Accepted Manuscript

Extracellular trap-like fiber release may not be a prominent defence response in snails: Evidence from three species of freshwater gastropod molluscs

Vladimír Skála, Anthony J. Walker, Petr Horák

PII: S0145-305X(17)30370-1

DOI: 10.1016/j.dci.2017.10.011

Reference: DCI 3007

To appear in: *Developmental and Comparative Immunology*

Received Date: 14 July 2017

Revised Date: 18 October 2017

Accepted Date: 19 October 2017

Please cite this article as: Skála, Vladimí., Walker, A.J., Horák, P., Extracellular trap-like fiber release may not be a prominent defence response in snails: Evidence from three species of freshwater gastropod molluscs, *Developmental and Comparative Immunology* (2017), doi: 10.1016/j.dci.2017.10.011.

Short Communication

Extracellular trap-like fiber release may not be a prominent defence response in snails: evidence from three species of freshwater gastropod molluscs

Vladimír Skála[1,2,*], Anthony J. Walker[3] and Petr Horák[1]

[1] Charles University, Faculty of Science, Department of Parasitology, Prague, Czech Republic

[2] Charles University, First Faculty of Medicine, Institute of Immunology and Microbiology, Prague, Czech Republic

[3] Kingston University, School of Life Sciences, Pharmacy and Chemistry, Molecular Parasitology Laboratory, Kingston upon Thames, Surrey, United Kingdom

*Corresponding author.
E-mail address: mich007@email.cz (V. Skála)

tel.: +420 221 951 822; fax: +420 224 919 704

Abstract

The discovery that mammalian neutrophils generate extracellular chromatin fibers that entrap/kill bacteria supported a new paradigm for innate immunity in animals. Similar findings in other models across diverse taxa have led to the hypothesis that the phenomenon is ancient and evolutionary conserved. Here, using a variety of synthetic (e.g. peptidoglycan) and biological (e.g. trematode larvae) components to investigate extracellular trap-like (ET-like) fiber production *in vitro* by haemocytes of *Lymnaea stagnalis*, *Radix lagotis* and *Planorbarius corneus* snails, ET-like fibers were rarely observed. We suggest, therefore, that ET-like fibers play a marginal role in defence of these snail species and thus the fiber production may not be a critical process underpinning immunity in all invertebrate species.

Keywords: Extracellular trap-like fiber (ET-like fiber); *Lymnaea stagnalis*; *Radix lagotis*; *Planorbarius corneus*; snail immunity; haemocytes.

1. Introduction

Reticulated DNA fibers produced by neutrophils (neutrophil extracellular traps; NETs), eosinophils (extracellular traps; ETs) and other cells of the vertebrate innate immune system are considered important structures that facilitate the elimination of bacteria and eukaryotic unicellular/multicellular parasites extracellularly (von Köckritz-Blickwede and Nizet, 2009; Zawrotniak and Rapala-Kozik, 2013; Hermosilla et al. 2014). In invertebrates, immunity typically relies on haemocytes that cooperate with humoral recognition factors such as lectins and fibrinogen-related proteins to deliver the defence response. While extracellular nucleic acids can bolster immunity as shown in the greater wax moth *Galleria mellonella* (Altincicek et al., 2008), ET-like fibers resembling NETs of vertebrates have recently also been found to mediate defence of *Litopenaeus vannamei* (Ng et al., 2013) and *Carcinus maenas* (Robb et al., 2014) haemocytes. Interestingly, mesogleal cells of the sea anemone *Actinia equina* (Robb et al., 2014), and sentinel cells of the social amoeba *Dictyostelium discoideum* (Zhang et al., 2016) have also been shown to release DNA fibers extracellularly. In molluscs, ET-like fibers have been reported in bivalves (*Mytilus edulis*, *Crassostrea gigas*) (Robb et al., 2014; Poirier et al., 2014), and gastropods (*Arion lusitanicus*, *Limax maximus* and *Achatina fulica*) in which the fibers entrapped metastrongyloid larvae (Lange et al., 2017). In the latter case, different types of ET-like fibers (i.e. aggregated, spread and diffuse) were observed, with histones and myeloperoxidase as fiber constituents (Lange et al., 2017).

