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ORIGINAL PAPER

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

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Abstract

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

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

Introduction

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

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

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

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

Materials and methods

Ethics approval statement

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

Gastropod maintenance under standardized and parasite-free conditions

Slugs

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

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

Giant snails

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

Generation of parasite-free gastropod F0

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

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

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

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

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

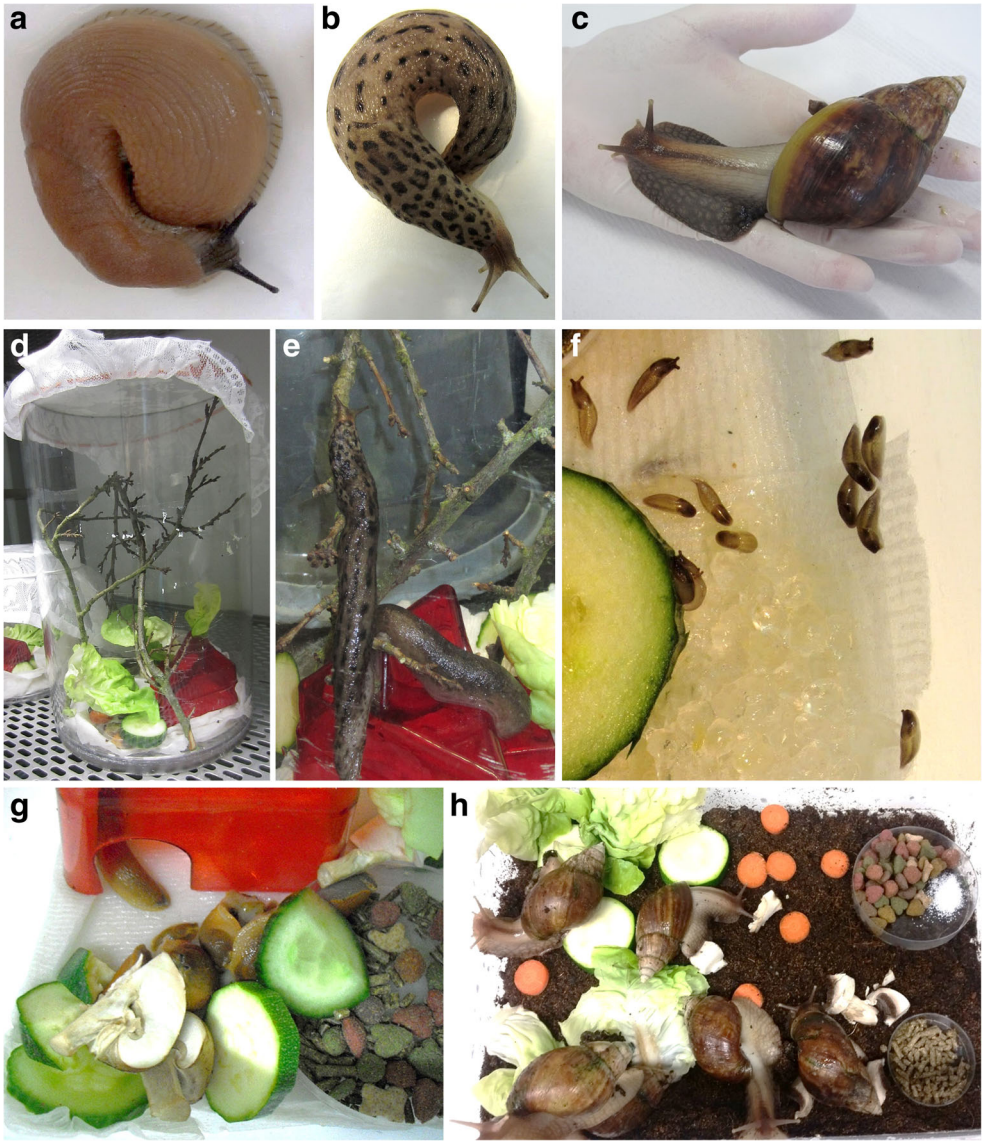

Fig. 1 Terrestrial gastropods maintained under controlled standardized conditions. Adult specimens of (**a**) the Iberian slug (*Arion lusitanicus*), (**b**) the giant garden slug (*Limax maximus*), and (**c**) the giant African snail (*Achatina fulica*). (**d**) Glass container used for reproduction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

