozymes, peroxidases] and own memory functions [49,50].

This study aimed to investigate early gastropod haemocyte-mediated innate immune reactions induced by the lungworm *A. vasorum* by utilizing the Giant African snail as a suitable model [33]. Here, *L. fulica* haemocytes were stimulated with *A. vasorum* soluble antigen (AvAg) as well as *A. vasorum* L1 and thereafter assessed for activation, ROS production and InEPT extrusion. Haemocyte-parasite interactions were analyzed via flow cytometry, live cell 3D-holotomographic microscopy and scanning electron microscopy (SEM). A novel technique of intracardiac puncture is here presented to obtain considerable haemolymph volumes and haemocyte numbers under standardized in vitro gastropod breeding conditions. Current findings provide novel insights into *L. fulica* haemocyte reactions against *A. vasorum*, which might translationally help to better understand zoonotic-relevant *A. cantonensis* and *A. costaricensis* infections. Taking the increasing popularity of *L. fulica* as pets into account [51,52], raising awareness of the potential health risks associated with handling the snails is essential.

2. Materials and Methods

2.1 Standardized gastropod maintenance

Giant African snails (*L. fulica*) were kept under standardized conditions in a fully automated climate chamber (ECP01E, Snijders Scientific B.V. Tilburg, the Netherlands). Humidity and temperature mimicked the gastropod's natural habitats in the tropics by applying temperature ranges from 20 to 26 °C and 50% humidity [53]. The lighting cycle consisted of 10 h of light and 10 h of darkness, with 2 h for dawn and dusk each. Snails were kept in plastic boxes with a 5 cm layer of commercial coconut soil (Kokosfaser–Humusziegel, TropicShop, Germany) and fed with lettuce (*Lactuca sativa*), carrots (*Daucus carota*), cucumber (*Cucumis sativus*), zucchini (*Cucurbita pepo*), as well as commercial dog food (Premium Trockenfutter Romeo, Germany). Common cuttlefish shells (*Sepia officinalis*) (Sepiaschalen, TropicShop, Germany) were offered ad libitum as a natural calcium source.

2.2 Haemolymph extraction and haemocyte isolation

A total of eight adult snails were used for haemolymph extraction. These specimens ranged in age from one to five years. Haemolymph extraction was performed by intracardiac puncture. Briefly, adult *L. fulica* were carefully washed with tap water and dried using paper towels before cardiac puncture. A hole was carefully drilled close to the pneumostome using a commercial drilling instrument (Dremel 3000, Dremel) until the shell was fully perforated (see supplementary video 1). Afterwards, a sterile syringe (23 G) was inserted to puncture the gastropod heart (see supplementary video 2). A haemolymph volume of up to one percent of the snail's body weight (BW) was extracted (i. e. 500-1000 μ l) and immediately mixed 1:1 with an anticoagulant gastropod haemolymph buffer (186 mM NaCl, 98 mM NaOH, 1.7 mM EDTA and 41 mM citric acid, pH 4.5) containing 3% penicillin (500 U/ml; Sigma-Aldrich) and streptomycin (500 μ g/ml; Sigma-Aldrich, Darmstadt, Germany) according to Lange et al. [34]. Afterwards, haemocytes present in the haemolymph sample were counted using a Neubauer chamber (Hecht assistant, Germany).

2.3 Preparation of *Angiostrongylus vasorum* L1 antigen (AvAg)

Vital *A. vasorum* L1 were isolated from the faeces of a naturally infected dog via the Baermann funnel technique. Briefly, 5 g of faeces were placed on a sieve with a 100 μ m mesh size. The sieve was mounted on a funnel and the funnel outlet was linked to a plastic tube closed with a metal clamp. The funnel was filled with water, until the faecal material in the sieve was half covered. After 24 h of incubation, the clamp on the tube was carefully opened, and 10 ml of faecal fluid containing viable *A. vasorum* L1 was extracted and washed 3 times with sterile PBS. Afterwards, 1% penicillin (500 U/ml; Sigma-Aldrich) and streptomycin (500 μ g/ml; Sigma-Aldrich, Darmstadt, Germany) were added to the sample, which was then stored at 4 °C for 2 days. Larvae were then washed thrice in PBS and homogenized via a sonifier (Branson Ultrasonics SFX150). A 60% pulse force was applied for one minute, during which the samples were kept on ice. Afterwards, the samples were centrifuged at 1300 rpm for 5 min at 4 °C. The pellet was discarded, and the soluble AvAg (supernatant) was stored at -80 °C. The protein concentration was measured using the Pierce BCA Protein Assay Kit (ThermoFisher).

2.4 Quantification of reactive oxygen species (ROS) by flow cytometry

For ROS detection, haemocytes were loaded with 10 μ M DCFH-DA (2,7-dichlorodihydrofluorescein diacetate) for 15 min at 22 °C. Unstained haemocytes served as controls for autofluorescence and background fluorescence signal detection. To assess the effect of AvAg stimulation on haemocyte ROS production, 1×10^3 haemocytes were stimulated for 60 and 120 min with soluble AvAg (1, 10, 100, and 1000 μ g/ml) and the fluorescence intensity (FL1) was thereafter registered at room temperature (RT). For positive and negative controls, haemocytes were

stimulated for the same time with the calcium ionophore A23187 5 μ M (Sigma-Aldrich, Germany) or left in RPMI medium, respectively. Data acquisition was performed using a BD Accuri C6 plus flow cytometer (BD Biosciences, Heidelberg, Germany), analyzing approximately 400 cells per time point and experimental condition. After the corresponding gating, the percentage of DCFH-DA⁺ cells was determined.

2.5 Live cell 3D-holotomography

For live cell 3D-holotomography, isolated haemocytes (n = 2) in sterile PBS, pH 7.4, were placed into a 35-mm imaging dish plate (Ibidi, Martinsried, Germany) in a top-stage incubator (Ibidi, Martinsried, Germany). Refractive index (RI)-based 3D-holotomographic images were obtained by using a 3D Cell Explorer-Fluo (Nanolive, Switzerland) microscope equipped with a \times 60 magnification (λ = 520 nm, sample exposure 0.2 mW/mm²) and a field depth of 30 μ m. Images were captured and analyzed using STEVE software (Nanolive, Switzerland) to obtain an RI-based z-stack. All images are displayed as maximum z-projections, and gamma, brightness, and contrast were adjusted (identically for compared image sets) using Fiji software (ImageJ, version 2.16.0).

2.6 Scanning electron microscopy (SEM)

Isolated haemocytes were left in RPMI medium or stimulated for 60 min at RT with LPS (0.1 ng/ μ l; Sigma-Aldrich) for negative and positive controls, respectively, and exposed to *A. vasorum* L1 and *A. vasorum* L1 antigen (*AvAg*, 10 μ g/ml). For fixation, 2.5% glutaraldehyde (Merck; Darmstadt, Germany) was used. Samples were washed in distilled water, dehydrated, critical point dried by CO₂ treatment, and finally sputtered with gold. Afterwards, the samples were analyzed by a scanning electron microscope (Philips XL30, Eindhoven, the Netherlands) at the Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany.

3. Results

3.1 Visualization of haemocyte activation via live cell 3D-holotomography

To characterize haemocyte baseline morphology and behavior, we analyzed the cells unstimulated by antigen or larvae. Haemocyte activation progressed asynchronously and spontaneously, with different morphological cell changes appearing at different stages of the response (Fig. 1). At the early stages of activation, the formation of few pseudopodia and an increased cell mobility and attachment was observed (Fig. 1b), whereas later stages, haemocytes showed extensive restructuring of the cytosol, the increased visibility of intracellular granules of various sizes and larger

pseudopodia arrangements (Fig. 1c, d). In some instances, we observed that pseudopodia from neighbouring haemocytes appeared to merge, thereby forming larger structures. In addition, broad veil-like extensions attached to the substrate were illustrated in multiple activated haemocytes (Fig. 1d).

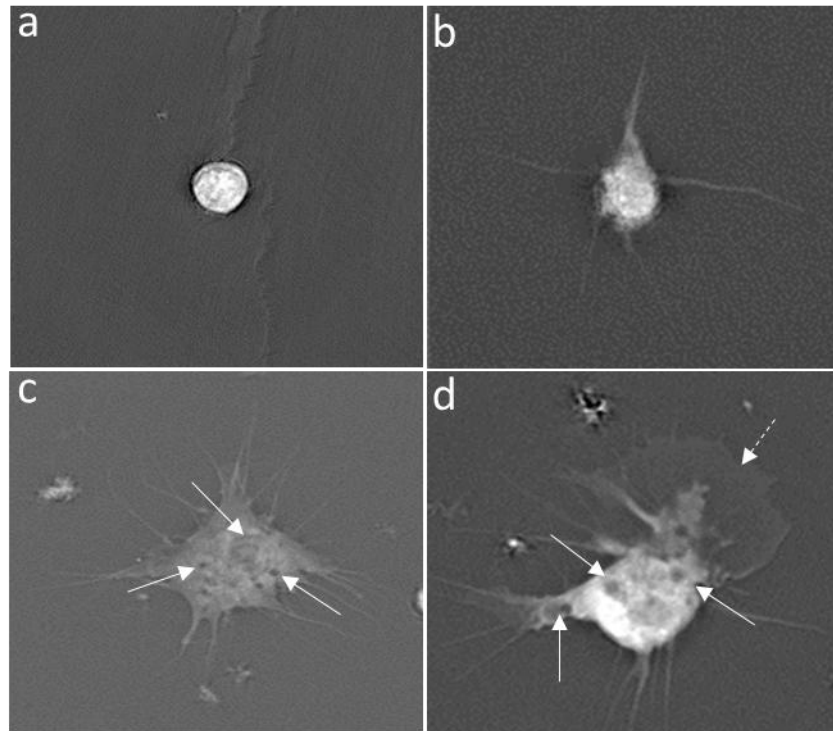


Figure 1. *Lissachatina fulica* haemocytes during different stages of spontaneous cell activation. a: inactive round haemocyte with flat surface; b: start of cell activation with attachment and pseudopod formation; c-d: fully activated, firmly attached haemocytes with pseudopod formation containing granules (solid arrows); d: veil-like extensions (dashed arrow).

