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1 **AUTHOR CONTRIBUTIONS**

2

3 Conceptualization: AGR, APD; Data curation: APD, NAdH; Formal analysis: APD,
4 NAdH; Funding acquisition: AGR, APD; Investigation: NAdH; Methodology: APD,
5 AGR, APV, CTR. NAdH; Project administration: AGR, APD; Resources: AGR, APD,
6 CTR; Writing - original draft: APD, NAdH; Writing - review & editing: APD, AGR,
7 APV, CTR. NAdH

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RELEASE OF CHROMATIN EXTRACELLULAR TRAPS BY PHAGOCYTES OF ATLANTIC SALMON, *Salmo salar* (LINNAEUS, 1758)

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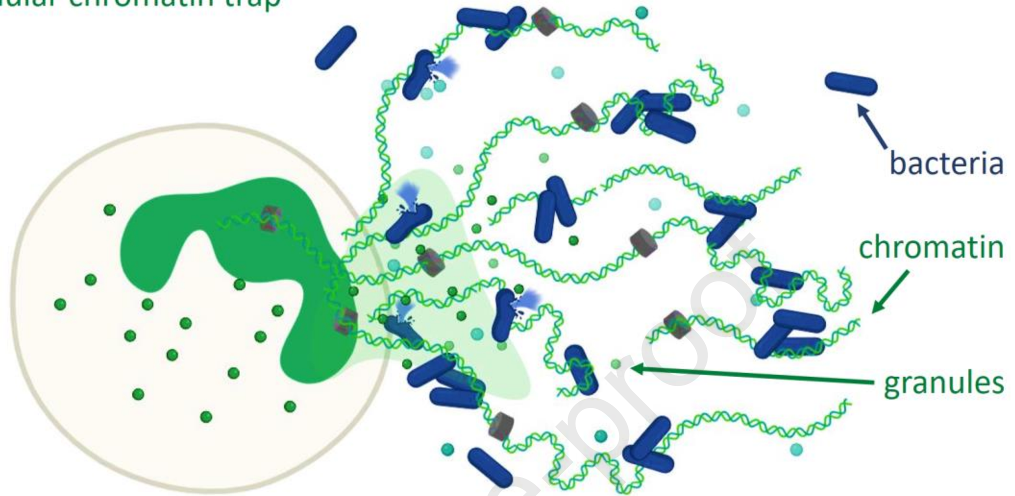
20 **GRAPHICAL ABSTRACT**

21



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23 **HIGHLIGHTS**

24

25 Chromatin extracellular traps (ETs) were released by salmon neutrophils

26

27 Calcium ionophore was a powerful inducer of ET release from neutrophils

28

29 ETs were decorated with histones H1 and H2A and neutrophil elastase

30

31 Bacteria induced ET release and association with ETs was observed

32

33 ET structures were observed in monocyte/macrophage suspensions

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34 **ABSTRACT**

35

36 Neutrophils release chromatin extracellular traps (ETs) as part of the fish innate immune
37 response to counter the threats posed by microbial pathogens. However, relatively little
38 attention has been paid to this phenomenon in many commercially farmed species, despite
39 the importance of understanding host-pathogen interactions and the potential to influence
40 ET release to reduce disease outbreaks. The aim of this present study was to investigate
41 the release of ETs by Atlantic salmon (*Salmo salar* L.) immune cells. Extracellular
42 structures resembling ETs of different morphology were observed by fluorescence
43 microscopy in neutrophil suspensions *in vitro*, as these structures stained positively with
44 Sytox Green and were digestible with DNase I. Immunofluorescence studies confirmed
45 the ET structures to be decorated with histones H1 and H2A and neutrophil elastase,
46 which are characteristic for ETs in mammals and other organisms. Although the ETs were
47 released spontaneously, release in neutrophil suspensions was stimulated most
48 significantly with 5 µg/ml calcium ionophore (CaI) for 1 h, whilst the fish pathogenic
49 bacterium *Aeromonas salmonicida* (isolates 30411 and Hooke) also exerted a stimulatory
50 effect. Microscopic observations revealed bacteria in association with ETs, and fewer
51 bacterial colonies of *A. salmonicida* Hooke were recovered at 3 h after co-incubation with
52 neutrophils that had been induced to release ETs. Interestingly, spontaneous release of
53 ETs was inversely associated with fish mass ($p<0.05$), a surrogate for age. Moreover,
54 suspensions enriched for macrophages and stimulated with 5 µg/ml CaI released ET-like
55 structures that occasionally led to the formation of large clumps of cells. A deeper
56 understanding for the roles and functions of ETs within innate immunity of fish hosts,
57 and their interaction with microbial pathogens, may open new avenues towards protecting
58 cultured stocks against infectious diseases.

59

60 **KEYWORDS:**

61

62 ETosis; macrophage; NETosis; neutrophil extracellular traps; polymorphonucleocyte

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63 **1. INTRODUCTION**

64

65 Various cell types recognise and respond to microbial invaders as part of the fish innate
66 immune response. The polymorphonuclear cells (PMNs; also known as granulocytes),
67 such as neutrophils, basophils and eosinophils, play a particularly important role and they
68 act through a series of mechanisms to counter microbial threats (Grayfer et al., 2014;
69 Rieger & Barreda, 2011). PMNs, particularly neutrophils, perform phagocytosis, where
70 microbes are internalised and inactivated in phagosomes by reactive oxygen species
71 (ROS) and other antimicrobial compounds (Havixbeck & Barreda, 2015; Havixbeck et
72 al., 2016, 2017). Like other granulocytes, neutrophils release antimicrobial compounds
73 into the extracellular space by the process of degranulation where they can then act
74 against their targets (Grayfer et al., 2014; Yin & Heit, 2018). Finally, neutrophils can
75 release extracellular traps (ETs), which are structures composed of decondensed nuclear
76 chromatin embedded with antimicrobial proteins, including neutrophil elastase,
77 myeloperoxidase (MPO) and histone fragments (Fernandes et al., 2002; Sollberger et al.,
78 2018; Urban et al., 2009; Van et al., 2020; Wen et al., 2018). ETs exert their antimicrobial
79 action by trapping microbes to prevent or delay their dissemination around the host
80 sufficiently to allow for other immune cells to be recruited to assist in preventing an
81 infection (Lázaro-Díez et al., 2017; O'Brien et al., 2017). Moreover, ETs may themselves
82 be directly antimicrobial because the chromatin structure provides a means to bring the
83 microbes into close proximity with the decorating proteins such as neutrophil elastase,
84 MPO and histone fragments that can exert these activities (Azzouz et al., 2018; Wang et
85 al., 2020; Zhao et al., 2017).

86

87 Much of the knowledge on ETs derives from studies of mammals and relatively few
88 studies have examined the phenomenon in fish hosts (Neumann et al., 2020). Still, ETs
89 have been observed to be released by PMNs of fathead minnow (*Pimephales promelas*)
90 (Palić et al., 2007a), zebrafish (*Danio rerio*) (Palić et al., 2007b), common carp (*Cyprinus*
91 *carpio*) (Brogden et al., 2012; Zhang et al., 2021), turbot (*Scophthalmus maximus*) (Chi
92 & Sun, 2016), sole (*Cynoglossus semilaevis*) (Zhao et al., 2017), rainbow trout
93 (*Oncorhynchus mykiss*) (Van et al., 2020) and Atlantic salmon (*Salmo salar*) (Alarcon et
94 al., 2021). In addition, Pijanowski et al. (2015) detected ETs in monocyte/macrophage
95 preparations from common carp, thus demonstrating other immune cells to be capable of
96 releasing the traps. Even with these studies, knowledge of teleost ETs remains nascent

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97 and differences in responses observed between species remain to be understood, including
98 the pathways controlling in initiation and release, the interaction with and effects on
99 microbes, and the range of cell types capable of this response. Moreover, there is little
100 understanding for variability observed between individual fish in their propensity to
101 release ETs (Van et al., 2020).
102
103 Though there is increasing recognition for the importance of ETs in humans and other
104 mammals in immunity and the negative impacts associated with dysfunctional control of
105 their release, few of the economically most important fish species have yet to be
106 investigated for the existence, control and role of ET release. Despite a recent observation
107 of ET release in Atlantic salmon (Alarcon et al., 2021), a species farmed intensively in
108 the waters of northern Europe and in Chile, very little is known in particular for the
109 constituents decorating the ET structures and the compounds that modulate ET release.
110 Global production of *S. salar* reached 2.4 million tonnes in 2018 (FAO, 2020) and the
111 success of this industry is underpinned by the ability to prevent and control diseases,
112 particularly those caused by bacteria. Vaccination programmes have contributed
113 significantly to infection control but outbreaks requiring antibiotic therapy can still occur
114 where a vaccine does not confer protection (Adams, 2019; Gravningen et al., 2019).
115 However, antibiotic use can increase the risk of selecting for resistant strains that has
116 associated detrimental consequences (Chuah et al., 2016; Higuera-Llantén et al., 2018;
117 Miranda et al., 2018), and a better understanding of the salmon innate immune system
118 may uncover alternative solutions to disease prevention and control .
119
120 Therefore, the aim of this present study was to investigate the release of ETs by Atlantic
121 salmon neutrophils and other immune cells, including the detection of characteristic
122 components of the structures, the actions of various modulators on release, and the
123 effectiveness of the traps against bacteria.
124
125 **2. MATERIALS AND METHODS**
126
127 **2.1 Fish and isolation of immune cells**
128
129 Apparently healthy pre-smolt salmon (determined by gross examination) were used in all
130 experiments. Fish were maintained at the Niall Bromage Freshwater Research Unit

