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Triploid animals, a potential model for ETosis research: Influence of polyploidy on the formation and efficacy of extracellular traps in the eastern oyster

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ABSTRACT

Decondensation and the subsequent release of chromatin from specific immune cells in response to inflammatory stimuli is a highly conserved aspect of the innate immune system and leads to the formation of extracellular traps, observable in nearly all forms of multicellular life. This process is known as ETosis, with the release of DNA and its associated antimicrobial proteins physically capturing and neutralizing pathogens following an infection or tissue damage. Despite the universality of this response, data concerning extracellular traps in non-model organisms is limited, with most invertebrate studies doing little more than proving their existence due to difficulties in stimulation and high interindividual variability in trap production. This study provides a novel, simple, and inexpensive method for the consistent stimulation of extracellular traps in eastern oyster (*Crassostrea virginica*) hemocytes. Using the methods described in this study, we compared how ploidy impacts the rate, size, and efficacy of extracellular traps. Findings demonstrated that hemocyte extracellular traps were potent antimicrobials against both Gram-positive and Gram-negative bacteria. Furthermore, we provide evidence to suggest that agranulocytes may be the primary ETosis effector cells in *C. virginica.* This study is the first to describe extracellular traps in *C. virginica* and highlights the possible benefits of using triploid animals to gain a further understanding of ETosis and the factors that regulate its induction and efficacy.

1. Introduction

The innate immune system consists of numerous cell types with highly specialized functions allowing the optimal response to immune insults. One such function is the ability of immune cells to undergo a form of programmed cell death dubbed "ETosis", in which cellular chromatin is decondensed and discharged out of the cell in response to inflammatory stimuli. The released DNA strands form dense net-like structures known as extracellular traps (ETs). ETs are decorated with antimicrobial products such as histones and enzymes (e.g., myeloperoxidase) resulting in the capture and neutralization of pathogens such as viruses, bacteria, fungi, and parasites [1,2]. This broad-acting response was first observed in human neutrophils in 2004 by Brinkmann et al., but has since been reported in a wide variety of taxa including mammals [3], birds [4], cnidaria, mollusks [5], plants [6], and colonial amoeba [7]. Etosis can be triggered by a diverse array of biotic and abiotic stimuli including but not limited to Phorbol 12-myristate 13-acetate (PMA; standard stimulus in vertebrate systems), lipopolysaccharide (LPS), live or dead bacteria, calcium ionophore, zymosan, physical trauma, UV light and surgery [8–10]. Excessive or improper ET production can lead to host tissue damage and inflammation, with ETs implicated in numerous autoimmune disorders [11] review). As such, etosis is a tightly regulated process with specific cell subpopulations (e.g., older neutrophils in humans) predisposed and primarily responsible for producing ETs [11]. Similar processes and specialization are also believed to be occurring in invertebrate immune cells [5]. Due to both the protective and antagonistic nature of ETs, etosis has now been extensively studied in vertebrates; however, most of the work in invertebrates has done little more than prove the existence of ETs across different species.

The invertebrate immune system is entirely reliant upon innate defenses, which consist of physical barriers and cellular processes primarily carried out by blood cells (hemocytes). Hemocytes perform a remarkably broad set of functions in invertebrates, such as the transport of nutrients, shell formation, and wound healing [12–14]. Moreover, hemocytes are the primary immune effector cells responsible for the

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phagocytosis of microbes, secretion of humoral factors, encapsulation of foreign bodies, and the production of reactive oxygen species (ROS) [15,16]. ETs were found to be implicated in some of these immune processes as removal of ETs (via DNAse I or pathway inhibitors) disrupted cell aggregation, nodule formation, and the encapsulation of pathogens in earthworms, shore crabs, cockroaches, and marine mussels [5,17–19]. ETs are frequently associated with the production of ROS in both vertebrates [20] and invertebrate systems [5,10,17,19,21], although NADPH-independent ETs have also been observed depending on the stimuli used, and the species assessed [10,17]. Direct interaction and neutralization of bacteria via ETs have been demonstrated in a few invertebrate systems [18,19,22], however high variability in the ability to stimulate ET production (in invertebrates) between and within species has hampered deeper investigation. Consequently, knowledge concerning the impacts of life history traits such as ploidy (DNA quantity), reproductive activity, or sex on the production and efficacy of ETs remains unaddressed.

In oysters (and fish), triploidization is becoming an increasingly popular technology, in which oysters are intentionally produced with an extra set of chromosomes leading to sterility and increased growth rates in the animals [23]. The sterility associated with these animals results in greater energy reserves [24], while the extra set of chromosomes causes a total increase in cell volume to accommodate the increased DNA content [25,26]. The influence of increased nuclear and total cell volume has been seldom addressed [27], however, the limited data concerning triploid immunology suggests that these oysters may present more stable immunological performances, as triploid blood cells undergo less seasonal variation in concentration and mortality throughout the year [28] while also presenting enhanced cellular ROS production [26]; reviewed in Ref. [27]. These traits along with the increased DNA content of triploids could directly benefit the study of invertebrate ETs, particularly when addressing how DNA content impacts ET quantity and efficacy.

In this study, triploid and diploid eastern oysters (*Crassostrea virginica*) were induced to produce ETs via a novel and reliable method, based on tissue damage, allowing the comparison of individual oysters and traits associated with ET production. As far as we know, this represents the first work to demonstrate ETs in the eastern oyster, as well as the only study to assess how life history traits of an invertebrate can influence ETs. The present study then sought to address if ETs produced from the eastern oyster were effective in the neutralization of bacteria (Gram-positive and Gram-negative). Moreover, the relationships between ET efficacy, hemocyte subpopulations, and their ability to produce ROS were examined. These results provide evidence that physically induced stress can be a potent inducer of ETs and contributes to the growing body of literature concerning ETs.