3.2 Exposure of haemocytes to *A. vasorum* L1 drives the formation of invertebrate extracellular phagocyte trap '(InEPT)-like' structures

SEM analysis illustrated phenotypic changes of haemocytes in response to LPS (0.1 ng/ μ l), *A. vasorum* L1 and soluble AvAg, respectively. (Fig 2). After the stimulation of haemocytes with *A. vasorum* L1, the formation of haemocyte clusters (Fig. 2a) and of both 'spread InEPT-like' ('sprInEPT') and 'diffuse InEPT-like' ('diffInEPT') (Fig. 2b) was observed, independent of direct contact with the larvae. Moreover, AvAg stimulation led to the formation of haemocyte aggregates with surface blebbing and to enhanced pseudopod formation. In some areas, veil-like structures attaching to the substrate were observed as well (Fig. 2c). After LPS stimulation haemocytes were

activated, attached to the substrate, formed pseudopodia and extruded fine 'diffInEPT'-like structures (Fig. 2d).

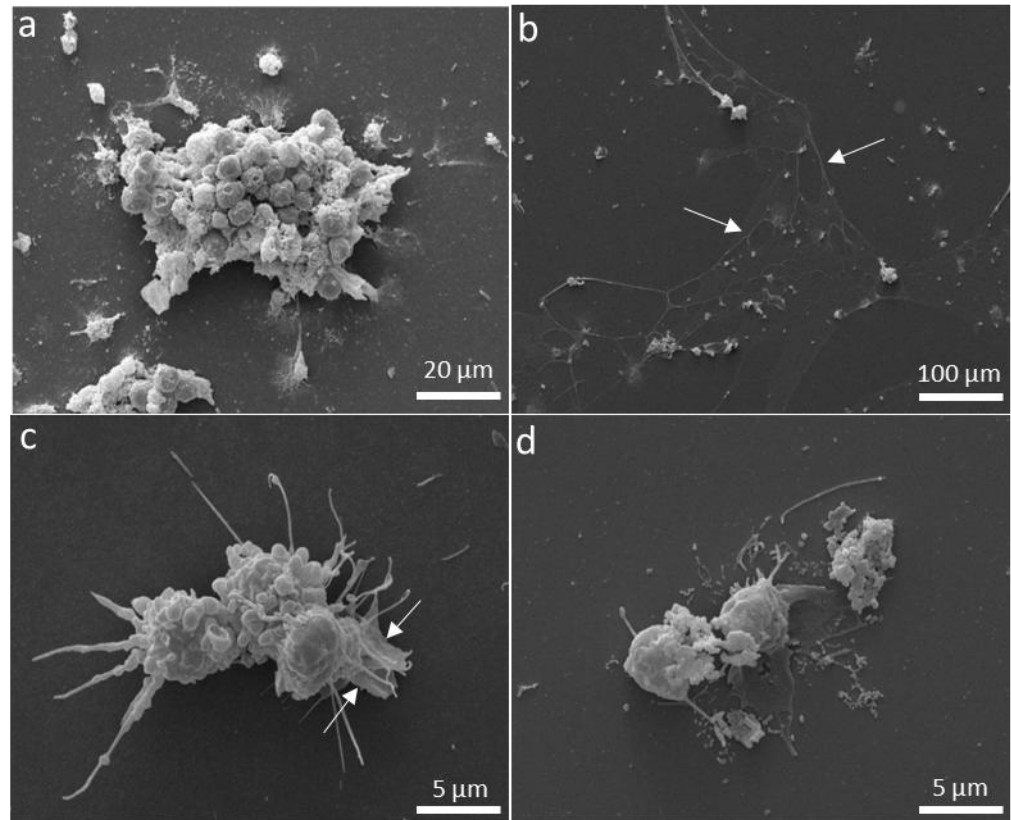


Figure 2. SEM images of *L. fulica* haemocytes reacting to different stimuli. *Angiostrongylus vasorum* L1 induced a: haemocyte aggregation, b: the formation of 'spread InEPT-like' ('sprInEPT') phenotypes (indicated by white arrows); c: AvAg-induced haemocyte pseudopod formation and irregular surfaces with membrane blebbing and veil-like structures (indicated by white arrows); d: LPS-stimulation mediated haemocyte activation with cell attachment, 'diffInEPT'-like structures and pseudopod formation.

3.3 AvAg stimulation induces ROS generation in gastropod haemocytes

To assess inherent oxidative responses, gastropod haemocytes were stimulated for 5, 60 and 120 min with AvAg and tested for intracellular ROS generation via flow cytometry (for gating strategy and single histograms, see Supplemental Figure 1). Here, haemocytes showed time- and antigen concentration-dependent reactions. Hence, an AvAg antigen concentration of 1000 μg/ml was toxic for haemocytes, since it resulted in cell fragmentation. At a concentration of 100 μg AvAg /ml, haemocytes responded with increased ROS production after 120 min of exposure when compared to medium controls (Fig. 4). Notably, haemocyte stimulation with the calcium ionophore A23187 (5

μM), commonly used as ROS inducers in the mammalian system, did not induce any changes in ROS production (Fig. 3).

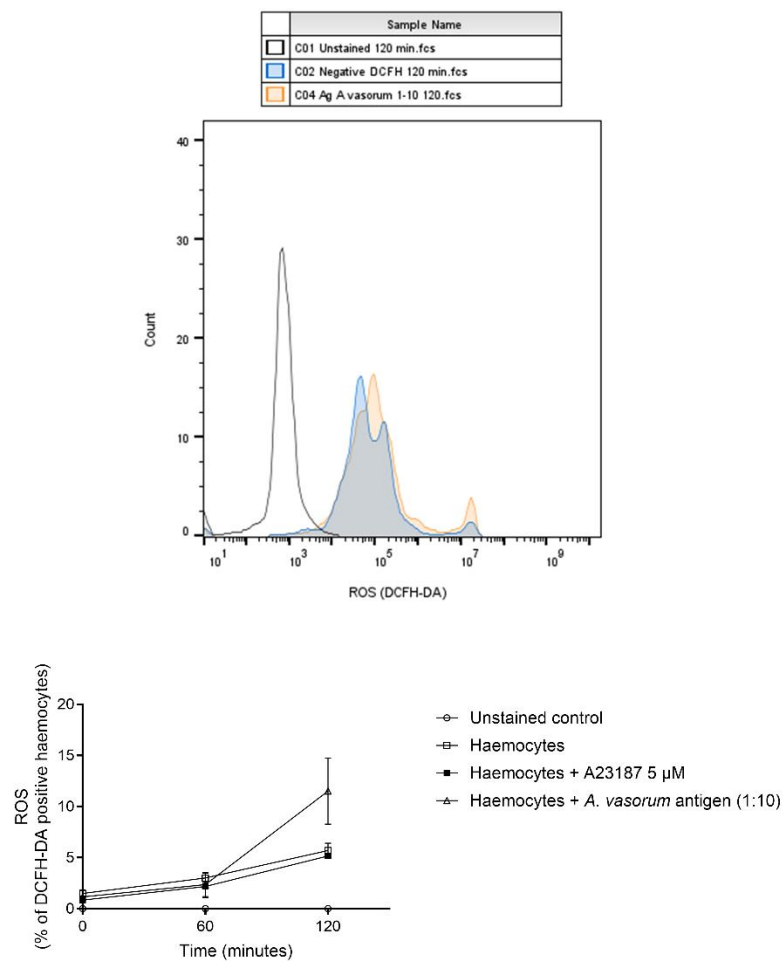


Figure 3. *A. vasorum* antigen stimulates intracellular ROS production by *Lissachatina fulica* haemocytes. For ROS detection, haemocytes were loaded with 10 μM DCFH-DA (2,7-dichlorodihydrofluorescein diacetate) for 15 min at 22 °C. Unstained haemocytes served as controls for autofluorescence and background signal detection. To assess the effect of AvAg on ROS production, 1×10^3 haemocytes were stimulated with soluble AvAg (100 $\mu\text{g}/\text{ml}$) and the fluorescence intensity (FL1) was registered at 60 and 120 min of incubation at RT. For controls, haemocytes were stimulated for the same time with the calcium ionophore A23187 5 μM or left in plain medium. Data acquisition was performed using a BD Accuri C6® plus flow cytometer, analyzing approximately 400 cells per time point and experimental condition. After gating, the percentage of DCFH-DA+ cells was determined.