In the current study, we employed haemocytes of *Lymnaea stagnalis* and two other species of freshwater gastropod snails, *Radix lagotis* and *Planorbarius corneus* to elucidate ET-like fiber production in snails that serve as intermediate hosts of trematode larvae. For comparative purposes, we used *Mytilus edulis* haemocytes that are known to release ET-like fibers.

2. Materials and methods

2.1. ET-like fiber release by Mytilus edulis *haemocytes*

Haemocytes of *M. edulis* were utilized for initial experiments. Haemolymph was extracted and haemocyte monolayers were prepared as previously described (Robb et al., 2014) in 96-well tissue culture plates (Nunc) employing 250 µl haemolymph/well diluted (1:1) with 0.05 M Tris-HCl buffer, pH 7.6, supplemented with 2% glucose, 2% NaCl, 0.5% EDTA. Haemocytes were incubated with 20 µM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) at 10 °C for 48 h, stained with 1 µM Sytox green (Thermo Fisher Scientific) that effectively binds DNA of dead cells (Thakur et al., 2015) and examined for ET-like fiber release under a fluorescence microscope (Olympus IX71).

2.2. Snails and haemocytes

Laboratory-reared *L. stagnalis* and *R. lagotis* were maintained at 19-22 °C in aerated aquaria, and fed fresh lettuce *ad libitum*. *Planorbarius corneus* snails were obtained from a local pond (Prague) and examined for cercarial shedding; infected snails were excluded from experiments. Haemolymph from snails was extracted according to Sminia (1972). Samples from *L. stagnalis* and *P. corneus* were pooled on ice, diluted 2:1 with sterile snail saline (SSS; Adema et al., 1991) and 250 µl transferred into individual wells of a 96-well plate. Experiments with *P. corneus* were also conducted in Chernin´s balanced salt solution (CBSS; Chernin, 1963). Haemolymph from *R. lagotis* was handled as described previously (Skála et al., 2014). The haemocyte number per well was approx. 2.8×10^5 for *L. stagnalis*, 6×10^4 for *R. lagotis* and 1.2×10^5 for *P. corneus*, enumerated using a Bürker haemocytometer.

2.3. Preparation of parasite material

Miracidia of *Trichobilharzia regenti* were obtained *via* the laboratory life cycle according to Horák et al. (1998), fixed in 2% (v/v) paraformaldehyde for 30 min and free aldehyde groups blocked in 1% glycine at 4 °C overnight (Zahoor et al., 2008). The larvae were then washed twice with SSS and stored at -20 °C. Homogenised miracidia were prepared by sonicating miracidia for three cycles (7W, 20 s each, Vibracell-72405 100-W ultrasonicator, Bioblock Scientific, France) in SSS followed by determination of protein concentration using Quant-iT Protein Assay Kit (Invitrogen).

2.4. Haemocyte exposure

Lymnaea stagnalis haemocyte monolayers were treated with SSS containing peptidoglycan (PGN; 0.1, 1.0 and 10.0 µg/ml), *E. coli* lipopolysaccharide serotype 0111:B4 (LPS; 0.1, 1.0 and 10.0 µg/ml), PMA (0.1, 1.0, 10.0 µM), galactose or fucose (200, 400, 800 nM, 1 and 10 µM), galactose-fucose in combination (800 nM of each in SSS) (all purchased from Sigma-Aldrich), live/heat-killed *Staphylococcus saprophyticus* at ~10, ~100 and ~1000 bacteria/haemocyte, or miracidial homogenate (1 or 10 µg/ml). All incubations were performed at room temperature (e.g. Plows et al., 2005) for 3 h and 24 h. Three independent experiments were performed with one replicate for each condition/duration. The haemocytes were then stained with 1 µM Sytox Green in SSS for 20 min and the entire cell populations examined visually under the fluorescence microscope; haemocytes producing ET-like fibers were enumerated.

For intact parasite exposure, 200 miracidia in 100 µl SSS were transferred to individual wells of a chamber slide (Lab-Tek); 200 µl complete *L. stagnalis* haemolymph were added and after 1 h incubation, 100 µl supernatant were replaced by 100 µl fresh haemolymph. This step was done to enhance the continuous migration of haemocytes towards

the parasite. Incubation times/Sytox green staining were as above; the experiments were performed twice independently. Finally, specimens were embedded in Vectashield (Vector Laboratories), examined using a Zeiss LSM880 laser scanning confocal microscope, and images analysed using FIJI Image J (Schindelin et al., 2012).

