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Chromatin extracellular trap release in rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792)

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ABSTRACT

Neutrophils release nuclear chromatin decorated with antimicrobial proteins into the extracellular milieu as an innate immune defence mechanism to counter invading microbes. These chromatin structures, called extracellular traps (ETs) and released by a process called NETosis, have been detected in mammals, certain invertebrates and some fish species, including fathead minnow, zebrafish, common carp, turbot, sole and barramundi. However, there have been no previous studies of ETs in the Salmonidae. ETs are released in response to chemical and biological stimuli, but observations from different fish species are inconsistent, particularly regarding the potency of various inducers and inhibitors. Thus, this present study aimed to describe ET release in a salmonid (rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792)) and uncover the inducers and inhibitors that can control this response. Highly enriched suspensions of polymorphonuclear cells (PMNs; mainly neutrophils) were prepared from head kidney tissues by a triple-layer Percoll gradient technique. ET structures were visualised in PMN-enriched suspensions through staining of the chromatin with nucleic acid-specific dyes and immunocytochemical probing of characteristic proteins expected to decorate the structure. ET release was quantified after incubation with inducers and inhibitors known to affect this response in other organisms. Structures resembling ETs stained positively with SYTOX Green (a stain specific for nucleic acid) while immunocytochemistry was used to detect neutrophil elastase, myeloperoxidase and H2A histone in the structures, which are diagnostic proteinaceous markers of ETs. Consistent with other studies on mammals and some fish species, calcium ionophore and flagellin were potent inducers of ETs, while cytochalasin D inhibited NETosis. Phorbol 12-myristate 13-acetate (PMA), used commonly to induce ETs, exerted only weak stimulatory activity, while heat-killed bacteria and lipopolysaccharide did not induce ET release. Unexpectedly, the ET-inhibitor diphenyleneiodonium chloride acted as an inducer of ET release, an observation not reported elsewhere. Taken together, these data confirm for the first time that ETs are released by salmonid PMNs and compounds useful for manipulating NETosis were identified, thus providing a platform for further studies to explore the role of this mechanism in fish immunity. This new knowledge provides a foundation for translation to farm settings, since manipulation of the innate immune response offers a potential alternative to the use of antibiotics to mitigate against microbial infections, particularly for pathogens where protection by vaccination has yet to be realised.

1. Introduction

Polymorphonuclear cells (PMNs), such as neutrophils, basophils and eosinophils (known collectively as granulocytes), function in innate immunity to protect fish from microbial threats. Like mammalian neutrophils, fish counterparts recognise pathogen-associated molecular patterns (PAMPs) on microorganisms and respond by initiating processes to inactivate the threat [1–3]. PMNs, particularly neutrophils, may internalise microbes into the phagosome by phagocytosis, where reactive oxygen species (ROS), produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, act to kill the microbes [4]. Alternatively, basophils and eosinophils in particular, may undergo degranulation to release compounds toxic to surrounding microbes, including proteins that compromise microbial membranes. Similarly, neutrophils contain high concentrations of antimicrobial proteins including neutrophil elastase (NE), myeloperoxidase (MPO), cathepsin G, proteinase 3, defensins and lysozyme [5]. Neutrophils may also release structures called extracellular traps (ETs) composed of decondensed nuclear chromatin (DNA and histones) in association with the granule proteins, NE and MPO [6,7]. ETs act to trap pathogens to prevent

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dissemination around the host and provide sufficient delay for the recruitment of immune cells to combat the microbial invaders [8–10]. ETs may exert direct bactericidal activity due to certain proteins that decorate the structure, such as NE and MPO, as well as histones and histone fragments [11–18], and ETs can also function against parasitic protozoa and helminth larvae [19].

In fish, ET-like structures were first described in PMN-enriched cell suspensions isolated from the head kidney of fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*) [20,21]. Thereafter, ET-like structures have been reported for the common carp (*Cyprinus carpio*) [22] and flatfish such as turbot (*Scophthalmus maximus*) [9] and sole (*Cynoglossus semilaevis*) [10]. However, ET release has not been characterised in salmonids, a commercially important family of fish responsible for 18% of the total value of internationally traded fish products [23]. Salmonids, such as rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*), are farmed intensively in Canada, Chile, Iran, Norway, Turkey and the United Kingdom [23–25].

The signalling cascades and compounds triggering ET release have been studied mainly in human neutrophils. In fish, the literature describes little consistency between the potency of inducers and inhibitors for ET release across different species, perhaps indicating a diversity of responses between fish groups and/or reflecting the lack of understanding of the signals mediating this process [20,21]. In general, two pathways are implicated in initiating ET release, with both causing intracellular ROS generation that acts as a primary signal for the cell to undergo NETosis, a cell death outcome distinct from apoptosis and necrosis [7,26]. The first pathway initiates ET release after activation of protein kinase C (PKC) leads to phosphorylation of NADPH oxidase (p47phox), which generates the intracellular ROS required for respiratory burst [27–30]. Bacteria [29,31], bacterial lipopolysaccharide (LPS) [32,33], flagellin [34,35], parasites [19,36] and fungal β-glucan [37,38] induce ET release through this NADPH oxidase pathway in mammalian neutrophils, while the PKC activator, phorbol 12-myristate 13-acetate (PMA), is used commonly to induce ET release in experiments involving many species [7,30,35,39,40]. Other biological inducers of ET release operating via this pathway include host-derived cytokines and inflammatory mediators, such as IL-1β, IL-8 and TNFα [41–43], and the synthetic analogue of double-stranded RNA, poly I:C [44]. In fish, exposure of tongue sole PMN-enriched cell suspensions to bacteria leads to an increase in ET release [10], while PMA is a potent inducer of ET release in common carp [44] and tongue sole [10], although this compound induces ET release only weakly in fathead minnow and zebrafish [21]. Diphenyleneiodonium chloride (DPI) is an inhibitor of NADPH oxidase that reduces ROS generation, which is required for PMA or PAMP-induced ET release [7,29,30,45]. Exposure of tongue sole PMN-enriched cell suspensions to NADPH oxidase inhibitors results in reduced ET release induced by PMA and bacterial suspensions [10]. Furthermore, DPI inhibits PMA-induced ET release in common carp [44], indicating that PMA or bacteria can stimulate ET release through an NADPH oxidase-dependent system.

In the second ET release initiation pathway, intracellular calcium influx induces mitochondrial-generated ROS through activation of the calcium-activated potassium channel of small conductance (SK channel) [39], and this rapid influx of Ca^{2+} into the mitochondrial matrix generates ROS independently of NADPH oxidase [39,46]. Calcium ionophore A23187 (CaI) is a potent inducer of mitochondrial-generated ROS, as it binds extracellular Ca^{2+} to form stable divalent cations that allows them to cross cell membranes and open the SK channels at the mitochondria [47]. In zebrafish and fathead minnow, CaI is a more potent inducer of ET release than PMA, suggesting this NADPH oxidase-independent mechanism for ET release is more important in these species, though the use of CaI to induce ET release has not been reported for most other fish species [20,21].

Following ROS generation by either pathway, NE localises to the nucleus to degrade the linker histones leading to decondensation of the chromatin, a process assisted by MPO, another protein that migrates to the nucleus [48]. Fish PMNs also require ROS signalling and chromatin processing by MPO to release ETs because NETosis in tongue sole PMNs is inhibited by 4-aminobenzoic acid hydrazide, an MPO inhibitor [10]. Initiation of ET release is observed through the loss of the lobed morphology of the nucleus, the breakdown of the nuclear membrane, followed by the release of chromatin into the extracellular milieu [6,7]. Inhibitors of cytoskeleton polymerisation, such as the mycotoxin cytochalasin D [6,45,49–51], can inhibit ET release because actin-mediated remodelling of the cytoskeleton is important during positioning of the nucleus close to the plasma membrane prior to the breakdown of the nuclear envelope and cell rupture [50].