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

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

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

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

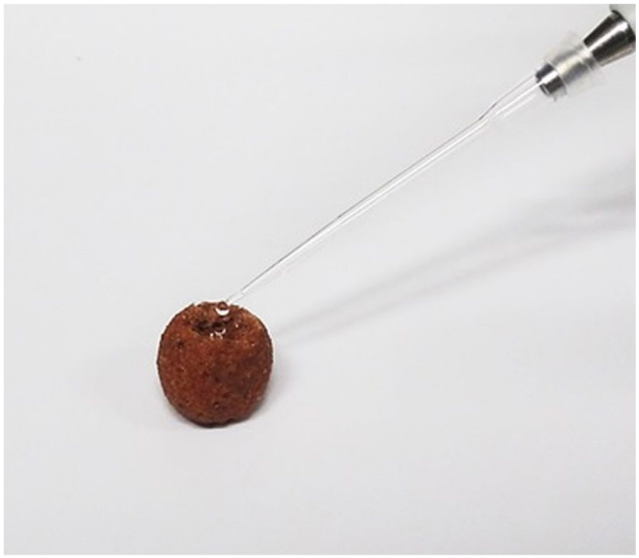

Fig. 2 Oral slug/snail infection. One hundred larvae suspended in 0.2-mL distilled water were deposited on a single dog food pellet