Haemocyte monolayers obtained from *R. lagotis* and *P. corneus* were incubated in SSS containing PMA (0.1, 1, 5, 10 µM), LPS (0.1, 1.0, 10.0 µg/ml), or heat-killed *S. saprophyticus* at ~100 bacteria/haemocyte.

3. Results and discussion

Initial experiments were performed with haemocytes of *M. edulis*, previously shown to produce ET-like fibers (Robb et al. 2014), to demonstrate fiber release in our laboratory. Similar to Robb et al. (2014), PMA clearly induced ET-like fiber release (Fig. 1A, B in Supplementary Materials) that was ETotic.

Next, snail haemocytes were exposed to PMA or LPS, compounds that were shown previously to stimulate effective NETs/ET-like fiber formation (von Köckritz-Blickwede and Nizet, 2009; Robb et al., 2014; Ng et al., 2013). Other components (e.g. L-fucose/D-galactose) were employed because they are linked to snail-trematode interactions (Plows et al., 2005).

The screening assays revealed that *L. stagnalis*, *R. lagotis* and *P. corneus* haemocytes produced only low numbers of extracellular DNA fibers (Table 1) and, therefore, other components associated with ET-like fibers such as histones (Ng et al., 2013; Robb et al., 2014) were impossible to investigate. However, given that occasional DNA fibers were observed in all species studied (Fig. 1) we define the fibers as 'ET-like' as in other invertebrates (Ng et al., 2013; Robb et al., 2014; Poirier et al., 2014; Lange et al., 2017).

That compounds such as PGN or PMA failed to elicit robust ET-like fiber production in *L. stagnalis* was surprising (Table 1). Similarly, PMA did not stimulate ET-like fiber formation by *C. gigas* haemocytes (Poirier et al., 2014). Exposure of haemocytes to 20 µM PMA in SSS or in modified SSS (SSS supplemented with D-trehalose (1g/L), D-glucose (1g/L) (Sigma-Aldrich) and antibiotics (penicillin/streptomycin; Lonza)), enabling longer-term *L. stagnalis* haemocyte survival for 48 h also did not evoke haemocyte ETotic responses (data not shown). On the other hand, *M. edulis* haemocytes produced fibers when exposed to 50 µM PMA for 48 h (Robb et al., 2014). In *R. lagotis*, haemocytes exposed to PMA produced only few ET-like fibers (Table 1, Fig. 1H). This finding was unexpected because PMA induces the respiratory burst in *R. lagotis* haemocytes (Skála et al., 2014), a reaction considered essential for ET-like fiber formation (Robb et al., 2014; Poirier et al., 2014).

Although LPS significantly induced NETs/ET-like fiber formation in mammalian neutrophils or shrimp haemocytes (von Köckritz-Blickwede and Nizet, 2009; Ng et al., 2013), only two ET-like fibers were produced by *L. stagnalis* haemocytes (Table 1, Fig. 1A). Additionally, no ET-like fibers were observed when these haemocytes were treated with 25 µg/ml LPS in modified SSS for 24h, and the protocol of Brinkmann et al. (2010) was used to visualise the fibers (data not shown). With *P. corneus*, one ET-like fiber was observed when haemocytes were exposed to 10 µg/ml LPS in CBSS for 24 h (Fig. 1F) whereas nine fibers were observed in SSS (Table 1). Thus, these different culture media did not seem to largely influence the outcome with respect to ET-like fiber formation.

PMA and LPS activate protein kinase C (PKC) in *L. stagnalis* haemocytes (Walker and Plows, 2003; Wright et al., 2006), which stimulates NO production (Wright et al., 2006). Such responses might, at least in part, explain the inability of PMA and LPS to effectively promote ET-like fiber production. However, D-galactose and L-fucose attenuate PKC and extracellular-signal regulated kinase (ERK) activation in *L. stagnalis* haemocytes, with

subsequent suppression of phagocytosis (Plows et al., 2005). These sugars are present on the surface of the helminth *T. regenti* (Blažová and Horák, 2005; Chanová et al., 2009), an incompatible parasite that penetrates but does not survive in *L. stagnalis*. However, exposure to these sugars did not affect ET-like fiber production (Table 1).