Deeper understanding of innate immunity in fish, including ET release, offers opportunities to manipulate and improve these responses, thus providing additional means to protect fish stocks against infections in aquaculture. Such new approaches could complement existing mitigation strategies, such as high-welfare husbandry and stock management practices and vaccination programmes, and this may be particularly important where vaccine development for pathogens has proven especially challenging. In many cases, antibiotics and anti-parasitic agents are the only treatment option when a disease outbreak occurs; however, the development of resistance to these drugs is of growing global concern [52,53]. New therapies that enhance the innate immune response offer an alternative to complement the existing range of disease mitigation measures in aquaculture.

The aim of this present study was to describe ET release in rainbow trout and to identify chemical and biological inducers and inhibitors that can affect this process, thus improving knowledge of the mechanisms mediating this response in salmonids.

2. Materials and methods

2.1. Reagents and bacteria

The following reagents were purchased from Sigma Aldrich Ltd (Gillingham, UK): benzocaine, 10 × Hank's balanced salt solution (HBSS) without calcium chloride or magnesium sulphate and sodium bicarbonate, heparin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Leibovitz's L-15 media (with L-glutamine and phenol red, but without sodium bicarbonate), penicillin-streptomycin antibiotic (Pen-Strep; per mL: 10,000 U penicillin and 10 mg streptomycin), Percoll at pH 8.5–9.5 (20 °C), and phorbol 12-myristate 13-acetate (PMA). The following reagents were purchased from Thermo Fisher Scientific (Loughborough, UK): calcium ionophore A23187 (CaI), 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) dilactate, heat-inactivated foetal bovine/calf serum (FCS), Hoechst 33342 trihydrochloride trihydrate, Roswell Park Memorial Institute-1640 medium (RPMI; supplemented where stated with FCS and Pen-Strep), and SYTOX Green. Phosphate buffered saline (PBS, pH 7.4; consisting 8 g NaCl, 0.2 g KCl, 1.78 g $Na_2HPO_4.H_2O$ and 0.24 g KH_2PO_4 per litre) was sterilised by autoclaving at 121 °C, 15 PSI for 20 min.

Vibrio anguillarum ATCC 43310 and NCIMB 1873 (a key Gram-negative pathogen affecting many fish species) were revived from cryopreserved glycerol stocks at −80 °C on tryptone soy agar supplemented with 1.5% NaCl (TSA; Thermo Fisher Scientific). Single colonies were inoculated into universal bottles containing 5 mL NaCl-supplemented tryptone soy broth (TSB; Thermo Fisher Scientific) and cultured for 16 h at 22 °C (150 rpm) to late-exponential phase. Then, the bacteria were pelleted by centrifugation (2600 × *g*, 15 min, 4 °C), the supernatant was discarded and the bacteria resuspended in 5 mL PBS by vortex. This wash step was performed three times, following which the absorbance of the bacterial suspension was measured with a spectrophotometer (CE 2041; Cecil Instruments Ltd, Cambridge, UK) at 600 nm (A_{600}) using PBS as the blank. Bacterial suspensions were diluted with PBS to 10^7 colony-forming units (CFU) mL^{-1}, and then serially diluted in quadruplicate and plated across TSA for incubation (24 h, 22 °C) to confirm CFU mL^{-1}. The bacteria suspension was heat-inactivated in a

glass universal tube in a water bath for 25 min at 60 °C and then diluted for experiments accordingly. Heat inactivation was confirmed by the absence of colonies forming on TSA after incubation for 48 h at 22 °C.

2.2. Source of fish and husbandry

Apparently healthy (determined by gross examination) triploid rainbow trout (*O. mykiss*) from a local fish farm were transported to the Institute of Aquaculture (IoA) immediately after being euthanised by a Schedule 1 technique (overdose of benzocaine and destruction of the brain) as described in the Animals (Scientific Procedures) Act 1986, or transported live for housing in the flow-through cold-water aquarium at the IoA for subsequent use. The fish farm operates a flow-through system (variable flow rate) with water intake from a nearby reservoir and the fish were exposed to constant light. Oxygen concentration in the water is detected by an automated probe and maintained at ≥6.5 mg/L. Stocking density is maintained at < 60 kg m^{-3}. Fish (each weighing 50–500 g; mean = 95 g) were collected for experiments between February 2016 to March 2016 and February 2017 to April 2017 when mean water temperature at the farm was 5.5 °C. At the IoA, water temperature was recorded from tanks on days when fish were sampled, and mean water temperature during the year was 9.8 °C (5.7–12.8 °C). Fish were fed by hand two to three times daily with a commercial feed.

2.3. Separation and enrichment of PMNs

Each euthanised fish was bled through a caudal venepuncture using a 2.5-mL syringe and 25G × 5/8 needle (Terumo, Surrey, UK), before the entire head kidney (HK) was removed by dissection using disinfected scalpel and tweezers in a Class II flow hood (BS5726; Gelaire, Sydney, Australia). The HK was transferred into a 100-μm mesh cell strainer (Falcon, Fisher Scientific, Loughborough, UK) in a 6-cm Petri dish containing 5 mL RPMI supplemented with 1% FCS and 0.01 U mL^{-1} heparin to prevent clotting, and the tissue was broken up by gently passing through the mesh with the plunger of a 5-mL syringe (Terumo, Tokyo, Japan). The resultant cell suspension was pipetted onto a discontinuous triple-layer Percoll gradient consisting of 5-mL layers at 1.060, 1.072, and 1.084 g mL^{-1} in a 50-mL centrifuge tube [54] and then centrifuged (400 × *g*, 35 min, 4 °C) to separate the PMNs from other leukocytes. A band enriched for PMNs forms at the interface of the 1.060 and 1.072 g mL^{-1} Percoll layers, and these cells were collected with a sterile Pasteur pipette and transferred to a 50-mL centrifuge tube. The PMNs were washed once by adding 20 mL of ice-cooled RPMI, centrifuging (800 × *g*, 7 min, 4 °C), and then resuspending the cell pellet in 2 mL RPMI + 1% FCS + Pen-Strep. The cells in the PMN-enriched suspension were counted with the aid of a light microscope and a Neubauer haemocytometer, and adjusted to ca. 4×10^5 cells mL^{-1} with RPMI.

2.4. Determination of cell types

Cytospin slides were prepared to observe and determine the different cell types in each PMN-enriched suspension. Briefly, a glass microscope slide was loaded into the Cytospin chamber (GMI Inc, Ramsey, MN, USA) and then 100 μL FCS and 100 μL cell suspension was added to the collection bucket. The chamber was centrifuged (1,000 rpm, 5 min, room temperature) and then slides air dried for 15 min at room temperature. The cells on the slides were stained with a commercial Romanowsky staining kit (TCS Biosciences, Buckingham, UK), rinsed gently with tap water, dried for 1 h at room temperature, and then observed by light microscopy at 20× magnification or greater (Olympus CH-2, or BX-51; Olympus, Southend-on-Sea, UK). The BX-51 microscope had an Axiocam MRC camera (Zeiss, Cambridge, UK) attached for imaging, and images were collected with the Axiovision software (v.4.8; Zeiss, Cambridge, UK). Mean relative abundances of PMNs and monocyte/macrophage (Mo/M) cells in suspensions were determined as the proportions of total cells observed in at least three randomly-selected fields of view from images acquired of Cytospin slides (one slide per fish). PMNs were identified as cells having a lobed, dark purple-stained nucleus; a pink-blue cytoplasm containing a few purple granules; and a cell diameter of 10–20 μm [55–57], while Mo/Ms were identified as cells containing a kidney-shaped, purple-stained nucleus; grey-blue cytoplasm lacking stained granules; and a cell diameter of 10–15 μm [58]. The separation procedure yielded cell suspensions containing ca. 60% PMNs and ca. 10% Mo/M, while the remaining 30% of cells were primarily lymphocytes and thrombocytes.