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

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

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

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

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

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

Results and discussion

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

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

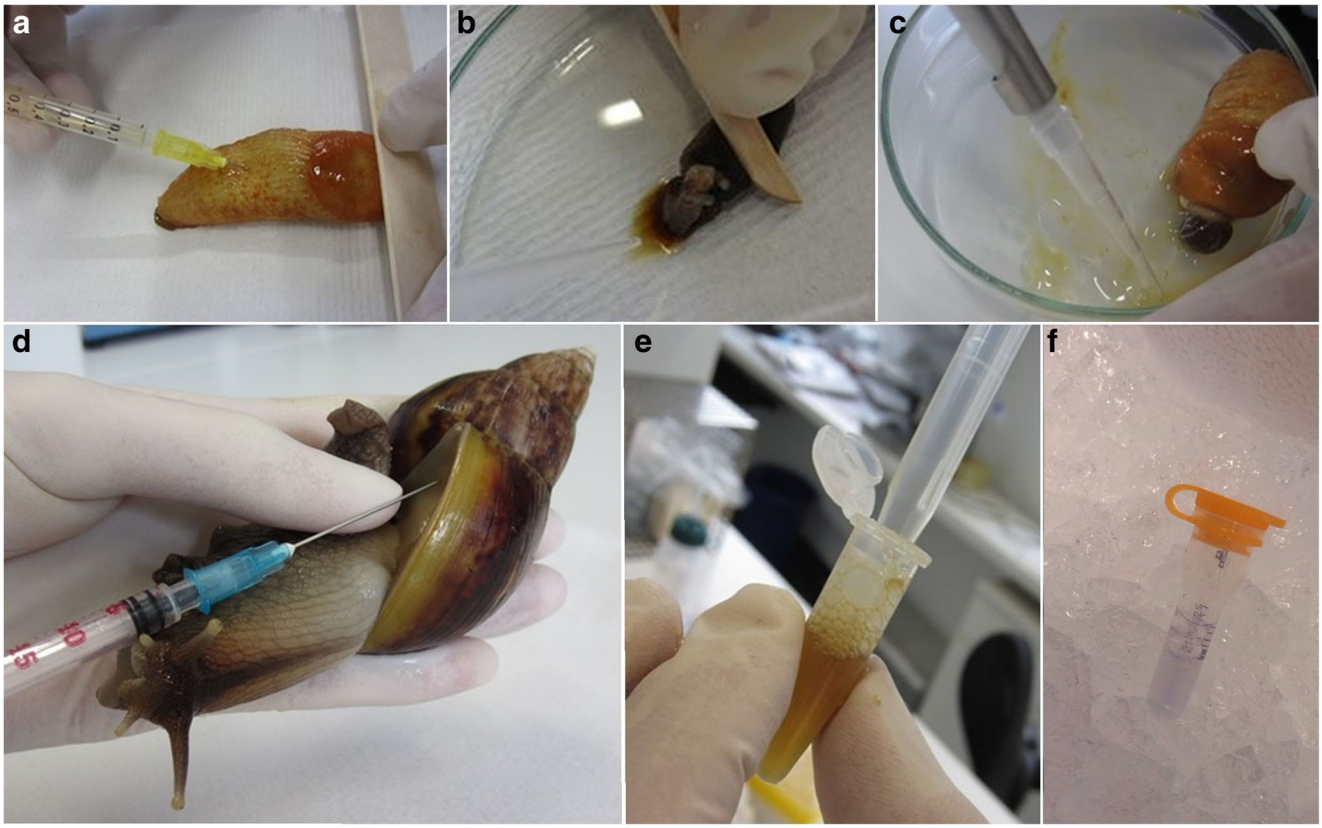

Fig. 3 Haemolymph extraction process. (**a**) Injection of collection solution (~ 10% of the body weigh). (**b**) Haemolymph recovery from *A. lusitanicus* and (**c**) *L. maximus.* (**d**) haemolymph collection via aspiration close to the pneumostome. (**e**) Haemolymph samples from slugs and (**f**)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

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

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

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

Table 1 Reproduction of gastropod species in fully automated climate chambers

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

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

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

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

Haemolymph extraction from slugs and snails

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

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

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

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

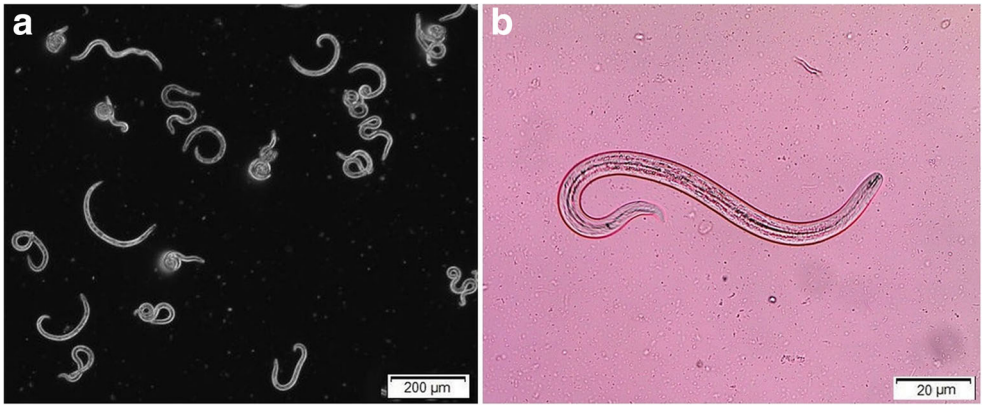

Fig. 4 Axenic culture of *Angiostrongylus vasorum*. (**a**) L1 isolated from feces of experimentally infected foxes. (**b**) L3 isolated from experimentally infected slugs *Limax maximus*

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

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

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

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

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

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

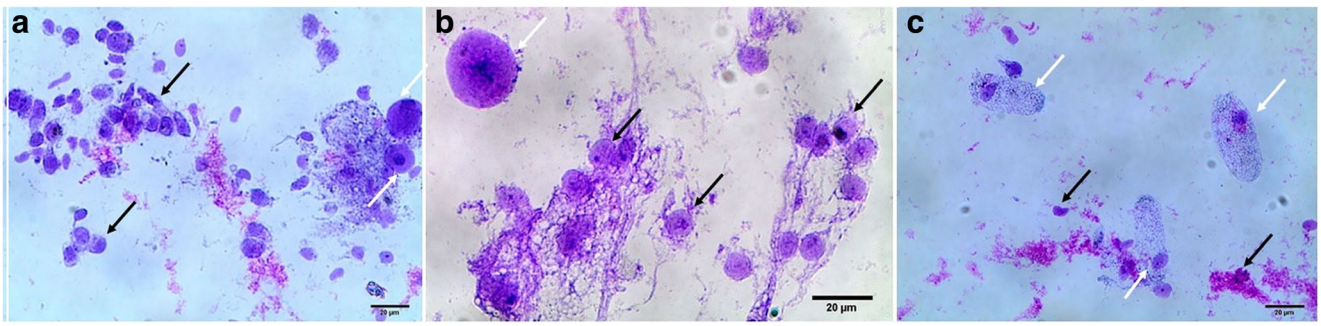

Fig. 5 Haemolymph smears stained by Giemsa. (**a**) Haemocytes of *Arion lusitanicus* (400×), (**b**) haemocytes of *Limax maximus* (400×) and (**c**) haemocytes of *Achatina fulica* (400×). Black arrows indicate type I (small), and white arrows indicate haemocyte type II (large)