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131 (University of Stirling) in 25 m^3 tanks at 30–50 kg/ m^3, with the system operating at a
132 flow rate of 50–70 l/min, oxygen concentration >7 mg/l, and typical mean monthly water
133 temperature of 8.3±1.3°C (± standard error, SEM). Constant light was provided post-
134 hatch from January until switching to a natural photoperiod in July, and fish were fed a
135 commercial pelleted feed (Skretting) *ad libitum*. Typically, fish were ca. 30–80 g but
136 larger fish (100–300 g) were also sampled occasionally. PMNs and mononuclear
137 leukocytes (i.e., monocytes/macrophages) were isolated from head kidney tissue
138 according to the triple-layer Percoll gradient procedure of Van et al. (2020). A band
139 enriched for PMNs formed at the interface of the 1.060 and 1.072 g/ml Percoll layers,
140 whilst the mononuclear leukocytes collected at the interface of the 1.072 and 1.084 g/ml
141 Percoll layers. Peripheral blood leukocytes (PBLs) were isolated from blood collected
142 aseptically from the caudal vein using a 2.5-ml syringe and 25G × 5/8 needle (Terumo,
143 Surrey, UK). Blood was diluted 5-fold in RPMI-1640 medium supplemented with 40
144 U/ml heparin and loaded onto a 54% Percoll layer. PBLs were collected from the
145 interphase region. All cell types were washed with refrigerated RPMI-1640 for 10 min
146 prior to use in experiments. Aliquots of washed cells were used for total cell counts,
147 determinations of viability, and Rapi-Diff II staining (Vetlab Supplies, Ltd., Pulborough,
148 UK) to visualise cell morphologies. PBL suspensions were also used for cell sorting and
149 purity assessments by immunostaining (see Section 2.9). Cell suspensions were adjusted
150 to 4×10^5 cells/ml and seeded into cell culture plates (Cell+; Sarstedt, Nümbrecht,
151 Germany) containing RPMI-1640 medium supplemented with 1% (v/v) fetal bovine
152 serum (FBS; Thermo Fisher Scientific, Loughborough, UK) and 0.5% (v/v) penicillin-
153 streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin; Sigma-Aldrich Ltd,
154 Gillingham, UK) and maintained at 15°C for the further experimental procedures.
155
156 **2.2 ET release assay**
157
158 When quantifying ET release, 50 μL of cell suspension (PMNs, mononuclear leukocytes
159 or PBLs) was seeded at 4×10^5 cells/ml in 96-well cell culture plates and allowed to settle
160 for 30 min at 15°C before being used for experiments. Blank control wells (lacking cells)
161 contained RPMI-1640 medium and corresponding volumes of diluents only. Plates were
162 incubated at 15°C (typically for 1 h), before staining for 20 min with Sytox Green (a
163 nucleic acid-specific dye; Invitrogen, Loughborough, UK) that was added to a final well

164 concentration of 5 µM to permit quantification of the fluorescence in each well according
165 to Van et al. (2020). Visualisation of ETs was performed with an Olympus BX-51
166 epifluorescence microscope equipped with an Axiocam MRC camera (Zeiss, Cambridge,
167 UK) and the Axiovision imaging software (v.4.8; Zeiss).
168

169 **2.3 DNA composition of the ET structures**

170

171 To confirm the DNA composition of the material released from the cells, PMN
172 suspensions (prepared in 96-well plates as described in Section 2.2) containing cells that
173 had released ET structures spontaneously were incubated with 200 U/ml DNase I
174 (prepared in the buffer containing $MgCl_2$ supplied with the enzyme; Thermo Fisher
175 Scientific) for 30 min and stained with Sytox Green as before. Differences in fluorescence
176 were quantified in wells between treatment and controls with a microplate reader and
177 changes in cell morphology were assessed by fluorescence microscopy. Moreover, by
178 combining fluorescence and phase-contrast microscopy, the relative percentages of cells
179 having undergone ETosis was calculated as a percentage of total cells (i.e., viable, dead
180 and ETotic cells).
181

182 **2.4 Effects of chemical and biological modulators of ET release**

183

184 The effects on ET release of a range of chemical and biological compounds known to
185 modulate ET release in fish and mammals was investigated by exposing PMN and
186 mononuclear leukocyte cell suspensions (prepared in 96-well plates as described in
187 Section 2.2) to calcium ionophore A23187 (CaI; Thermo Fisher Scientific), phorbol 12-
188 myristate 13-acetate (PMA; Sigma Aldrich Ltd, Gillingham, UK), lipopolysaccharide
189 (LPS from *E. coli* O111:B4; Sigma Aldrich), polyinosinic–polycytidylic acid sodium salt
190 (Poly I:C; Sigma Aldrich; PMNs only) and diphenyleneiodonium chloride (DPI; Sigma
191 Aldrich), and ETs quantified by fluorescence according to Section 2.2. The final
192 concentrations added to the wells of the 96-well plates were 5 µg/ml CaI, 10 nM PMA,
193 10 µg/ml LPS, 40 µg/ml Poly I:C, or 10 µM DPI.
194

195 **2.5 Immunofluorescence antibody test (IFAT)**

196

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197 To characterise the decoration of the chromatin composing the ETs, a panel of antibodies
198 was evaluated against conserved markers of ETs. Specifically, these were mouse to
199 human histone H2A (L88A6) (#3636; Cell Signaling Technology, London, UK), mouse
200 to human histone H1/DNA (MAB3864; Millipore, Watford, UK), and rabbit to human
201 neutrophil elastase (ab21595; Abcam, Cambridge, UK). Secondary conjugated antibody,
202 Alexa Fluor 488, and 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) nuclear dye
203 were sourced from Invitrogen. PMNs prepared in 96-well cell culture plates as described
204 in Section 2.2 were induced with 5 µg/ml CaI or 10 nM PMA (1 h, 15ºC) to release ETs
205 and then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After
206 fixing, cells were washed with PBS and non-specific binding was blocked with 3% bovine
207 serum albumin (BSA; Merck, Watford, UK) in PBS for 1 h. Then, primary antibodies
208 were added at appropriate dilutions (histone H2A, 1:200; H1/DNA, 1:200; neutrophil
209 elastase, 1:20) and left to incubate overnight at 4ºC. After three washes with PBS,
210 conjugated antibodies (diluted 1:300 in PBS) supplemented with 3% BSA were added for
211 90 min. Cells were washed thrice with PBS to remove excess antibodies and then
212 incubated with 300 nM DAPI to counterstain the DNA present. Images were acquired
213 immediately with an EVOS FL cell imaging system (Thermo Fisher Scientific) equipped
214 with bright field, 357/44 nm and 470/22 nm excitation LED lights, ×20 and ×40 objective
215 lenses, and processed using the NIS-Elements 3.2 software.
216
217 **2.6 Effect of bacteria on ET release by PMNs**
218
219 To assess whether bacteria could induce ET release, 100 µl of PMN suspension prepared
220 as Section 2.1 was seeded at 4×10^5 cells/ml in 24-well cell culture plates (Cell+; Sarstedt,
221 Nümbrecht, Germany) and then 100 µl of either of two *Aeromonas salmonicida* subsp.
222 *salmonicida* isolates (*A. salmonicida* Hooke or *A. salmonicida* 30411) was added to the
223 wells to 4×10^7 colony-forming units (CFU)/ml. The bacteria had been cultured from
224 single colonies in 5 ml tryptic soy broth (TSB; Oxoid, Basingstoke, UK) overnight at
225 22ºC with orbital shaking at 150 rpm. Bacteria were harvested in exponential phase by
226 centrifugation (2700 ×g, 15 min, 4ºC), washed once with PBS and resuspended to the
227 desired CFU/ml. Control PMN cultures were incubated with RPMI-1640 medium and
228 diluent only. After centrifugation (800 ×g; 10 min; 22ºC) to allow the bacteria to come
229 into contact with the cells and materials at the bottom of the wells, cultures were incubated
230 for 2 h at 22ºC. Following this, Sytox Green was added to the cultures to a final well

231 concentration of 5 μM for 20 min, before the fluorescence of each well was quantified
232 with a microplate reader and well contents observed under the fluorescence microscope
233 as above.
234

235 **2.7 Bacteria interaction with ETs**

236

237 To visualise the interaction between the bacteria and the ETs, PMNs at 4×10^5 cells/ml in
238 the wells of a 24-well cell culture plate were induced to release ETs by exposure to 5
239 μg/ml CaI for 1 h at 15ºC, before staining with 5 μM Sytox Green as above. Meanwhile,
240 washed bacterial suspensions of *A. salmonicida* Hooke or 30411 were stained with 300
241 nM DAPI for 30 min at 4ºC, washed with PBS at 2600 ×g for 10 min and resuspended in
242 PBS. Then, the bacterial suspensions were added to the PMN cell suspensions to ca. 4
243 $\times 10^7$ CFU/ml. After centrifugation as described in Section 2.6, the cultures were
244 incubated at 22ºC for 2 h and then well contents were observed under the fluorescence
245 microscope with images acquired as above.
246

247 **2.8 Effect of ETs on bacterial viability**

248

249 To assess the effect of the ETs on bacterial viability, PMN cell suspensions were prepared
250 and induced to release ETs as described in Section 2.7, and then *A. salmonicida* Hooke
251 was added to ca. 4×10^7 CFU/ml. In controls, the PMNs were incubated for 30 min in
252 medium supplemented with 200 U/ml DNase I to digest away any ETs present before
253 bacteria were added. Plates were centrifuged for 10 min at 800 ×g to encourage contact
254 between the bacteria and the ETs, before a sub-sample was taken to determine CFU/ml
255 (time = 0) by serial dilution in PBS and plating on TSA to allow for colony counts. The
256 plate containing bacteria and PMNs was incubated for 3 h at 22ºC at which point the
257 contents of each well were collected and plated to determine CFU/ml. Preliminary studies
258 had determined that DNase I and CaI at the concentrations used had no effect on bacteria
259 viability (data not shown).
260

261 **2.9 Magnetic-activated cell sorting (MACS)**

262

263 Specific monoclonal antibodies mAb-8 and mAb-42 raised against membrane
264 glycoproteins of trout thrombocytes (Köllner et al., 2004) were provided kindly by Dr

265 Bernd Köllner (Institute of Immunology, Friedrich-Loeffler Institute, Riems, Germany).
266 Atlantic salmon PBLs purified from blood according to Section 2.1 were resuspended in
267 an Eppendorf tube to a density of 4×10^5 cell/ml and incubated with 0.4 µg/ml of mAb-8
268 and mAb-42 for 30 min on ice. After washing twice with RPMI-1640, cells were
269 resuspended in 160 µl culture medium plus 40 µl of MACS microbeads coupled to a goat
270 anti-mouse IgG antibody (Miltenyi Biotec, GmbH, Germany) for 30 min at 4ºC. After
271 washing twice more, samples were resuspended in RPMI-1640 containing 2 mM
272 ethylenediaminetetraacetic acid and 10% FBS and then loaded onto a mini MACS column
273 (Miltenyi Biotec, Bergisch Gladbach, Germany) to purify antibody-positive cells.
274 Unlabelled leukocytes flowing through the column were discarded. After one wash step
275 with RPMI-1640, the column was detached from the magnetic separator and the bound
276 cells were eluted with 1 ml of culture medium. The thrombocyte-enriched population was
277 quantified with a haemocytometer by light microscopy, and this suspension was used to
278 assess the effects of CaI and PMA on ET release, with assays prepared and performed as
279 described in Sections 2.2 and 2.4. To assess purity of the original cell suspension before
280 MACS, an aliquot was incubated with either mAb-8 and mAb-42 and stained with Alexa
281 488 goat-anti-mouse IgG for 90 min, with 300 nM DAPI used to counterstain the cell
282 nuclei before evaluation by fluorescence microscopy.
283
284 **2.10 Statistical analyses**
285
286 All statistical analyses were conducted and data plotted with GraphPad Prism v.7.04
287 software. Spearman's rank and Pearson coefficient were used to assess the significance
288 of the correlations between fish mass and yield of PMNs or the $\log_{10}$ of fluorescence
289 values resulting from spontaneous ET release, respectively. Differences between
290 treatment groups in experiments were assessed by Student's t-test (two-tailed). Statistical
291 significance was achieved at $p<0.05$, with multiple comparisons accounted for by Holm's
292 correction.
293
294 **2.11 Ethics statement**
295
296 All procedures were conducted in accordance with the European Directive 63/2010/EU
297 on the protection of laboratory animals used for scientific purposes. The study was
298 approved by the Ethics Committee of the Institute of Aquaculture at the University of