2.5. Quantification of ET release

A microtitre plate assay was developed for observing and quantifying ET release in PMN-enriched suspensions. SYTOX Green, a cell membrane-impermeable nucleic acid stain, was employed for ET quantification as the intensity of the fluorescence signal from this dye is proportional to ET release [59–61] (Supplementary Fig. 1). For quantification, to each well of a flat-bottomed 96-well culture plate (Cell+; Sarstedt, Nümbrecht, Germany) was added 50 μL of PMN-enriched suspension. The plate was incubated (30 min, 15 °C) to allow the cells to adhere to the bottom of the wells. Then 10 μL CaI in RPMI, a known inducer of ETs in other fish [20,21], was added to give final well concentration of 5 μg mL^{-1}; to control wells was added 10 μL RPMI. Plates were incubated for up to 12 h at 15 °C in the dark, before observation and/or quantification of ET release. After incubation, 10 μL SYTOX Green (to give final well concentration of 5 μM; Thermo Fisher Scientific) was added to each well and the plate incubated for 5 min at room temperature in the dark. Then, the fluorescence emitted from each well (attributable to stained extracellular DNA including chromatin) was measured with a spectrofluorimeter (excitation = 485 nm, emission = 528 nm; BioTek Synergy HT, Swindon, UK). Mean fluorescence was calculated from triplicate wells for each treatment group, and then mean fluorescence of control wells was subtracted from the mean fluorescence of each treatment group. Well contents were also observed directly using an inverted fluorescent microscope (Olympus IX-70; Olympus) through the LUCPlanFLN 20 × objective (PH2 Phase Plan Achromat; Olympus). Images were acquired by the Axiocam MRC camera operating Axiovision v.4.8 software (Carl Zeiss AG, Oberkochen, Germany) using phase contrast and fluorescent filters, and then overlaid and processed using ImageJ (v1.50i; https://imagej.net/).

2.6. Digestion with DNase-I

To confirm that the extracellular structures that stained positively with SYTOX Green in the PMN-enriched suspensions were composed of DNA, DNase-I was added to investigate whether this degraded the structures because this enzyme cleaves DNA without sequence specificity [62]. The PMN-enriched suspension was prepared in a microtitre plate as Section 2.5. After 3 h incubation, the contents of each well were treated with 10 μL DNase-I (made up in DNase buffer containing $MnCl_2$ and then diluted in water; Thermo Fisher Scientific) to give final well concentrations of 0.01, 0.1, 1, 10, and 100 U mL^{-1}, while control wells received DNase buffer diluted in water. After incubation for 3 h at 15 °C, the well contents were stained with SYTOX Green and fluorescence quantified as Section 2.5. Mean fluorescence was calculated from replicate wells for each treatment group (n = 5) and then normalised to the respective controls to yield fold-change values (mean fluorescence of treatment well divided by mean fluorescence of control well). Well contents were observed directly by microscopy as Section 2.5.

2.7. Immunocytochemistry of diagnostic markers

Immunocytochemistry (ICC) was performed to determine the presence of MPO, NE and histone H2A proteins in association with the ET-like structures released from trout PMN-enriched cell suspensions. The

primary antibodies diluted in PBS (without Ca^{2+} or Mg^{2+} ions) were polyclonal rabbit-anti-MPO IgG1 (at 1:20; ab9535; Abcam, Bristol, UK), polyclonal rabbit anti-NE IgG1 (1:20; ab21595; Abcam) and mouse anti-histone H2A (at 1:200; Mouse mAb #3636; Cell Signalling Technology, London, UK), while the secondary antibodies were polyclonal Alexa Fluor 488 goat anti-rabbit IgG (at 1:300; A32723; Thermo Fisher Scientific) and polyclonal goat anti-mouse IgG (at 1:300; A11001; Thermo Fisher Scientific). The PMN-enriched suspension was prepared in a microtitre plate as Section 2.5. After 3 h incubation, the contents of each well were fixed by removing the medium with a pipette and adding 100 μL of 4% paraformaldehyde (Sigma Aldrich Ltd), followed by incubation for 20 min at room temperature. Then each well was washed three times by removing the culture medium with a pipette, adding 100 μL of PBS (without Ca^{2+} or Mg^{2+} ions) and incubating for 5 min at room temperature. Then blocking was performed to reduce non-specific binding by removing the PBS and adding 100 μL of blocking buffer in PBS, which comprised 2% goat serum, 2% FCS and 0.2% Triton X-100 (Sigma Aldrich Ltd), and incubating for 30 min at room temperature. The blocking buffer was removed and 100 μL of the desired primary antibody was added, and the plate incubated for 90 min at room temperature. The wells were washed three more times with PBS as before, and then 100 μL of the desired secondary antibody was added, and the plate incubated again (45 min at room temperature). The PBS washing was repeated as before and then extracellular DNA and DNA in the cell nuclei were stained by adding 100 μL DAPI in water at 1 nM, and incubation for 5 min at room temperature. Images were acquired for each well in FITC (excitation = 488 nm, emission = 523 nm) and DAPI (excitation = 360 nm, emission = 460 nm) channels, allowing detection of both secondary antibodies using Olympus IX-70 or Olympus IX-73 microscopes (with a LUCPlanFLN 40 × objective lens), and then images were processed as Section 2.5.

2.8. Inducers on ET release

The effects of known inducers of ET release in fish and other systems were tested on PMN-enriched suspensions prepared in a microtitre plate as Section 2.5, with different inducers of ET release added after initial cell adherence. The inducers and final well concentrations were: CaI at 0.1, 1, 5, 10, and 25 μg mL^{-1}, recombinant flagellin from *Yersinia ruckeri* at 100 ng mL^{-1} [63], ultra-pure LPS (pLPS, purified by ion exchange chromatography; Sigma Aldrich Ltd) at 0.1, 1, 10, 25, and 50 μg mL^{-1}, PMA at 1, 10, 20, 40, and 60 nM, and the heat-inactivated bacteria, *V. anguillarum* ATCC 43310 or *V. anguillarum* NCIMB 1873, at 10^3, 10^5, or 10^7 CFU mL^{-1}. Each inducer was solubilised in RPMI or according to manufacturer instructions, and to controls were added appropriate concentrations of RPMI and any other diluents. For each inducer, a different plate was prepared for each time point (i.e., non-repeated measures). After incubation for up to 12 h, the well contents were stained with SYTOX Green and fluorescence read as Section 2.5. Mean fluorescence was calculated from triplicate wells for each treatment group and then normalised to the respective controls to yield fold-change values. Well contents were observed directly by microscopy as Section 2.5. This experiment was performed for four fish in total (except PMA at 40 nM and 60 nM, where six fish were used). To account for nucleic acid contamination in the reagents that could affect the fluorescence readings, CaI, flagellin, pLPS, PMA and heat-inactivated bacteria were incubated with RPMI and stained with SYTOX Green. Triplicate wells of each treatment (and concentration) were prepared, and mean fluorescence emitted from these wells lacking cells was subtracted from each well containing PMN-enriched cell suspension. Notably, there was no significant difference in fluorescence signal in wells containing the inducers compared to negative control wells containing medium only (Student's t-test; $p < 0.05$; data not shown).