2. Methods

2.1. Oysters

Adult (2-year-old) half-sibling diploid and triploid *C. virginica* (shell lengths, mean ± SD: diploid = 86 ± 8 mm, triploid = 90 ± 12 mm) with shared maternal genetics were collected from a local oyster farm located in Great South Bay (Islip, New York, USA: 40.6871° N, 73.1114° W). Triploid oysters were produced using the tetraploid crossing method (tetraploid sperm mixed with diploid eggs; see Ref. [27]; for greater details) and resulted in 100% triploid progeny, assessed using flow cytometry analysis of DNA content [29]. Once collected, oysters were transported back to Stony Brook University (Stony Brook, New York, USA; 40.9060° N, 73.1191° W) and immediately cleaned, labeled, and placed into the same recirculating aerated tank with filtered (0.2 μm) and UV-sterilized seawater at 20 °C and 25 practical salinity units (PSU). Oysters were maintained for a minimum of one week and a maximum of two weeks with regular water changes before the start of any experiments. They were fed daily using Reed Mariculture Shellfish Diet 1800® following the manufacturer's recommendations. Oysters were starved for 24 h before all experiments.

2.2. Hemocyte collection, characterization, and measurement of reactive oxygen species (ROS)

Hemolymph was withdrawn from the adductor muscle of triploid and diploid oysters through a notch in the posterior valve margins of the shell made either five days prior (puncture stimulated oysters, detailed below) or immediately before sampling (external stimulants or non-stimulated controls, detailed below). Hemolymph samples from individual oysters were immediately diluted (1:5) with chilled sterile artificial seawater (SASW; 25 PSU), then divided into two aliquots. One of the two subsamples was held on ice for etosis experiments, while the second aliquot was used to calculate cell concentration, characterize cell subpopulations (agranulocytes and granulocytes), and measure intracellular reactive oxygen species (ROS) using a 4-color BD FACSCalibur flow cytometer (Becton Dickinson Biosciences). ROS was calculated by measuring green fluorescence five and 30 min after the addition of 2′,7′-dichlorofluorescein diacetate (final concentration 1 μM; ThermoFisher Scientific, Waltham, MA, USA) to the hemolymph subsample held at room temperature. Forward and side scatter parameters allowed the assessment of cell subpopulations and concentrations [30]. The cell concentrations from hemolymph subsamples held on ice were then adjusted to 3×10^5 cells/ml using SASW and used for all etosis experiments.

2.3. Stimulation of etosis and visualization of ETs

2.3.1. External stimulants

Several stimuli were tested at various concentrations for their capacity to trigger etosis in hemocytes following methods modified from Refs. [10,21] with the details listed in Table 1. Briefly, diluted hemocyte samples (collected from diploid or triploid oysters as detailed below) were seeded in 96-well cell culture plates at 3×10^5 cells/ml. DNAse I (1 mg/ml final concentration; Sigma-Aldrich, Saint Louis, MO, USA) was added to appropriate control wells. Cells were then allowed to adhere for 25 min at 20 °C after which stimuli were added at desired concentrations in triplicate (12 wells total per stimuli tested: three stimulated, three unstimulated, three DNAse I and stimulated, three DNAse I and unstimulated) to assess if stimuli-induced ETs exceeded spontaneous etosis, which is defined here as the basal rate of etosis occurring in the absence of any stimuli. Plates were then incubated for 10 min, then centrifuged for 5 min at 250g and 20 °C to bring the nets (which are tri-dimensional) to the bottom of the plate and facilitate visualization. Wells were then allowed to incubate for an additional 10 min after which membrane impermeable DNA-binding dye SYTOX green (5 μM final concentration; ThermoFisher Scientific, Waltham, MA, USA) was added allowing for the visual inspection of ETs using a Nikon Eclipse TE 2000-S microscope equipped with NIS-Elements D imaging software. When initially assessing stimuli, the production of ETs was monitored for 0.5–5 h after stimulation, with peak etosis times visually assessed and recorded (Table 1).

2.3.2. Physical stimulation: puncture method

The ability of physical stress to produce ETs was also investigated. Five days before experiments, the posterior valve margins of both diploid and triploid adult *C. virginica* were notched. Using the notch, the animal's adductor muscle was punctured three consecutive times with a 20-gauge needle (notched but without tissue puncture was also tested, Table 1), with each puncture slightly displaced to make three separate wounds. Upon the third puncture wound, 250 μl of hemolymph was withdrawn and discarded. Oysters were then placed back into their holding tanks. Water changes were performed as needed to maintain optimal water conditions. A subset of oysters collected at the same time were left unstimulated to serve as controls. After five days of recovery

Table 1
The stimuli used to induce ETs in oyster hemocytes. The value for concentrations indicates the final concentration of each stimulant used. ET stimulation refers to the number of ETs present in a well compared to the unstimulated control wells as well as the reproducibility of the assay. Least potent stimuli to most potent stimuli are ranked in order from none, rare, few, moderate, and numerous. Triploid and diploid oysters were used equally throughout these trials for each stimulant (minimum of 3 oysters), with each animal treated and assessed individually. NA: not applicable.