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

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

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

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

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

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

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

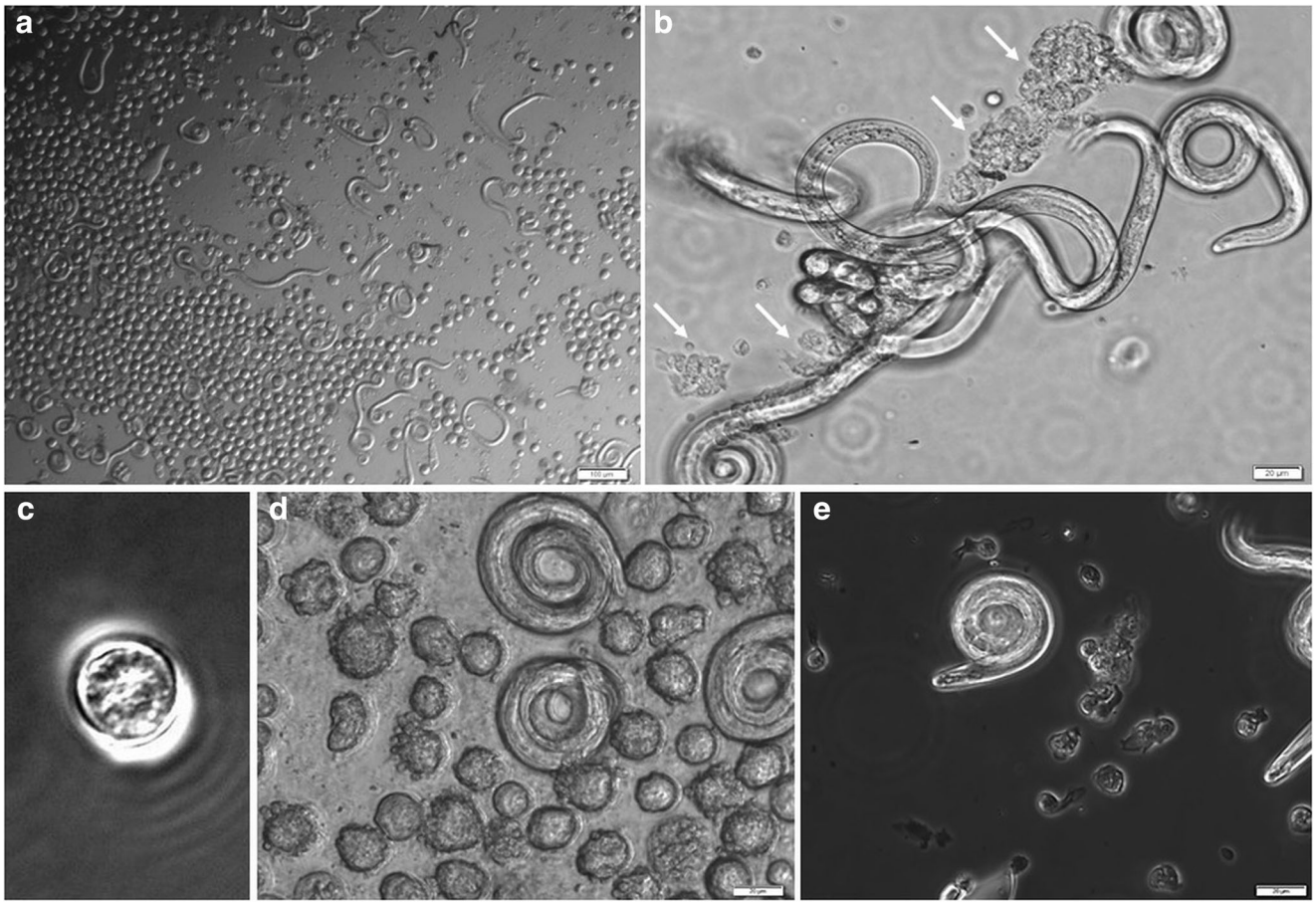

Fig. 6 Co-cultures of gastropod haemocytes and *A. vasorum* larvae. (**a**) *Limax maximus* haemocytes co-cultured with axenic and viable *A. vasorum* L1. (**b**) Cluster of *Arion lusitanicus* haemocytes acting against *A. vasorum* L1s forming aggregated material (arrows, 30 min). (**c**) Non-activated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

