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

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1   Short Communication

2   **Extracellular trap-like fiber release may not be a prominent defence**

3   **response in snails: evidence from three species of freshwater gastropod**

4   **molluscs**

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14 **Abstract**

15 The discovery that mammalian neutrophils generate extracellular chromatin fibers that

16 entrap/kill bacteria supported a new paradigm for innate immunity in animals. Similar

17 findings in other models across diverse taxa have led to the hypothesis that the phenomenon is

18 ancient and evolutionary conserved. Here, using a variety of synthetic (e.g. peptidoglycan)

19 and biological (e.g. trematode larvae) components to investigate extracellular trap-like (ET-

20 like) fiber production *in vitro* by haemocytes of *Lymnaea stagnalis*, *Radix lagotis* and

21 *Planorbarius corneus* snails, ET-like fibers were rarely observed. We suggest, therefore, that

22 ET-like fibers play a marginal role in defence of these snail species and thus the fiber

23 production may not be a critical process underpinning immunity in all invertebrate species.

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.

71   *2.3. Preparation of parasite material*

72   Miracidia of *Trichobilharzia regenti* were obtained *via* the laboratory life cycle

73   according to Horák et al. (1998), fixed in 2% (v/v) paraformaldehyde for 30 min and free

74   aldehyde groups blocked in 1% glycine at 4 °C overnight (Zahoor et al., 2008). The larvae

75   were then washed twice with SSS and stored at -20 °C. Homogenised miracidia were

76   prepared by sonicating miracidia for three cycles (7W, 20 s each, Vibracell-72405 100-W

77   ultrasonicator, Bioblock Scientific, France) in SSS followed by determination of protein

78   concentration using Quant-iT Protein Assay Kit (Invitrogen).

79   *2.4. Haemocyte exposure*

80   *Lymnaea stagnalis* haemocyte monolayers were treated with SSS containing

81   peptidoglycan (PGN; 0.1, 1.0 and 10.0 µg/ml), *E. coli* lipopolysaccharide serotype 0111:B4

82   (LPS; 0.1, 1.0 and 10.0 µg/ml), PMA (0.1, 1.0, 10.0 µM), galactose or fucose (200, 400, 800

83   nM, 1 and 10 µM), galactose-fucose in combination (800 nM of each in SSS) (all purchased

84   from Sigma-Aldrich), live/heat-killed *Staphylococcus saprophyticus* at ~10, ~100 and ~1000

85   bacteria/haemocyte, or miracidial homogenate (1 or 10 µg/ml). All incubations were

86   performed at room temperature (e.g. Plows et al., 2005) for 3 h and 24 h. Three independent

87   experiments were performed with one replicate for each condition/duration. The haemocytes

88   were then stained with 1 µM Sytox Green in SSS for 20 min and the entire cell populations

89   examined visually under the fluorescence microscope; haemocytes producing ET-like fibers

90   were enumerated.

91   For intact parasite exposure, 200 miracidia in 100 µl SSS were transferred to

92   individual wells of a chamber slide (Lab-Tek); 200 µl complete *L. stagnalis* haemolymph

93   were added and after 1 h incubation, 100 µl supernatant were replaced by 100 µl fresh

94   haemolymph. This step was done to enhance the continuous migration of haemocytes towards

95   the parasite. Incubation times/Sytox green staining were as above; the experiments were

96   performed twice independently. Finally, specimens were embedded in Vectashield (Vector

97   Laboratories), examined using a Zeiss LSM880 laser scanning confocal microscope, and

98   images analysed using FIJI Image J (Schindelin et al., 2012).

99   Haemocyte monolayers obtained from *R. lagotis* and *P. corneus* were incubated in

100   SSS containing PMA (0.1, 1, 5, 10 µM), LPS (0.1, 1.0, 10.0 µg/ml), or heat-killed *S.*

101   *saprophyticus* at ~100 bacteria/haemocyte.

102   **3. Results and discussion**

103   Initial experiments were performed with haemocytes of *M. edulis*, previously shown to

104   produce ET-like fibers (Robb et al. 2014), to demonstrate fiber release in our laboratory.