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299 Stirling. Euthanasia of the fish was performed in accordance with Schedule 1 of the
300 Animals (Scientific Procedures) Act 1986 (United Kingdom).
301
302 **3. RESULTS**
303
304 **3.1 ET structures released by salmon PMNs**
305
306 Preparations of cells isolated from the head kidney were highly-enriched in PMNs
307 (**Figure 1**); these cells were non-adherent in culture, round in appearance, and contained
308 a polymorphic nucleus surrounded by granulocytic and basophilic cytoplasm (**Figure 1**),
309 which are characteristics consistent with mammalian neutrophils. The PMN cell
310 suspensions contained few other phagocytes (**Figure 1**). Fish mass correlated directly and
311 significantly with the proportion of PMNs in the cell suspensions ($p<0.05$) (**Figure 1**).
312
313 Web-like and streaky extracellular structures that stained positively with Sytox Green and
314 resembling ETs were observed in PMN suspensions during culture *in vitro*, presumably
315 resulting from spontaneous release (**Figure 1**). Interestingly, spontaneous release of ETs
316 (as quantified by fluorescence) was inversely associated with fish mass ($p<0.05$) and there
317 appeared to be greater variability in response in the suspensions from fish <120 g (**Figure**
318 **1**).
319
320 When the PMN suspensions were incubated with DNase I, there was a significant
321 decrease in the fluorescence signal detected in the wells and the web-like structures were
322 no longer observed by microscopy (**Figure 2**). Indeed, the percentage of cells that had
323 released observable ETs in the PMN population following treatment with DNase I was
324 reduced significantly compared to untreated controls (**Figure 2**).
325
326 Fluorescence was quantified in PMN-enriched cell cultures exposed to various
327 modulators of ET release (**Figure 3**). There was a significant increase in the fluorescence
328 released by PMNs treated with 5 µg/ml CaI for 1 h compared to the untreated control
329 (**Figure 3**). Fluorescence microscopy confirmed the presence of structures resembling
330 ETs in the wells in this treatment group, and these exhibited different morphologies and
331 stained positively with Sytox Green (**Figure 3**). Furthermore, there was a small but
332 significant increase in fluorescence in wells containing PMNs exposed to 10 µg/ml LPS

333 for 1 h, while exposure to 40 µg/ml Poly I:C led to a significant decrease in fluorescence
334 compared to untreated controls. Exposure of PMN cell suspensions to 10 nM PMA or 10
335 µM DPI for 1 h showed no significant difference in fluorescence compared to untreated
336 controls, and this was consistent with microscopy observations that revealed the presence
337 of few ETs (**Figure 3**).

338

339 **3.2 Detection of conserved markers of ETs**

340

341 Immunofluorescence studies to analyse the composition of the chromatin structures
342 released from salmon PMNs confirmed that these were decorated with proteins
343 characteristic of ETs in other species (**Figure 4**). Neutrophil elastase, a major component
344 of the granules in neutrophils, was observed throughout the complex structure of the
345 extruded chromatin (**Figure 4**). The nuclear protein, histone H2A, co-localised with the
346 DNA (staining positive with DAPI) in the extracellular environment, confirming a
347 nuclear origin for the chromatin (**Figure 4**). We also observed co-localisation of histone
348 H1/DNA in the extracellular strands by using a specific antibody directed against this
349 immunogen (**Figure 4**).

350

351 **3.3 Induction of ET release from PMNs by *A. salmonicida***

352

353 Next, the ability of live bacteria to induce ET release from salmon PMNs was examined.
354 Microscopy observations indicated that PMNs extruded the characteristic web-like
355 structures after interaction with two isolates of *A. salmonicida* and, compared to untreated
356 controls, there was a significant increase in fluorescence in wells containing PMNs
357 exposed to *A. salmonicida* Hooke (233±22.5 a.u.) and *A. salmonicida* 30411 (332.7±46.5
358 a.u.) (**Figure 5**).

359

360 **3.4 Effect of ETs on bacteria viability**

361

362 After co-incubation of bacteria with cells that had been induced to release ETs, fewer
363 bacterial colonies of *A. salmonicida* Hooke were recovered at 3 h after inoculation (1.27
364 $\times 10^6$ CFU/ml), though this difference was not significant when accounting for multiple
365 comparisons (**Figure 6**). However, when the ETs had been digested away by the addition

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366 of recombinant DNase I, the abundance of bacterial colonies remained similar to the
367 inoculum (2.30×10^6 CFU/ml at 3 h compared with 2.52×10^6 CFU/ml at inoculation).
368

369 **3.5 ETs released by other cells of myeloid origin**

370

371 Cells in suspensions enriched for macrophages (68.7±4.9%) were round and had a
372 characteristic bean-shaped nucleus surrounded by a large volume of cytoplasm. After 1 h
373 stimulation with 5 µg/ml CaI, fluorescence from the wells increased significantly
374 (493.7±76.7 a.u.) compared to untreated controls (202.5±22.83 a.u.) (**Figure 7**).
375 Microscopy confirmed that the macrophages appeared to have released structures
376 resembling ETs that stained positively with Sytox Green, which occasionally led to the
377 formation of large clumps (**Figure 7**). Macrophage-enriched cell suspensions exposed to
378 10 nM PMA, 10 µg/ml LPS, or 10 µM DPI showed no significant change in fluorescence,
379 which is consistent with no increase in ET release and microscopy observations
380 confirmed this (**Figure 7**).

381

382 Regarding PBLs, the isolated cells in the enriched suspension were small and round with
383 a large round nucleus, common characteristics that make it difficult to differentiate
384 lymphoid and myeloid subsets. However, by using specific antibodies against surface
385 markers of trout thrombocytes and MACS, salmon thrombocytes were successfully
386 isolated (**Supplementary figure**). Exposure of these cells to 10 nM PMA or 5 µg/ml CaI
387 resulted in non-significant increases in fluorescence from the suspensions compared to
388 the untreated controls (**Supplementary figure**). Visualisation by fluorescence
389 microscopy showed mainly non-viable and lysed cells in untreated controls and cell
390 suspensions treated with CaI (i.e., intact nuclei), with just a few 'streaky' structures
391 observed in the cell suspensions incubated with PMA and further experiments are needed
392 to confirm whether these structures are ETs (**Supplementary figure**).

393

394 **4. DISCUSSION**

395

396 Innate immunity is the first line of the fish host defence against pathogens, with
397 neutrophil-like cells in particular playing a critical role in this response. These phagocytes
398 contribute to antimicrobial defences by eliminating microbes by phagocytosis,
399 undergoing oxidative burst and, in many vertebrates, through the release of chromatin by

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400 the mechanism of ETosis. This present study is the most comprehensive so far on the
401 phenomenon of ET release by immune cells of Atlantic salmon, an important farmed
402 species.
403
404 ETs consist of decondensed chromatin (DNA and histones) in close association with
405 cytoplasmic granules containing proteases and anti-microbial peptides (Brinkmann et al.,
406 2004; Fuchs et al., 2007), and these structural features are conserved in vertebrates and
407 invertebrates (Chuammitri et al., 2009; Homa, 2018; Robb et al., 2014; Zhang et al., 2016;
408 Zhao et al., 2017). Structures consistent with ETs were detected in enriched suspensions
409 of neutrophil-like cells prepared from healthy salmon pre-smolts. The extracellular DNA
410 scaffold of the ET was visualised by staining with nucleic acid-specific dye, Sytox Green,
411 while these structures could be digested completely within minutes after exposure to the
412 nuclease, DNase I. Immunofluorescently-labelled antibodies specific for histone H2A
413 and histone H1 in association with DNA confirmed the extracellular DNA to be chromatin
414 and thus of nuclear origin. Finally, IFAT also confirmed the presence of neutrophil
415 elastase within the ET structure, which is a key diagnostic marker, and confirmed the
416 extrusion of nuclear material together with cytoplasmic granules into the extracellular
417 environment (Brinkmann et al., 2004, 2012; Brinkmann & Zychlinsky, 2012; Fuchs et
418 al., 2007; Havixbeck & Barreda, 2015; Metzler et al., 2011; Okada, 2017;
419 Papayannopoulos & Zychlinsky, 2009; Wernersson et al., 2006).
420
421 The ETs released in highly enriched cell suspensions of Atlantic salmon neutrophil-like
422 cells formed spontaneously, which is consistent with observations on similar cell
423 suspensions isolated from rainbow trout (Van et al., 2020) and mammals (Fuchs et al.,
424 2007; Hoffmann et al., 2016; Jeffery et al., 2016; Maini et al., 2016; Pilsczek et al., 2010).
425 Spontaneous release of ETs in cultures *in vitro* may be due to the unfavourable nature of
426 these conditions, and fish immune cells are short-lived outside the host. Still, it was
427 possible to stimulate ET release from the Atlantic salmon neutrophils by exposure to CaI,
428 which induces the release of ROS, and this conforms with findings from other studies on
429 immune cells from fish (Palić et al., 2007a, 2007b; Van et al., 2020). In mammals, CaI
430 stimulates ET release via an NADPHox-independent cascade, by activating potassium
431 channels and inducing mitochondrial ROS production (Nobuhiro Douda et al., 2015;
432 Parker et al., 2012). Meanwhile, under the experimental conditions of this present study,
433 PMA (a stimulant of ET release in many species, particularly mammals) did not cause a