Inducer	Concentration	ET stimulation/ reproducibility	Time till peak ET (hrs)	Assessment methods
Lipopolysaccharide (LPS)	1 mg/ml 0.5 mg/ml 0.1 mg/ml	None	NA	Visual
Zymosan A	1 mg/ml 0.5 mg/ml 0.1 mg/ml	None	NA	Visual
Phorbol 12-myristate 13-acetate (PMA)	50 ng/ml 25 ng/ml	None	NA	Visual
Calcium ionophore (A23187)	16 μM 8 μM	Moderate	1–2	Visual, & bacterial neutralization
Vibrio coralliilyticus RE98	1:10 MOI 1:50 MOI 1:100 MOI	Few Few Rare	1–2	Visual
Vibrio coralliilyticus RE22	1:10 MOI 1:50 MOI 1:100 MOI	Few Few Rare	0.5–1.5	Visual
Vibrio anguillarum	1:10 MOI 1:50 MOI 1:100 MOI	Rare-none	2–3	Visual
Vibrio brasiliensis	1:10 MOI 1:50 MOI 1:100 MOI	Rare-none	3	Visual
Vibrio harveyi	1:10 MOI 1:50 MOI 1:100 MOI	Rare-none	3	Visual
Perkinsus marinus	1:10 MOI	None	NA	Visual
Physical stress (notching alone)	Shell notched 5 days prior	None	NA	Visual, & bacterial neutralization
Physical stress (puncture and hemolymph withdrawal)	Punctured and 250 μl removed	Numerous	0.5–1	Visual, & bacterial neutralization

(incubations shorter than 4 days or longer than 6 resulted in poorer ET stimulation), hemocytes from punctured and unstimulated oysters were collected and treated (incubation times, DNAse I controls, centrifugation, SYTOX green, 495 nm excitation, 525 nm emission) in the same manner outlined above; however, no further stimuli were used to enhance ET production in these oyster hemocytes. Cells were fixed with 1% formalin 30 min after the addition of SYTOX green to prevent differences in etosis evaluation due to the time between the first and last photos collected. Cell fixation did not appear to alter the SYTOX signals during this period. Hemocytes from non-stimulated, non-punctured oysters served as controls allowing the assessment of how physical stress (tissue wound) impacts ET production.

2.3.3. Visual confirmation and quantification

A combination of phase contrast and fluorescence microscopy (495 nm excitation, 525 nm emission) were used to calculate the percentage of viable cells vs etotic cells, as well as ET size. Viable cells were calculated by a lack of SYTOX green binding to their nuclei, and etotic cells were differentiated from dead cells by their characteristic thread-like shapes. Three separate fields of view (0.22 mm) were photographed per well (3 control and 3 experimental wells for a total of 18 photos per oyster) for etosis rate calculations. Photos were taken ~1.5 h after initial hemolymph collection. ET size data was calculated using these photos by converting images to grayscale, then using the threshold function in ImageJ [31] to record ET area in pixels on 100 independent non-touching cells from oysters within each ploidy.

2.4. Evaluation of antibacterial activity associated with etosis

2.4.1. Bacterial neutralization assay design

The puncture method outlined in 2.3.2 was found to be the most effective method used for ET stimulation based on visual assessment, thereafter all bacterial neutralization experiments followed the puncture method. The first bacterial neutralization (*Aliiroseovarius crassostreae;* section 2.4.2) assays were used to validate visual observations made using SYTOX green comparing puncture vs non-punctured oysters. The second set of bacterial neutralization assays (*Enterococcus faecalis;* section 2.4.3) were carried out for ploidy comparisons. During these experiments, separate well plates were prepared in tandem, to allow for visual assessment and confirmation of ETs (following methods in 2.3.3) without disrupting bacterial neutralization experiments.

In both experiments, the neutralization of bacteria via ETs was examined following the general methods described by Refs. [19,32] with slight modifications. In brief, 96-well plates were seeded with 100 μl of oyster hemocytes (3×10^5 cells/well, 6 wells per tested oyster) suspended in SASW (25 PSU). DNAse I was added to half of the wells (3 of 6 wells per oyster) with oyster hemocytes to degrade ETs and serve as internal controls. Wells were then incubated for 25 min at 20 °C allowing hemocytes to adhere. Following the incubation, bacteria (either *A. crassostreae* or *E. faecalis*, see below) were added to all wells at a concentration of ~4000 CFU per well (measured using OD 600). Plates were centrifuged at 250g for 10 min to increase bacteria and ET contact then incubated at 20 °C for 5 h. Contents from replicate wells (3 per treatment, per oyster) were then pooled together (to provide a more stable observation per oyster) with 100 μl of the combined well content removed, appropriately diluted, and plated onto species-appropriate agar.

2.4.2. Aliiroseovarius crassostreae

A. crassostreae is a Gram-negative ecologically relevant bacteria known to be the causative agent of juvenile oyster disease. This species was used in the first round of bacterial neutralization experiments to assess the puncture method. Prior to experimentation, bacteria were grown on marine agar for 2 days at 28 °C. Experiments were performed

as outlined above. Well contents for this assay were plated on marine agar and incubated at 28 °C for 48 h after which colonies were counted. Both punctured and non-punctured diploid and triploid oysters were used in this experiment. Experiments occurred during May.

2.4.3. *Enterococcus faecalis*

The second set of ET bacterial neutralization assays occurred between June and August and were performed utilizing Gram-positive spectinomycin-resistant *E. faecalis*. *E. faecalis* was grown on spectinomycin supplemented brain heart infusion agar (SSBHI) for 24 h at 37 °C prior to the experiment. Only puncture stimulated oysters were compared using *E. faecalis* assays. Slight modifications were made during these assays such as the addition of spectinomycin (0.12 mg/ml final concentration, ThermoFisher Scientific, Waltham, MA, USA) and Cytochalasin D (10 μg/ml, ThermoFisher Scientific, Waltham, MA, USA) which were added at the same time as *E. faecalis*. Cytochalasin D inhibits phagocytosis of bacteria, therefore eliminating bacterial reduction due to internalization and intracellular killing and further helping to isolate the impact of ETs on bacterial viability. Pretreatment of neutrophils with Cytochalasin D has been shown to negatively impact ET formation [33], which is why Cytochalasin D was added at the same time as the bacteria after peak etosis periods had already been reached to avoid such interference. Spectinomycin was used to remove any native bacteria acquired during hemolymph collection helping to reduce variability. Following the 5-h incubation of bacteria with ETs, well contents were again pooled between replicates, diluted, and plated onto SSBHI agar. Inoculated plates were incubated for 24 h at 37 °C after which colonies were counted. Previous work showed that the viability of the *E. faecalis* strain used was not impacted by prolonged incubations (>5 h) in SASW at various salinities (15–35 PSU) (unpublished results).