4. Discussion

A broad range of gastropod species act as obligatory intermediate hosts of the highly pathogenic vascular nematode *A. vasorum*, which may cause life-threatening disease in dogs. So far, little is known about innate immune reactions generated by *A. vasorum*-infected gastropods. In the current study, we showed that gastropod haemocytes indeed responded to AvAg and *A. vasorum* L1 stimulation by upregulating both the formation of 'InEPT-like' structures and intracellular ROS production. ROS synthesis and release by innate immune cells is a well-conserved effector mechanism, widely utilized by professional phagocytes among the animal kingdom, and which has already been described before for other invertebrate species, including the fruit fly *Drosophila* sp. [54], oysters or other bivalves [55,56] and the freshwater snail *Biomphalaria glabrata* [57]. However, to the best of our knowledge, findings on ROS production by *L. fulica* haemocytes are scarce [58], and data on nematode-driven reactions or on ROS dynamics in this mollusk species remain limited. Hence, present findings expand current knowledge on this effector mechanism in *L. fulica* haemocytes within the neglected field of invertebrate immunology and related effector molecules. Notably, the stimulation of haemocytes with soluble AvAg drove ROS generation, highlighting the versatility of haemocyte ROS production and confirming these effector molecules as a general defense mechanism against various stimuli, as previously reported for various organisms, including mammals, fish, insects and plants [59,60]. Likewise, oxidative burst activities generally play a key role in the innate immune system of gastropods, defending the organism against various pathogens, such as parasites and bacteria [61,62]. Notably, the adverse effects of ROS on nematode viability have been demonstrated in other host contexts [63].

The formation of extracellular traps (ETs) is a well-documented effector mechanism of mammalian phagocytes utilized in the innate defense against nematode parasites [64–66]. A prerequisite for both ET formation and ROS production is the activation of phagocytes. In line, SEM analyses phenotypically illustrated haemocyte activation after exposure to all *A. vasorum* L1, soluble AvAg and LPS. Hence, in all cases, the haemocyte surface changed from smooth to rough and the cells extended their membranes by forming pseudopodia. The presence of InEPT in the absence of direct contact with *A. vasorum* L1 suggests that InEPT formation occurred as a response to excretory/secretory products present in the well. Moreover, enhanced adhesion to the underlying matrix and haemocyte aggregation was recorded. Notably, and as documented by live cell 3D-holotomography, haemocytes also showed spontaneous activation after ex vivo extraction, but at a minor level. This basal activity likely reflects an inherent patrolling and phagocytic readiness but may also have been driven by mechanical irritation due to the isolation process, such as the extraction itself, the mixing with the anticoagulant buffer and the contact with glass trays. In addition, *L. fulica* snails may come into contact with some microorganisms even under current standardized maintenance, like bacteria brought into the enclosure with food or soil. Furthermore, the current procedure of haemolymph extraction by cardiac puncture may not be entirely sterile, even though

sterile syringes and anticoagulant buffer with antibiotics were used, especially when considering bacteria from the natural microbiome of invertebrate haemolymph [67,68]. However, since only a small proportion of ex vivo-extracted haemocytes showed spontaneous activation, we consider the current isolation protocol as suitable. Moreover, as illustrated in the supplementary videos, the procedure of haemolymph extraction also proved to be mild and non-irritating for snail individuals, not at all affecting their well-being and viability (please see supplementary footage material).

SEM analysis enabled a detailed illustration of 'InEPT-like' formations by activated haemocytes. Thin and elongated fibers forming extracellular 'net-like' DNA structures in response to *A. vasorum* L1 were observed. Morphological heterogeneity was evident, with different 'InEPT-like' structures being documented, i. e. 'sprInEPT'- and 'diffInEPT'-like structures in response to haemocyte *A. vasorum* L1 stimulation. Whether these parasite-induced extracellular structures own the same immunomodulatory properties as mammalian NETs (e. g. anti-inflammatory properties of aggNETs [69,70]) awaits further investigations. Finally, holotomography further complemented conventional microscopic techniques by enabling 3D live cell imaging of haemocytes under almost physiological conditions, i. e., without stressing haemocytes by staining or other experimental procedures, potentially resulting in artificial cell activation, as previously reported [71,72].

However, several limitations must be considered in the present haemocyte study. Firstly, the number of haemocytes isolated per *L. fulica* specimen is still low and not at all comparable with the mammalian system, thereby hampering extensive analyses on molecular mechanisms, signaling cascades, metabolic signatures, receptor analyses and InEPT formation. Secondly, SEM analyses exclusively provide ultrastructural information on the haemocyte surface but do not allow conclusions on molecular composition or expression of receptors. Moreover, so far, the actual impact of 'InEPT-like' structures on invading lungworm larvae remains unclear. However, an excessive InEPT formation may also harm gastropod tissues as reported for mammalian ETs [73,74]. Thirdly, the currently used method for haemocyte isolation from haemolymph does not apply physiological pH and oxygen concentrations, which both were reported to affect innate leukocyte biology [75–77]. Future experimentation will pursue obtaining higher numbers of haemocytes for more physiological experimental conditions to better understand gastropod-mediated innate immunity.

5. Conclusions

In conclusion, we here demonstrate that *A. vasorum* L1 and AvAg exposure to haemocytes leads to the activation of the gastropod haemocytes, being accompanied by both ROS production and 'InEPT-like' formation. Overall, this work deepens the understanding of the complex gastropod-parasite interactions and of the gastropod innate immune system in general. In addition, these findings highlight *L. fulica* as a promising model organism for studying invertebrate innate immune reactions not only against invading parasites but also other pathogens like bacteria or viruses.

Further malacological research is needed to study the complex innate immune system and its impact on the transmission of gastropod-borne diseases worldwide.

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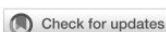
8.2 Patent *Troglostrongylus brevior*-, *Aelurostrongylus abstrusus*-, *Angiostrongylus* sp.-, and *Crenosoma* sp. infections in wild Eurasian lynxes (*Lynx lynx*) and their habitat-sharing gastropod intermediate hosts

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Haas et al. *Frontiers in Veterinary Science* (2025),
<https://doi.org/10.3389/fvets.2025.1515507>

Eigener Anteil an der Publikation:

- Projektplanung: 10 %, zusammen mit Betreuern
- Durchführung Versuche: 20 %, zusammen mit Ko-Autoren
- Auswertung der Experimente: 20 %, zusammen mit Ko-Autoren
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OPEN ACCESS

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RECEIVED 23 October 2024
ACCEPTED 23 May 2025
PUBLISHED 03 July 2025

CITATION
Haas M, Segeritz L, Anders O, Middelhoff TL,
Myat Tun A, Hasheminasab SS, Cocchiararo B,
Dusch A, Taubert A and Hermosilla C (2025)
Patent *Troglostrongylus brevior*-,
Aelurostrongylus abstrusus-, *Angiostrongylus*
sp.-, and *Crenosoma* sp. infections in wild
Eurasian lynxes (*Lynx lynx*) and their
habitat-sharing gastropod intermediate hosts.
Front. Vet. Sci. 12:1515507.
doi: 10.3389/fvets.2025.1515507

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Patent *Troglostrongylus brevior*-, *Aelurostrongylus abstrusus*-, *Angiostrongylus* sp.-, and *Crenosoma* sp. infections in wild Eurasian lynxes (*Lynx lynx*) and their habitat-sharing gastropod intermediate hosts

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The formerly widely spread Eurasian lynx (*Lynx lynx*) nowadays represents an endangered large wild felid species in Germany. Recent and ongoing conservation efforts have succeeded in establishing small but stable lynx populations in distinct parts of Germany. However, very little is known on the occurrence of neglected and re-emerging gastropod-borne cardiopulmonary nematodes in wild *L. lynx* populations in Europe. Therefore, the aim of current study was to estimate metastrongyloid infections in, a group of seven free-ranging, (sub-) adult Eurasian lynxes from the Harz Mountains (Germany) which were equipped with GPS/GSMS collars and in resident gastropod intermediate host populations. Both, lynx scat samples ($n = 24$) and terrestrial gastropods ($n = 153$) were collected in close proximity to prey remains left behind by Eurasian lynxes respectively in natural habitats in a non-invasive and un-molested manner. Fresh fecal samples were analyzed for the presence of metastrongyloid first-stage larvae (L1) by standard Baermann funnel technique and morphologically identified to genus level. Morphological metastrongyloid L1 were additionally investigated by PCR for final species identification. Terrestrial gastropods (i.e., slugs, semi-slugs, snails) were morphologically identified to genus level, thereafter artificially digested and analyzed for the presence of lungworm larvae. This work delivers a first report on the occurrence of patent *Troglostrongylus brevior*-, and *Crenosoma* sp.-infections in wild Eurasian lynxes in Germany and re-confirms recent findings on *Aelurostrongylus abstrusus*- and *Angiostrongylus* sp. infections in these lynxes. Overall, a total lungworm occurrence of 37.5% (9/24) was detected in assessed Eurasian lynx samples and 51.1% (4/7) of lynxes showed patent metastrongyloid infections. In digested terrestrial gastropods, 1.3% (2/153) contained *A. vasorum* larvae, underlining a successful propagation of *A. vasorum* life cycle in the Harz Mountains. Hence, we recommend regular

monitoring for metastrongyloid infections not only in wild Eurasian lynxes but also in obligate intermediate hosts to better understand their impact on animal and population health to support current conservation efforts on this endangered large felid species in Europe.