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434 significant increase in ET release from the Atlantic salmon neutrophils and it seems to be
435 only a weak stimulant in fish (Chi & Sun, 2016; Palić et al., 2007a, 2007b; Van et al.,
436 2020). Moreover, although purified LPS caused a significant increase in ET release from
437 Atlantic salmon neutrophils, this was to a far lesser extent than CaI. In contrast, purified
438 LPS failed to induce ET release in carp phagocytes (Pijanowski et al., 2015) and rainbow
439 trout PMNs (Van et al., 2020), whilst ET release was stimulated in tongue sole neutrophils
440 (Wen et al., 2018) and carp phagocytes when exposed to a less pure preparation of LPS
441 (Pijanowski et al., 2015). Lipopolysaccharides are sensed through toll-like receptor 4
442 (TLR-4) that activates the MyD88-dependent pathway and the transcription factor NF-
443 κB involved in ROS formation, which underlies the stimulatory role of LPS in ET release
444 in mammals (Khan et al., 2017). However, TLR-4 in teleosts has been described only in
445 catfish, zebrafish and other cyprinid species (Zhang et al., 2014), thus perhaps explaining
446 the at best modest effect of LPS on the induction of ET release from salmonid neutrophils.
447 The stimulatory effects of LPS on ET release may be due to impurities found in this
448 reagent (Pijanowski et al., 2015). DPI is used commonly to inhibit ET release because it
449 inhibits the required ROS generated by NADPH oxidase. However, Van et al. (2020)
450 found DPI to be an inducer of ET release from PMNs isolated from rainbow trout. This
451 present study found that DPI did not stimulate ET release from Atlantic salmon
452 neutrophils, thus the initial findings of Van et al. (2020) were not corroborated, though
453 this may be in part due to differences in exposure concentrations and incubation times.
454 Of note, Alarcon et al. (2021) found increasing ET release was not associated with a
455 significant increase in ROS in leukocyte preparations from salmon, while Yirong et al.
456 (2020) and Pijanowski et al. (2019) have reported ROS generation to be important in ET
457 release by carp neutrophils. Seemingly contrasting findings like these further underscores
458 our lack of understanding of the underlying pathways of ET release in fish and highlights
459 the need for further study to elucidate the mechanisms underlying ET release.
460
461 Exposure of Atlantic salmon neutrophils to live *A. salmonicida*, the Gram-negative
462 bacterium responsible for furunculosis, led to increased ET release. This is consistent
463 with observations on other fish hosts in response to bacteria, including tongue sole PMNs
464 responding to *Edwardsiella tarda*, *Vibrio harveyi* and *Pseudomonas fluorescens* (Zhao et
465 al., 2017), turbot neutrophils responding to *P. fluorescens* (Chi & Sun, 2016), and Atlantic
466 salmon PMNs responding to the intracellular bacterium *Piscirickettsia salmonis* (Alarcon
467 et al., 2021). Furthermore, recombinant flagellin from *Yersinia ruckeri* was a potent

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468 inducer of ET release from rainbow trout PMNs (Van et al., 2020). As such, bacterial
469 pathogens and antigens appear to be strong candidates as natural triggers of ET release
470 from fish neutrophils *in vivo*, similar to the case for mammals (Ashar et al., 2018; Jenne
471 et al., 2013; Urban et al., 2009) and invertebrates (Poirier et al., 2014; Robb et al., 2014;
472 Zhang et al., 2016).
473
474 Interestingly, cells derived from larger fish (i.e., older) were less likely to release ETs
475 spontaneously, as fluorescence values from spontaneous ET release in PMN suspensions
476 associated inversely with fish mass. This observation may underlie at least some of the
477 variation seen between individual fish (e.g., Van et al. 2020), and several studies of non-
478 fish species have associated ageing with reduced capacity to form ETs under certain
479 circumstances. In adult humans with chronic periodontal infection, neutrophils from older
480 adults are less likely to form ETs (Hazeldine et al., 2014), whilst Tseng et al. (2012)
481 reported ageing-associated impairment of ET release in mice both *in vitro* and *in vivo*,
482 with the latter associated with marked bacteraemia in a model of *Staphylococcus aureus*
483 infection (Tseng et al., 2012). In addition, differences in neutrophil yields and the
484 likelihood to release ETs spontaneously in the Atlantic salmon could be linked to the
485 considerable physiological changes associated with onset of smoltification in these
486 animals, which may occur at ca. 120 g and significantly impacts immunity (Johansen et
487 al., 2016). Further experiments with closely genetically-related fish cohorts maintained
488 under identical culture conditions would be warranted to confirm age-related effects on
489 ET release in fish.
490
491 One focus of the present study was to assess the interaction between bacteria and ETs
492 released by Atlantic salmon neutrophils. ETs act as part of the innate response to counter
493 bacterial invaders by trapping bacteria to prevent dissemination around the host and/or
494 exerting direct antimicrobial action through bringing trapped microbes into contact with
495 the decorating proteins that can exert such activities (Brinkmann et al., 2004). The close
496 interaction between ETs and bacteria was apparent by fluorescence microscopy. A
497 trapping assay was performed to test whether the ETs from Atlantic salmon neutrophils
498 trapped or inactivated *A. salmonicida*, where the bacteria were brought into close contact
499 with intact ETs or ETs that had been digested away with DNase and then the abundance
500 of CFU determined after 3 h incubation. Fewer CFU were recovered from cultures
501 containing intact ETs, which may indicate trapping of the bacteria within the mesh of the

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502 ET or inactivation of bacteria by ET components. The antimicrobial components of
503 digested ETs would still have been present in the control cultures but these may have
504 been unable to act against the bacteria due to being distributed throughout the medium
505 and not brought into close proximity with the bacteria for a sufficient time. Though the
506 reduction in CFU due to the ETs just failed to reach statistical significance, this
507 observation warrants further investigation and other studies have found ETs released by
508 fish to exert trapping and antimicrobial actions. Entrapment of bacteria and inhibition of
509 replication has been reported for turbot neutrophils incubated with *P. fluorescens* (Chi &
510 Sun, 2016), and carp neutrophils incubated with *Aeromonas hydrophila* (Brogden et al.,
511 2014).
512
513 Finally, the capabilities of other phagocytes to release ETs was examined in Atlantic
514 salmon, specifically for monocyte/macrophages and thrombocytes, as mammalian
515 eosinophils, basophils and macrophages have shown ET release previously (Boe et al.,
516 2015; Doster et al., 2018; Liu et al., 2014; Morshed et al., 2014; Pertiwi et al., 2019; Yang
517 et al., 2018; Yousefi et al., 2012, 2015), whilst carp macrophage preparations have also
518 been reported to contain ETs (Pijanowski et al., 2015). These other cell types share a
519 common myeloid progenitor with neutrophils and so the ability to release ETs may be
520 retained (Rombout et al., 2005). Consistent with Pijanowski et al (2015), the salmon
521 macrophage suspensions contained ET-like structures that stained positively with Sytox
522 Green after, in our case, stimulation with CaI. Though unlikely, given the purity of the
523 preparations, it cannot be excluded that the ET-like structures observed could have
524 derived from other cell types contaminating the suspension. Thrombocytes were also
525 examined for their ability to release ETs, as these cells are nucleated and phagocytic in
526 fish (Stosik et al., 2019), unlike their mammalian counterparts the platelets.
527 Thrombocytes are the most abundant blood cell type after the erythrocytes in most teleosts
528 (Rey Vázquez & Guerrero, 2007) and these cells contribute to the immune response of
529 lower vertebrates by performing phagocytosis and expressing immune-relevant genes and
530 enzymatic activities (Köllner et al., 2004; Shigdar et al., 2009). Indeed, thrombocytes
531 from cyprinid species and flounder play roles to counter bacteria (Nagasawa et al., 2014;
532 Stosik et al., 2002), whilst in mice platelets interact with neutrophils to cause ETs to be
533 released *in vivo* into the vasculature (Jenne et al., 2013). Salmon thrombocytes, isolated
534 by MACS expressed conserved membrane glycoprotein domains similar to those on
535 rainbow trout thrombocytes (Köllner et al., 2004), were incubated with PMA and CaI;

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536 however, there was little evidence for ET release besides some 'streaky' structures in
537 fluorescence microscope images from PMA-exposed cells.
538
539 Fluorescence assays were used to quantify ET release from cells throughout this present
540 study, but these assays may be influenced by dead or membrane-compromised cells in
541 the suspensions that may be dying by apoptosis or necrosis, as nuclei from these cells will
542 also stain positively with Sytox Green. Controlling for cells that die by these other
543 pathways is difficult and serves to emphasise the importance of including microscopy
544 observations to support findings relying on fluorescence data quantification of ETs. A
545 further limitation of the present study is that all experiments were performed *in vitro*, and
546 *in vivo* trials will be essential if the roles and functions of ETs in the fish innate response
547 are to be fully understood.
548
549 **5. CONCLUSION**
550
551 To conclude, this present study demonstrates that ETs are released by salmon phagocytes,
552 including neutrophils, thus providing a foundation for further study. The ET structures
553 and the modulators of their release are consistent with reports for immune cells isolated
554 from other species of fish, thereby supporting the evolutionarily conserved nature of this
555 defence response. A deeper understanding for the roles and functions of ETs within innate
556 immunity of fish hosts, and their interaction with microbial pathogens, may open new
557 avenues towards protecting cultured stocks against infectious diseases.
558
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570

