

Journal of Innate Immunity

Manuscript:	JIN-2017-8-3/R1
Title:	Echinochrome A release by red spherule cells is an iron-withholding strategy of sea urchin innate immunity
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Keywords:	antimicrobial, coelomocytes, damage response, degranulation, invertebrate immunity
Type:	Research Article

This is the peer-reviewed but unedited manuscript version of the following article: Coates CJ, McCulloch C, Betts J & Whalley T, 'Echinochrome A Release by Red Spherule Cells Is an Iron-Withholding Strategy of Sea Urchin Innate Immunity', *Journal of Innate Immunity*, 2018;10:119–130 (DOI: 10.1159/000484722)]. The final, published version is available at <http://www.karger.com/?doi=10.1159/000484722>

Echinochrome A release by red spherule cells is an iron-withholding strategy of sea urchin innate immunity

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Running title: Immune-competence of red spherule cells

Abstract

Cellular immune defences in sea urchins are shared amongst the coelomocytes – a heterogeneous population of cells residing in the coelomic fluid (blood equivalent) and tissues. The most iconic coelomocyte morphotype is the red spherule cell (or amebocyte), so named due to the abundance of cytoplasmic vesicles containing the naphthoquinone pigment, echinochrome A. Despite their identification over a century ago, and evidence of anti-septic properties, little progress has been made in characterising the immune-competence of these cells.

Upon exposure of red spherule cells from sea urchins, *Paracentrotus lividus* and *Psammechinus miliaris*, to microbial ligands, intact microbes and damage signals, we observed cellular degranulation and increased detection of cell-free echinochrome in the coelomic fluid *ex vivo*. Treatment of the cells with ionomycin, a calcium-specific ionophore, confirmed that an increase in intracellular levels of Ca²⁺ is a trigger of echinochrome release. Incubating Gram-positive/negative bacteria as well as yeast with lysates of red spherule cells led to significant reductions in colony-forming units. Such antimicrobial properties were counteracted by the addition of ferric iron (Fe³⁺), suggesting that echinochrome acts as a primitive iron chelator in echinoid biological defences.

Keywords: coelomocytes; antimicrobial; damage response; degranulation; invertebrate immunity; *Paracentrotus lividus*; *Psammechinus miliaris*;

1. Introduction

Lacking adaptive immune capabilities, invertebrates such as insects and decapod crustaceans are used routinely to study the mechanisms and biological complexities of innate immunity. Unlike those invertebrates, sea urchins are deuterostomes – placing them on the same ancestral branch of life as chordates prior to the divergence of these metazoan lineages. The fully sequenced genome of the purple sea urchin, *Strongylocentrotus purpuratus*, has revealed the shared origin of many immune gene families and the genetic synonymy between vertebrates and echinoderms [1, 2]. The canonical view of invertebrate innate immunity describes three arms of defence – (1) physical barriers such as the exoskeleton, (2) cellular activities within the equivalents of blood, namely coelomic fluid or haemolymph, and (3) humoral factors that include (but are not limited to) antimicrobial peptides, lysozyme and complement-like proteins [reviewed by 3, 4, 5]. Cell-derived immunity in sea urchins is provided by the coelomocytes – a heterogeneous population consisting of four distinct morphotypes: phagocytes, vibratile cells, colourless and red spherule cells. The former can be subdivided into discoidal, polygonal and small phagocytes, which express a myriad of immune effectors belonging to the (Sp)Transformer gene family [6, 7]. The phagocytes are tasked with identifying, ingesting and destroying invading pathogens, whereas the vibratile cells are said to be involved in hemostasis [8, 9, 10]. The immunological function of colourless spherule cells (CSCs) remains unclear, although some evidence supports a cytotoxic role [11].

Despite progress being made in enhancing our understanding of sea urchin immunity over the past 50 years, little is known about the pigmented coelomocytes in adult coelomic fluid, namely red spherule cells (RSCs). RSCs owe their distinct colouration to echinochrome A, a 1,4-naphthoquinone packaged within cytoplasmic vesicles (or granules) [12]. Initial studies on RSCs provided circumstantial evidence in support of immune-competence [13, 14, 15], further strengthened when Service and Wardlaw (1984) [16] deduced the antibacterial activity of echinochrome A from the edible sea urchin, *Echinus esculentus*. Following this, Gerardi *et al.* (1990) [17] fractionated *Paracentrotus lividus* coelomocytes and monitored bactericidal activity of RSC lysates toward several marine *Vibrio* species (100% inhibition of bacterial growth was achieved within 12 hours). The authors confirmed that RSC immune activity was independent of lysozyme, but the mechanism of inhibition of microbial growth remained unknown. More recently, the levels of RSCs in the coelomic fluid of *P. lividus* have been

proposed as a good indicator of environmental stress due to their enhanced presence in coelomic fluid in animals living in waters contaminated with heavy metals and/or xenobiotics [18, 19, 20].

Renewed interest in culturing *P. lividus* and its continued development as an ecotoxicology model presents a greater need to document the immune-capacity and health status indicators of this commercial shellfish. The overall aim of our study was to assess the putative role of RSCs in innate immunity. This was addressed by interrogating (1) the physiological responses of RSCs in the presence of microbes, their exoplasmic sugar moieties (ligands) and damage-related signals, and (2) the nature of the anti-infective properties of liberated contents of cytoplasmic vesicles, i.e. echinochrome A. Our findings demonstrate a capacity of RSCs to respond to pathogen/damage-associated molecular patterns (e.g. lipopolysaccharides) by undergoing exocytosis through a mechanism most likely involving Ca^{2+} influx. The extracellular echinochrome A targets bacteria and yeast *in vitro*, leading to reductions in colony forming units. The broad antimicrobial activity of RSC lysates can be offset by the addition of iron – leading us to surmise that echinochrome’s iron chelating properties impede microbial colonisation of the sea urchin host.