KEYWORDS

Aelurostrongylus abstrusus, *Angiostrongylus*, *Crenosoma*, Eurasian lynx, *Lynx lynx*, *Troglostrongylus brevior*, wildlife

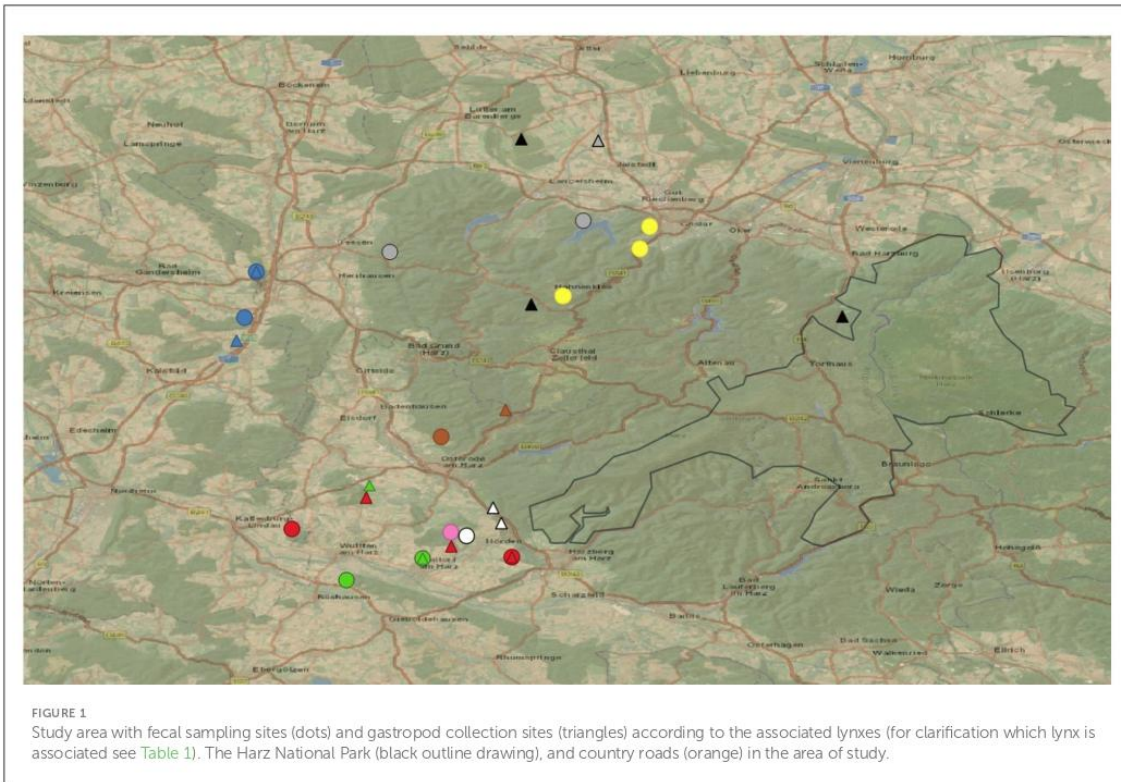
1 Introduction

The Eurasian lynx (*Lynx lynx*) represents the largest felid apex predator in Europe and, in consequence, plays a fundamental role in the maintenance of ecosystem health by not only influencing food web composition but also increasing biodiversity in natural biomes (1–7). Consistently, as a natural apex predator, *L. lynx* might affect prey behavior, number and composition and thereby indirectly influencing flora and fauna biodiversity (5–7). Additionally, the surrounding of the remains of Eurasian lynx prey, also referred to as killing sites, can be recognized as such for several weeks, since lynx feed on their prey multiple times, depending on the prey size and eventual disturbances. Killing sites are nowadays considered as important micro-ecosystems influencing environmental biomes (5–8). Consistently, killing sites can provide valuable data on predator-prey relationships, on complex host-parasite interactions, and additionally serve as nutritional sources for numerous vertebrates, invertebrates and microbes (8–16). Despite a positive impact of wild Eurasian lynxes on ecosystem preservation, their crucial role in biodiversity improvement is generally underestimated in Germany and elsewhere (2–7).

Two hundred years ago, the geographic distribution of Eurasian lynxes ranged from the European mainland to Central Asia and from the Tibetan plateau of China to Eastern parts of Russia (17–22). The Eurasian lynx has the IUCN (International Union for the Conservation of Nature) status least concern and is strictly protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), the Council Regulation (EC) 338/97 and Federal Species Protection Regulations (FSPR) in face of its population vulnerability (19, 21). However, according to the IUCN, the total Eurasian lynx population is listed as “least concern” since it shows a wide distribution in sparsely populated geographic areas of Eastern Europe and Asia (19, 21, 23). In contrast, in Germany, Eurasian lynxes are still facing extinction and are therefore listed as “critically endangered” according to the IUCN and the German Red List Centre (GRLC), based on their low individual numbers and critical population fragmentation (18–25). Main threats for *L. lynx* survival in Central Europe are poaching, traffic accidents, habitat fragmentation, habitat loss and insufficient presence of prey. Moreover, infectious diseases like viral infections (e.g., canine distemper, FeLV, FIV) and parasite infestations (e.g., *Sarcoptes*) negatively impact small Eurasian lynx populations as it is the case for the re-introduced Harz Mountain population (23, 26). Given that ecto- and endoparasitoses are well-known as causes of suffering and decline (27, 28), regular monitoring seems relevant for conservation issues.

Accordingly, feline gastropod-borne metastrongyloid nematodes, such as *Aelurostrongylus abstrusus*, *Angiostrongylus chabaudi*, *Crenosoma vismani*, and *Troglostrongylus brevior*, can cause bronchopneumonia and cardiopulmonary disorders in various definite hosts, including wild felids and lynxes (29–32). Alongside, some of these lungworm species parasitize domestic/feral cats (*Felis catus*) and wild cats (*Felis silvestris*) (33–42). Nonetheless, very little is currently known on these infections in free-ranging Eurasian lynxes. The crenosomatid lungworm species *T. brevior* and *C. vismani* parasitize the bronchi and bronchioles of feline definite hosts whilst *A. abstrusus* resides in subpleural parenchyma and alveoli (32, 37, 43, 44). Conversely, the angiotropic nematode *A. chabaudi* parasitizes the pulmonary arteries and the right heart of mainly wild cats (30, 33, 45). Embryonated metastrongyloid eggs are deposited, first-stage larvae (L1) hatch and migrate via lung tissues to larynx/pharynx, are swallowed and shed via defecation into the environment during patency. Thereafter, exogenous L1 infect terrestrial gastropods (i.e., slugs, semi-slugs, snails) acting as obligate intermediate hosts. In gastropods, L1 develop into second- (L2) and infective third- (L3) larval stages within approximately 2–4 weeks, depending on the parasite species. Eurasian lynxes become infected either after ingesting L3-infected gastropods or via consumption of paratenic hosts (amphibians, reptiles, birds, rodents) carrying infective L3. Alternatively, but more unlikely, wild Eurasian lynxes might become infected from standing waters containing dead intermediate hosts and/or released infective L3 (46). In case of *T. brevior*, also lactogenic transmission was recently demonstrated for domestic cats in Italy (42).

Feline troglostrongylosis has gained scientific interest in Europe where it is considered as a spill-over event from wild cats (*F. silvestris*) to feral cats (30, 32, 37, 42, 47–52). Pathological alterations of troglostrongylosis in lynx include multifocal, consolidated, firm tan to gray areas in various lung lobes with thickened alveoli walls, filled with necrotic debris, leukocyte infiltration and degenerated inflammatory cells, as well as parasite larvae and eggs and a lung oedema (32), thereby corroborating histopathological findings of *T. brevior*-infected wild cats (*F. silvestris*) (35, 36, 53). In line, proteinaceous lung oedema was described in wild bobcats (*Lynx rufus*) infected with closely related *Troglostrongylus wilsoni* (54). Of note, a recent study identified *T. brevior* in terrestrial gastropods in South America thereby expanding its geographic distribution (55). Obviously, spill-over of metastrongyloid infections from feral cats to free-ranging Eurasian lynxes or wild cats may occur when sharing the same biome (31). As already stated, only few studies exist on patent metastrongyloid infections in wild Eurasian lynxes



and on their impact on population health (29, 31, 32, 37, 38). Therefore, the current study aims to add epizootiological data by evaluating not only patent metastrongyloid infections in free-living Eurasian lynxes but also in gastropod intermediate hosts in the Harz Mountains, being habitat of the largest *L. lynx* population in Germany.

2 Material and methods

2.1 Study area

Collection sites were allocated along the western part of the Harz Mountains nearby the Harz National Park (HNP; 51.6946953, 10.5674415) in Germany. This mountainous area outside the HNP is characterized by opened landscapes, vast forested and meadow areas, provincial towns and some large country roads. An illustration of the study area is given in Figure 1, the respective geographic map was generated by QGIS V.3.28.1 (QGIS Geographic Information System. QGIS Association. <http://www.qgis.org>).

2.2 Collection of scat samples and gastropods at Eurasian lynx killing sites

Wild Eurasian lynxes sampled in this study included 6 young/sub-adult animals and an adult male. Four of these lynxes

(F10, M18, M19, and M20) were rehabilitated animals, which were originally found as orphans and raised in enclosures before being released again into the wild. The other three animals (F11, F12, and M22) were captured weak, and were rehabilitated before release, none of them showed any clinical respiratory signs. All animals were equipped with GPS/GSM-transmitting collars (VECTRONIC AEROSPACE, Berlin) by the staff of the HNP before release (see Figure 2). Moreover, parasitological examinations of collared juvenile Eurasian lynxes were performed, which excludes M22 which was captured as an adult animal. All juvenile animals were tested with copromicroscopy and were all found positive for *Toxocara cati* and thus received anthelmintic treatments (Ivomec[®], ivermectin, 0.5 mg/kg, Boehringer Ingelheim), before being released again. Lynx killing sites were identified using two handheld GPS devices (GPSMAP[®] 64s and GPSMAP[®] 65s, Garmin, Olathe, USA) and by profiting from received repeated collar-transmitted signals from distinct geographic spots, thereby proving the presence of lynxes at this locations (see above). In the current study, hidden prey animals included roe deer (*Capreolus capreolus*), hares (*Lepus europaeus*) and red foxes (*Vulpes vulpes*). A variety of arthropods (e.g., flies, maggots, carrion beetles, ants and isopods) as well as different terrestrial gastropods (i.e., slugs, semi-slugs and snails) were found in close proximity to killing sites (please refer to Figures 3, 5). In total, 41 killing sites or Eurasian lynx habitats were identified via GPS tracking and on 25 sites, scat-and/or gastropod samples were successfully collected.