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572

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575

576 **CONFLICTS OF INTEREST**

577

578 The authors confirm they have no known conflicts of interest.

579

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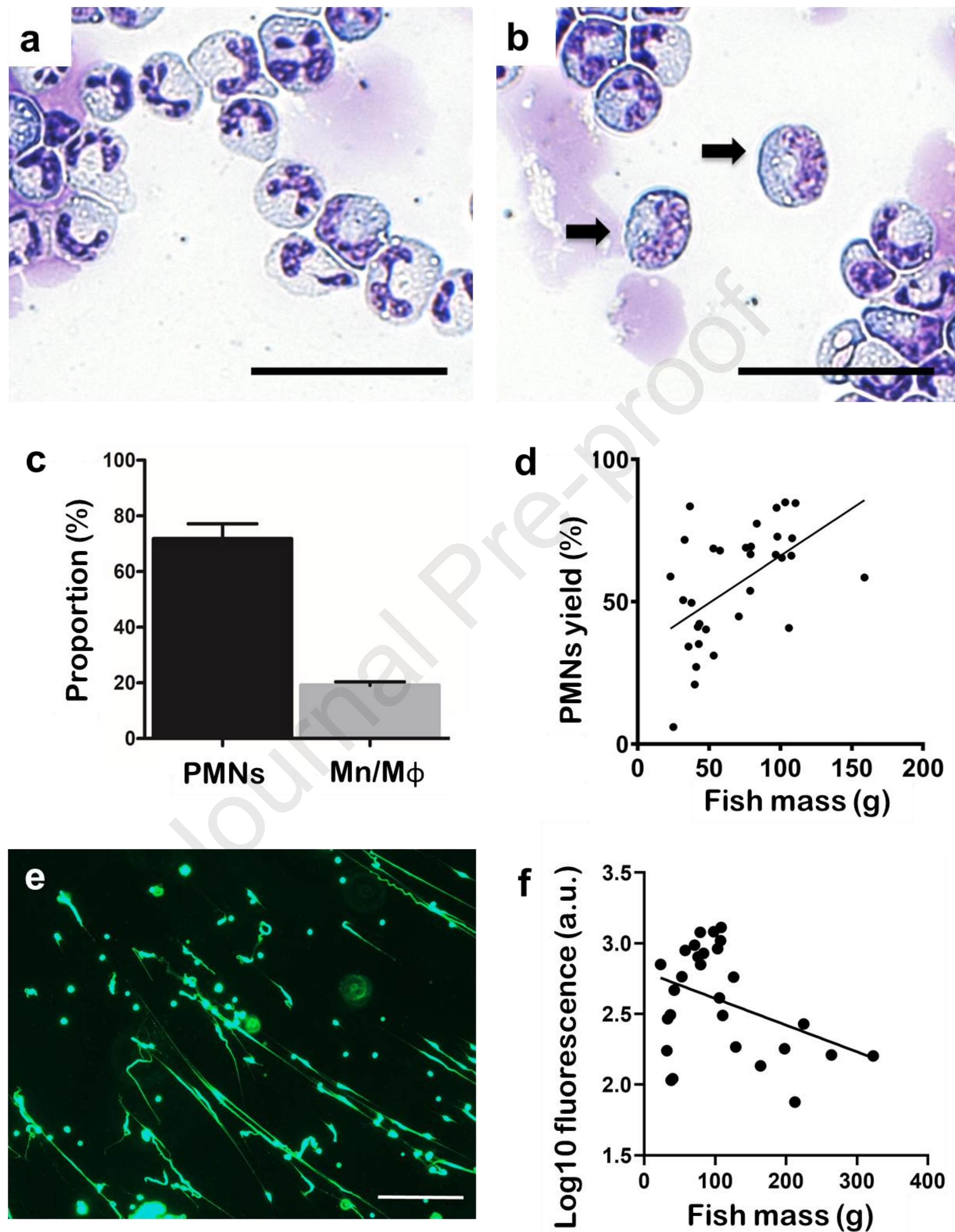
866 **FIGURES AND TABLES**

867

868 Figure 1



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869

870 **Fig. 1. Neutrophil-like cells isolated from Atlantic salmon head kidney**
871 **spontaneously released structures resembling extracellular traps**
872 **(ETs)**. The neutrophil-enriched cell fraction collected at the interface of the 1.060 and
873 1.072 g/ml Percoll layers was non-adherent in culture and characterised by

874 cytology. **a.** Representative cytological spin slide of the isolated cells stained with Rapi-
875 Diff II. Cells presented a characteristic eosinophilic polymorphonuclear morphology,
876 with the nucleus divided into several lobes, and granulocytic cytoplasm; scale bar, 50
877 µm. **b.** Magnification of an optical field showing mononuclear cells infiltrating the
878 cytology sample, with arrows indicating cells with mononuclear morphology; scale bar,
879 50 µm. **c.** Bar chart showing percentages of the different myeloid leukocyte subsets in the
880 isolated cell population (mean ± SEM, n = 6 fish). Polymorphonuclear cells (PMNs; i.e.,
881 neutrophils) were the predominant subset (>70%) in the isolated population, followed by
882 monocyte/macrophages (Mn/Mϕ). **d.** Correlation between the mass of each fish and yield
883 of PMNs (mean percentage in each cell preparation) obtained from individual fish (r_s =
884 0.4824, p (two-tailed) = 0.0052, n = 32). **e.** Fluorescence microscopy image of neutrophil-
885 enriched cell fraction stained with 5 µM Sytox Green, showing the spontaneous release
886 of ETs *in vitro*; scale bar, 100 µm. **f.** Association between the mass of each fish and the
887 spontaneous release of ETs as measured by fluorescence (log_{10} values used due to
888 heteroscedasticity and non-linear decay shape to curve); r = -0.3814, p (two-tailed) =
889 0.0452, n = 28.

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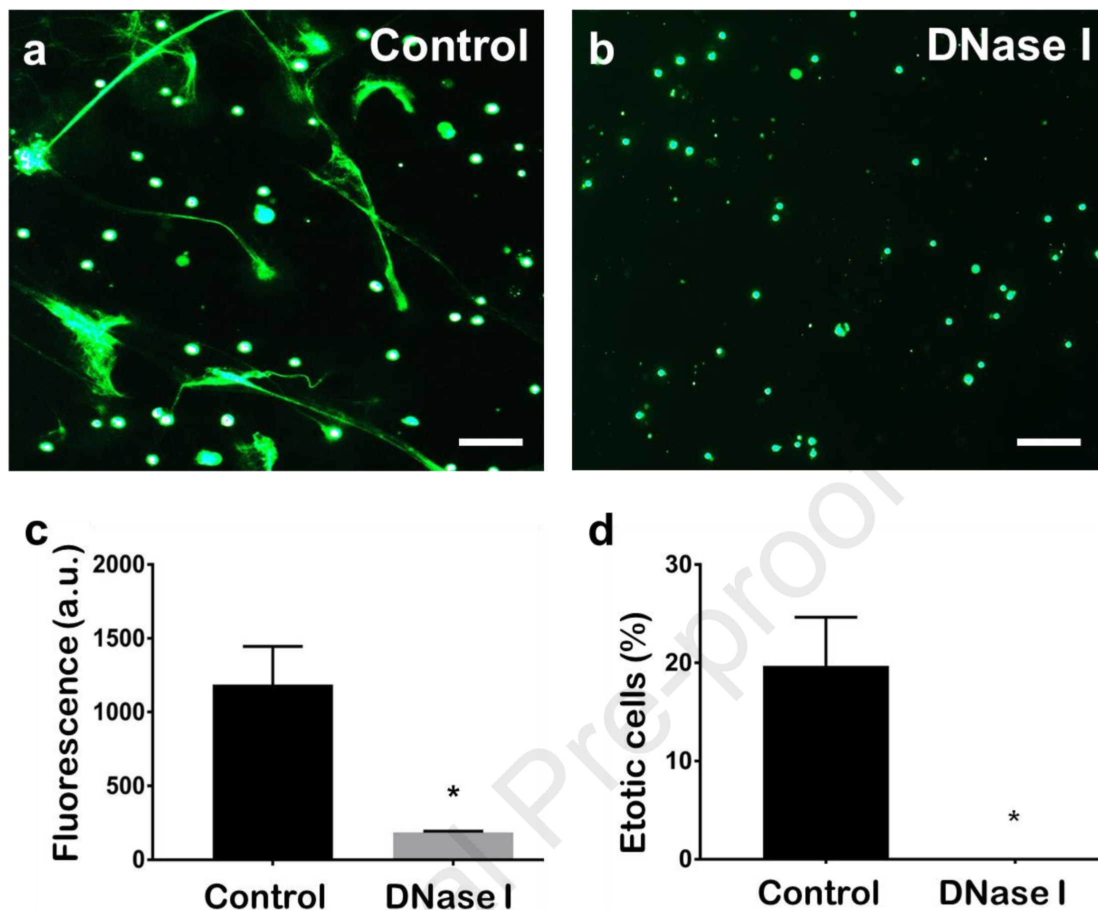
890 Figure 2



891

892 **Fig. 2. Nucleic acid structure of the extracellular trap (ET)-like**
893 **structures confirmed by enzymatic digestion with DNase I. a–b**. Fluorescence
894 microscopy images of neutrophil-enriched cell suspensions from Atlantic salmon
895 cultured *in vitro* and stained with 5 µM Sytox Green. **a**. After settling (30 min, 15ºC),
896 control cells were incubated with RPMI-1640 culture medium for 30 min; scale bar, 100
897 µm. **b**. The nucleic acid nature of the structures was confirmed by degradation with
898 medium containing 200 U/ml DNase I for 30 min; scale bar, 100 µm. **c**. Bar chart of
899 fluorescence (mean ± SEM) of neutrophil-enriched cell suspensions incubated with
900 culture medium lacking or supplemented with 200 U/ml DNase I for 30 min and stained
901 with 5 µM Sytox Green; * indicates a significant difference from the untreated control (t
902 = 3.86046, p = 0.0048, n = 5). **d**. Bar chart showing the percentage of
903 ETotic cells (mean ± SEM) in the neutrophil-enriched cell suspension following
904 incubation with culture medium lacking or supplemented with 200 U/ml DNase I for 30
905 min; * indicates a significant difference from the untreated control (t = 3.8871, p =
906 0.0177, n = 3; percentage data were arcsine transformed before statistical testing).