2. Materials and Methods

2.1 Maintenance of sea urchins

Paracentrotus lividus adults (37.4 ± 1.7 cm test diameter) were obtained from FAI Ardtoe Marine Research Facility, Ardtoe, UK. *Psammechinus miliaris* adults (32.6 ± 2.7 cm test diameter) were collected from coastal waters near Oban and Millport, UK. In the laboratory, sea urchins were maintained in closed circulation tanks (30 individuals per 80 L) between 6°C and 10°C containing a mixture of artificial (Instant Ocean) and filtered seawater, and fed dried kelp *ad libitum*. Particulates were siphoned daily in addition to 25% of seawater being exchanged weekly.

2.2 Coelomocyte removal and preparations

All chemicals and reagents (including microbial ligands and membrane phospholipids) of the highest purity available were purchased from Sigma Aldrich (Dorset, UK) unless stated otherwise.

Coelomic fluid (up to 5 mL) was extracted from sea urchins using a 26-gauge hypodermic needle attached to a sterile syringe containing an equal volume of pre-chilled anti-coagulant (20mM Tris-HCl, 0.5M NaCl, 70mM EDTA, pH 7.5). Each animal was sprayed on the oral (ventral) surface with 70% ethanol prior to needle insertion through the peristomial membrane. Extracted coelomocytes were enumerated using an improved Neubauer haemocytometer or plastic counting chambers (FastRead counting slides, Immune Systems, Torquay, UK). Further cytology work was performed using an Axiovert 135 inverted microscope.

Continuous 40-60% Percoll gradients were used for cell fractionation. Gradients were prepared in sterile Beckman polyallomer tubes using 4 mL Percoll diluted with an equal volume of 2x anti-coagulant (40mM Tris, 1M NaCl, 140mM EDTA, pH 7.5). The mixture was centrifuged at 30,000 x g using a fixed angle rotor (23.5°) for 30 minutes at 4°C. The coelomic fluid extract and anti-coagulant mixture were layered onto gradients and centrifuged at 400 x g using a swing-out rotor (JS 24.15) for 15 minutes at 4°C. Polyallomer tubes were pierced using sterile 26-gauge hypodermic needles and fractions were collected (1mL) into pyrogen-free, conical tubes containing 4 mL anti-coagulant buffer. Samples were further centrifuged for 10 minutes at 500 x g (4°C), the supernatant was discarded and coelomocytes were re-suspended in 500 µL artificial coelomic fluid (ACF) (10mM CaCl₂, 14mM KCl, 50mM MgCl₂, 398mM NaCl, 1.7mM NaHCO₃, 25mM Na₂SO₄, 10mM HEPES, pH 7.4; [21]). The homogeneity of each fraction was assessed by microscopy – only those populations consisting of >95% red spherule cells were used.

2.3 Effect of microbial and damage-related ligands on coelomocytes *in vitro*

Approximately $2.5 \times 10^4 \pm 3.9 \times 10^3$ isolated RSCs (in 500 µL ACF) were seeded into each well of a 24-well (pyrogen-free) culture plate and left for 30 minutes at room temperature ($\leq 20^\circ\text{C}$) to settle before centrifugation at 250 x g for 5 minutes at 4°C with no braking. After centrifugation, microbial ligands ranging in concentration from 15–75 µM (mannan from *Saccharomyces cerevisiae*, laminarin from *Laminaria digitata*, lipopolysaccharides from *Escherichia coli* and lipoteichoic acids from *Staphylococcus aureus*) and inner membrane phospholipids at 25–50 µM (phosphatidylserine and phosphatidylethanolamine) were added to each well and incubated at room temperature for 1 hour. Controls, absent ligands, were run concurrently. Cellular activity was recorded by calculating the percentage of RSCs that released echinochrome (fully de-granulated). For each well, randomly chosen fields of view were selected until 200-300 cells had been assessed. N.B. the viability of extracted (unstimulated)

coelomocytes at room temperature (<20°C) was monitored *in vitro* over a 4 hour period using trypan-blue exclusion (0.2% w/v, [22]). Colourless spherule cells were selected for this task due to the technical challenges encountered when staining the pigmented RSCs. Cells staining blue were recorded as dead.

Overnight cultures of Gram-positive bacteria (*Bacillus megaterium*, *Bacillus subtilis*), yeast (*Saccharomyces cerevisiae* strain AH22) and Gram-negative bacteria (*Escherichia coli* strain M15) were used to challenge isolated RSCs *in vitro*. *S. cerevisiae* was cultured at 30°C in YEPD broth (1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, 2% (w/v) D-glucose, pH7) and all bacteria were grown at 37°C in Lysogeny broth (1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 1% NaCl, pH7). Optical density readings at 600 nm were recorded for each microbe using a Novaspec-4049 Spectrophotometer. An OD₆₀₀ value of 1.0 is equal to ~3 x10⁷ cells/mL for *S. cerevisiae* and ~1.2 x10⁹ cells/mL for *E. coli*, *B. megaterium* and *B. subtilis* [22]. Microbial cultures (1 mL) were centrifuged at 1000 x g for 5 minutes (4°C) and re-suspended in 1ml PBS pH 7.4 prior to dilution into pre-prepared culture wells containing RSCs (2.5 x10⁴ ± 3.9 x10³). Sea urchin coelomocytes (suspended in ACF) were incubated in the presence of bacteria (2 x10⁶ cells/ml) or yeast (1 x10⁶ cells/mL) for 1 hour at room temperature (20°C) and responses to each microbe (i.e. de-granulation) were quantified as stated above.

The calcium-specific ionophore, ionomycin (Tocris, Avonmouth, UK), was used to test whether RSCs are reliant on elevated intracellular levels of Ca²⁺ for degranulation. RSCs were maintained *ex vivo* in ACF as detailed above, treated with 2, 4 or 10 µM ionomycin (stock solution prepared at 2 mM in DMSO) and observed at 0 and 60 minutes using microscopy. All images were captured using a 63x 1.2 NA objective on a Zeiss Axiovert 135 microscope attached to an AxioCam MRc camera system and analysed using Zen (Zeiss) and/or ImageJ software. Trypan-blue exclusion assays were used to determine viability of RSCs at 2 hours post-activation with 10 µM ionomycin.