Overall, 24 individual fecal samples originating from the seven individuals mentioned above, were collected during six



FIGURE 2
A wild Eurasian lynx (*Lynx lynx*) wearing a GPS collar.

visits of killing sites between March 2022 and February 2023. As mentioned before these samples could be associated with the different individual lynx by the sampling methods here used. Following this 3/24 samples originated from M18 (12,5%), 6/24 from M19 (25%), 1/24 from M20 (4,2%), 3/24 from M22 (12,5%), 3/24 from F10 (12,5%), 4/24 from F11 (16,6%), 1/24 from F12 (4,2%) and 3/24 from a meeting point of F12 + M22 (12,5%). Scat samples were identified based on characteristic morphology, size, composition (mainly containing roe deer hair), emission of lynx-specific odor, as well as the feline specific covering behavior of the feces with surrounding material. All the before mentioned characteristics in combination lead to the diagnosis of lynx feces. Collected scat samples were labeled, kept at 4°C and immediately transferred to the Institute of Parasitology of the Justus Liebig University Giessen for further parasitological analysis. In cases of uncertainty of the scat origin, fecal samples were additionally analyzed by molecular approaches at the Centre for Wildlife Genetics, Senckenberg Research Institute and Natural History Museum, Gelnhausen, Germany (for detailed description of this methodology refer to Section 2.3).

Besides scat samples, a total of 153 terrestrial gastropods were collected during 2021 and 2023 at 14 previously selected GPS-tracked killing sites or in Eurasian lynx habitats during scat sampling (please refer to Table 1, Figures 1, 5), and surrounding areas (up to 1 km radius). All gastropods were collected manually by wearing gloves in search for humid mollusc hiding places (i.e.,

beneath leaves, rocks or rotten wood) or in close proximity to killing sites. Some slugs were directly collected from carcasses, bones or beneath carcasses, as illustrated in Figure 3. All GPS-identified lynx killing sites were visited during daytime to avoid disturbance of nocturnal Eurasian lynxes and prey animals and with a time delay of at least 3 days to not disturb lynxes in their feeding behavior.

2.3 Molecular analysis of lynx feces

At the Centre for Wildlife Genetics (Senckenberg Research Institute and Natural History Museum, Gelnhausen, Germany) DNA from fecal samples were extracted, this and the following steps were performed by using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) and using the Qiacube-Robotic-System (Qiagen, Germany). Two mitochondrial markers were employed for species identification. The first marker consisted of the two primers L15995 (5'-CTCCACTATCAGCACCCAAAG-3') and H16498 (5'-CCTGAAGTAAGAACCAGATG-3') (56) and was used for general confirmation of a mammal species. The second marker consisted of the two primers LF4 (5'-GACATAATAGTGCTTAATCGTGC-3') (57) and H16498 (5'-CCTGAAGTAAGAACCAGATG-3') (58) detecting particularly members of the family Felidae. For PCR, 5 µl SensiFAST SYBR No-ROX Kit (Biocat, Germany), 0.4 µl of the respective primer



FIGURE 3

A typical Eurasian lynx (*Lynx lynx*) killing site at the Harz Mountains. (A) Leftovers of a killed roe deer (*Capreolus capreolus*). Initially the carcass was found entirely covered with grass, leaves, branches and soil particles; (B) a red-breasted carrion beetle (*Oiceoptoma thoracicum*) feeding on meat leftovers of a roe deer pelvis. (C) An excavated Eurasian lynx faecal sample which was also found entirely hidden under grass, leaves and branches. (D) Terrestrial slug (*Arion* sp.; indicated by red circle) found on a bone.

pair, 1.2 μ l of nucleic acid-free Water (Carl Roth, Germany) and 3 μ l of DNA extract were mixed. The cycling protocol included 1 \times 3 min at 95°C, 40 \times 95°C for 5 s and 60°C for 30 s, 10°C for storage. Before Sanger sequencing, PCR products were purified by ExoSAP-IT (ThermoFisher Scientific, USA) following the manufacturer's instructions. The BLAST tool (59) was used for species determination of the obtained sequences.

2.4 Detection of metastrongyloid first-stage larvae in fecal samples

Fecal samples were processed at the day of collection by using the standard Baermann funnel technique (60, 61). After 24 h of incubation, samples were microscopically analyzed using an Olympus BH-2[®] light microscope (Olympus, Tokyo, Japan) equipped with a digital camera (SC30[®], Olympus, Tokyo, Japan). Metastrongyloid L1 were morphologically characterized, and in

cases of high larval motility, treated with Lugol's iodine solution [iodine-potassium iodide solution according to Lugol (1% iodine), Carl Roth, Germany] to immobilize larvae (62). Lungworm larvae were identified to genus level according to their typical morphological and morphometric characteristics using several larvae per sample [i.e., body length, detail of anterior extremity, oesophageal shape (non-rhabditiform) and length (1/3–1/2 the length of larvae), and typical tail morphology] (31, 32, 34, 35, 47, 48, 63, 64) (see Figure 4).

2.5 Gastropod digestion for metastrongyloid larvae detection

Gastropods were first identified to genus level via morphological characteristics, then cryo-euthanized and stored at –20°C until further processing (55), artificially digested and sieved according to Penagos-Tabares et al. (55). In brief, frozen

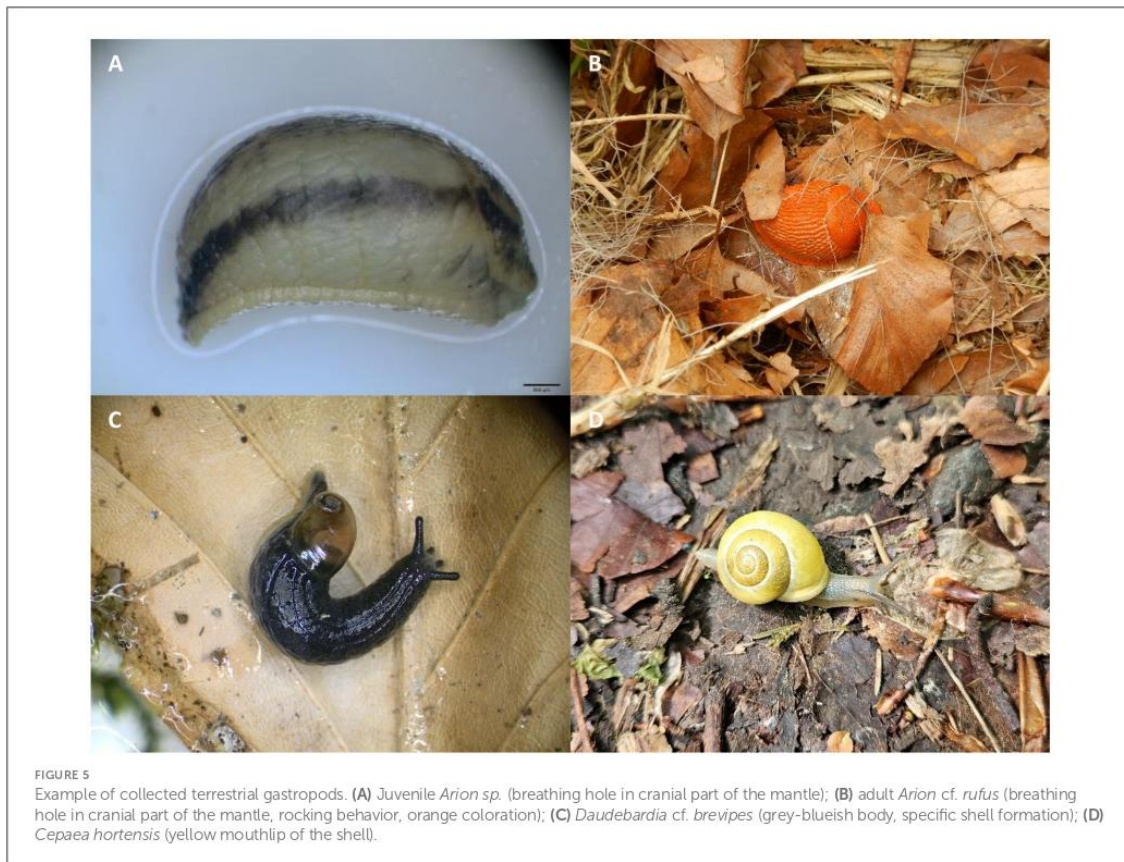


gastropods were cut into small pieces and immersed in a digestion solution [10 g pepsin powder 2000 FIP-U/g (Carl Roth, Germany), 8.5 g NaCl, 30 mL HCl 37% (Carl Roth, Germany) adjusted to 1 L by distilled water] for 3 h at 37°C in 50 ml sterile plastic tubes (Greiner) under permanent shaking. After digestion, samples were sieved through a 300 µm pore-sized metal sieve (Retsch) to remove undigested material/debris and then passed through a 25 µm pore-sized metal sieve (Retsch). Remnants of the latter sieving process, were then transferred to 10 ml tubes and sedimented at 800 g for 5 min at room temperature (RT). The sediments were examined microscopically for the presence of metastrongyloid larvae (Olympus BH-2®, Olympus, Tokyo, Japan).