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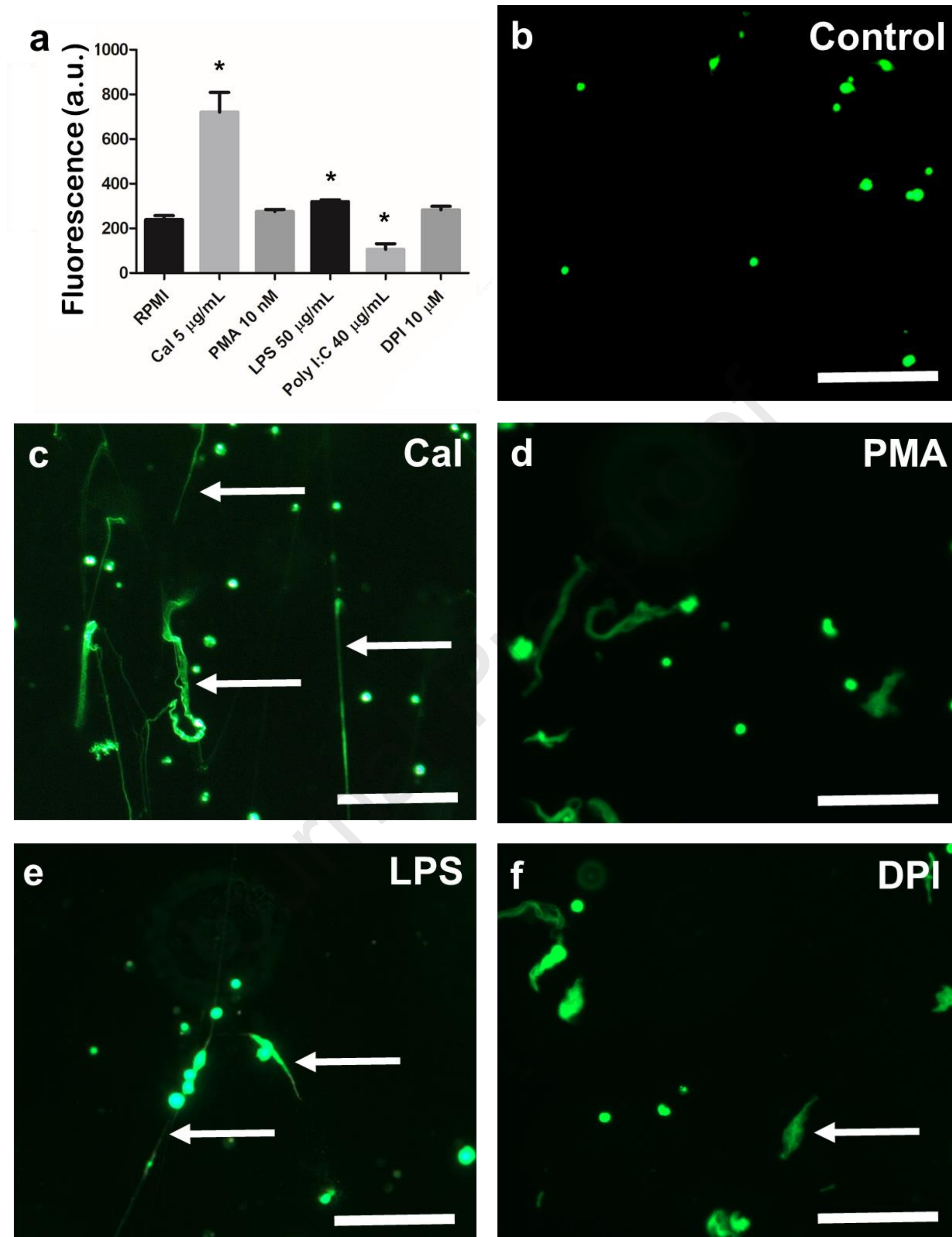
907 Figure 3



908

909 **Fig. 3. Effects of previously characterised stimulants on extracellular traps (ETs)**
910 **released from neutrophil-enriched cell suspensions from Atlantic salmon. a.** Bar
911 chart showing fluorescence (mean ± SEM) of neutrophil-enriched cell suspensions after
912 incubation (1 h, 15°C) with various proposed inducers and inhibitors of ETosis and
913 stained with 5 μM Sytox Green showing cultures exposed to calcium ionophore (CaI) and
914 LPS had significantly greater fluorescence compared to the untreated control (indicated

915 by *; CaI: t = -5.3387, p = 0.0007, n = 5; lipopolysaccharide [LPS]: t = -3.9831, p =
916 0.0040, n = 5), which was not the case for phorbol 12-myristate 13-acetate (PMA) and
917 diphenyleneiodonium chloride (DPI) (PMA: t = -1.7787, p = 0.1132, n = 5; DPI: t = -
918 1.8394, p = 0.1031, n = 5), whilst exposure to polyinosinic–polycytidylic acid sodium
919 salt (Poly I:C) yielded a significant reduction in fluorescence (t = 4.2372, p = 0.0028, n =
920 5). **b–f**. Fluorescence microscopy images of neutrophil-enriched cell suspensions
921 incubated with different compounds (1 h, 15ºC); scale bars, 100 µm. **b.** Untreated
922 controls. **c.** Incubation with µg/ml CaI. **d.** Incubation with 10 nM PMA. **e.** Incubation
923 with 50 µg/ml LPS. **f.** Incubation with 10 µM DPI. Note that the Poly I:C treatment image
924 resembled closely the untreated controls (not shown).

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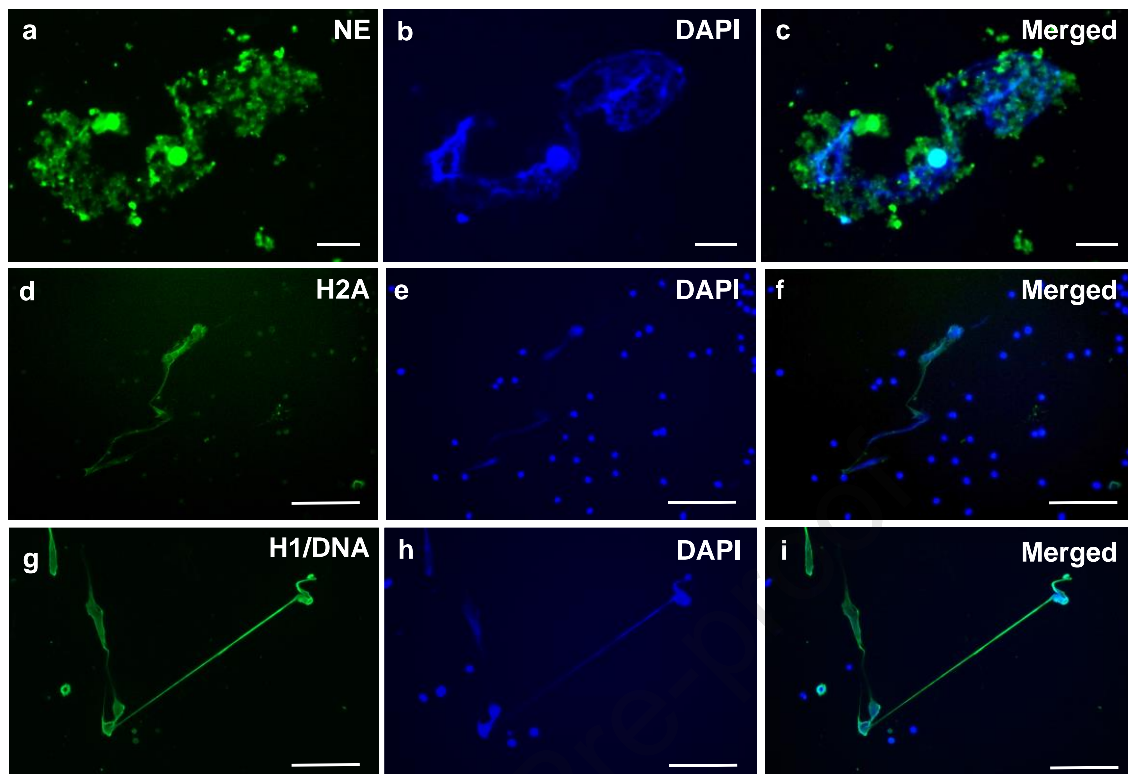
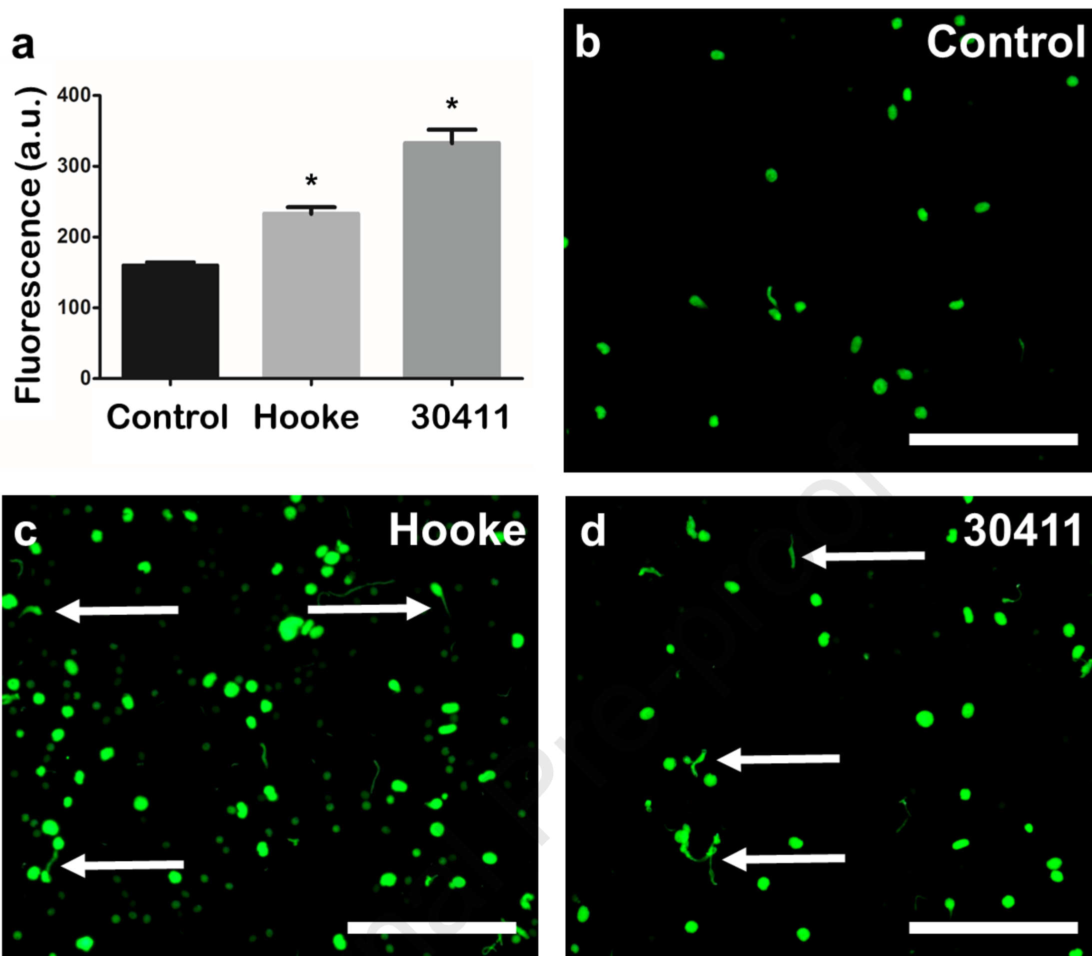
925 Figure 4



926

927 **Fig. 4. Confirmation of decoration of the extracellular trap (ET)-like structures with**
928 **characteristic protein signatures by immunostaining**. Fluorescence microscopy
929 images of neutrophil-enriched cell suspensions from Atlantic salmon cultured *in vitro*
930 with 5 µg/ml CaI (**a**–**f**) or 10 nM PMA (**g**–**h**) for 1 h at 15°C. **a–c.** Immunocytochemical
931 detection of neutrophil elastase in ETotic neutrophils; scale bars, 50 µm. **a.** Localisation
932 of neutrophil elastase by rabbit to human neutrophil elastase and stained with conjugated
933 Alexa-488 (green). **b.** DNA stained blue with 4′,6-diamidino-2-phenylindole (DAPI). **c**.
934 Merge of a–b. **d–f.** Immunocytochemical detection of histone H2A in different ETotic
935 neutrophils; scale bars = 100 µm. **d.** Localisation of histone H2A by mouse to human
936 histone H2A and stained with conjugated Alexa-488. **e.** DNA stained with DAPI. **f.** Merge
937 of d–e. **g–h.** Immunocytochemical detection of histone H1 with DNA in an extended
938 extracellular strand interlinking two cells; scale bars, 100 µm. **g.** Localisation of H1 with
939 DNA by mouse to human histone H1/DNA and stained with conjugated Alexa-488. **h.**
940 DNA stained with DAPI. **i.** Merge of g–h.