2.9. Inhibitors of ET release

The effects of known inhibitors of ET release in fish and other organisms were tested on PMN-enriched suspensions prepared in a microtitre plate as previously described for inducers in Section 2.5, but with different inhibitors of ET release being added after initial cell adherence. The inhibitors and final well concentrations were: cytochalasin D from *Zygosporium mansonii* (Sigma Aldrich Ltd) at 0.1, 1, 5, 10 and 20 μM and diphenyleneiodonium chloride (catalogue number D2926; Sigma Aldrich Ltd) at 0.1, 1, 5, 10 and 25 μM. Again, the inhibitors were solubilised in RPMI or according to manufacturer instructions, and to controls were added appropriate concentrations of RPMI and any other diluents. For each inhibitor, a different plate was prepared for each time point (i.e., non-repeated measures). The microtitre plates were incubated for 30 min and then 10 μL of CaI was added to each well to give a final well concentration of 5 μg mL^{-1}. After incubation for up to 6 h, the well contents were stained with SYTOX Green and fluorescence read as Section 2.5. Mean fluorescence was calculated from triplicate wells for each treatment group and then normalised to the respective controls to yield fold-change values. Well contents were observed directly by microscopy as Section 2.5. This experiment was performed for four fish in total (except for DPI at 10 μM and 25 μM, where six fish were used). Nucleic acid contamination in the reagents was accounted for according to Section 2.8, and there was no significant difference in fluorescence signal in wells containing DPI and cytochalasin D compared to negative controls containing medium only (Student's t-test; $p < 0.05$; data not shown). A follow up experiment was performed without induction of ET release by CaI for DPI at 0.1, 1, 5, 10 and 25 μM, as described in Section 2.8.

2.10. Statistics

Statistical analyses were carried out using Prism v.5.1 (GraphPad, La Jolla, USA). For comparisons of mean fluorescence data from multiple groups, normality and equality of variances were assessed by Kolmogorov–Smirnov and Bartlett's tests, respectively. As the data on DNase digestion were non-normal, multiple Mann-Whitney *U* test were performed where each treatment group was compared to the control, with multiple comparisons accounted for by applying the Bonferroni correction. For all other data, two-way ANOVA was performed in conjunction with Bonferroni's post-hoc test to compare relative mean fluorescence of the treatment groups against the controls. For all analyses, $p < 0.05$ was accepted to be the threshold of statistical significance.

2.11. Ethics statement

All experiments were approved by the Institute of Aquaculture Ethics Committee or the University of Stirling Animal Welfare Ethical Review Body.

3. Results

3.1. ET-like structures observed in PMN-enriched suspensions

In a microtitre plate assay, ET-like structures staining positively with SYTOX Green were observed in PMN-enriched suspensions in wells that had not been exposed to an inducer of ET release, which indicated spontaneous release under the experimental conditions (Fig. 1). However, far more ET-like structures were observed in PMN-enriched suspensions that had been exposed to CaI, a known inducer of ETs in fish and other organisms (Fig. 1).

3.2. ET-like structures composed of DNA

To confirm the DNA nature of the observed ET-like structures, PMN-

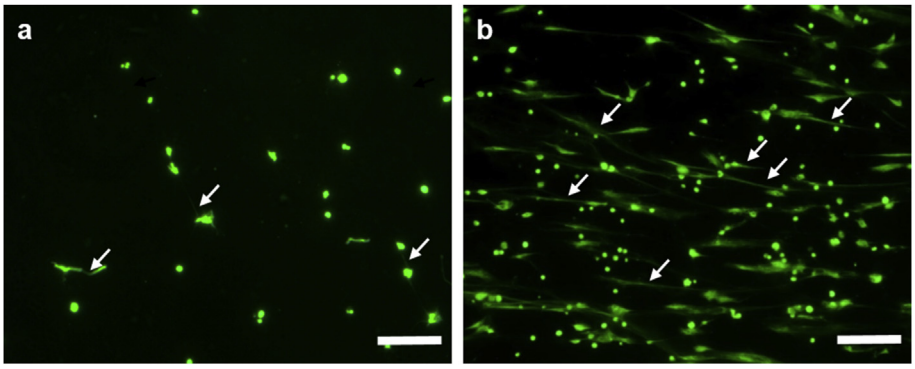

Fig. 1. Spontaneous release of ET-like structures and induction by exposure to CaI. Representative fluorescence microscopy images of ET-like structures released by PMN-enriched cell suspensions (white arrows). PMNs were isolated by triple-layer Percoll gradients, seeded into 96-well culture plates at 4×10^5 cells mL^{-1} and incubated for 6 h at 15 °C with (a) RPMI only (control); (b) RPMI supplemented with CaI at 5 μg mL^{-1}. DNA was stained with SYTOX Green (5 μM, 5 min, room temperature) and observations were made with a fluorescence microscope (20 × objective lens). Scale bars = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

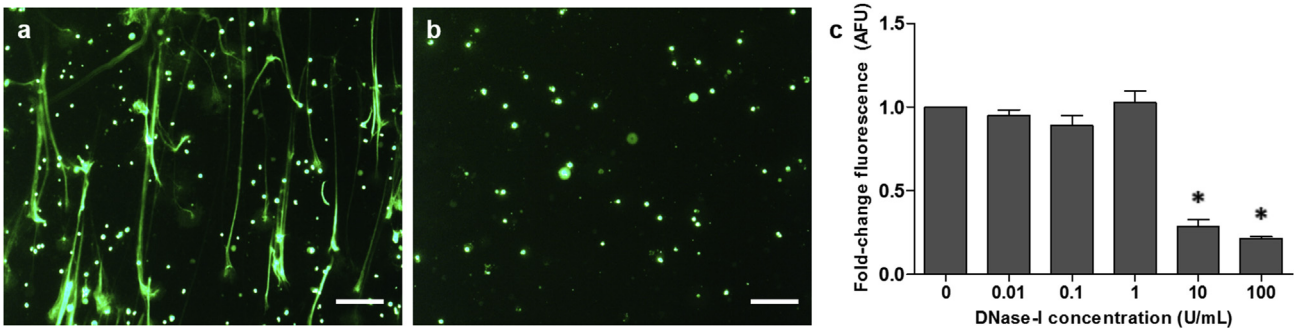

Fig. 2. Degradation of ET-like structures with DNase-I confirms their DNA composition. Representative fluorescence microscopy images of ET-like structures released from PMN-enriched cell suspensions (4×10^5 cells mL^{-1}) exposed to CaI (5 μg mL^{-1}, 3 h, 15 °C) and then treated for 3 h at 15 °C with DNase buffer supplemented with (a) dH_2O (control); (b) 100 U mL^{-1} DNase-I. DNA was stained with SYTOX Green (5 μM, 5 min, room temperature) and observed with a fluorescence microscope (20 × objective lens). Scale bars = 100 μm. (c) Bar chart showing mean fold-change in fluorescence of wells containing PMN-enriched cell suspensions (4×10^5 cells mL^{-1}) after exposure to CaI (5 μg mL^{-1}, 3 h, 15 °C) to induce the release of ET-like structures, and then to DNase-I (0.01–100 U mL^{-1}, 3 h, 15 °C). Exposure of ET-like structures to DNase-I at 10 and 100 U mL^{-1} resulted in significant reductions in fluorescence attributable to the ET-like structures. Mann-Whitney *U* test; *$p < 0.05$ compared to control; error bars are s.e.m.; $n = 5$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

enriched suspensions that had been induced with CaI to release ET-like structures were stained with SYTOX Green and then treated with DNase-I, an endonuclease that degrades nucleic acids. The fluorescence signal emitted from wells containing ETs was reduced significantly after treatment with ≥10 U mL^{-1} DNase-I for 3 h, and fluorescence microscopy observations confirmed the lack of ET-like structures in treated wells (Fig. 2).

3.3. ET-like structures contain MPO, NE and histone H2A

To further confirm that the observed ET-like structures were ETs, ICC was performed to detect the presence of MPO, NE and histone H2A in association with the chromatin, as these important proteins are known to associate with ETs and are used as diagnostic markers. Crucially, FITC-conjugated anti-MPO, NE and histone H2A antibodies were each found in association with the DAPI-stained DNA of the ET-like structures in PMN-enriched suspensions induced with CaI (Fig. 3).