105   Similar to Robb et al. (2014), PMA clearly induced ET-like fiber release (Fig. 1A, B in

106   Supplementary Materials) that was ETotic.

107   Next, snail haemocytes were exposed to PMA or LPS, compounds that were shown

108   previously to stimulate effective NETs/ET-like fiber formation (von Köckritz-Blickwede and

109   Nizet, 2009; Robb et al., 2014; Ng et al., 2013). Other components (e.g. L-fucose/D-

110   galactose) were employed because they are linked to snail-trematode interactions (Plows et

111   al., 2005).

112   The screening assays revealed that *L. stagnalis*, *R. lagotis* and *P. corneus* haemocytes

113   produced only low numbers of extracellular DNA fibers (Table 1) and, therefore, other

114   components associated with ET-like fibers such as histones (Ng et al., 2013; Robb et al.,

115   2014) were impossible to investigate. However, given that occasional DNA fibers were

116   observed in all species studied (Fig. 1) we define the fibers as 'ET-like' as in other

117   invertebrates (Ng et al., 2013; Robb et al., 2014; Poirier et al., 2014; Lange et al., 2017).

118   That compounds such as PGN or PMA failed to elicit robust ET-like fiber production

119   in *L. stagnalis* was surprising (Table 1). Similarly, PMA did not stimulate ET-like fiber

120   formation by *C. gigas* haemocytes (Poirier et al., 2014). Exposure of haemocytes to 20 µM

121   PMA in SSS or in modified SSS (SSS supplemented with D-trehalose (1g/L), D-glucose

122   (1g/L) (Sigma-Aldrich) and antibiotics (penicillin/streptomycin; Lonza)), enabling longer-

123   term *L. stagnalis* haemocyte survival for 48 h also did not evoke haemocyte ETotic responses

124   (data not shown). On the other hand, *M. edulis* haemocytes produced fibers when exposed to

125   50 µM PMA for 48 h (Robb et al., 2014). In *R. lagotis*, haemocytes exposed to PMA

126   produced only few ET-like fibers (Table 1, Fig. 1H). This finding was unexpected because

127   PMA induces the respiratory burst in *R. lagotis* haemocytes (Skála et al., 2014), a reaction

128   considered essential for ET-like fiber formation (Robb et al., 2014; Poirier et al., 2014).

129   Although LPS significantly induced NETs/ET-like fiber formation in mammalian

130   neutrophils or shrimp haemocytes (von Köckritz-Blickwede and Nizet, 2009; Ng et al., 2013),

131   only two ET-like fibers were produced by *L. stagnalis* haemocytes (Table 1, Fig. 1A).

132   Additionally, no ET-like fibers were observed when these haemocytes were treated with 25

133   µg/ml LPS in modified SSS for 24h, and the protocol of Brinkmann et al. (2010) was used to

134   visualise the fibers (data not shown). With *P. corneus*, one ET-like fiber was observed when

135   haemocytes were exposed to 10 µg/ml LPS in CBSS for 24 h (Fig. 1F) whereas nine fibers

136   were observed in SSS (Table 1). Thus, these different culture media did not seem to largely

137   influence the outcome with respect to ET-like fiber formation.

138   PMA and LPS activate protein kinase C (PKC) in *L. stagnalis* haemocytes (Walker

139   and Plows, 2003; Wright et al., 2006), which stimulates NO production (Wright et al., 2006).

140   Such responses might, at least in part, explain the inability of PMA and LPS to effectively

141   promote ET-like fiber production. However, D-galactose and L-fucose attenuate PKC and

142   extracellular-signal regulated kinase (ERK) activation in *L. stagnalis* haemocytes, with

143 subsequent suppression of phagocytosis (Plows et al., 2005). These sugars are present on the

144 surface of the helminth *T. regenti* (Blažová and Horák, 2005; Chanová et al., 2009), an

145 incompatible parasite that penetrates but does not survive in *L. stagnalis*. However, exposure

146 to these sugars did not affect ET-like fiber production (Table 1).