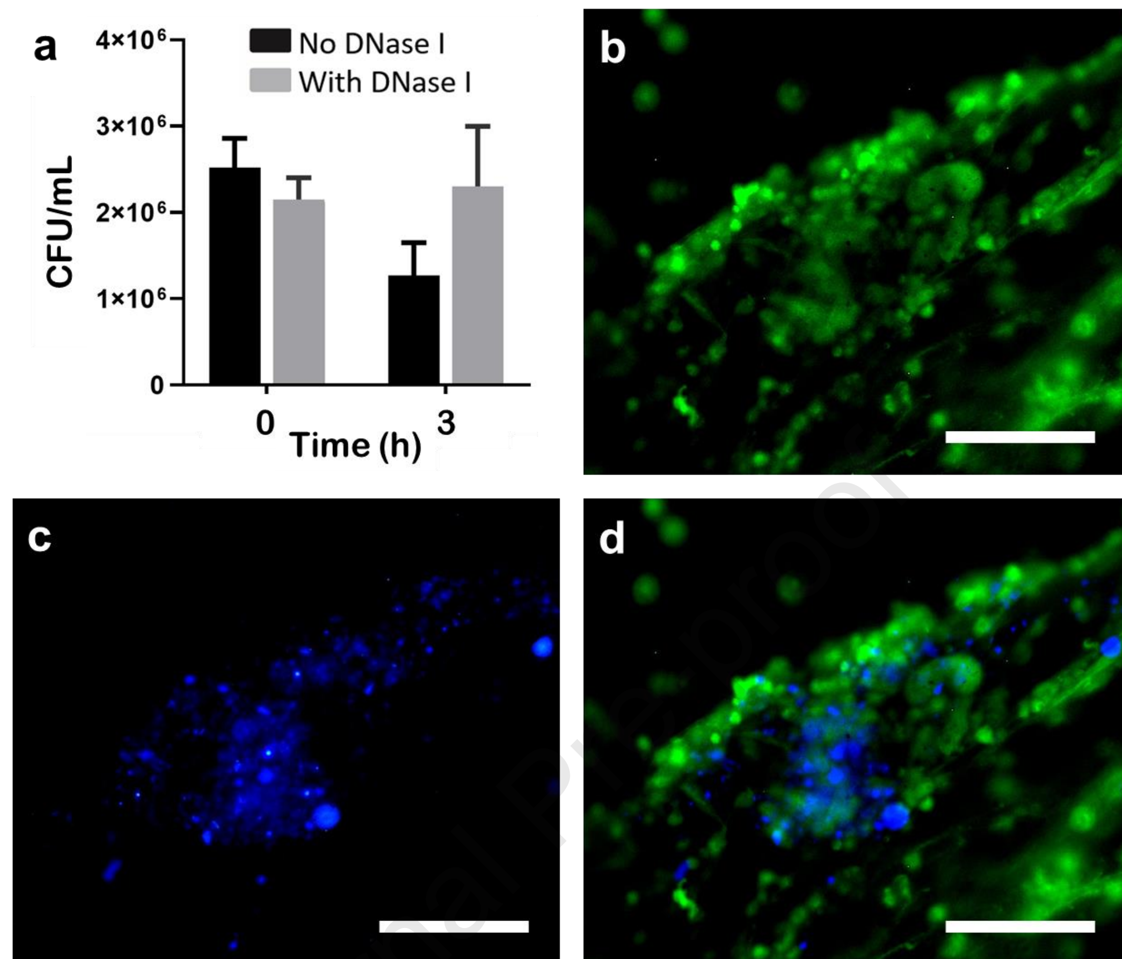
941 Figure 5



942

943 **Fig. 5. *Aeromonas salmonicida* induced extracellular trap release in neutrophil-**
944 **enriched cell suspensions from Atlantic salmon**. After settling (30 min, 15ºC),
945 neutrophil-enriched cell suspensions were incubated with *A. salmonicida* 30411 or Hooke
946 at a multiplicity of infection of ca. 100 colony-forming units for 2 h at 22ºC. **a.** Bar chart
947 showing fluorescence (mean ± SEM) of neutrophil-enriched cell suspensions incubated
948 with bacteria and stained with 5 µM Sytox Green showing cultures exposed to *A.*
949 *salmonicida* Hooke and 30411 had significantly greater fluorescence compared to the
950 untreated control (indicated by *; Hooke: $t = 7.1644$, $p = 0.0000$, $n = 6$; 30411: $t = 8.8521$,
951 $p = 0.0000$, $n = 6$). **b–d.** Fluorescence microscopy images of neutrophil-enriched cell
952 suspensions after staining with 5 µM Sytox Green; scale bars, 100 µm. **d.** Untreated
953 control neutrophil-enriched cell suspensions contained few ETs. **c.** Extracellular
954 chromatin was extruded by neutrophils after incubation with *A. salmonicida* Hooke. **d.**
955 Extracellular chromatin was extruded by neutrophils after incubation with *A. salmonicida*
956 30411.

957 Figure 6



958

959 **Fig. 6. Interaction between bacteria and extracellular traps (ETs).** Neutrophil-
960 enriched cell suspensions from Atlantic salmon were incubated with 5 µg/ml calcium
961 ionophore (1 h, 15ºC) to induce ET release and then *Aeromonas salmonicida* Hooke was
962 added at a multiplicity of infection of ca. 100 colony-forming units (CFU). **a**. Bar chart
963 showing CFU (mean ± SEM) recovered from the wells containing ETs (No DNase I) or
964 that had been digested away with 200 U/ml DNase I for 30 min (With DNase I) showing
965 there was no significant change in CFU/ml for either treatment at 3 h when accounting
966 for multiple comparisons (No DNase I: t = 2.6311, p = 0. 0301, n = 5; With DNase I: t =
967 -0.2414, p = 0.8153, n = 5). **b–d.** Neutrophil-enriched cell suspensions were incubated
968 with *A. salmonicida* Hooke for 2 h and then their interaction was visualised by
969 fluorescence microscopy; scale bars, 100 µm. **b**. DNA was stained with 5 µM Sytox
970 Green. **c**. *A. salmonicida* Hooke was stained with 300 nM 4′,6-diamidino-2-phenylindole
971 (DAPI). **d**. Merge of b–c.

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973 Figure 7

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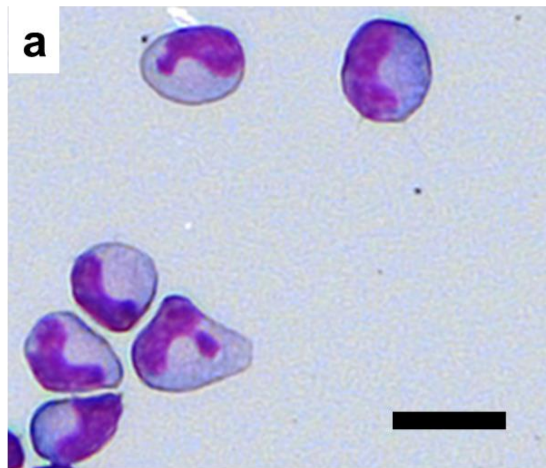