Additionally, an interspecies comparison between mixed coelomocyte populations extracted from *P. lividus* and *P. miliaris* was performed *in vitro*. Coelomocytes were removed from sea urchins and processed as mentioned above but were not fractionated. Instead, ~4x 10⁵ cells were exposed to 25 µM of each microbial ligand (LPS, LTA, mannan) or 10 µM ionomycin and left for 30 minutes at room temperature in sterile 15 mL centrifuge tubes (and agitated gently).

Post-incubation, 15 μ L of the coelomocyte suspension was assessed for the proportion of intact (pigmented) red spherule cells using brightfield microscopy (x40).

2.4 Spectrophotometric detection of Echinochrome A release

Sea urchins were challenged with 3 μ g LPS per mL of coelomic fluid (\sim 5 μ M) via injection into the coelomic cavity through the peristomial membrane using a 26-gauge hypodermic needle. The amount of LPS injected was standardised using a modified formula presented in Smith et al. (1995) [23]: weight of sea urchin(g) \times 0.18 = X mL coelomic fluid. The accuracy of this formula to predict dosages was confirmed by removing all coelomic fluid (exsanguination) from a sub-sample of *P. lividus* ($n = 8$). Surfaces were sterilised with 70% ethanol pre- and post-treatment. Control injections consisted of ACF only. At 1 and 24 hours post inoculation, 1 mL of coelomic fluid was removed. Differential cell counts were performed using 15 μ L coelomic fluid, with the remaining sample volume (\sim 985 μ L) being centrifuged at 10,000 \times g for 5 min to remove the coelomocytes. The acellular coelomic fluid (i.e. supernatant) was placed in a quartz cuvette (1 cm path length) and absorbance values across the range, 300–700 nm, were recorded using an Ultrospec 2100 pro UV/Vis spectrophotometer. The effect of immune challenge on the acellular coelomic fluid was monitored via absorbance peaks at 346 nm and 480 nm, which are indicative of echinochrome A [16, 24].

2.5 Antimicrobial properties of red spherule cell-derived echinochrome A

Bacteria and yeast were grown as stated above; 1 mL of each culture was centrifuged, resuspended in PBS pH 7.4 and subsequently diluted to 1×10^6 microbes per mL. RSC fractions (\geq 98% homogenous) were centrifuged at 10,000 \times g (4°C) for 10 min, re-suspended in 1mL deionized water and vortexed to lyse the cells. Post-lysis, cell debris was pelleted using centrifugation (4,000 \times g for 5 minutes at 4°C) and the supernatant was retained on ice. Three assays were prepared for each microbe: (1) microbes alone (negative control), (2) microbes treated with 100 μ L RSC lysate (1×10^5), or, (3) 50 mM EDTA (positive control). Subsamples of RSC-lysates were spread onto agar to check for potential contamination.

After microbes were incubated at room temperature for 1 hour, samples were diluted in PBS so that \sim 200 colony-forming units (CFUs) were plated onto pre-prepared 2% agar (in YEPD for yeast and LB for bacteria). Two types of agar recipes were used for each treatment, one

containing regular medium and another supplemented with 0.05% (w/v) ferric ammonium citrate (FAC; Fe³⁺). FAC was selected as this form of iron is more readily available for microbes to utilise (in addition to being a hematinic). The inoculated plates were incubated for 24–48 hours; *S. cerevisiae* at 30°C, *E. coli*, *B. megaterium* and *B. subtilis* at 37°C. Absorbance readings of RSC lysates from 300 nm – 550 nm were recorded in the absence and presence of 200 µM FAC to assess whether iron formed complexes with echinochrome A.

2.6 Data handling

All data were gathered from experiments performed on at least three independent occasions (see individual figure legends for sample sizes), and are represented by mean values with 95% confidence intervals. Assays concerning ligands, microbes and damage signals *in vitro* were run in triplicate (3 technical replicates per biological sample). Analysis of variance (1- or 2-way) with Tukey's multiple comparison tests were utilised to assess data for significant differences at $p \leq 0.05$. Statistical analyses and figure preparation were carried out using GraphPad Prism v7.

3. Results

Response of red spherule cells to immune-stimulants in vitro

On average, 6.62×10^6 coelomocytes per mL of coelomic fluid were extracted from sea urchin (*P. lividus*; Figure 1A) adults, consisting of 70.7% phagocytes (55–84 %), 16.1% colourless spherule cells (7–24%), 9.5% red spherule cells (1.5–25.9 %), and 3.7% vibratile cells (<1–10.2%) (Figure 1B). Fractionation of mixed coelomocyte populations was achieved using 4060% Percoll gradients. Four cellular bands were observed in addition to a diffuse layer of debris found at the Percoll-coelomic fluid interface (Figure 1C & 1D). Bands 1, 2 and 3 consisted mainly of phagocytes, vibratile and colourless spherule cells, respectively. Homogeneity ranged from 82% to >95%. Phagocytes are generally sub-divided into discoidal, polygonal and small morphotypes, but an enumeration of these sub-types was not within the scope of our experimentation. RSCs made-up $\geq 98\%$ of band 4, with colourless spherule cells found to be the only contaminant (<2%).

Isolated RSCs responded to the presence of various immune-stimulants *in vitro* through the apparent exocytosis of cytoplasmic vesicles containing echinochrome A (Figures 2 – 4). The proportions of cellular degranulation in control samples ranged from 8.8–11.5%, whereas treatment of RSCs with either microbial ligands (LPS, LTA, mannan, laminarin), intact microbes (*E. coli*, *B. megaterium*, *B. subtilis*, *S. cerevisiae*) or inner membrane phospholipids (PS, PE), led to significant increases in echinochrome A release (ligands, $F_{(4, 30)} = 65$, $p < 0.001$; microbes, $F_{(4, 10)} = 32.27$, $p < 0.001$; damage, $F_{(2, 12)} = 96.59$, $p < 0.001$). Lipoteichoic acid (LTA) from Gram-positive bacteria was the most potent activator of RSCs (47%) across the concentration range 15–75 μM (Figure 2A). LPS from Gram-negative bacteria and β glucan (i.e. laminarin) from brown algae were not as effective as LTA at the highest dose tested (75 μM), 27.8% and 30.4% respectively, but were found to be significantly different to the control. On average, the presence of intact microbes led to a significant 2.5-fold increase in the proportion of de-granulated RSCs compared to the control (Figure 2B; *E. coli* > *S. cerevisiae* > *B. megaterium* > *B. subtilis*). No internalisation of targets (i.e. phagocytosis) was observed in these particular coelomocytes.