3.4. Induction of ET release by CaI, flagellin and PMA, but not by pLPS and heat-inactivated bacteria

The effects of known inducers of ET release in fish and other systems were tested on PMN-enriched suspensions by measuring changes in the fluorescence signal and by microscopy observations. CaI induced ET release within 1 h exposure, with CaI at 1, 5, 10, and 25 μg mL^{-1} resulting in 2.38 ± 0.26, 3.16 ± 0.30, 3.35 ± 0.21 and 3.33 ± 0.27-fold increases in fluorescence in the treatment well compared to negative controls (Fig. 4). Observations by fluorescence microscopy confirmed high abundances of ETs stained positively by SYTOX Green in the wells treated with CaI at ≥1 μg mL^{-1}, while only the round nuclei of a few cells with compromised cell membranes were stained in control wells (Fig. 4). Longer incubation of the PMN-enriched suspensions with CaI at ≥1 μg mL^{-1} showed little, if any, further increases in fluorescence compared to 1 h, before appearing to decline at 12 h, suggesting that all the cells capable of releasing ETs had already done so (Fig. 4). The PMN-enriched suspensions treated with CaI at 0.1 μg mL^{-1} did not show significant change in fluorescence signal during 12 h (Fig. 4). A closer investigation of the timing of ET release after exposure to CaI at 5 μg mL^{-1} through measurement of fluorescence every 10 min during 1 h incubation revealed that there was a significant increase in fluorescence attributable to ETs immediately after exposure (1.32 ± 0.04-fold change in fluorescence compared to negative controls), with fluorescence in the PMN-enriched suspensions gradually increasing thereafter (Fig. 5).

After exposure to flagellin at 100 ng mL^{-1}, the fluorescence signal from PMN-enriched suspensions increased at 1 h by 3.34 ± 0.81 compared to negative control (Fig. 4). Similarly to CaI, there was little increase in fluorescence signal from wells at subsequent exposure times, except for the final reading at 6 h that was lower than the reading at 5 h, again suggesting that the maximum response had been achieved (Fig. 4). Notably, the flagellin had caused the ETs and cells within the suspension to form very large clumps when observed by microscopy (Fig. 4).

At 6 h, PMA at 40 and 60 nM caused an increase in well fluorescence due to the release of ETs and these increases were significant at 9 h, with respective 2.26 ± 0.72 and 2.54 ± 0.88-fold increases in fluorescence compared to negative controls (Fig. 4); ETs stained with SYTOX Green were observed by microscopy in these wells. Lower concentrations of PMA (1, 10, and 20 nM) had little effect on well fluorescence values during 12 h (Fig. 4).

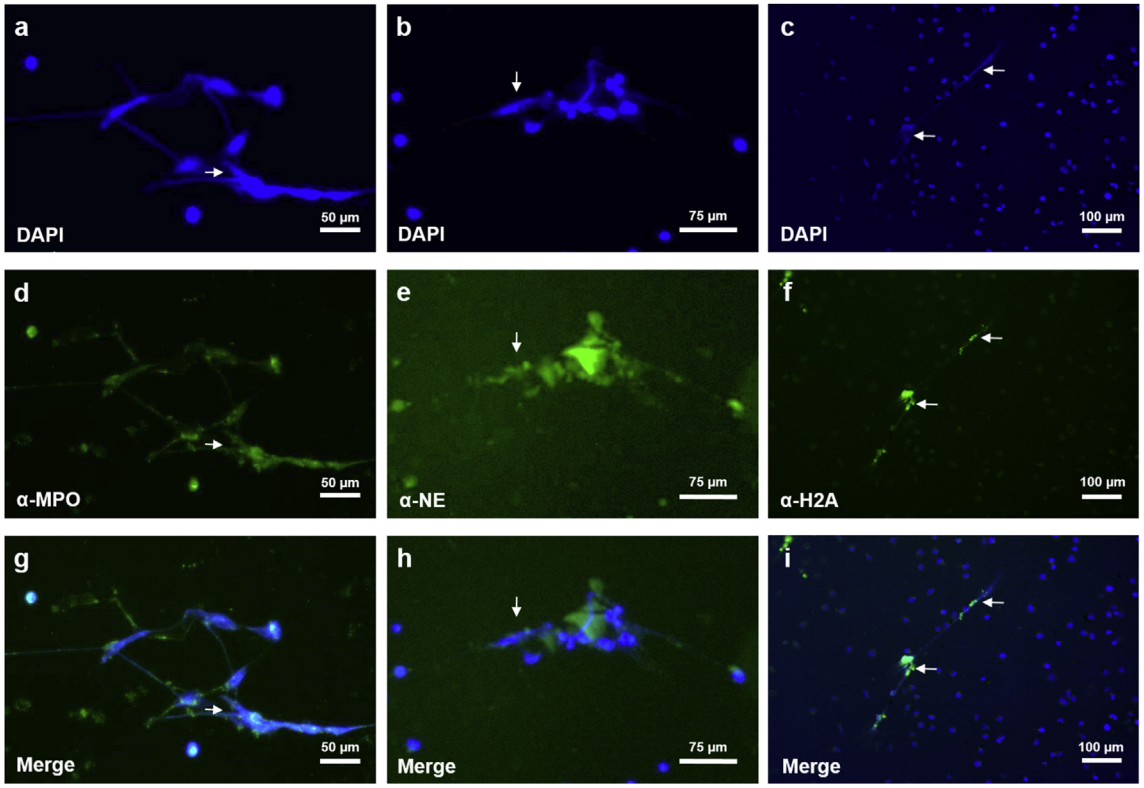

Fig. 3. Localisation of extracellular DNA with three characteristic protein constituents of ETs: MPO, NE and histone H2A. Representative fluorescence microscopy images of immunocytochemically stained ET-like structures released from PMN-enriched cell suspensions (4×10^5 cells mL^{-1}) exposed to CaI (5 μg mL^{-1}, 3 h, 15 °C). (a–c) staining of ET-like structures (white arrows) with the membrane-permeable nucleic acid-specific DAPI; (d–f) staining with FITC-conjugated anti-MPO (1:20), NE (1:20), and histone H2A antibodies (1:200), respectively (white arrows); (g–i) merged DAPI and FITC channels confirming the association of ET-like structures stained with DAPI in conjunction with MPO, NE and histone H2A, respectively (white arrows). a, b, d, e, g, h are 40 × objective lens; c, f, i are 20 × objective lens. No positive staining was observed in controls lacking the primary antibodies (PBS only instead), indicating that the secondary FITC-conjugated antibodies were specific to the primary markers, and were not cross-reacting with other non-specific targets.

Interestingly, exposure of cells to pLPS at ≤50 μg mL^{-1} for up to 12 h did not result in a change in fluorescence compared to negative controls not exposed to pLPS, suggesting little to no release of ETs in the PMN-enriched suspensions (Fig. 4). Fluorescence microscopy observations confirmed the absence of ETs in these wells and the contents appeared similar to the control wells. Moreover, two isolates of heat-inactivated *V. anguillarum* (at up to 10^7 CFU mL^{-1} for 12 h) did not induce the release of ETs from PMN-enriched suspensions, as there was no difference in fluorescence compared to the negative controls (Fig. 4).

3.5. Cytochalasin D inhibited ET release but DPI induced NETosis

To assess the effects of cytochalasin D and DPI, two known inhibitors of ET release, on ET release in PMN-enriched suspensions, the cells were pre-incubated for 30 min with the inhibitors prior to exposure to 5 μg mL^{-1} CaI to induce ET release. At 3 h post-exposure to CaI, PMN-enriched suspensions that had been pre-incubated with cytochalasin D at 10 and 20 μM showed significant reductions in fluorescence attributable to ET release compared to negative controls, and at 6 h the suspensions pre-incubated with cytochalasin D at 5 μM also yielded reduced fluorescence, indicative of ET release inhibition (Fig. 6). Fluorescence microscopy observations confirmed the presence of fewer ETs in PMN-enriched suspensions that had been treated with cytochalasin D compared to negative controls (Fig. 6).