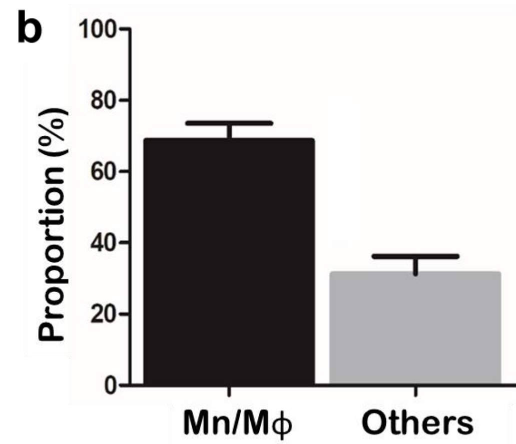

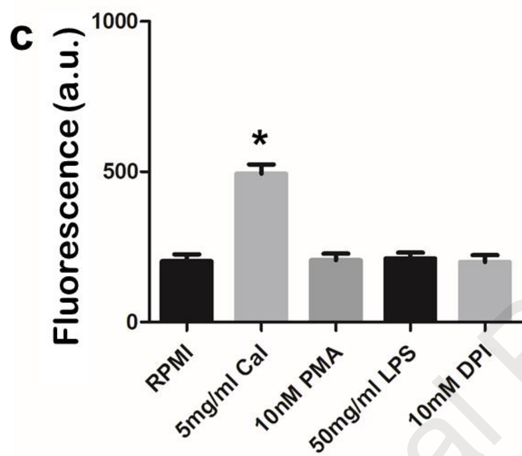

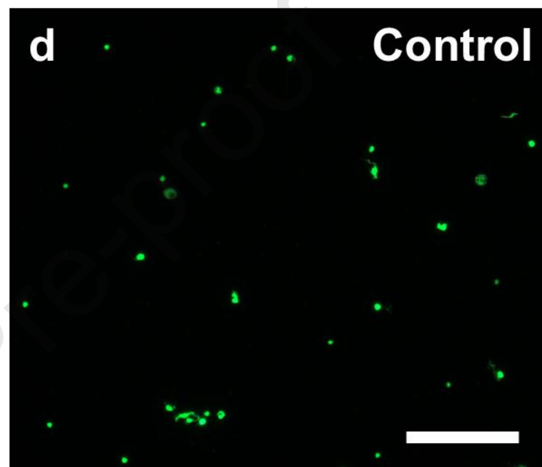

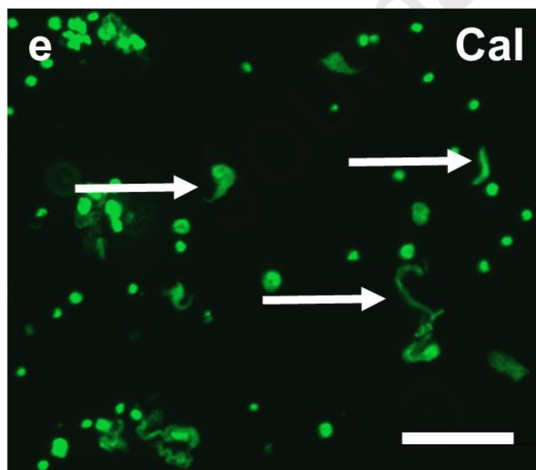

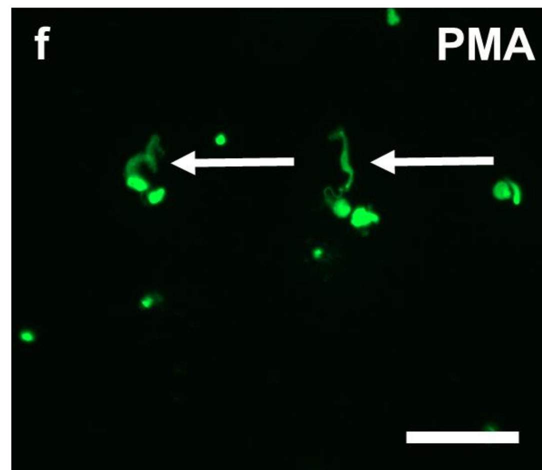

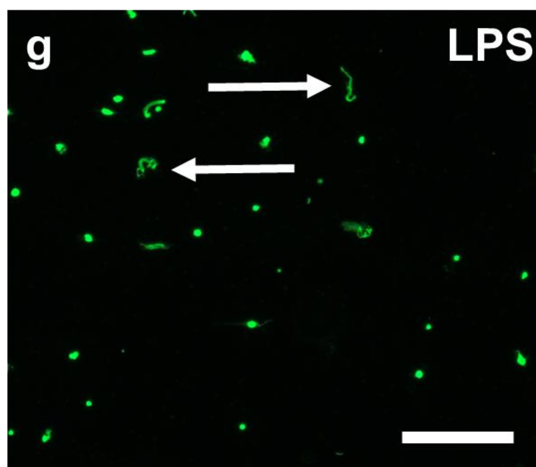

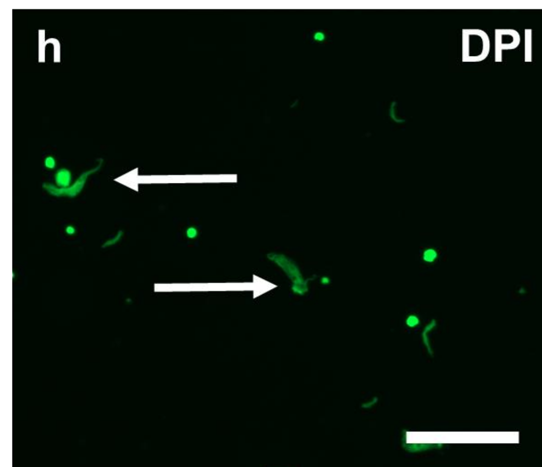

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975 **Fig. 7. Effects of previously characterised stimulants on extracellular traps (ETs)**
976 **released from macrophage-enriched cell suspensions from Atlantic salmon. a.** Light
977 microscopy image of cytospin slide of isolated mononuclear cells stained with Rapi-Diff
978 II; scale bar, 20 µm. **b.** Bar chart showing the percentage of monocyte/macrophages
979 (Mn/Mϕ) in the isolated cell population (mean ± SEM, n = 9). **c.** Bar chart showing
980 fluorescence (mean ± SEM) of macrophage-enriched cell suspensions after incubation (1
981 h, 15ºC) with various proposed inducers and inhibitors of ETosis and stained with 5 µM
982 Sytox Green showing cultures exposed to calcium ionophore (CaI) had significantly
983 greater fluorescence compared to the untreated control (indicated by *; CaI: $t = -7.0314$,
984 $p = 0.0001$, n = 5), which was not the case for the other compounds (phorbol 12-myristate
985 13-acetate [PMA]: $t = -0.1033$, $p = 0.9202$, n = 5; lipopolysaccharide [LPS]: $t = -0.1841$,
986 $p = 0.8585$, n = 5; diphenyleneiodonium chloride [DPI]: $t = 0.0819$, $p = 0.9367$, n = 5).
987 **d–h.** Fluorescence microscopy images of macrophage-enriched cell suspensions
988 incubated with different compounds (1 h, 15ºC); scale bars, 100 µm. **d.** Untreated controls
989 spontaneously released a low abundance of ETs. **e.** Incubation with 5 µg/ml CaI. **f.**
990 Incubation with 10 nM PMA. **g.** Incubation with 50 µg/ml LPS. **h.** Incubation with 10
991 µM DPI.

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992 **Supplementary figure**

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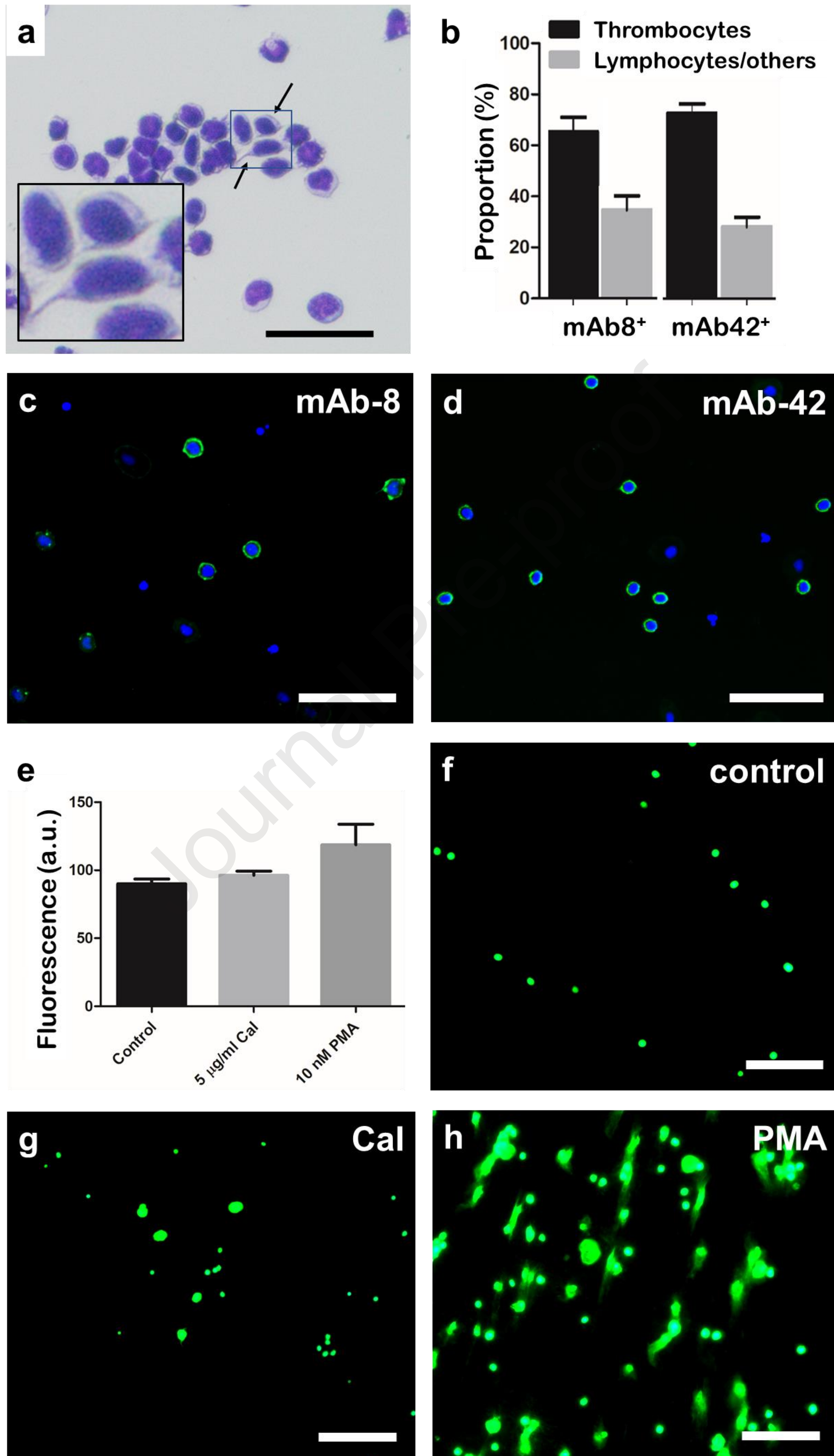

993

994

995 **Supplementary figure. Effects of previously characterised stimulants on**
996 **extracellular traps (ETs) released from thrombocyte-enriched cell suspensions from**
997 **Atlantic salmon.** Peripheral blood leukocytes were sorted to positively select for
998 thrombocytes and incubated with proposed inducers of ETosis (1 h, 15ºC). **a.** Cytological
999 spin slide of the isolated cells collected as described in Section 2.1 and stained with Rapi-
1000 Diff II. The cells presented a basophilic mononuclear morphology, with a round central
1001 nucleus or the characteristic spindle shape nucleus (arrows); scale bar, 50 µm. **b.** Before
1002 immunomagnetic sorting, 65% of cells in the suspension stained positively with mAb-8,
1003 whilst 72% stained positively with mAb-42. **c.** Microscopic image of thrombocytes
1004 stained with mAb-8 (Köllner et al., 2004) and conjugated Alexa-488, with nuclei
1005 counterstained with 4′,6-diamidino-2-phenylindole (DAPI). **d.** Microscopic image of
1006 thrombocyte membrane glycoproteins stained with mAb-42 and conjugated Alexa-488,
1007 with DAPI-counterstained nuclei. **c–d** Scale bars, 50 µm. **e.** Bar chart showing
1008 fluorescence (mean ± SEM; n = 3) of salmon thrombocyte cell suspensions (enriched by
1009 magnetic-activated cell sorting) incubated with 5 µg/ml calcium ionophore (CaI) or 10
1010 nM phorbol 12-myristate 13-acetate (PMA). There were no significant differences
1011 between the CaI or PMA treatments and the untreated controls (CaI: t = -1.3571, p = 0.
1012 2463, n = 3; PMA: t = -1.8360, p = 0. 1402, n = 3). **f–h.** Fluorescence microscopy images
1013 of thrombocyte-enriched cell suspensions incubated with proposed inducers of ETosis
1014 and stained with 5 µM Sytox Green; scale bars, 100 µm. **f.** Untreated controls. **g.** Cells
1015 incubated with 5 µg/ml CaI. **h.** Cells incubated with 10 nM PMA.

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