The second most potent inducer of RSCs *in vitro* was the negatively charged phospholipid, phosphatidylserine (PS). PS stimulated a 2.5-fold increase in RSC activity when compared to the control (10.3%; Figure 3). A second phospholipid, namely phosphatidylethanolamine (PE), was less effective than PS yet still activated 24.8% of the RSCs when applied at the same concentration of 25 μM . Upon doubling the concentration of PS to 50 μM , a reciprocal increase (39.7%) in RSC degranulation was observed, however, this was not the case for PE. Examination of extracted RSCs pre-activation, revealed an abundance of refractile, reddishbrown granules (containing echinochrome A) clearly visible within the cytosol (Figures 1D & 2). Following exposure to pathogen- or damage-associated molecular patterns (PAMPs, DAMPs), the RSCs emptied their cytoplasmic cargo into the surrounding milieu, flattened, and were no longer refractile (Figures 2 and 3B). The extent of the mass exocytosis can be seen in Figure 3B, where vacuole-like compartments occupy the seemingly quiescent RSC.

Role of calcium in red spherule cell degranulation

To further interrogate the degranulation process in *P. lividus* RSCs, we employed the Ca^{2+} -specific ionophore, ionomycin. Exposure to 2 μM ionomycin led to ~32% of RSCs releasing their granular content *in vitro*. This proportion increased to ~90% when the concentration of ionomycin was doubled to 4 μM (Figure 3), thereby suggesting an increase in intracellular levels of calcium $[\text{Ca}^{2+}]_i$ was required for echinochrome release. When coelomocytes were loaded with the Ca^{2+} chelator BAPTA (20 μM ; as the membrane permeant AM-ester) prior to exposure to ionomycin or immune stimulants, there was no release of granular contents (data not shown). A comparison of mixed coelomocyte populations removed from *P. lividus* and the green sea urchin, *Psammechinus miliaris*, verified that RSCs responded to bacterial cell wall components (LTA, LPS) and ionomycin in a similar manner (Figure 4). Conversely, RSCs in the mixed populations from both species were unresponsive to mannan from *S. cerevisiae* ($p > 0.05$). Preliminary experiments to visualise the increase in $[\text{Ca}^{2+}]_i$ into RSCs were performed using the fluorescent indicator, Fluo-3 AM (Supp. Figure 1). Upon addition of 10 μM ionomycin there was a clear increase in fluorescence within vesicular structures and the cytosol, indicative of Ca^{2+} influx. At such a high concentration of ionomycin, degranulation of echinochrome A was observed in ~99% of RSCs within 60 seconds. The viability of RSCs 2 hours after 10 μM ionomycin was >93%. Notably, removal of Ca^{2+} from the artificial coelomic fluid (ACF) interfered with the activation of RSCs despite the presence of immune-stimulants (Supp. Figure 2).

Antimicrobial activity of red spherule cell lysates

Intra-coelomic injection of LPS (3 μg per mL coelomic fluid) into *P. lividus* adults led to significant increases in the proportions of circulating RSCs within 1 hour ($p < 0.001$) in contrast to coelomocyte numbers from control animals over the same experimental period ($p = 0.98$) (Figure 5A and 5B). RSCs increased to 21.9% between 0 and 30 min, and then fell to 17.2% at 60 minutes. Cell numbers correlated inversely with the amount of soluble echinochrome A detected in the coelomic fluid – monitored via absorbance maxima at 346 nm and 480 nm (Figure 5C). A hyperchromic effect (2-fold increase) was noted at 346 nm in the coelomic fluid of LPS-stimulated sea urchins within 1 hour. LPS caused an initial increase (at 30 minutes) in the proportion of RSCs within the circulating coelomocyte population, which subsequently underwent degranulation (Figure 5D). The levels of RSCs and soluble echinochrome A in

challenged sea urchins recovered by 24 hours, in line with data from control animals having received an injection of ACF only.

In vitro antimicrobial activity of RSC lysates was tested against Gram-positive and Gram-negative bacteria as well as yeast. Lysates from 1×10^5 RSCs were incubated with 1×10^6 of each microbe for 1 hour at room temperature prior to plating ~ 200 CFUs onto agar with/without ferric ammonium citrate (FAC). The number of viable microbes (i.e. CFUs) decreased significantly to 17.8% for *E. coli* ($p < 0.001$), $\sim 45\%$ for *Bacillus sp.* ($p < 0.001$), and 61% for *S. cerevisiae* ($p = 0.003$) when compared to untreated (control) microbes (Figure 6A). CFUs recovered to $>80\%$ for each treated microbe when grown on agar supplemented with iron (FAC) as opposed to standard agar recipes. Notably, complete recovery of CFUs (97.8–104.9%) was achieved when microbes were treated with FAC and RSC lysates simultaneously, prior to plating (Supp. Figure 3). Microbes that were exposed to a known antimicrobial iron chelator, namely EDTA, displayed similar trends of CFU mortality (Figure 6A). EDTA-treated microbes recovered to $>95\%$ viability when cultured on FAC-agar, which was similar to the data for microbes treated with RSC lysates.

To test whether RSC lysates (i.e. echinochrome A) inhibited microbial growth via iron deprivation, we studied the spectral properties of lysates incubated with ferric iron (Fe^{3+}). A hypochromic effect was observed in the absorbance spectrum at 480 nm upon incubation with 200 μM FAC for 15 minutes (Figure 6B). Additionally, the shoulder peak at ~ 525 nm was no longer distinguishable. These results suggested that iron and echinochrome formed complexes.