As the soluble compounds did not substantially stimulate ET-like fiber formation by the snail haemocytes, we tested pathogen-haemocyte combinations. Heat-killed *S. saprophyticus* bacteria were phagocytosed by the snail haemocytes (Fig. 1C in Supplementary Materials) whereas several ET-like fibers were produced with unclear function (Table 1); similar results were also obtained with live *S. saprophyticus* (data not shown). In contrast, ET-like fibers produced by *C. gigas* haemocytes were shown to entrap *Listonella anguillarum* (Poirier et al., 2014), whereas fibers produced by *L. vannamei* trapped and killed *E. coli* (Ng et al., 2013).

Experiments using fixed *T. regenti* miracidia and whole snail haemolymph showed that haemocytes encapsulate the parasite (Fig. 1D). Moreover, confocal microscopy revealed that several haemocytes expelled ET-like fibers against *T. regenti* during 3 h exposure (Fig. 1D-E). In gastropods, haemocyte derived ET-like fibers were demonstrated previously in *A. fulica*, which trapped viable *Angiostrongylus vasorum* larvae *in vitro* (Lange et al., 2017). Release of ET-like structures was also observed *in vivo* in the mucous extrapallial space of *L. maximus* in response to invading *A. vasorum* (Lange et al., 2017). However, in our study, only a few *L. stagnalis* haemocytes produced ET-like fibers against *T. regenti* (Fig. 1D-E) and thus the fibers are unlikely the main defence tool for parasite elimination. Although attempted, evaluation of *T. regenti* and *L. stagnalis* interactions in snail histological sections was technically demanding (results not shown) and, therefore, the extent of ET-like fiber production *in vivo* remains unknown. Finally, homogenised *T. regenti* miracidia did not stimulate significant ET-like fiber production (Table 1, Fig. 1B-C).

To conclude, we examined the ability of several compounds and pathogens to elicit ET-like fiber production in the freshwater snails *L. stagnalis*, *R. lagotis* and *P. corneus in vitro*. ET-like fiber production has previously been reported in several invertebrates including molluscs. Together with reports on vertebrates, it is postulated that NETs/ET-like fiber release is a widely shared and effective defence mechanism among animals. The findings presented here highlight variation in ET-like fiber-based innate immune mechanisms in invertebrates, since no significant ET-like fiber production was achieved with the investigated haemocytes following exposure to a wide range of stimulants used in other studies. For further confirmation, *in vivo* studies are required.

Funding: The study was financially supported by the Charles University (Prague) research programmes UNCE-204017, SVV 244-260432/2017, PROGRES Q43 and by a research grant of the Malacological Society of London to VS.

Acknowledgments:

We would like to thank Veronika Siegelová (Charles University) for laboratory maintenance of snails and parasites, and Veronika Harsová (Charles University) for excellent technical support. We also would like to thank Dr. Petr Petráš (The National Institute of Public Health) for kind provision of *Staphylococcus saprophyticus*.

References:

Adema, C., M., van Deutekom-Mulder, E., C., van der Knaap, W., P., Meuleman, E., A., Sminia, T., 1991. Generation of oxygen radicals in hemocytes of the snail *Lymnaea stagnalis* in relation to the rate of phagocytosis. Dev. Comp. Immunol., https://doi.org/10.1016/0145-305x(91)90043-X

Altincicek, B., Stötzel, S., Wygrecka, M., Preissner, K., T., Vilcinskas, A., 2008. Host-derived extracellular nucleic acids enhance innate immune responses, induce coagulation, and

prolong survival upon infection in insects. J. Immunol., https://doi.org/10.4049/jimmunol.181.4.2705

Blažová, K., Horák, P. 2005. *Trichobilharzia regenti*: the developmental differences in natural and abnormal hosts. Parasitol. Int., https://doi.org/10.1016/j.parint.2005.03.003

Brinkmann, V., Laube, B., Abed, U.A., Goosmann, C., Zychlinsky, A., 2010. Neutrophil extracellular traps: how to generate and visualize them. J. Visual. Exp., https://doi.org/10.3791/1724

Hermosilla, C., Caro, T., M., Silva, L., M., Ruiz, A., Taubert, A., 2014. The intriguing host innate immune response: novel anti-parasitic defence by neutrophil extracellular traps. Parasitol., https://doi.org/10.1017/S0031182014000316

Horák, P., Kolářová, L., Dvořák, J., 1998. *Trichobilharzia regenti* n. sp. (Schistosomatidae, Bilharziellinae), a new nasal schistosome from Europe. Parasite, https://doi.org/10.1051/parasite/1998054349