147   As the soluble compounds did not substantially stimulate ET-like fiber formation by

148 the snail haemocytes, we tested pathogen-haemocyte combinations. Heat-killed *S.*

149 *saprophyticus* bacteria were phagocytosed by the snail haemocytes (Fig. 1C in Supplementary

150 Materials) whereas several ET-like fibers were produced with unclear function (Table 1);

151 similar results were also obtained with live *S. saprophyticus* (data not shown). In contrast,

152 ET-like fibers produced by *C. gigas* haemocytes were shown to entrap *Listonella anguillarum*

153 (Poirier et al., 2014), whereas fibers produced by *L. vannamei* trapped and killed *E. coli* (Ng

154 et al., 2013).

155   Experiments using fixed *T. regenti* miracidia and whole snail haemolymph showed

156 that haemocytes encapsulate the parasite (Fig. 1D). Moreover, confocal microscopy revealed

157 that several haemocytes expelled ET-like fibers against *T. regenti* during 3 h exposure (Fig.

158 1D-E). In gastropods, haemocyte derived ET-like fibers were demonstrated previously in *A.*

159 *fulica*, which trapped viable *Angiostrongylus vasorum* larvae *in vitro* (Lange et al., 2017).

160 Release of ET-like structures was also observed *in vivo* in the mucous extrapallial space of *L.*

161 *maximus* in response to invading *A. vasorum* (Lange et al., 2017). However, in our study, only

162 a few *L. stagnalis* haemocytes produced ET-like fibers against *T. regenti* (Fig. 1D-E) and thus

163 the fibers are unlikely the main defence tool for parasite elimination. Although attempted,

164 evaluation of *T. regenti* and *L. stagnalis* interactions in snail histological sections was

165 technically demanding (results not shown) and, therefore, the extent of ET-like fiber

166 production *in vivo* remains unknown. Finally, homogenised *T. regenti* miracidia did not

167 stimulate significant ET-like fiber production (Table 1, Fig. 1B-C).

168   To conclude, we examined the ability of several compounds and pathogens to elicit

169 ET-like fiber production in the freshwater snails *L. stagnalis*, *R. lagotis* and *P. corneus in*

170 *vitro*. ET-like fiber production has previously been reported in several invertebrates including

171 molluscs. Together with reports on vertebrates, it is postulated that NETs/ET-like fiber release

172 is a widely shared and effective defence mechanism among animals. The findings presented

173 here highlight variation in ET-like fiber-based innate immune mechanisms in invertebrates,

174 since no significant ET-like fiber production was achieved with the investigated haemocytes

175 following exposure to a wide range of stimulants used in other studies. For further

176 confirmation, *in vivo* studies are required.

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258 **Captions:**

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

262 **Figure 1.** Extracellular trap-like (ET-like) fiber production by haemocytes of *Lymnaea*
263 *stagnalis* (**A-E**), *Planorbarius corneus* (**F-G**) and *Radix lagotis* (**H**). Green fluorescence
264 represents DNA positive material - cell nuclei and ET-like fibers (arrows). (**A**) Low
265 magnification of haemocyte monolayer shows that one cell produces ET-like fiber when
266 treated with LPS (1 µg/ml) for 3h. (**B, C**) ET-like fibers produced after the treatment of cells
267 with homogenised *Trichobilharzia regenti* miracidia (10 µg/ml) for 3h (**B**) and 24h (**C**). (**D,**
268 **E**) Encapsulation of *T. regenti* miracidia (arrowheads) by snail haemocytes, and expulsion of
269 ET-like fibers (arrows) against the parasite during 3h confrontation; detailed view in the
270 insets. (**F**) ET-like fiber produced after the treatment of haemocytes with LPS (10 µg/ml) in
271 CBSS for 24h. (**G**) ET-like fiber formed in the presence of *Staphylococcus saprophyticus* for
272 3h. (**H**) ET-like fiber produced after the treatment of cells with PMA (5µM) for 3h.