2.5. Immunofluorescence microscopy

An immunofluorescence assay was performed to identify histones in extracellular traps and confirm that the nets detected represent authentic etotic nets. Puncture stimulated hemocytes were seeded in 24-well plates containing poly-D-lysine-coated glass coverslips (Bioland Scientific, Paramount, CA, USA). Following peak etotic time points (assessed previously using SYTOX green on separate wells) cells were fixed using 2% paraformaldehyde, then washed (3 times) using phosphate buffered saline (PBS), permeabilized with 0.1% Triton x-100 for 5 min and washed 3 times in PBS. Cells were then blocked with 2% bovine serum albumin (BSA) and 0.1% saponin in PBS for 1 h at 4 °C. Cells were incubated overnight at 4 °C in a solution of 1% BSA, and 0.1% saponin with rabbit anti-H2A IgG (GeneTex, Irvine, CA, USA) diluted 1:200 in PBS. The following day, cells were washed three times with PBS and incubated with the secondary antibody (goat anti-rabbit IgG, labeled with DyLight488, GeneTex, Irvine, CA, USA) diluted (1:300) in PBS. Finally, cells were washed (3 ×) with PBS, mounted onto glass slides with VECTASHIELD containing 4′,6-diamidine-2′-phenyl-indole dihydrochloride (DAPI; Vector Laboratories, Newark, CA, USA), and examined by fluorescence microscopy using a Nikon Eclipse TE 2000-S microscope equipped with NIS-Elements D imaging software. Co-localization of histones with DNA from extruded chromatin is a commonly used feature for the differentiation of ETs from non-etotic cell death pathways.

2.6. Statistical analysis

All data were analyzed and are presented as the mean ± the standard deviation unless otherwise stated. Assumptions of normal distribution and homoscedasticity were tested using Shapiro–Wilk and Bartlett's tests, respectively. All percentage data (bacterial neutralization, cell proportions, etotic rate) were arcsine square root transformed. Significant differences were determined using a Student's t-test or a one-way analysis of variance (ANOVA) for parametric data, while a Kruskal-Wallis test was used when data failed to meet parametric assumptions. The relationship of bacterial neutralization to hemocyte subpopulations and ROS production were investigated using a Spearman's rank correlation. All statistical analyses were performed using R statistical software [50] with differences considered significant if p values were less than 0.05. All animals were treated separately with the number used in each experiment specified in figure legends.

3. Results

3.1. Stimulation of extracellular traps in the eastern oyster

Numerous stimuli were tested for their ability to stimulate ETs in diploid and triploid eastern oysters, with ET visualization assessed using DNA binding stain SYTOX green. When added to eastern oysters hemocytes in vitro, the vast majority of stimuli previously used in other organisms were incapable of producing ETs in a reliable and repeatable manner that surpassed the background rate of spontaneous etosis, regardless of ploidy. Qualitative assessments of the potency of tested stimuli to produce ETs are summarized in Table 1. Of the previously published stimuli, calcium ionophore (A23187) and live bacteria (*Vibrio coralliilyticus* specifically) were the two best performing stimuli, occasionally producing visible ETs in stimulated wells, however, these stimuli were found to be too variable to be reliably used. Among all the stimuli tested, the muscle puncture (in vivo stimulation) method was found to be the most reliable, and consistently able to produce etotic cells without the need for further stimulation (ETs measured in vitro). This method produced ETs relatively rapidly (peak ET production < 1-h post hemolymph withdrawal) and consistently (all punctured oysters presenting some ETs). ETs formed structures similar to those described in other organisms, being primarily diffuse "puffball-like" (Fig. 1A) or "comet-like" (Fig. 1B–D), were often aggregated in the wells, and occasionally aligned with directionality (Fig. 1E). The co-localization of DNA and H2a histone was also observed using anti-histone H2a specific fluorescent antibodies (Fig. 1F&G). In all trials, there was a basal rate of etosis observed to occur in unstimulated hemocytes (spontaneous etosis), which accounted for about 4.36% (±1.3%) of dead hemocytes among diploid and triploid oysters combined. When oysters were stimulated using the puncture protocol, spontaneous etosis significantly increased (Kruskal-Wallis test, $p < 0.005$, n = 23) to an average of 17.58% (±13.7%) of dead hemocytes among diploid and triploid combined (Fig. 2). No ETs were observed in any DNAse I treated controls.

3.2. Effect of ploidy on extracellular trap size and rate

Triploid oysters were observed to produce significantly (Kruskal-Wallis test, $p = 0.042$, n = 15) more ETs than diploid oysters with 23.2% (±14.5%; 0.8% of total cell count) of dead triploid hemocytes and 11.1% (±7.7%; 1.6% of total cell count) of dead diploid hemocytes being etotic after puncture stimulation (Fig. 2). In addition to producing ETs more frequently, the surface areas of ETs produced by triploid oysters were also significantly larger (Student's t-test, $p < 0.005$, n = 200) than those produced by diploid oysters with average ET size being 272 μm^2 (±112.6 μm^2) and 169 μm^2 (±69.8 μm^2) per etotic hemocyte, respectively (Fig. 3).