Chanová, M., Bulantová, J., Máslo, P., Horák, P., 2009. In vitro cultivation of early schistosomula of nasal and visceral bird schistosomes (*Trichobilharzia* spp., Schistosomatidae). Parasitol. res., https://doi.org/10.1007/s00436-009-1343-y

Chernin, E., 1963. Observations on hearts explanted in vitro from the snail *Australorbis glabratus*. J. Parasitol., https://doi.org/10.2307/3275797

Lange, M., K., Penagos-Tabares, F., Muñoz-Caro, T., Gärtner, U., Mejer, H., Schaper, R., Hermosilla, C., Taubert, A., 2017. Gastropod-derived haemocyte extracellular traps entrap metastrongyloid larval stages of *Angiostrongylus vasorum*, *Aelurostrongylus abstrusus* and *Troglostrongylus brevior*. Parasite Vector, https://doi.org/10.1186/s13071-016-1961-z

Ng, T., H., Chang, S., H., Wu, M., H., Wang, H., C., 2013. Shrimp hemocytes release extracellular traps that kill bacteria. Dev. Comp. Immunol., https://doi.org/10.1016/j.dci.2013.06.014

Plows, L., D., Cook, R., T., Davies, A., J., Walker, A., J., 2005. Carbohydrates that mimic schistosome surface coat components affect ERK and PKC signalling in *Lymnaea stagnalis* haemocytes. Int. J. Parasitol., https://doi.org/10.1016/j.ijpara.2004.11.012

Poirier, A., C., Schmitt, P., Rosa, R., D., Vanhove, A., S., Kieffer-Jaquinod, S., Rubio, T., P., Charrière, G., M., Destoumieux-Garzón, D., 2014. Antimicrobial histones and DNA traps in invertebrate immunity EVIDENCES IN *CRASSOSTREA GIGAS*. J. Biol. Chem., https://doi.org/10.1074/jbc.M114.576546

Robb, C., T., Dyrynda, E., A., Gray, R., D., Rossi, A., G., Smith, V., J., 2014. Invertebrate extracellular phagocyte traps show that chromatin is an ancient defence weapon. Nat. Comm., https://doi.org/10.1038/ncomms5627

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J., Y., White, D., J., Hartenstein,

V., Eliceiri, K., Tomancak, P, Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Met., https://doi.org/10.1038/nmeth.2019

Sminia, T., 1972. Structure and function of blood and connective tissue cells of the fresh water pulmonate *Lymnaea stagnalis* studied by electron microscopy and enzyme histochemistry. Z. Zellforsch. Mikrosk. Anat., https://doi.org/10.1007/BF00307004

Skála, V., Černíková, A., Jindrová, Z., Kašný, M., Vostrý, M., Walker, A., J., Horák, P., 2014. Influence of *Trichobilharzia regenti* (Digenea: Schistosomatidae) on the defence activity of *Radix lagotis* (Lymnaeidae) haemocytes. PLOS ONE, https://doi.org/10.1371/journal.pone.0111696

Thakur, S., Cattoni, D., I., Nöllmann, M., 2015. The fluorescence properties and binding mechanism of SYTOX green, a bright, low photo-damage DNA intercalating agent. Eur. Biophys. J., https://doi.org/10.1007/s00249-015-1027-8

Von Köckritz-Blickwede, M., Nizet, V., 2009. Innate immunity turned inside-out: antimicrobial defense by phagocyte extracellular traps. J. Mol. Med., https://doi.org/10.1007/s00109-009-0481-0

Walker, A., J., Plows, L., D., 2003. Bacterial lipopolysaccharide modulates protein kinase C signalling in *Lymnaea stagnalis* haemocytes. Biol. Cell, https://doi.org/10.1016/j.biolcel.2003.09.001

Wright, B., Lacchini, A., H., Davies, A., J., Walker, A., J., 2006. Regulation of nitric oxide production in snail (*Lymnaea stagnalis*) defence cells: a role for PKC and ERK signalling pathways. Biol. Cell, https://doi.org/10.1042/BC20050066

Zawrotniak, M., Rapala-Kozik, M., 2013. Neutrophil extracellular traps (NETs)-formation and implications. Acta Biochim Pol, 60, 277-84.