Discussion

RSCs are often identified near damaged spines, encapsulated bacteria and infested epidermal tissues of sea urchins [13–15, 25, 26], yet until now evidence supporting a role for RSCs in immunity has been lacking. Due to the distinct morpho-functional properties of echinoid coelomocytes and the convenience of density separation media (Figure 1), we were able to examine RSCs ($>98\%$ homogeneity) *in vitro*. The introduction of immune-stimulants (microbes, PAMPs) and membrane phospholipids triggers the exocytosis of echinochrome-containing vesicles in up to 50% of RSCs (Figures 2–4). Direct injection of lipopolysaccharides into the coelom mobilises RSCs to release echinochrome A *in vivo*

(Figure 5). These data indicate RSCs recognise ‘non-self’ motifs leading them to undergo morphological and physiological changes associated with enhanced antimicrobial defence (Figure 6; Supp. Video 1).

Responses of invertebrate immune cells to PAMPs and DAMPs are well characterised across diverse taxa, however, sea urchin RSCs are an exception [9, 10, 23; 27]. When insect and crustacean hemocytes encounter pathogens they release a battery of immune effectors (through exocytosis) to immobilise/entrap the intruders as part of their inflammatory programmes [28]. RSCs alone, and in mixed coelomocyte populations from *P. lividus* and *P. miliaris*, degranulate when presented with Gram-negative and Gram-positive bacteria as well as yeast (Figures 2 and 4). The inner-membrane phospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE), also stimulate echinochrome A release (Figure 3). PS location is restricted to the cytoplasmic membrane of healthy coelomocytes. Its relocation onto the cell surface is a hallmark of cell death (apoptosis) in metazoans [29], drawing attention to defective (immune) cells and those tissues compromised by pathogens. Surveys of wounded sea urchins consistently find elevated levels of RSCs (>40%) in the coelomic fluid compared to ‘healthy’ conspecifics (~10%) [13, 25, 26, 30]. These animals play host to noxious bacteria, fungi and algae – organisms that we have proven RSCs react to (Figures 2 and 3). Amorphous red materials and friable layers coincide with hemostasis and spine regeneration, hinting that RSCs deposit echinochrome A to prevent the loss of coelomic fluid during infection [13, 25, 26, 30]. Such barriers are found in ‘hanging-drop’ preparations of purple (*S. purpuratus*) and red (*Mesocentrotus franciscanus*) sea urchin coelomocytes where

RSCs form ‘palisades’ on the edges of clots and bacterial aggregates [13]. Our data reinforce these early studies, and indicate that RSCs are recruited to injury sites (Figures 3 and 5), recognise antigens (Figures 2 and 4), and release echinochrome A. Additionally, we present new evidence for echinochrome A having another, more direct role to play in echinoid immune defence (see next section).

The effects of ligands/microbes/phospholipids can be mimicked by ionomycin, indicating that elevated intracellular Ca^{2+} is the trigger for echinochrome A release (Figures 3 and 4). Mechanistic aspects of degranulation events and cell-derived immunity in invertebrates (e.g. *Drosophila* hemocytes) are largely modulated by calcium [31]. We used the Ca^{2+} -indicator dye Fluo-3 to determine if the release of echinochrome A following application of ionomycin correlates with intracellular Ca^{2+} in RSCs. Shortly after the addition of ionomycin there is

enhanced Fluo-3 fluorescence, meaning Ca^{2+} concentration has indeed increased (Supp. Figure 1). Spatial distribution of Fluo-3 fluorescence reveals an increase in intra-vesicular Ca^{2+} accompanies the morphological changes of RSCs. This accumulation of Ca^{2+} in cytoplasmic compartments, as well as in the cytosol, is not unheard-of (reviewed by [32]). Firstly, although ionomycin is a Ca^{2+} -specific ionophore, it is relatively non-selective regarding membranes into which it can insert. Secondly, Ca^{2+} indicator dyes such as Fluo-3 AM can accumulate in secretory vesicles, especially those that are acidic [32]. These characteristics of ionomycin likely account for the compartmentalised fluorescent signal visible in activated RSCs. Our observations on the requirement for external Ca^{2+} (Supp. Figure 2) and the blocking effect of intracellular BAPTA on RSC responsiveness further support a role for Ca^{2+} in echinochrome A discharge.

By inoculating *P. lividus* adults with LPS, we have gained rare insight into RSC activities *in vivo* (Figure 5). Within 30 minutes, the proportion of circulating RSCs doubles – likely due to cells making their way into circulation from neighbouring tissues where they carry out immune-surveillance. This short period of time is not sufficient for hematopoiesis to occur. By 60 minutes, there is a noticeable drop in RSC numbers but an increase in absorbance at 346 nm, a signal that more cell-free echinochrome A is in the coelomic fluid (Figure 5D; Supp. Figure 4). Activation of invertebrate defences generally leads to an increase in freefloating immune cell numbers and the liberation of bioactive compounds (e.g. antimicrobial peptides and lysozyme) [33]. Likewise, exposure of sea urchin phagocytes to LPS *in vitro* induces cellular aggregation (reminiscent of encapsulation) and *de novo* synthesis of SpTransformer proteins (formerly Sp185/333) (Majeske et al., 2013). Intriguingly, both phagocytes and RSCs from the green sea urchin (*Strongylocentrotus droebachiensis*) increase gene expression of a defensin-like antimicrobial peptide, namely strongylocin 2, when exposed to *E. coli in vitro* [34]. Whether the mRNA is translated into a functional peptide or degraded in the cytoplasm remains to be determined. Nevertheless, if RSCs can produce AMPs in addition to echinochrome A, then both could be released to combat sepsis.