Somewhat unexpectedly, DPI acted as an inducer not an inhibitor of ET release in PMN-enriched suspensions, and so the effect of this compound alone on ET release was assessed for up to 12 h. The induction effect was dose-dependent, with greater concentrations of DPI associated with greater fluorescence attributable to ETs (Fig. 6). The increase in fluorescence from PMN-enriched suspensions was significantly different from controls for the 25 μM DPI group at ≥3 h, and ≥6 h and 12 h for the 10 μM and 5 μM DPI groups respectively (Fig. 6). Again, fluorescence microscopy observations confirmed increased ET release in PMN-enriched suspensions exposed to sufficient concentrations of DPI.

4. Discussion

This present study sought to characterise the release of chromatin-composed ETs by salmonid PMNs for the first time using *O. mykiss* as the study species, which provides a key first step in gaining an understanding of the role of this innate response mechanism in protecting these fish against microbial infection. The experiments conducted have confirmed the presence of ETs in highly-enriched PMN cell suspensions isolated from head kidney preparations through: i) confirmation of ET-like structures to be composed of DNA; ii) immunocytochemical detection of diagnostic proteins in close association with the extracellular chromatin; iii) modulation of ET release by the PMNs through exposure to recognised inducers and inhibitors of this process in other species.

A key piece of evidence in confirming the presence of ETs in rainbow trout PMN cell suspensions was the detection of NE and MPO in close association with decondensed chromatin (composed of DNA and histones), as these characteristics are consistent with ETs released by neutrophils of other species and satisfy the commonly accepted diagnostic definition of an ET [6,7,48,64–69]. The presence of extracellular DNA was confirmed through the detection of material that stained positively with SYTOX Green, a dye that intercalates with nucleic acid, and by rapid degradation of these structures with the DNA-

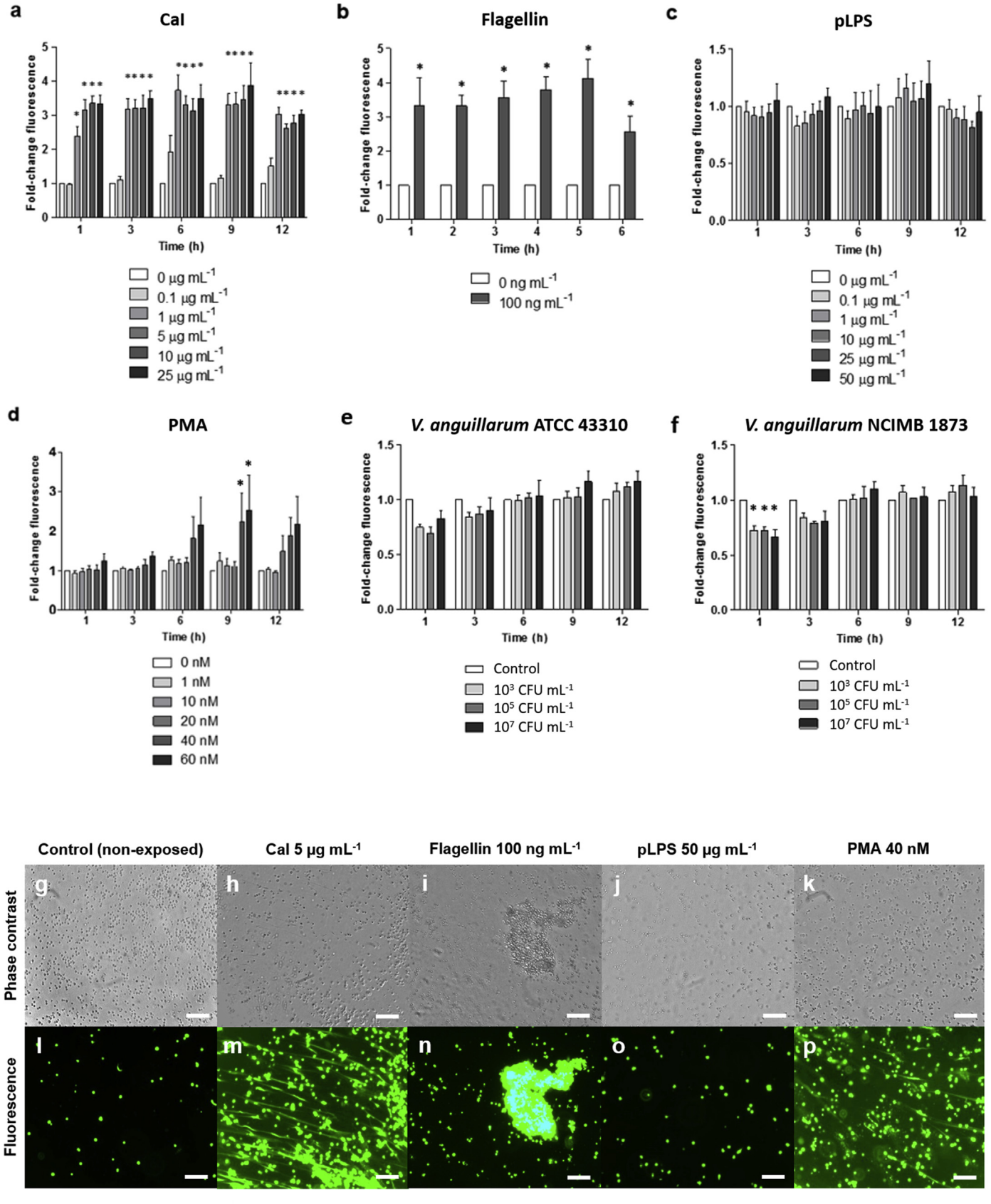

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Fig. 4. CaI, flagellin and PMA induce ET release but LPS and heat-inactivated bacteria do not. Bar charts showing mean fold-changes in fluorescence of wells containing PMN-enriched cell suspensions (4×10^5 cells mL^{-1}) after exposure for up to 12 h at 15 °C to: (a) CaI, (b) flagellin, (c) pLPS, (d) PMA, (e) heat-inactivated *V. anguillarum* ATCC 43310 and (f) heat-inactivated *V. anguillarum* NCIMB 1873, and then stained with SYTOX Green (5 μM, 5 min, room temperature). Exposure of PMN-enriched cell suspensions to CaI at ≥1 μg mL^{-1} at ≥1 h, flagellin at 100 ng mL^{-1} at ≥1 h, or ≥40 nM of PMA at 9 h resulted in significant increases in fluorescence attributable to the ET-like structures. Note that there were significant reductions in fluorescence in the wells exposed to *V. anguillarum* NCIMB 1873 for 1 h, but this results from the subtraction of the fluorescence in the bacteria only negative control wells from the experimental wells. Two-way ANOVA with Bonferroni's post-hoc test; *$p < 0.05$ compared to control; error bars are s.e.m.; n = 4 fish, except PMA at 40 nM and 60 nM (n = 6). Representative images of same field of view acquired by phase contrast (g–k) and fluorescence microscopy (i–p) of ET-like structures (white arrows) released from PMN-enriched cell suspensions (4×10^5 cells mL^{-1}) after exposure for 6 h at 15 °C to: (g,i) no inducer; (h,m) CaI (5 μg mL^{-1}); (i,n) flagellin (100 ng mL^{-1}); (j,o) pLPS (50 μg mL^{-1}); (k,p) PMA (40 nM), and then stained with SYTOX Green (5 μM, 5 min, room temperature). Images acquired with 20 × objective lens; scale bars = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