2.6 Molecular identification of metastrongyloid species

All larvae-positive samples ($n = 11$, 9 fecal- and 2 gastropod samples) were additionally analyzed by molecular techniques.

Therefore, all larvae from each Baermann sediment were collected via careful pipetting and each sediment were analyzed individually by metastrongyloid-specific PCRs, and finally sequenced to species level. Therefore, DNA was isolated from larvae using a commercial kit (DNeasy Blood and Tissue Kit®, Qiagen, Hilden, Germany). PCRs were performed using the universal nematode primers NC1 (5'-ACGCTCTGGTTCAGGGTTGTT-3') and NC2 (5'-TTAGTTTCTTTCTCCGCT-3') (65). Using a total reaction volume of 50 µl, HOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia) and 5 µl of DNA template, cycling was performed at the following conditions: denaturation at 95°C for 15 min, 35 cycles of denaturation at 95°C for 20 s, annealing at 52°C for 30 s and extension at 72°C for 30 s, followed by a final elongation step at 72°C for 5 min as reported elsewhere (65). In cases when metastrongyloid PCRs yielded negative or inconclusive results, and the quantity of amplicon-DNA from initial PCR was low, a second nested conventional PCR was conducted using the primers NC1 (5'-ACGCTCTGGTTCAGGGTTGTT-3') and MetR (5'-CCGCTAAATGATATGCTTA-3') (66). Obtained



amplicons were purified via gel electrophoresis, sent to a commercial sequencing service (LGC Genomics, Berlin, Germany) and analyzed by BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>; accessed on 15 December 2022).

3 Results

3.1 Locations of GPS-tracked killing sites

GPS-tracked lynx killing sites included areas of wastewater treatment plants, abandoned quarries, private shooting grounds and former ammunition depots, all in vicinity to wooded areas (see Figure 1).

3.2 Occurrence of metastrongyloid infections in wild Eurasian lynxes

Out of 24 lynx fecal samples, 37.5% (9/24) revealed positive for metastrongyloid L1 and 57.1% (4/7) Eurasian lynxes showed metastrongyloid-positive fecal samples for at least one lungworm species (for details see Table 1). All L1 were identified as parasitic nematode larvae belonging to the family Metastrongylidae. In total, four different cardiopulmonary parasites were identified

to genus level: *Aelurostrongylus*, *Angiostrongylus*, *Crenosoma*, and *Troglostrongylus* (see Figure 4).

Three PCR products obtained from Eurasian lynx fecal samples proved positive for feline metastrongyloid-specific DNA and were thereafter analyzed by sequencing. Based on molecular analyses, two parasitic larvae were additionally identified to species level as *T. brevior* and *A. abstrusus*. However, the molecular identification of *Angiostrongylus* and *Crenosoma* L1 remained un-conclusive. The gene sequencing results were deposited at GenBank® under the accession numbers: OQ225253 (for *A. abstrusus*) and OQ222066 + OQ222065 (for *T. brevior*), respectively. In one Eurasian lynx (M20), a patent co-infection with *A. abstrusus* and *Angiostrongylus* sp. was detected. One lynx (M19) showed in one sample *Angiostrongylus* sp. L1 whereas in a later scat sample this finding could not be reconfirmed. Another lynx (M22) showed *Crenosoma* sp. L1 and *T. brevior* L1 in two different samples from the same location, in a later sample *T. brevior* L1 could be reconfirmed.

3.3 Gastropod species diversity and metastrongyloid infections in gastropods

The most common gastropod species found at lynx killing sites or in the surroundings were slugs of the genus *Arion* (59.5%; 91/153), followed by snails of the genus *Cepaea* (11.1%; 17/153)

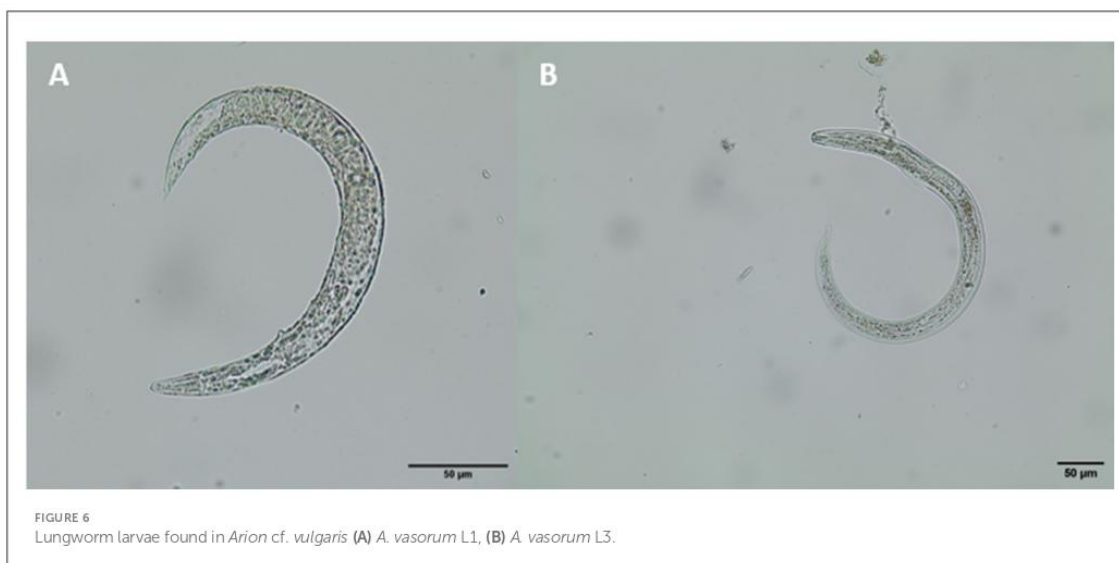
TABLE 1 Sample collection sides with associated lynxes.

Sampling side associated lynx	Collection date	Collection side	Fecal sample	Gastropod species (number)	Morphological results (number of samples) in lynx fecal sample = (L); in gastropod sample = (G)	Molecular results (number of samples) in lynx fecal sample = (L); in gastropod sample = (G)	Prey species and other information on sampling side
M18 ● ▲							
	4/8/2022	51.83913°, 10.10655°	Yes (1x)	/	/	/	Roe deer
	4/9/2022	51.87350°, 10.11538°	Yes (2x)	Unidentified gastropod (1)	/	/	Roe deer
	4/24/2022	51.82179°, 10.10059°	/	<i>Arion</i> sp. (3), <i>Arion vulgaris</i> (1)	/	/	Roe deer
M19 ● ▲							
	4/10/2022	51.71321°, 10.20021°	/	<i>Cepaea</i> sp. (6), <i>Discus rotundatus</i> (4), <i>Limax maximus</i> (1)	/	/	/
	4/23/2022	51.65810°, 10.24001°	Yes (1x)	<i>Cepaea</i> sp. (3), <i>Arion</i> sp. (1), <i>Deroceras reticulatum</i> (1), <i>Limax cinereoniger</i> (1), <i>Limax maximus</i> (1)	<i>Angiostrongylus</i> sp. (1) (L)	/	3 red foxes
	2/13/2023	51.64184°, 10.18289°	Yes (5x)	/	/	/	/
M20 ● ▲							
	3/17/2022	51.770390°, 10.302170°	/	<i>Arion</i> sp. (3)	/	/	
	3/21/2022	51.75012°, 10.253870°	Yes (1x)	/	<i>A. abstrusus</i> (1) (L), <i>Angiostrongylus</i> sp. (1) (L)	<i>A. abstrusus</i> (1)(L)	
M22 ● ▲							
	3/27/2022	51.68020°, 10.14202°	Yes (2x)	/	<i>Crenosoma</i> sp. (L) (1), <i>T. brevior</i> (L) (1)	/	Roe deer
	4/10/2022	51.667140°, 10.261500°	/	<i>Arion</i> sp. (3), <i>Cepaea</i> sp. (2), <i>Discus rotundatus</i> (1), <i>Limax cinereoniger</i> (1)	/	/	Roe deer
	4/21/2022	51.70369°, 10.19776°	/	<i>Arion</i> sp. (2), <i>Arion</i> cf. <i>rufus</i> (1)	/	/	Roe deer; <i>Arion</i> cf. <i>rufus</i> see Figure 5
	4/24/2022	51.65908°, 10.30684°	Yes (1x)	<i>Limax cinereoniger</i> (2)	<i>T. brevior</i> (1) (L)	/	Bird
F10 ● ▲							
	3/27/2022	51.91183°, 10.35997°	Yes (1x)	/	<i>T. brevior</i> (1) (L)	<i>T. brevior</i> (1) (L)	Hare
	4/8/2022	51.88828°, 10.21545°	Yes (2x)	/	<i>T. brevior</i> (L) (2)	/	Roe deer + resting place
	4/22/2022	51.97175°, 10.37115°	/	<i>Cepaea</i> sp. (1)	/	/	Roe deer
F11 ● ▲							
	4/11/2022	51.85538°, 10.34501°	Yes (1x)	/	/	/	Roe deer

(Continued)

TABLE 1 (Continued)