Echinochrome A is a putative immune factor in sea urchins

The cytoplasmic granules of RSCs are replete with the pigment echinochrome A (6-ethyl2,3,5,7,8-pentahydroxy-1,4-naphthoquinone; Figure 6C). Service and Wardlaw (1984) [16] first assigned antimicrobial properties to echinochrome A using ethanol/acetone extractions of whole coelomocyte lysates from *E. esculentus*. Antibacterial activity of

echinochrome A toward *Pseudomonas* strain 111 was concentration (20 – 200 μM) and time (4 – 48 hours) dependent [16]. Following this, lysates of fractionated RSCs (1×10^5) from *P. lividus* were found to be 100% effective at killing marine bacteria (*Vibrio* species, *Photobacterium* sp. strain 56) over a 12 hour period at 20°C [17]. More recently, extracts of EchinochromeA:SpinochromeC (75:25) from the tests/spines of several tropical sea urchin species were found to be effective at killing *E. coli*, *B. subtilis* and *Shewanella oneidensis* [35]. An EC_{50} value of 61 μM has been calculated for the metal reducing bacterium, *S. oneidensis*. None of these studies investigated the mechanism behind RSC and echinochrome's anti-infective characteristics. That said, lysozyme (muramidase activity) was ruled out as a contributing factor in RSC lysates [16, 17, 35]. Based on our evidence, we argue that iron-chelating properties of echinochrome A underpin these earlier observations (Figure 6). By following the protocol of Gerardi et al. (1990) [17], RSC-lysates inhibited CFUs by 82.2% in *E. coli*, 53.1% in *B. megaterium*, 56.3% in *B. subtilis* and 38.9% in *S. cerevisiae* after 1 hour incubation at 20°C (Figure 6, Supp. Figure 3). The microbicidal nature of RSC-lysates can be counteracted by plating the treated microbes onto iron-supplemented agar (0.05% FAC). Re-introducing iron reduces CFU mortalities to 13.3–19.1%. Also, treating bacteria and yeast with RSC-lysates in the presence of Fe^{3+} prior to plating has no (measurable) negative impact on microbial growth (Supp. Figure 3). In its purified form, echinochrome A is a potent antioxidant and metal chelator capable of scavenging oxidising/nitrosative radicals and forming complexes with ferric/ferrous states of iron in a molar ratio of 1:2 (echinochrome : iron) [36]. The addition of iron ($\leq 80 \mu\text{M}$ FeSO_4) alters the absorbance profile of purified echinochrome A ($\sim 40 \mu\text{M}$) from *S. intermedius* [36]; a result comparable to the effects of ferric ammonium citrate (200 μM) on RSC lysates containing ($\sim 27 \mu\text{M}$) echinochrome A observed here (Figure 6B). The free ortho-hydroxyl groups and ketol structure of echinochrome A facilitates the chelation of iron (Figure 6C). Collectively, these data suggest that echinochrome A from RSC lysates gathers unbound iron from the environment, thereby depriving microbes of this essential metal. Iron reintroduction posttreatment does not restore CFU viability to 100% (Supp. Figure 3). This is only achieved when excess iron (Fe^{3+}) is added to RSC-lysates during treatment, implying echinochrome A may also interfere with microbes directly – analogous to the antimicrobial mechanism of synthetic metal chelators like EDTA [37]. Given its hydrophobicity, it is also possible that echinochrome A directly enters microbes and has some intracellular effects. We postulate that the ability of echinochrome A to switch between oxidised and reduced forms (via hydroxyl groups; Figure 6C) benefits the sea urchin host through the disarmament of

reactive by-products (e.g. H_2O_2 , ONOO^-) caused by immune activities, i.e. phagocytosis-associated respiratory burst [38]. The withholding of metals, particularly iron, is an important component of innate immunity in vertebrates and invertebrates alike, because iron is an essential factor for microbial growth and pathogenicity [39]. We demonstrate that LPS activates *P. lividus* RSCs into releasing the metal-binding pigment, echinochrome A, *in vitro* and *in vivo* (Figures 2 - 5). LPS has also been shown to induce the synthesis of 60 stress and immune-related factors within the coelomic fluid of *S. purpuratus*, notably transferrin and ferritin [40]. These proteins have well-defined roles in immunity and metabolism as they coordinate the detoxification, transport and storage of iron.

Concluding remarks

Based on our observations, and after careful consideration of the available literature, we propose dual functionality of RSCs *in vivo*. First, RSCs detect and respond to microbes by releasing echinochrome A to sequester iron from the environment. Second, pathological trauma mobilises RSCs to prevent the systemic spread of microbes and deploy echinochrome A to disarm oxidising and nitrosative radicals produced as a consequence of immune vigour. The iron-chelating properties of echinochrome A would serve as a microbial deterrent and its ability to act as a chemical antioxidant would reduce the likelihood of collateral damage.

Acknowledgements

We are grateful to Prof. Andrew F. Rowley (Swansea University) for constructive comments on multiple versions of this manuscript, and to the two anonymous reviewers for their candour.

Funding

This research was supported financially by the University of Stirling and Swansea University.

Conflict of interest statement

The authors declare no conflicts of interest, financial or otherwise.

Author contributions

C.J.C. and T.W. designed the research. All authors performed the experiments. C.J.C. collated and analysed the data. C.J.C. prepared the manuscript with input from T.W.

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Figure legends

Figure 1 Density-dependent fractionation of sea urchin coelomocytes. **A)** *Paracentrotus lividus* adult. **B)** Total and differential coelomocytes per ml of coelomic fluid (mean +/- 95% CI, $n = 30$). **C)** 40-60% continuous Percoll gradient with extracted coelomic fluid, before and after centrifugation. Band 0 consists mainly of cellular debris. Band 1 contains phagocytes; bands 2 and 3 contain vibratile and colourless spherule cells (CSCs), respectively; band 4 is >98% red spherule cells (RSCs). **D)** Living coelomocytes removed from *P. lividus*. The red spherule cells are distinguishable due to the abundance of echinochrome-containing cytosolic vesicles (red arrow-heads). Colourless spherule cells, phagocytes and a vibratile cell are highlighted by white, black and blue arrow-heads respectively. Scale bar represents 20 μm .

Figure 2 Degranulation of red spherule cells in response to pathogen-associated molecular patterns. Isolated red spherule cells (RSCs) were exposed to increasing concentrations of **(A)** bacterial (LPS, lipopolysaccharide; LTA, lipoteichoic acid), fungal (mannan) and algal (laminarin, a β -glucan) ligands, or, **(B)** intact microbes (Grampositive/negative bacteria and yeast) for 1 hour *in vitro*. RSC responses to such challenges were recorded as percentage degranulation. All data are represented as mean values + 95% CI, $n = 3$. An asterisk (*) indicates a significant difference ($p < 0.05$) between the control and treatment. Inset; images depicting RSCs in the absence (control) or presence of an immunestimulant. Scale bar represents 10 μm .