3.3. Bacterial neutralization from extracellular traps

The puncture method was compared to unstimulated controls for its capacity to display bactericidal activity (commonly associated with ETs) via incubation with *A. crassostreae* to validate visual observations of ETs (performed on separate plates in tandem), using both diploid and triploid oysters. All bactericidal results are normalized to bacterial counts from an oyster's DNAse I control wells (counts from DNAse I plates are considered 100% viability). Following the 5-h incubation, bacterial counts from wells containing puncture-stimulated hemocytes showed

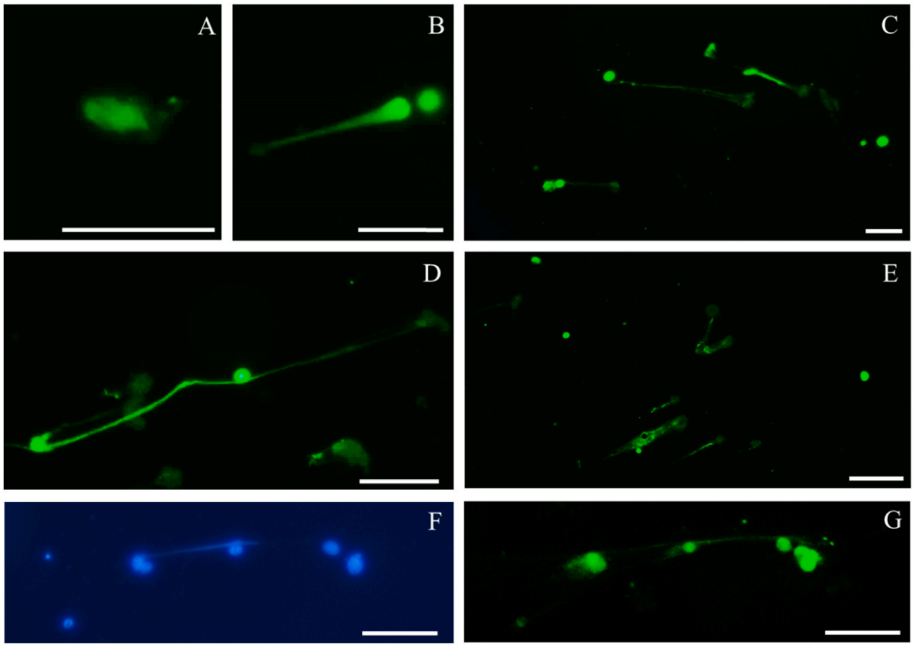

Fig. 1. Extracellular traps formed within an hour after hemolymph collection following puncture stimulation in both diploid and triploid eastern oysters (photographs presented here were taken from triploid oysters). (A) "Puffball-like" and (B–D) Comet-like ETs formed by oyster hemocytes and stained using SYTOX green. (E) ETs display moderate directionality and aggregation in the well plate. (F–G) Decondensed chromatin stained using DAPI (F, Blue) and decorated with H2a histones (G, green) labeled using immunofluorescence. Scale bars A-E represent 25 μm, F & G are 10 μm.

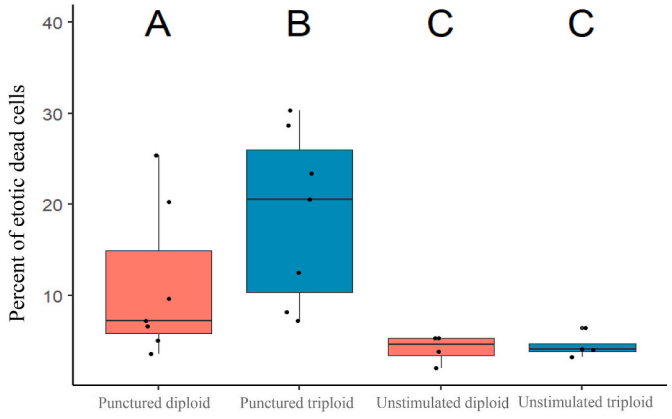

Fig. 2. Data presented using box and whiskey plots indicating the percentage of etotic cells normalized to the number of dead cells present in a well with the bars indicating their upper and lower quartile ranges. Black dots represent individual oyster measurements of etosis rates. The puncture method was the only stimulation used in this experiment. Different letters indicate statistically significant differences between treatments ($p < 0.05$, Kruskal-Wallis test).

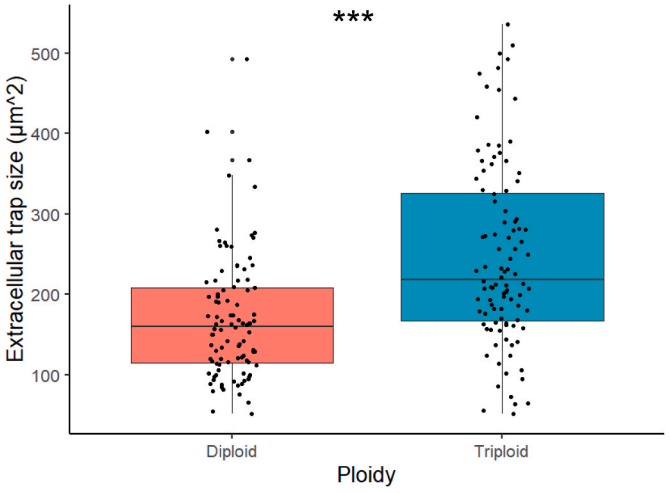

Fig. 3. Average surface area of ETs formed by diploid (red, n = 100), and triploid (blue, n = 100) hemocytes, 1 h after hemolymph collection following puncture stimulation. Only isolated (non-touching) ETs were measured to avoid accidental inflation of ET size and are represented as black dots. *** indicates a highly significant difference, and the bars represent the upper and lower quartiles of the data ($p < 0.005$, Student's t-test).

significantly greater reductions (Student's t-test, $p = 0.0127$, n = 23) in bacterial numbers than the unstimulated controls with an average reduction of 68.4% (±26%) and 38.5% (±28%) compared to their DNAse I controls, respectively (Fig. 4).

Follow up experiments utilizing Cytochalasin D to reduce the influence of phagocytosis, compared ploidy's impact on ET derived neutralization of *E. faecalis* after puncture stimulation. At 5 h post incubation of bacteria with ETs, there was a significant (Student's t-test, $p < 0.01$, n = 36) reduction in bacterial counts (56.4% ± 21.7% when combining diploid and triploid oyster results; Fig. 5) in ET positive wells compared to control wells incubated with DNAse I. There were no significant differences in bacterial neutralization between separate trials (from June to August; 1-way ANOVA), although there was a general trend of decreasing ET efficacy as time progressed (Supplemental Fig. 1). Triploid and diploid differences in bacterial neutralization from ETs were not significant from one another with an average of 56.8% (±19.4%), and 55.9% (±24.7%) of bacteria neutralized respectively.