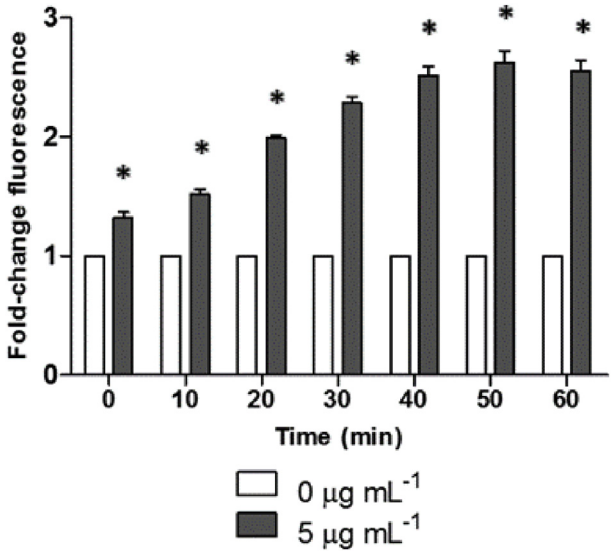

Fig. 5. CaI induces rapid ET release in PMN-enriched suspensions. Bar chart showing the mean fold-change in fluorescence of wells containing PMN-enriched cell suspensions (4×10^5 cells mL^{-1}) after exposure for ≤1 h at 15 °C to CaI (5 μg mL^{-1}), then stained with SYTOX Green (5 μM, 5 min, room temperature). Exposure of PMN-enriched cell suspensions to CaI at 5 μg mL^{-1} resulted in a significant increase in fluorescence attributable to the ET-like structures immediately after exposure. Two-way ANOVA with Bonferroni's post-hoc test; *$p < 0.05$ compared to control; error bars are s.e.m. n = 4 fish.

degrading enzyme, DNase-I. Meanwhile, the detection of histone H2A, a core histone, with the extracellular DNA confirmed the chromatin nature of the structures observed. The detection of NE and MPO with the chromatin indicates neutrophils in the PMN-enriched suspensions to be the cells most likely responsible for releasing the ETs because these cells contain high concentrations of these proteins, although ET release by other immune cells cannot be ruled out, given that this process has been reported for other granulocytes, mast cells and macrophages in other vertebrates [70,71]. Moreover, macrophages isolated from the common carp head kidney also release ET-like structures after incubation with LPS [72]. Despite previous reports on the presence of ETs in immune cell suspensions isolated from various fish species, few studies have confirmed the detection of each of the diagnostic protein markers, perhaps due to difficulty in achieving this given the lack of primary antibodies available to detect fish versions of the proteins that decorate the ET structure. Immunocytochemical staining of ETs released in PMN-enriched cell suspensions from fish showed that NE was detected in common carp [44], MPO was detected in fathead minnow [21] and common carp [44], and both of these markers were detected in zebrafish [20]. Brogden et al. [22] and Chi and Sun [9] detected histones in association with the extracellular DNA in common carp and turbot, respectively. In this present study, high titres of the primary antibodies were required for target detection (1:20 dilution for MPO and NE; 1:200 dilution for histone H2A), likely due to a lack of specificity of the commercially available antibodies for these proteins, which are raised against human homologues. The lack of such basic tools for identifying fish targets presents an ongoing challenge for researchers in this field.

ETs were released spontaneously in the PMN-enriched suspensions from rainbow trout and this is in line with previous reports in humans and dogs [59,73,74]. Nevertheless, NETosis was induced in the PMN-enriched suspensions through exposure to PMA, flagellin and CaI but was inhibited by cytochalasin D, which is in general agreement with observations made in other animals, including fish, suggesting that the molecular pathways underlying the initiation of ET release are evolutionarily ancient and conserved [21,45,75]. Even so, there is little consistency reported in the potency of inducers and inhibitors for ET release from neutrophils derived from different fish species, meaning that the signalling mechanisms mediating this process between species remains uncertain. ET release is induced after ROS generation via pathways dependent or independent of NADPH oxidase, an enzyme complex commonly found in the cell membranes of phagocytes [76]. Stimulation of the NADPH oxidase pathway is achieved via exposure to PMA, though PAMPs such as LPS or flagellin can also elicit NADPH oxidase-dependent ET release from human neutrophils [30,35,39]. In this present study, flagellin was a potent inducer of ETs from rainbow trout PMNs and caused the mixture of cells and ETs to form large clumps, which has been observed in other studies of ETs [77,78]. These observations strongly suggest flagellin to be an important natural signal during the initiation of NETosis and, consistent with this suggestion, this PAMP causes a strong pro-inflammatory response in rainbow trout macrophages through inducing the release of IL-6, IL-8 and TNF-α [63]. PMA was a relatively weak inducer of ETs from rainbow trout PMNs, but this is similar to observations of fathead minnow and zebrafish immune cells [20,21], whereas this compound exerts a more potent effect for ET release by PMNs from common carp and tongue sole [10,44]. The underlying reasons for these differences will need to be examined through further experimentation. DPI inhibits ROS generation by NADPH oxidase and it can reduce ET release by PMA or PAMP-induced neutrophils of mammals [7,29,30,45]. In carp, DPI similarly reduced PMA-induced ET release from PMNs [44], while exposure of tongue sole PMNs to NADPH oxidase inhibitors reduced ET release induced by PMA and bacterial suspensions [10], indicating ET release in these fish species operates through an NADPH oxidase-dependent system. In this present study, DPI did not inhibit ET release from rainbow trout PMNs but instead increased ET release, a surprising observation not reported elsewhere and thus deserving of further investigation. However, this unexpected result could be explained by the non-specific action of DPI, which can affect several cellular pathways [79]. Notably, pLPS and heat-inactivated bacteria did not induce ET release from rainbow trout PMN suspensions and this may be because fish do not detect and respond to LPS stimuli in the same way as mammals [80,81], possibly because many fish genomes lack toll-like receptor 4 (TLR-4) orthologs and essential costimulatory molecules required for activation of PMNs by LPS [80]. Still, it is possible that the heat inactivation process had denatured the epitopes sufficiently to prevent recognition of the bacteria by the PMNs, and therefore the use of live bacteria or alternative activation methods that leave bacterial surface structures in tact warrant consideration. In this respect, tongue