Figure 3 Degranulation of red spherule cells in response to damage-associated molecular patterns. **A)** Isolated red spherule cells (RSCs) were exposed to inner membrane phospholipids (PS, phosphatidylserine; PE, phosphatidylethanolamine) and the calcium ionophore, ionomycin, for 1 hour *in vitro*. RSC responses to such challenges were recorded as percentage degranulation. Data are represented as mean values + 95% CI, $n = 3$. An asterisk (*) indicates a significant difference ($p < 0.05$) between the control and treatment. **B)** Images depicting RSCs challenged with inner membrane phospholipids (PS, PE), and, when intracellular levels of Ca^{2+} increased due to the presence of an ionophore (ionomycin). Scale bar represents 20 μm .

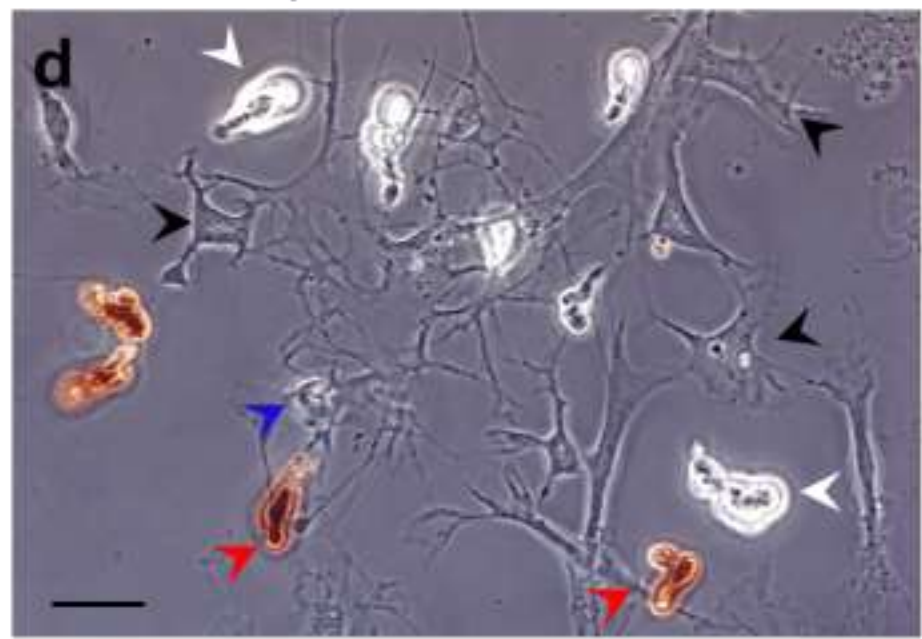
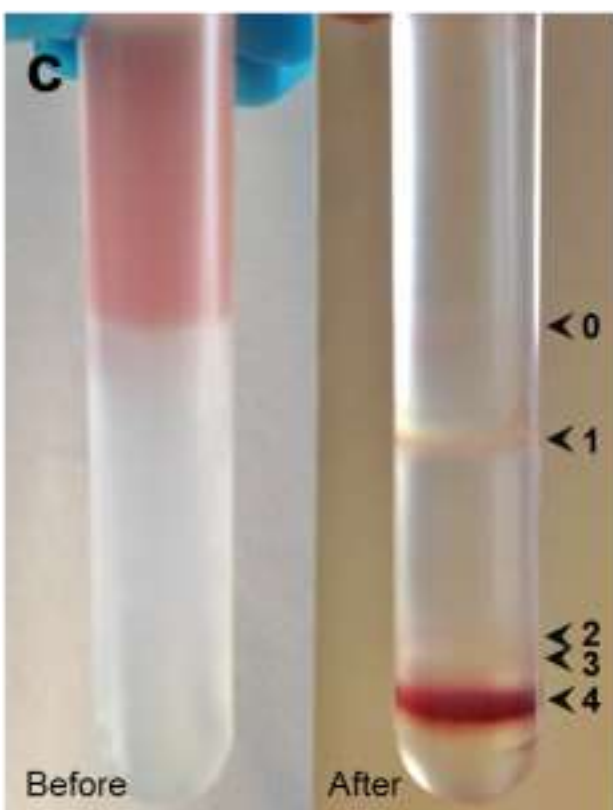
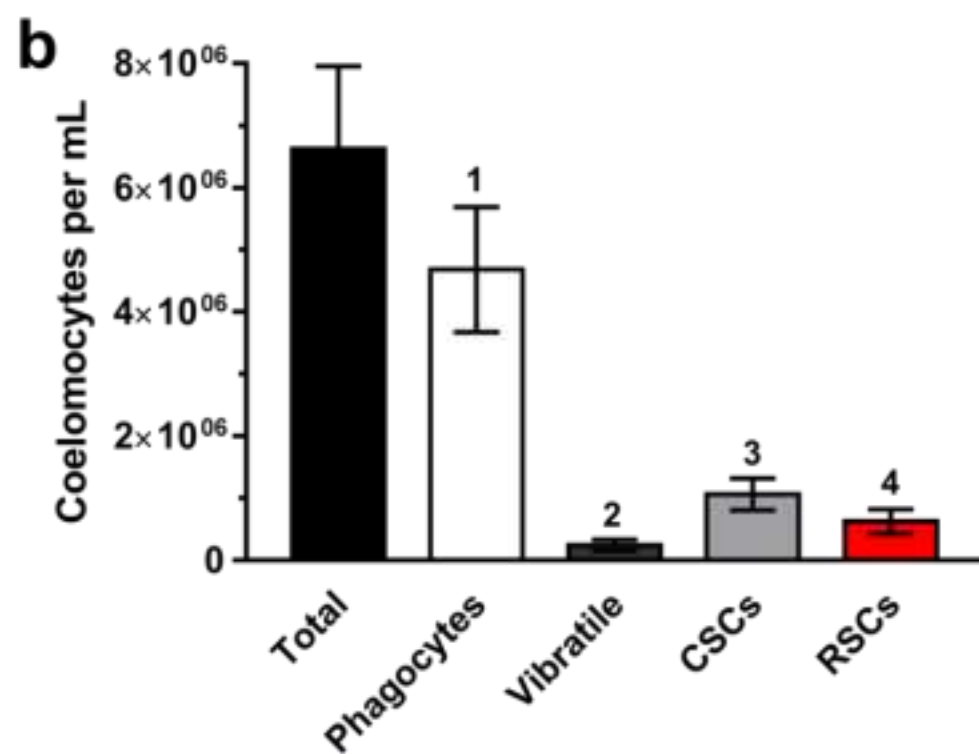
Figure 4 Degranulation of red spherule cells in mixed coelomocyte populations from (a) *Paracentrotus lividus* and (b) *Psammechinus miliaris*. Coelomocytes were extracted into an anti-coagulant, pelleted and re-suspended in artificial coelomic fluid, and seeded into wells of a 24-well culture plate without fractionation. Mixed coelomocyte populations were exposed to 25 μ M of each microbial ligand (LPS, lipopolysaccharides; LTA, lipoteichoic acids; mannan) and 10 μ M ionomycin (positive control). The reduction in red spherule cell numbers due to degranulation was recorded after 1 hour. All data are represented as mean values + 95% CI, $n = 5$ (for each species). An asterisk (*) indicates a significant difference ($p < 0.05$) between the control and treatment.