Sampling side associated lynx	Collection date	Collection side	Fecal sample	Gastropod species (number)	Morphological results (number of samples) in lynx fecal sample = (L); in gastropod sample = (G)	Molecular results (number of samples) in lynx fecal sample = (L); in gastropod sample = (G)	Prey species and other information on sampling side
	1/15/2023	51.89079°, 10.40236°	Yes (2x)	/	/	/	Roe deer
	1/15/2023	51.90740°, 10.40969°	Yes (1x)	/	/	/	Roe deer
F12 ◯ Δ							
	4/11/2022	51.68467°, 10.29901°	/	<i>Arion</i> sp. (6), <i>Cepaea</i> sp. (2), <i>Daudebardia</i> sp. (2), <i>Aegopinella</i> sp. (1), <i>Daudebarida brevipes</i> (1), <i>Daudebardia rufa</i> (1)	/	/	Hare; for <i>Daudebardia</i> sp. see Figure 5
	4/11/2022	51.69615°, 10.29277°	/	<i>Arion</i> sp. (6), <i>Limax cinereoniger</i> (1)	/	/	Hare
	1/17/2023	51.67504°, 10.27293°	Yes (1x)	/	/	/	Roe deer
F12+M22 ◯							
	3/20/2022	51.67781°, 10.261090°	Yes (3x)	/	<i>A. abstrusus</i> (L) (1)	<i>T. brevior</i> (L) (1)	Mating point F12 + M22; molecular result were found in positive fecal sample (L)
No associated lynx ▲							
	6/15/2021	51.97298°, 10.31394°	/	<i>Arion</i> sp. (30), <i>Helicodonta obvoluta</i> (4), <i>Limax maximus</i> (3), <i>Limax cinereoniger</i> (2), unidentified gastropod (2), <i>Cochlodina laminata</i> (1), <i>Discus rotundatus</i> (1)	<i>A. vasorum</i> (2) (G)	/	Langelsheim
	6/16/2021	51.83997°, 10.55407°	/	<i>Arion</i> sp. (32), <i>Cepaea</i> sp. (3), <i>Limax maximus</i> (3), <i>Discus rotundatus</i> (2), <i>Succinea putris</i> (2), <i>Cochlodina laminata</i> (1), <i>Limax cinereoniger</i> (1), unidentified gastropod (1)	/	/	Bad Harzburg
	4/11/2022	51.848972°, 10.321411°	/	<i>Arion</i> sp. (3)	/	/	Roe deer
	2022	/	/	<i>Limax maximus</i> (3)	/	/	Found in the Harz region but with no exact location



and the Leopard slug (*Limax maximus*, 10.5%; 16/153). Rare semi-slug species of the genus *Daudebarida* (2.6%; 4/153) with small translucent shells were also collected (see Figure 5). For more details on terrestrial gastropod species diversity please refer to Table 1.

Of digested terrestrial gastropods ($n = 153$), 1.3% of them (2/153) contained metastrongyloid larvae and were identified as *A. vasorum* (see Figure 6). All two positive slugs belonged to the genus *Arion*. Referring to slug larval burden, one *Arion* slug carried two *A. vasorum* larvae whilst the other one proved highly infected with a total larval burden of 34 larvae. Overall, all three larval development stages of *A. vasorum*, i.e., L1, L2, and L3, were found in the latter slug.

4 Discussion

The killing site-based, non-invasive sample collection included several advantages like an efficient, un-molested scat and gastropod collection leading to less harm and stress for both humans and animals and being in accordance to current animal welfare and wildlife conservation strategies in contrast to other sampling methods, which might require stressful animal capture. Thus, GPS-based identification of killing sites (see above) seems feasible for non-invasive scat sample- and terrestrial gastropod collections.

Based on the biological behavior of lynxes, actual killing sites were sometimes hard to discover in the field, since lynxes typically deeply hide their prey under thorny bushes, foliage and branches or deposit them in very remote areas. In most cases, feces were found entirely covered by leaves and branch piles reflecting typical feline behavior. Most of the places with killing sites share the characteristic of being human-dominated areas, with a low level of human interaction.

The current study is based on a lynx population consisting of sub-adult and adult individuals, which were re-introduced into nature after their capture and rearing (below 1 year). Proceedings

of re-introduction included obligatory anthelmintic treatments of sub-adult lynxes (M18, M19, M20, F10, F11, and F12) with ivermectin to eliminate *T. cati* and potential other nematode infections before release. It is to mention that currently there is no explicit study which shows the specific effectiveness of ivermectin against all the metastrongyloid lungworms, which were found in this survey. However there are different reports which show the effectiveness of ivermectin against lungworms of the genus *Crenosoma* (67, 68). Against *Aelurostrongylus abstrusus* ivermectin seems to have an incomplete effectiveness (69, 70). For the use of ivermectin against *Angiostrongylus* sp. and *Troglostrongylus* sp. in felids there is currently insufficient scientific knowledge. We therefore recommend to review reintroduction protocols for wild felids using ivermectin and maybe switch to better working compounds if the aim is to eradicate metastrongyloid lungworm infections. If ivermectin is to be used, the animals should be tested again with the Baermann funnel after treatment to detect possibly surviving metastrongyloid lungworms and if the result is positive, the animals should be treated with another more specific compound. A complete absence of metastrongyloid lungworm infections in the reintroduced lynxes, mentioned in this manuscript, prior their release into the wild, can therefore not be ruled out and given that the results of the copromicroscopic analysis yielded negative for metastrongyloid lungworm infections, it seems still highly feasible to assume that patent metastrongyloid infections detected in the current study were acquired within the study area either by ingesting metastrongyloid-infected gastropod intermediate hosts and/or after consumption of infected paratenic hosts including amphibians, reptiles, birds and rodents.

The metastrongyloid genera *Aelurostrongylus*, *Angiostrongylus*, and *Troglostrongylus* have previously been described not only in wild Eurasian lynxes but also in wild cats (*F. silvestris*) and domestic cats (30, 37, 50). In contrast, *Crenosoma* infections in wild felids were reported exclusively for *L. lynx* and identified as *C. vismani* (29). Of note, current parasitological results include the

broadest metastrongyloid species diversity for wild Eurasian lynxes compared to former reports (31, 37, 38, 71). This species diversity might be explained by the current method, i.e., examination of fresh feces by the Baermann funnel, which allows the detection of living larvae. Of note, this technique is still considered as gold standard for lungworm larvae diagnostics (72–80). In contrast, other studies on Eurasian lynx lungworms either analyzed preserved (i.e., fixed or frozen) fecal samples or examined carcasses and/or did not apply the Baermann funnel technique, thereby potentially reducing the sensitivity of lungworm detection (31, 38, 71, 81). Another explanation for current parasite diversity may be related to the age structure of analyzed Eurasian lynxes, consisting mainly of sub-adult animals. Correspondingly, juvenile domestic cats and young wild cats also showed a broader range of metastrongyloid species, thereby being predisposed for these cardiopulmonary infections by age, we would assume that this will be the case for Eurasian lynx as well (30, 48, 51, 82–84).

A recent study on endoparasites of free-ranging Eurasian lynxes ($n = 24$) of the Harz Mountains reported a prevalence of 12.5% (3/24) for metastrongyloid L1, among them *Angiostrongylus* spp.-like larvae and *A. abstrusus*, nonetheless neither *T. brevior* nor *Crenosoma* spp.-larvae were detected in this study (31). Interestingly, the present study revealed a lungworm species that has never been reported before in free-ranging German Eurasian lynxes, the metastrongyloid lungworm *Troglostrongylus brevior*. Moreover, current findings on troglostrongylosis represents the third-ever report in literature for this feline host species in Europe. Hence, the first and second report on *T. brevior* infections in Eurasian lynxes came from Bosnia and Herzegovina in 2015 (32) and from Romania in 2022 (37), respectively. Conversely, in North America the closely related species *T. wilsoni* was reported to occur in Canadian lynxes (*Lynx canadensis*) (85) and in wild bobcats (*Lynx rufus*) (54, 86–88). In line with rare reports on lynx troglostrongylosis, the occurrence of aelurostrongylosis in Eurasian lynxes has only been described for three countries so far, i.e., Switzerland, Poland and Germany (31, 38, 89). *Troglostrongylus* seems to be of more clinical concern, than *A. abstrusus* as they seem to show in general a more severe clinical picture (90–92). In Switzerland, analyses of 58 fecal samples from dead *L. lynx* were in five cases (9%) positive for *A. abstrusus*. Interestingly, in one of the examined Swiss Eurasian lynx, histopathological findings unveiled a multifocal mild granulomatous pneumonia (89). In Poland, a much higher *A. abstrusus* prevalence of 21% was reported (38). Current findings re-confirm *A. abstrusus* as circulating in free-ranging Eurasian lynxes in Germany and highlight the importance of regular monitoring on gastropod-borne aelurostrongylosis (31). Recently, feline crenosomosis was reported in wild Eurasian lynxes in Latvia. Based on morphological and morphometric characteristics, *C. vismani* was identified as the related infective pathogen (29). In the current study, we failed to identify the *Crenosoma* species for the detected larvae by molecular tools. It has to be noted that, it cannot be ruled out, that the here identified *Crenosoma*-L1 might also have originated from an infected prey animal [i.e., red fox (*Crenosoma vulpis*) or European hedgehog (*Crenosoma striatum*)] (41, 93–99). Accordingly, three carcasses of killed red foxes were found at one killing site. Equally, current findings on *Angiostrongylus*-L1 might have originated from other infected prey, predator/mesopredator

living within the same biome. Hence, interactions of wild Eurasian lynxes with badgers (*Meles meles*), racoons (*Procyon lotor*) and wild cats sharing the same habitats of the Harz Mountains might result in spill-overs and spill-backs of *Angiostrongylus* spp.-infections as previously postulated (27, 30, 31, 100, 101). Particularly feral cats and domestic dogs may pose a risk in reverse of spill-overs for endangered Eurasian lynxes (31). Wildlife studies have already demonstrated that especially feral cats play a significant role in transmitting parasites to wild lynx populations as reported for re-introduced Iberian lynxes in Spain and Portugal (27, 100, 101).