3.4. Relationship between hemocyte subpopulations and ROS activity with etotic activity

To understand what hemocyte subpopulations are primarily responsible for the efficacy of ETs as well as the involvement of cellular ROS, we related data collected from the flow cytometer to results from the bacterial neutralization assays. Diploid and triploid data were analyzed together as there was no significant difference in neutralization based on ploidy level. *E. faecalis* neutralization was observed to be significantly (Pearson's correlation, $p = 0.0190$, n = 36) and positively ($r^2 = 0.389$) associated with the percentage of agranulocytes present in the hemolymph (Fig. 6A). Granulocytes prevalence had a negative relationship of similar magnitude. If both bacterial neutralization assays (*A. crassostreae* and *E. faecalis*) are analyzed together, this relationship of

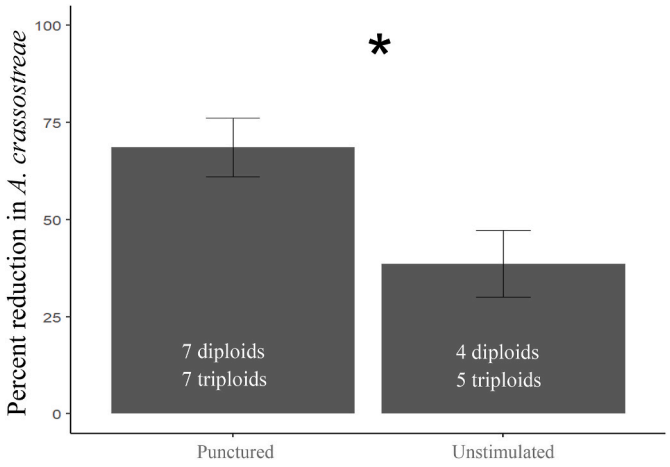

Fig. 4. Antibacterial activity presented as the percentage (± standard error) of *A. crassostreae* bacteria reduced in wells with ETs relative to wells with DNAse I. Bars represent the combined results from both diploid and triploid oysters. Hemocytes from oysters were either stimulated using the puncture method (left) or not (right). * Significant difference between stimulated and unstimulated hemocytes ($p = 0.0127$, Student's t-test, n = 23).

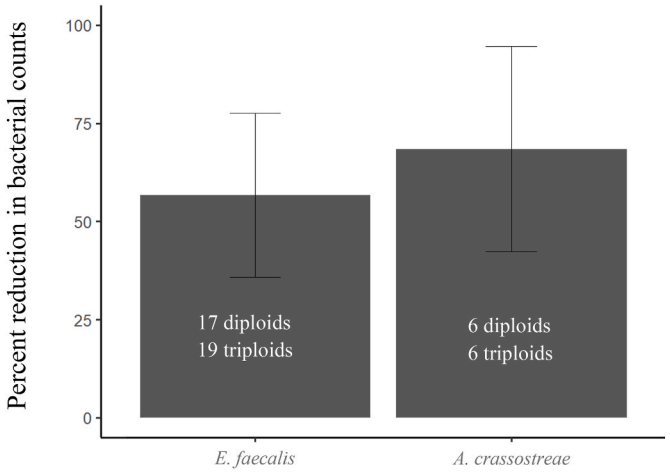

Fig. 5. Average (± standard error) ET derived neutralization of *E. faecalis* (n = 36) and *A. crassostreae* (n = 12) in vitro relative to their DNAse I controls after stimulation via the puncture method. Diploid and triploid results were graphed together, as ploidy differences were non-significant.

agranulocytes prevalence to neutralization is strengthened (Pearson's correlation, $p<0.005$, $r^2 = 0.413$, n = 48), however, this should be interpreted with caution due to differences between these assays.

The relationship between total cellular ROS and *E. faecalis* neutralization was found to be significantly and positively correlated for both agranulocytes ($p < 0.005$, $r^2 = 0.518$, n = 36) and granulocytes cell populations ($p < 0.05$, $r^2 = 0.441$, n = 36) although agranulocytes had the stronger relationship (Fig. 6B). In fact, agranulocyte and granulocyte ROS productions were strongly correlated with one another ($p < 0.005$, $r^2 = 0.746$, n = 36, S2). When comparing triploids and diploids, total ROS production at 30 min post incubation was consistently higher in triploids, but this trend was not significantly different for either granulocytes (Student's t-test, $p = 0.098$, n = 36) or agranulocytes (Student's t-test, $p = 0.111$, n = 36). Regardless of ploidy, granulocytes produced significantly more ROS than agranulocytes (Student's t-test, $p < 0.001$, n = 36, data not shown). Overall hemocyte size, and total cell concentrations (prior to standardization for the assays) were not significantly correlated with etosis efficacy.

4. Discussion

Marine bivalves inhabit volatile environments where they are frequently exposed to extreme environmental fluctuations and diverse microbial communities, and they rely on their robust immune responses to combat these stressors. The newly described cellular-mediated innate immune response, ETs, likely contributes to bivalve resistance; however, there are currently few studies addressing invertebrate ETs or their functionality. In this study, we assessed the potency of several stimuli to induce etosis in hemocytes from diploid and triploid eastern oysters, then linked organismal traits to the production and efficacy of ETs using a novel and reliable method of stimulation.