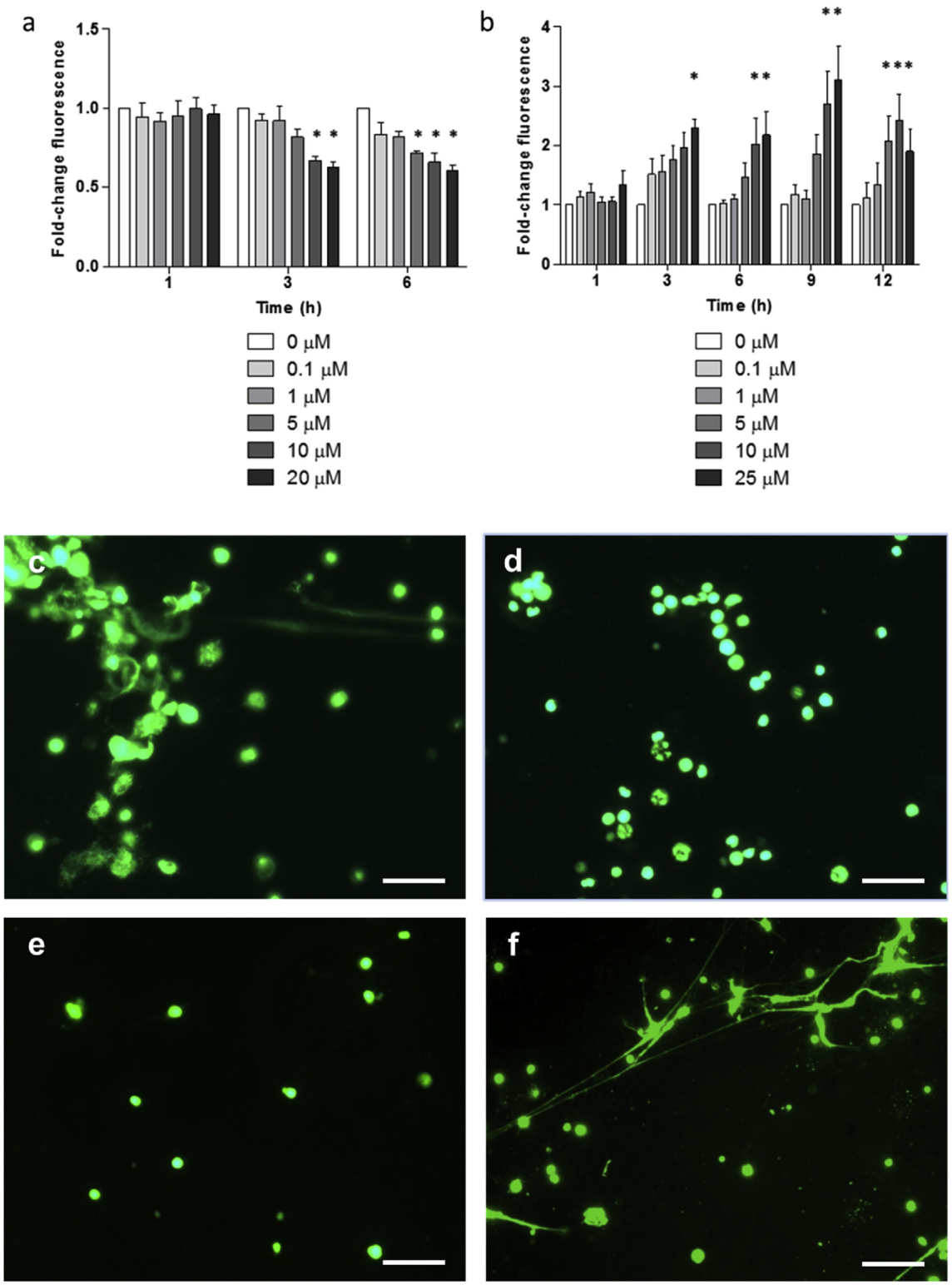

Fig. 6. Cytochalasin D inhibits ET release, while DPI induces ET release. Bar charts showing mean fold-changes in fluorescence of wells containing PMN-enriched cell suspensions (4×10^5 cells mL^{-1}) after initial exposure for 30 min at 15 °C to (a) cytochalasin D, and then exposed for ≤6 h at 15 °C to CaI (5 μg mL^{-1}) before being stained with SYTOX Green (5 μM, 5 min, room temperature); for (b) DPI, the PMN-enriched cell suspensions were exposed for ≤12 h before staining and ET release was not induced with CaI. Exposure of PMN-enriched cell suspensions to cytochalasin D at ≥10 μM at ≥3 h and ≥5 μM at ≥6 h, resulted in significant decreases in fluorescence attributable to the ET-like structures. Meanwhile, exposure to DPI at 25 μM at ≥3 h and 10 μM at ≥6 h and 5 μM at ≥12 h, resulted in significant increases in fluorescence attributable to the ET-like structures. Two-way ANOVA with Bonferroni's post-hoc test; *$p < 0.05$ compared to control; error bars are s.e.m.; $n = 4$ fish, except DPI at 10 μM and 25 μM ($n = 6$). Representative images acquired by fluorescence microscopy of ET-like structures released from PMN-enriched cell suspensions (4×10^5 cells mL^{-1}) after initial exposure for 30 min at 15 °C to: (c) no inhibitor and; (d) cytochalasin D at 10 μM, and then exposed for 3 h at 15 °C to CaI (5 μg mL^{-1}) before being stained with SYTOX Green (5 μM, 5 min, room temperature); (e) no inhibitor compared to (f) DPI at 10 μM (3 h at 15 °C). The fluorescence microscopy images confirm the inhibition of ET-like structures in PMN-enriched cell suspensions incubated with cytochalasin D and the induction of ET-like structures by DPI. Images acquired with 20 × objective lens; scale bars = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sole PMNs exposed to viable cells of *Pseudomonas fluorescens*, *Vibrio harveyi* or *Edwardsiella tarda* did release ETs within 2 h [10], indicating the potential of living bacteria to be an ET inducer in some fish.

Meanwhile, CaI is recognised to stimulate ET release via ROS generation independent of NADPH oxidase [29,39] and this compound is a potent inducer of ET release in zebrafish and fathead minnow, especially when compared to PMA, β-glucan and LPS [20,21]. In this present study, CaI was also a reliable and potent inducer of rapid ET release from rainbow trout PMNs, with 5 μg mL^{-1} exerting a potent effect. Finally, ET release by the rainbow trout neutrophils was inhibited in a dose- and time-dependent manner by cytochalasin D, which acts independently of NADPH oxidase by inhibiting the remodelling of the cytoskeleton necessary at various stages of ET release and this effect likely explains the observed inhibitory action [6,45,50]. ET release was also inhibited in zebrafish and fathead minnow PMNs by the actin polymerisation inhibitor cytochalasin B, indicating that fish PMNs also rely on cytoskeleton remodelling for ET release [20,21]. Taken together, these observations suggest that although ET release by rainbow trout PMNs can be triggered by NADPH-dependent and -independent pathways, there is a reduced dependence on the former for ET release compared to some mammals, most invertebrates and certain other fish species. This conclusion is consistent with observations on common carp where DPI inhibited ET release from PMNs by PMA but not by LPS or zymosan, indicative of triggering by NADPH oxidase-dependent and -independent pathways [44]. A better understanding of the signalling mechanisms leading to ET release in fish may improve our understanding of the evolution of this innate immune response, given the evolutionary divergence of teleosts [82].

The assays in this present study were performed with cell suspensions enriched for PMNs derived from the head kidney, and cell counts indicated that approximately 60% of the cells were PMNs based on morphological characteristics. However, the purity of neutrophils in the test suspensions could be improved by labelling the cells with a monoclonal antibody specific for rainbow trout neutrophils, such as E3D9, and performing a cell sorting protocol [83]. Moreover, the experiments herein relied upon the quantification of fluorescence signal produced by SYTOX Green, an assay used commonly to study ETs but one that has several drawbacks. When cell clumping occurs, such as when the PMNs were exposed to flagellin in particular, the fluorescence signal plateaus and this introduces a degree of inaccuracy into ET quantification. Furthermore, the assay cannot distinguish between the fluorescence signal derived from ETs and the SYTOX Green-stained nuclei of membrane-compromised or dead cells, although the small size of these nuclei compared to the diffuse structures of the ETs minimises their contribution to the overall well signal. In addition, care was taken to account for the fluorescence signals derived from nucleic acid contamination of reagents. Such uncertainties reinforce the importance of examining the wells by microscopy when using a SYTOX Green-based assay for ET quantification to support the well readings, as was performed routinely in this present study.

During this study, it became apparent that there was high variability between individual fish in terms of ET release, either spontaneously or when incubated with the various inducers and inhibitors. Variation in ET release between individuals has been reported previously [7,74] and the description of this variability, and investigation of the reasons underlying the differences, will be one focus of future experiments. Knowledge gathered on inducers and inhibitors in this present study will support further experiments aiming to determine the role of ETs in the innate response against microbes.

5. Conclusion

In conclusion, this present study demonstrates for the first time that rainbow trout PMNs have the capacity to release ETs, a mechanism shown in other organisms to exert a protective effect against invading pathogens, and this is an important first step towards understanding the role of ETs in this commercially important family of fish. From knowledge obtained in humans and mammals, improved understanding of ETs and identification of mechanisms to manipulate this response beneficially may enhance our ability to protect fish against diseases, thus improving fish health and welfare and farm productivity.

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Declaration of competing interest

The authors confirm they have no known conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.01.040.

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