273 **Supplementary Figure 1.** (**A-B**) Extracellular trap-like (ET-like) fiber production by
274 haemocytes of *Mytilus edulis* after the treatment of cells with PMA (20 µM) for 48 h. Green
275 fluorescence represents DNA positive material - cell nuclei and ET-like fibers (white arrows).
276 (**C**) Phagocytosis of *Staphylococcus saprophyticus* bacteria (black arrows) by *Lymnaea*
277 *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
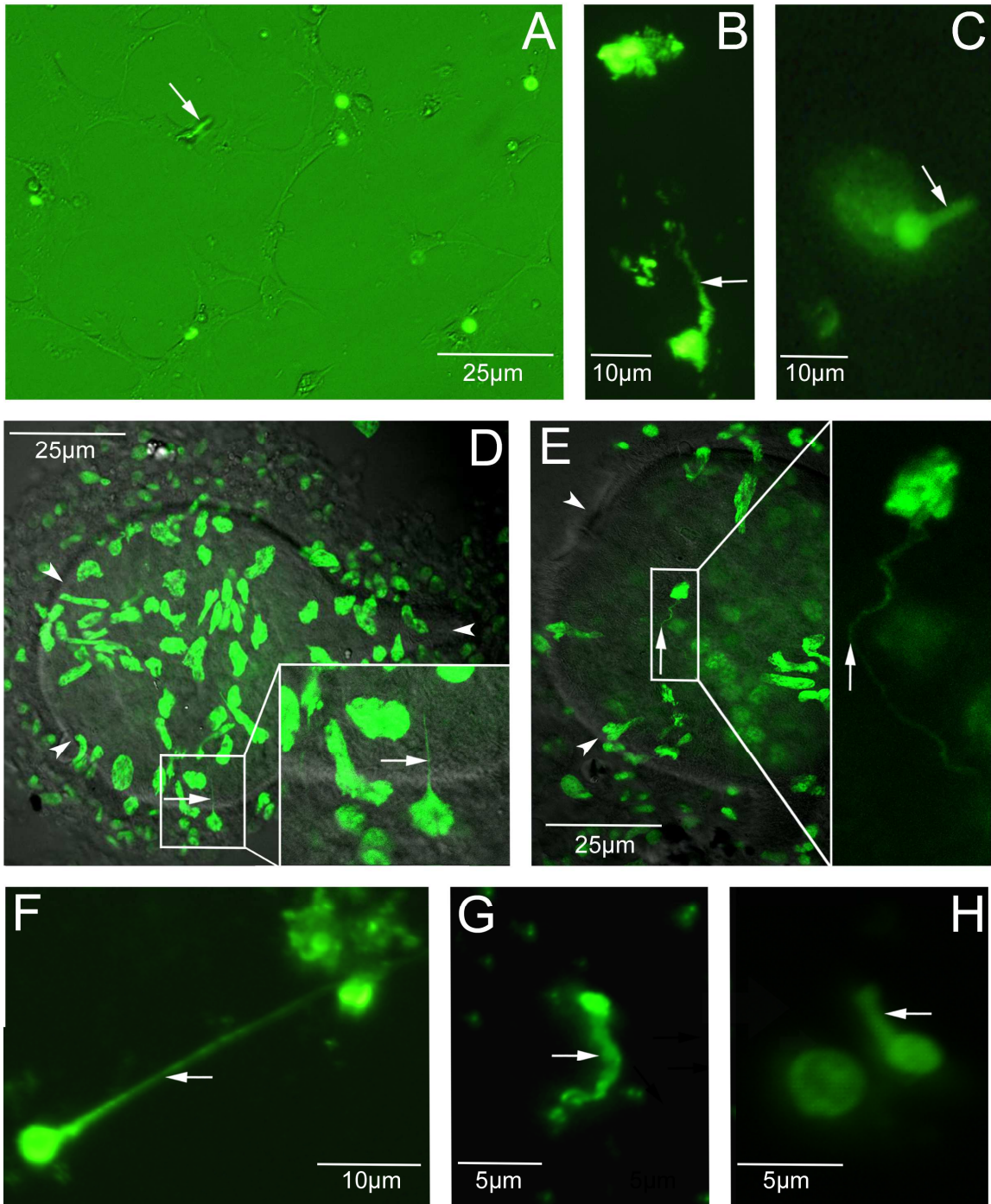
Highlights:

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.