Zahoor, Z., Davies, A., J., Kirk, R., S., Rollinson, D., Walker, A., J., 2008. Disruption of ERK signalling in *Biomphalaria glabrata* defence cells by *Schistosoma mansoni*: implications for parasite survival in the snail host. Dev. Comp. Immunol., https://doi.org/10.1016/j.dci.2008.05.014

Zhang, X., Zhuchenko, O., Kuspa, A., Soldati, T., 2016. Social amoebae trap and kill bacteria by casting DNA nets. Nat. Comm., https://doi.org/10.1038/ncomms10938

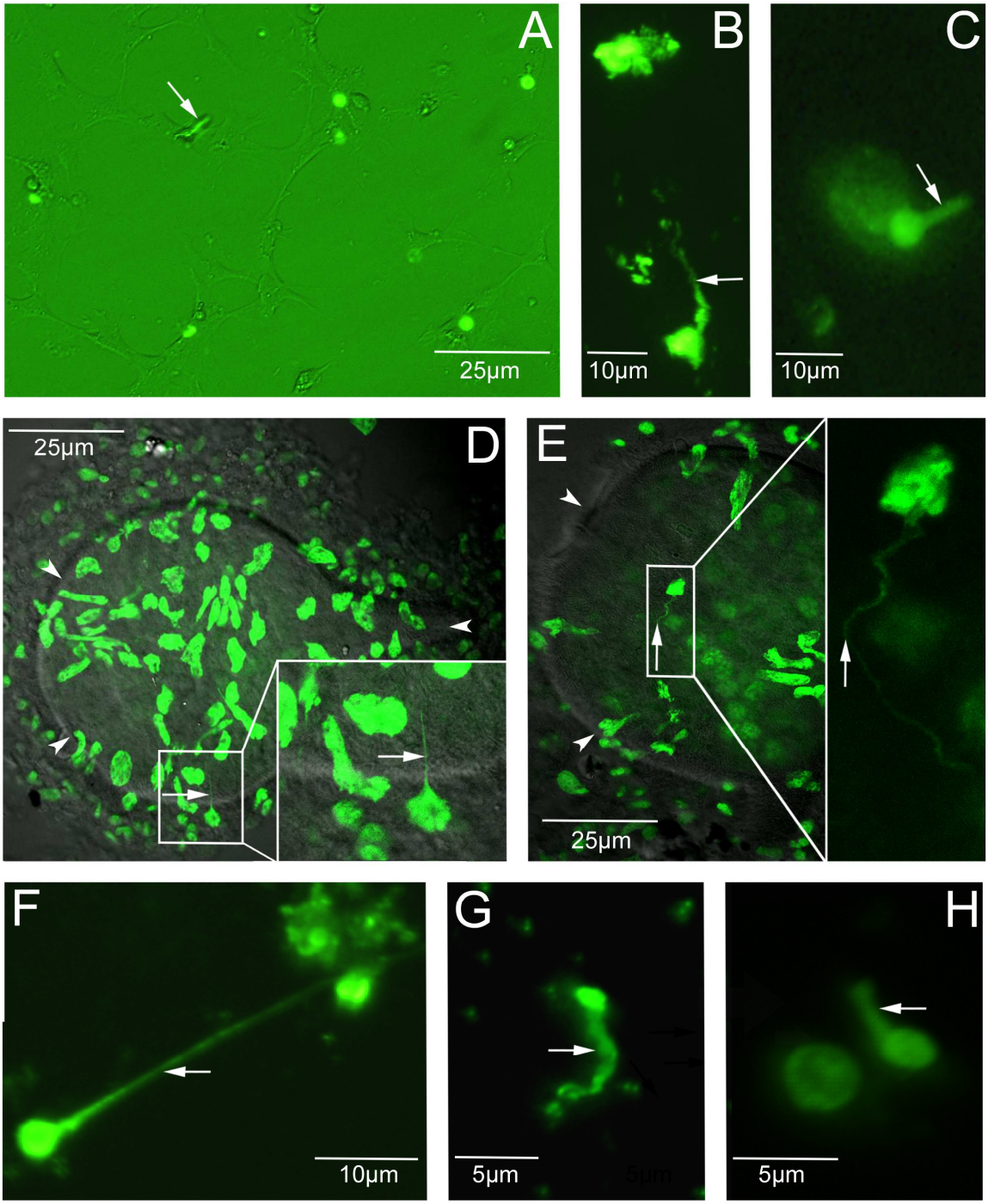
Captions:

Table 1. An overview of compounds/pathogens and conditions used to stimulate ET-like fiber production by haemocytes of the freshwater snail species *Lymnaea stagnalis*, *Radix lagotis* and *Planorbarius corneus*.