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

Conclusions and future perspectives

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

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

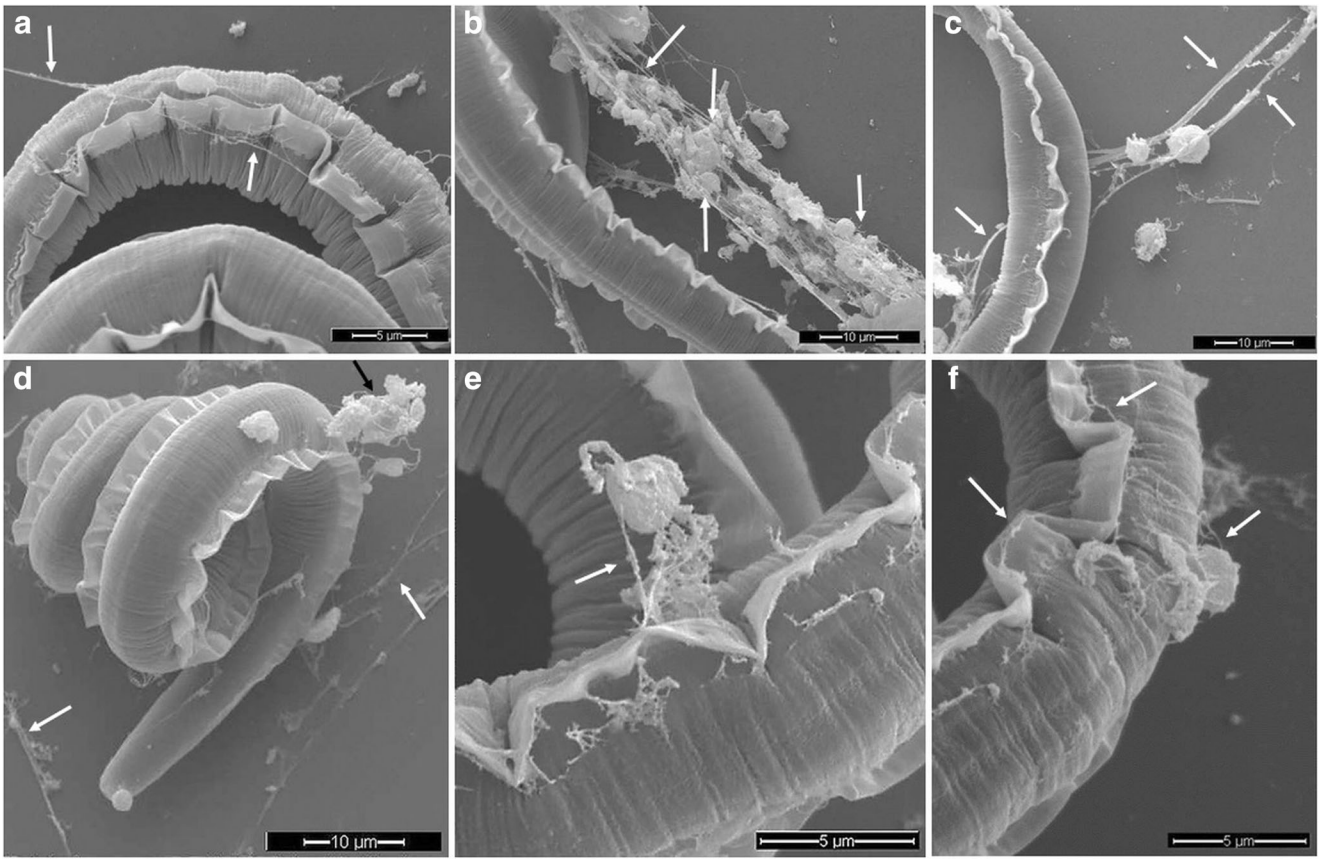

Fig. 7 Scanning electron microscopy analysis of gastropod haemocytes—*A. vasorum*-L1-co-cultures. (**a**) *Limax maximus* haemocytes co-cultured with axenic *A. vasorum* L3, spread ETs (*spr*InEPTs, arrows, 30 min). (**b**) *Limax maximus* haemocytes co-cultured with axenic *A. vasorum* L1, aggregated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

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

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

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Compliance with ethical standards

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

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

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

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

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