ET producing cells are typically one of the first cells to respond to tissue damage as ETs play a critical role in preventing the dissemination of pathogens, regulating inflammatory responses, and stimulating tissue growth factors [34]. As such, it is unsurprising that the puncture method outlined in this paper stimulated ET production in oyster hemocytes. *In vivo,* stimulation of hemocyte derived ETs following an injury was observed previously by Ref. [21]; in which etotic responses (revealed by histology and High-performance liquid chromatography) were present in the pacific oyster (*C. gigas*) following an adductor muscle puncture (via syringe). Our results build on this previous work with in vitro assays showing that the puncture method outlined here can significantly increase the basal rate of spontaneous etosis, measured both visually and via bacterial neutralization. Moreover, this manner of etosis stimulation produced ETs of similar shape and appearance relatively rapidly (<1 h), compared to previous studies in which peak etosis can be observed 4–24 h post-stimulation [5,10]. When testing previously reported stimuli, ET production was highly variable between individual oysters with highly responsive oysters (strong etotic response to biotic or abiotic stimuli) oftentimes observed to have relatively high rates of spontaneous etosis in unstimulated control wells. High interindividual variation has previously been documented among human donors [35] with host factors such as underlying inflammatory diseases [36], metabolic state [37], host microbiomes [38], and high glucose levels [39] contributing to the high interindividual variability. Thus, the high variance observed between individual oysters in this study could depend on differences in host-dependent variables like oyster energetic reserves. Resulting from their sterility, triploid oysters are known to have enhanced glycogen content [24] compared to diploids, possibly explaining the higher rates of spontaneous etosis in punctured triploids, although this requires further testing. Regardless, the puncture method was able to increase the basal rate of spontaneous etosis in both ploidies, making it useful for identifying traits associated with ET production in the eastern oyster.

Circulatory hemocytes in oysters represent a variety of cells with diverse functions primarily differentiated by intracellular complexity and oftentimes categorized as either granulocytes or agranulocytes [40]. Using this broad categorization of invertebrate blood cells, we were able to find a positive and significant association between the percentage of agranulocytes in oyster hemolymph and etosis efficacy (measured via *E. faecalis* neutralization), suggesting that these cells are the primary effectors of etosis in the eastern oyster. ETs in invertebrates are known to be critical during the encapsulation of foreign material [5,17–19] which aligns with previous studies reporting agranulocytes to be important for wound plugging in Pacific oysters [41], aggregation in Sydney rock oysters (*Saccostrea glomerata*; [42], and encapsulation in eastern oysters [43]. Furthermore, the positive (and significant) relationship between agranulocyte ROS production and ET efficacy suggests they may play a role in etosis, as the initiation of etotic pathways and ET formation is often associated with cellular ROS production in invertebrate and vertebrate systems [5,10,19,20]. Typically, granulocytes are observed to produce greater amounts of ROS compared to agranulocytes in oysters [44], and although this was also observed in this study, granulocyte ROS production had a weaker relationship to ET efficacy than agranulocytes further highlighting the role of agranulocytes in etosis. Granulocyte prevalence was negatively associated with ET efficacy, so the association