Figure 1. Extracellular trap-like (ET-like) fiber production by haemocytes of *Lymnaea stagnalis* **(A-E)**, *Planorbarius corneus* **(F-G)** and *Radix lagotis* **(H)**. Green fluorescence represents DNA positive material - cell nuclei and ET-like fibers (arrows). **(A)** Low magnification of haemocyte monolayer shows that one cell produces ET-like fiber when treated with LPS (1 µg/ml) for 3h. **(B, C)** ET-like fibers produced after the treatment of cells with homogenised *Trichobilharzia regenti* miracidia (10 µg/ml) for 3h **(B)** and 24h **(C)**. **(D, E)** Encapsulation of *T. regenti* miracidia (arrowheads) by snail haemocytes, and expulsion of ET-like fibers (arrows) against the parasite during 3h confrontation; detailed view in the insets. **(F)** ET-like fiber produced after the treatment of haemocytes with LPS (10 µg/ml) in CBSS for 24h. **(G)** ET-like fiber formed in the presence of *Staphylococcus saprophyticus* for 3h. **(H)** ET-like fiber produced after the treatment of cells with PMA (5µM) for 3h.

Supplementary Figure 1. (A-B) Extracellular trap-like (ET-like) fiber production by haemocytes of *Mytilus edulis* after the treatment of cells with PMA (20 µM) for 48 h. Green fluorescence represents DNA positive material - cell nuclei and ET-like fibers (white arrows). **(C)** Phagocytosis of *Staphylococcus saprophyticus* bacteria (black arrows) by *Lymnaea stagnalis* haemocytes during 3 h incubation.

species	compound/pathogen in SSS buffer	condition	duration (h)	no. of ET-like fibers observed
Lymnaea stagnalis	phorbol 12-myristate 13-acetate	0, 0.1, 1, 10 (µM)	3	0, 0, 0, 0
			24	0, 0, 0, 0
	lipopolysaccharide	0, 0.1, 1, 10 (µg/ml)	3	0, 1, 2, 2
			24	0, 1, 0, 0
	peptidoglycan	0, 0.1, 1, 10 (µg/ml)	3	0, 6, 7, 4
			24	1, 1, 6, 3
	D-galactose	0, 200, 400, 800 (nM); 1, 10 (µM)	3	2, 3, 4, 7; 0, 0
			24	2, 1, 8, 5; 0, 0
	L-fucose		3	2, 5, 0, 1; 0, 0
			24	2, 1, 1, 2; 0, 0
	D-galactose/L-fucose	0, 800/800 (nM)	3	2, 6
			24	2, 0
	S. saprophyticus	0, 10, 100, 1000 bacteria/haemocyte	3	0, 0, 0, 0
			24	0, 4, 0, 0
	homogenised *T. regenti* miracidia	0, 1, 10 (µg/ml)	3	1, 6, 7
			24	0, 3, 6
Radix lagotis	phorbol 12-myristate 13-acetate	0, 0.1, 1, 5, 10 (µM)	3	2, 1, 2, 3, 3
			24	2, 0, 0, 0, 0
	S. saprophyticus	0, 100 bacteria/haemocyte	3	2, 0
			24	0, 0
Planorbarius corneus	lipopolysaccharide	0, 0.1, 1, 10 (µg/ml)	3	0, 9, 0, 0
			24	0, 0, 0, 0
	S. saprophyticus	0, 100 bacteria/haemocyte	3	0, 1
			24	1, 0


A
25μm
B
10μm
C
10μm
25μm
D
E
25μm
F
10μm
G
5μm
H
5μm

Highlights:

Extracellular-trap like fiber production may be a limited response in certain invertebrate taxa.

Haemocytes of *Lymnaea stagnalis*, *Radix lagotis* and *Planorbarius corneus* snails produced only few ET-like fibers *in vitro*.

Lymnaea stagnalis haemocytes encapsulated *Trichobilharzia regenti* miracidia *in vitro* and expelled a limited number of ET-like fibers.