In the present study, we failed to molecularly confirm the endemic *A. chabaudi* parasite to species level for the current German lynx samples. Nevertheless, this species has already been reported for wild cats in Germany (30), and from other European countries (35, 36, 102). However, patent *A. chabaudi*-infections have not yet been detected in wild Eurasian lynxes. Given that wild cats and Eurasian lynxes cohabit the same biome in the Harz Mountains, transmission of *A. chabaudi* seems plausible through consumption of either infected intermediate hosts or paratenic hosts (31, 64, 103), but further investigations are needed for conclusive data on lynx angiostrongylosis in Germany.

In this study lungworm larvae only of the species *A. vasorum* were found in gastropods of the genus *Arion* sp. The origin of infection of the sampled lynxes, with the lungworms, which were found during the analysis of the Baermann funnel, has to be investigated in further studies. Until this time their way of infection is speculative. Felids tend to get infected with metastrongyloid lungworms by ingesting paratenic hosts (e.g., rodents and birds) rather than by ingesting gastropods, this case seems also feasible for Eurasian lynxes and is therefore a highly reasonable cause of infection (39, 104). Wild cats (*F. silvestris*) seem to be a reservoir for *T. brevior* (49), and as they are occurring syntop with the Eurasian lynx in the Harz Mountains they could be a source of infection. The majority of the collected slugs belonged to the species *Arion* cf. *vulgaris*, which is in accordance to other studies the most common slug species in Germany and mainly found in urbanized areas due to its synanthropic behavior (105). Nonetheless, also *Arion* cf. *rufus* slugs were here found in the lynx biomes of the Harz Mountains. Unfortunately, the species determination of collected *Cepaea* snails was impossible since pivotal morphological characteristics for proper identification (e.g., color of the mouthlip of the shell, internal morphology of reproductive organs) were missing due to beginning autolysis or fragmentation of the shell (106). The susceptibility of gastropod species to lungworm infections is explained by different factors e.g., intermediate host behavior and size of the gastropod (52, 107, 108). The exact parameters which influence the susceptibility of gastropod species have to be investigated in further studies, for example with a broader sample size or experimental infections. The prevalence of metastrongyloid lungworms in gastropod intermediate hosts has already been done in several studies (52, 55, 109).

Considering present metastrongyloid findings in digested gastropod intermediate hosts, exclusively *A. vasorum* larvae in two *Arion* cf. *vulgaris* slugs were found. Most probably these infections result from marked coprophagic behavior of this slug species (16, 110) when compared to other investigated gastropod genera (e.g., *Cepaea* and *Daudebardia*), what makes them good intermediate

hosts. When referring to definite hosts of *A. vasorum*, the most predominant species in the Harz Mountains are red foxes followed by gray wolves (*Canis lupus*), besides domestic dogs, while felines are usually considered as rare and inadequate hosts (111, 112). Especially red foxes are well-known to show high *A. vasorum* prevalences in Germany (93, 113). Moreover, it cannot be ruled out that the presence of *A. vasorum* larvae in *Arion* slugs was due to intermediation, where nematode stages are transferred from one infected gastropod to another by carnivorous behavior or by contact with L3 released from dead intermediate hosts (114, 115).

5 Conclusion

To the best of current knowledge, this work represents the first report on patent *T. brevior*-infections in wild Eurasian lynxes in Germany. Additionally, current data re-confirmed patent *A. abstrusus* infections to be circulating in the *L. lynx* population inhabiting the Harz Mountains, which is the largest one in Germany. Our findings emphasize the necessity for additional research on neglected cardiopulmonary diseases, such as feline angiostrongylosis and crenosomosis, in wild Eurasian lynxes to increase the current understanding on their epizootiology, pathogenesis, immunity, and clinical relevance. Moreover, the presence of *A. vasorum* larvae in gastropods was here reported for the first time for the Harz Mountains within the Federal State of Lower Saxony. Even though our study is limited in terms of low animal numbers, its significance lies in the importance of exploring wildlife-associated parasitoses in a non-disturbing manner to uncover potential threats to endangered large felid apex predators. Considering the limited knowledge on the pathological and clinical findings induced by lungworm infections in wild felids, regular veterinary monitoring is crucial to evaluate the population health status, which could additionally be done by the examination of samples from other regions as well as clinical assessments of animals in rescue centers or necropsies of dead animals to get broader insights in metastrongyloid lungworm infections in Eurasian lynxes. These regular veterinary monitoring programs will play a crucial role for future re-introduction programs and should encompass not only the target but also sympatric species.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because the study was done on fecal samples, which were gained from the field, with no disturbances of the animals.

Author contributions

MH: Investigation, Visualization, Writing – original draft, Writing – review & editing, Methodology. LS: Investigation, Writing – review & editing. OA: Methodology, Resources, Writing – review & editing. TM: Methodology, Resources, Writing – review & editing. AM: Investigation, Visualization, Writing – review & editing. SH: Writing – review & editing. BC: Methodology, Writing – review & editing. AD: Investigation, Methodology, Writing – review & editing. AT: Funding acquisition, Resources, Supervision, Validation, Writing – review & editing. CH: Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. The study was partially funded by the Institute of Parasitology, Faculty of Veterinary Medicine of the Justus Liebig University Giessen (JLU) and the Harz National Park (HNP), Germany.

Acknowledgments

We express our deepest gratitude to Dr. Manuel Uribe for assisting with mapping in QGIS and by providing valuable support in the field. Special thanks to all the land owners and hunting tenants for allowance to observe their areas for signs of Eurasian lynxes activities, as well as the authorities of the State Forests of Lower Saxony, for the allowance to pass through and search in their territories for killing sites. Our special appreciation extends to the authorities of the Harz National Park (HNP) for their constant and kind cooperation, logistical support, and the necessary driving permits. For extraordinary help in the determination of gastropod species we thank the malacologists Dr. Frank Walther and Dr. Vollrath Wiese. Further, we acknowledge Christine Henrich for her valuable assistance in conducting molecular analysis of collected metastrongyloid larvae. Finally, we thank Dr. Daniela Grob for her assistance in gastropod sampling.

Conflict of interest

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

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9. Eigenständigkeitserklärung

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig 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.

Alena Dusch

10. Danksagung

Ich möchte mich an dieser Stelle herzlich bedanken an meinem Doktorvater, Prof. Dr. habil. Prof. h.c. Carlos Hermosilla, der mich über meine ganze Zeit am Institut für Parasitologie der JLU Gießen mit hoher Motivation betreut hat. Er hatte immer ein offenes Ohr für Ideen und Probleme und hat mich immer tatkräftig unterstützt. Durch seine Unterstützung konnte ich mich persönlich und beruflich weiterentwickeln. Ohne ihn wäre die Anfertigung dieser Thesis nicht möglich gewesen.

Vielen Dank an die Institutsleiterin Prof. Dr. Anja Taubert für meine Aufnahme am Institut für Parasitologie und die Schaffung der Rahmenbedingungen, die die Durchführung meiner Arbeit ermöglicht haben. Besonders bedanken möchte ich mich auch bei Dr. Lisa Segeritz, die mich vor allem in den Anfangsphasen meines Projekts rat- und tatkräftig unterstützt hat. Mireille Ampama danke ich zutiefst für ihre Hilfe bei der Schneckenpflege und ihre wunderbare Unterstützung, die mich jeden Tag erneut motiviert hat. Vielmals danke ich auch Christine Henrich für ihre herausragende Durchführung und Unterstützung bei den beschriebenen molekularbiologischen Methoden. Ein großes Dankeschön geht an Dr. Zahady Velasquez und Dr. Iván Conejeros für die gemeinsamen Experimente im Bereich der Gastropoden-Immunologie.

Des Weiteren möchte ich mich bedanken bei allen Kolleginnen und Kollegen, die ich während meiner Arbeit am Institut kennenlernen durfte: Rashmi Nakate, Sagar Ajmera, Oliver Puckelwaldt, Piero Vitolo Sosa, Mireille Ampama, Max Möscheid, Constanza Salinas, Gabriel Espinosa, Lisbeth Rojas, Jobst Fischer, Thomas Nowicki und Hannah Salecker. Mit euch wurde jeder noch so lange Tag im Labor oder im Büro vor dem Computer zu einem besonderen Erlebnis.

Vielen Dank auch an alle motivierten Schneckensammler: Prof. Dr. Carlos Hermosilla, Honorine Mireille Nguete Ampama, Seyed Sajjad Hasheminasab, Manuel Uribe, Marcel Haas, Charlotte Scriba, Dorin Quernes, Piero Vitolo Sosa und Alicia Riedinger.

Für die herzliche Aufnahme am Erasmus Medical Centre in Rotterdam möchte ich besonders Joep van de Sanden und Prof. Dr. Verburg danken. Durch euch habe ich einen Einblick in völlig neue Methoden und ein spannendes Arbeitsfeld erhalten.

Zu guter Letzt möchte ich mich bei meiner Familie und meinen Freunden bedanken: Birgit Dusch, Markus Weyland, Tabea Dusch, Gisela Burger, Alicia Riedinger, Sarah Röser und Tim Schmiedel. Ohne eure tatkräftige Hilfe und unterstützenden Worte wäre es mir nicht möglich gewesen, meine Promotion zu beenden. Vielen Dank.