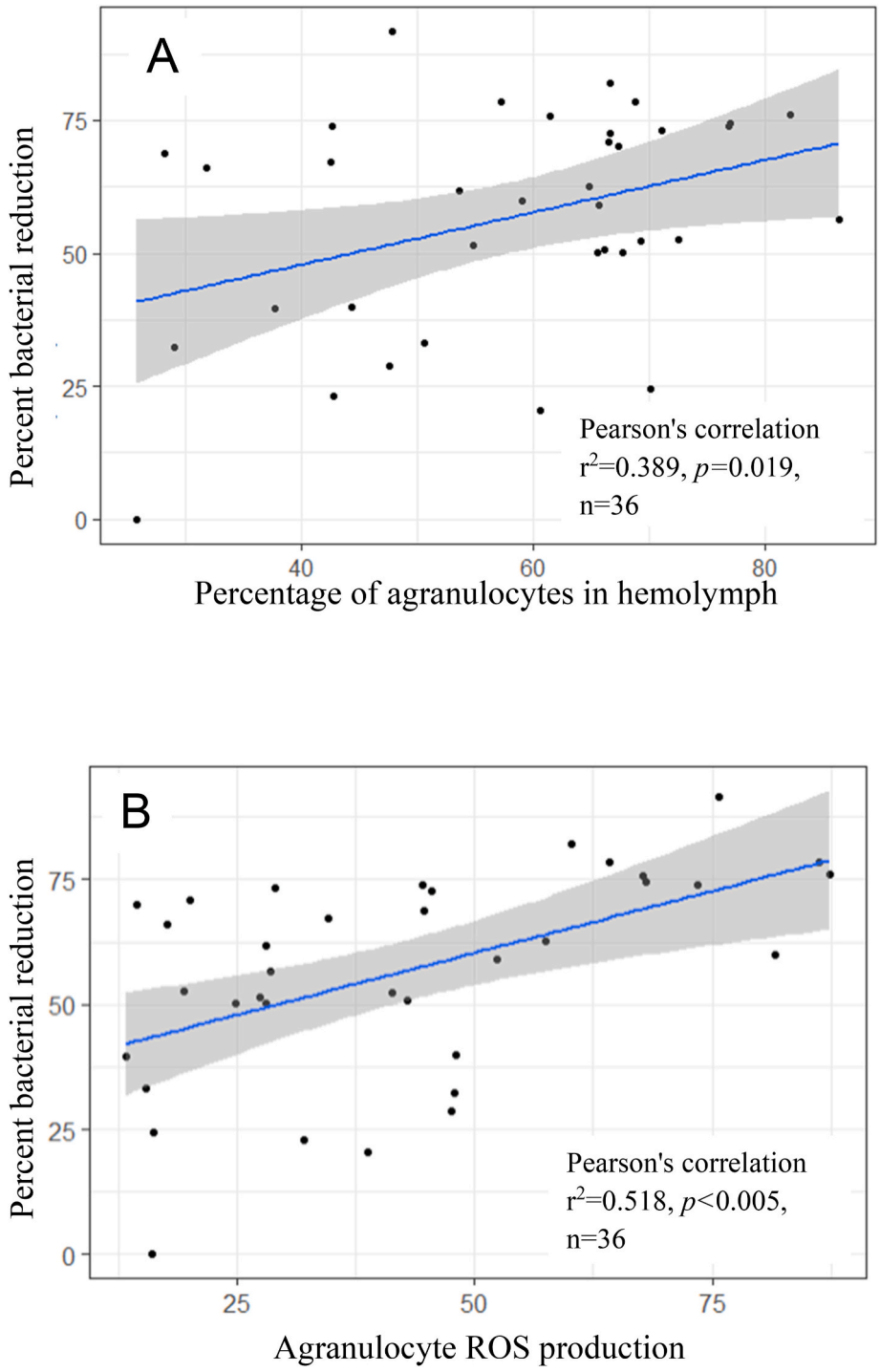

Fig. 6. A) Relationship between ET derived *E. faecalis* neutralization and the percentage of agranulocytes in oyster hemolymph (A) or ROS production by agranulocytes (B, presented as relative fluorescent units). Neutralization is defined as the percentage of bacteria removed in experimental wells compared to DNAse I controls.

of granulocyte ROS production to ET efficacy may be an artifact of a higher ROS baseline in animals with strong etotic responses. The specialization of hemocyte subtypes is expected, and more robust etosis assays may be achievable as methods to identify and isolate cell subtypes are improved upon, allowing for higher proportions of etotic cells to be assessed.

The strong antibacterial responses of ETs have been demonstrated in numerous systems [5,8,19,45], with our data demonstrating a similar antibacterial activity in the eastern oyster. Overall, ET production in the eastern oyster was found to reduce bacterial concentrations by more than 50% in Gram-positive bacteria and 65% in Gram-negative bacteria. All of the assays were performed during the summer, and ET derived neutralization of Gram-positive bacteria decreased as trials progressed (June–August), suggesting a possible seasonal influence on ET responses. The influence of seasons on immune responses in ectothermic organisms is well documented [28] as temperature, food availability, and gonad development strongly influence how an animal can respond to pathogens and stress. Triploidy had a positive effect on the production of ETs and the overall size of ETs produced, which surprisingly had no significant impact on their ability to neutralize bacteria. There are more

than 80 distinct proteins associated with ETs efficacy [45] and although triploids do have more DNA and presumably more histones, their larger cells may contain the same quantity of other proteins related to etosis antibacterial efficacy. For example, the larger cell size of triploids would theoretically be associated with an increase in the number of mitochondria present in a triploid cell, however, when compared to diploids mitochondrial counts were similar (unpublished preliminary results), demonstrating that factors other than size (nuclear or total cell) dictate intracellular composition. Additionally, antimicrobial products such as complement proteins [46–48], and lysozyme [46] are produced in the same quantities between diploid and triploid fish from several species, showing that ploidy may have limited influence on the production of antimicrobial factors. Consequently, triploid animals may serve as a unique tool to isolate the influence of increased DNA on certain microbes, particularly for pathogens able to evade or express virulence factors in response to ETs [49].

Although having measurable effects, one limitation of this study is that visualized etotic cells only accounted for a small percentage of the total cells present in oyster hemolymph even after stimulation. We believe that the low proportion of etotic cells is likely a result of two primary factors inherent to our study. The first is the short incubation period before etosis quantification. All visual assessments in this study were done within 2 h post hemolymph withdrawal, which is substantially shorter than the peak times used in previous studies working with invertebrates [10,17,21]. For example [5], observed significantly more chromatin expulsion in crab hemocytes incubated with 0.1 μM PMA after 24 h than at 4 or 14 h. While lengthening the incubation time might enable similar findings, we specifically used short incubation periods as etosis is considered a rapid innate immune response [45], and prolonged incubations may not accurately represent immunological relevance. Secondly, the lack of previous data concerning our system required the use of whole hemolymph, and as such ET rates could have been increased if work was done solely using agranulocytes. Regardless, our current findings showed promising results on ET production, which allows for further experiments to expand and focus on agranulocytes, a subtype historically believed to be less important as an active immune effector.

In conclusion, these results demonstrate that eastern oysters are capable of producing ETs, and tissue damage via a puncture wound is a reliable method for the induction of etosis. ETs stimulated via the puncture method were rapidly produced after hemolymph collection and were potently antimicrobial. Moreover, we identified that agranulocytes may be the primary initiators of etosis in oyster hemolymph, and their capacity to generate ROS is linked to their efficacy, although more studies are needed to confirm these findings. Surprisingly, no ploidy-based difference in bacterial neutralization was observed despite triploids producing more frequent and larger ETs. These data highlight our fragmentary understanding of what regulates ET production and efficacy, particularly in non-model organisms. As such, the increased rate and size of ETs produced by triploids (there are now numerous vertebrate and invertebrate species in which triploidy can be induced, see Ref. [27]) may aid in the identification of new stimuli (may be a needed step for new species), increase the sensitivity of assays (particularly visual based assays), and help determine the functional role of chromatin (as opposed to proteins present in the nets) in ET antimicrobial efficacy. Moreover, these traits may predispose triploids to the antagonistic features of ETs which could itself be useful as this is currently one of the most investigated aspects in the field of etosis with no invertebrate models currently identified. Future works should be conducted to validate the puncture method in other species, as well as identify what specific proteins may lead to ET efficacy. Additionally, far more work is needed to understand how ETs function in animals (both the protective and antagonistic features of ETs) and relate this information to their ecology. Triploid animals offer a unique tool for the study of etosis in addressing what components of ETs lead to their protective or antagonistic traits and the relationship between DNA content and ET functionality.

Authors statement

CB and BA designed the study. CB and YB performed the experiments. BA secured the funding. CB drafted the initial manuscript with guidance from YB and BA. All authors edited and approved the manuscript before submission.

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Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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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.2023.108992.

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