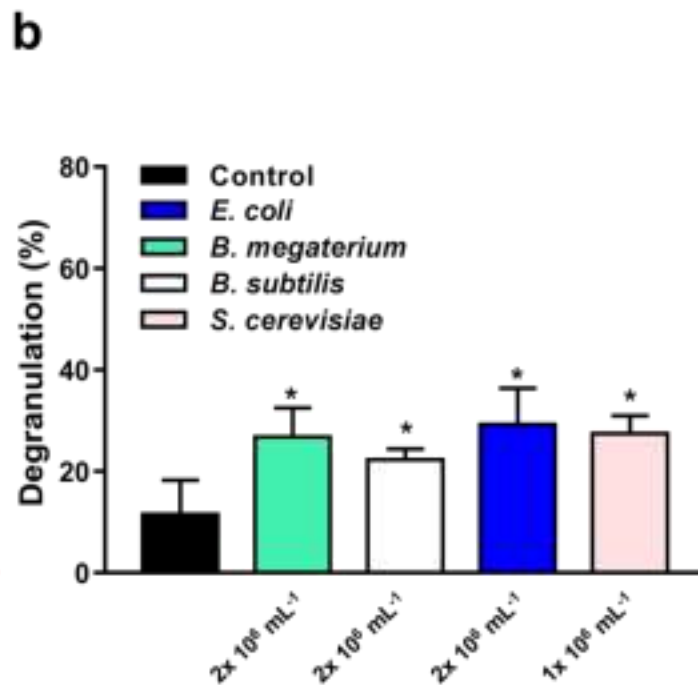
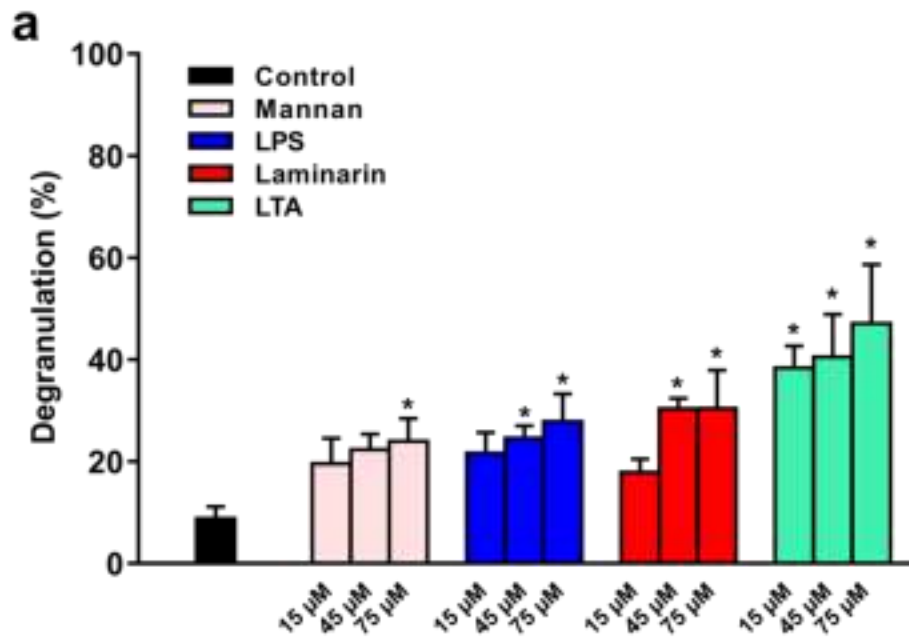
Figure 5 Response of red spherule cells to lipopolysaccharides *in vivo*. **A)** The proportion of red spherule cells in the free-floating coelomocyte population was determined upon inoculation with 3 μ g lipopolysaccharides (LPS) per mL coelomic fluid or artificial coelomic fluid (control). **B)** Living coelomocytes removed from ACF-injected *Paracentrotus lividus*. An intact red spherule cell can be seen alongside four colourless spherule cells. Scale bar represents 20 μ m. **C)** Absorbance spectrum of cell-free coelomic fluid from *P. lividus* after 1 hour post-challenge. The observed peaks at 346 nm and 480 nm are indicative of echinochrome a [see 16, 24]. The presented spectra are a representation of experiments carried out on three independent occasions. **D)** Peak absorbance values for echinochrome were monitored in cell-free coelomic fluid at 1 hour and 24 hours post-injection with LPS. Control values were recorded at 1 hour post-injection with ACF. Data displayed in **A** and **D** are mean values with 95% CI, $n = 5$. Unshared letters indicate significant differences ($p < 0.05$).

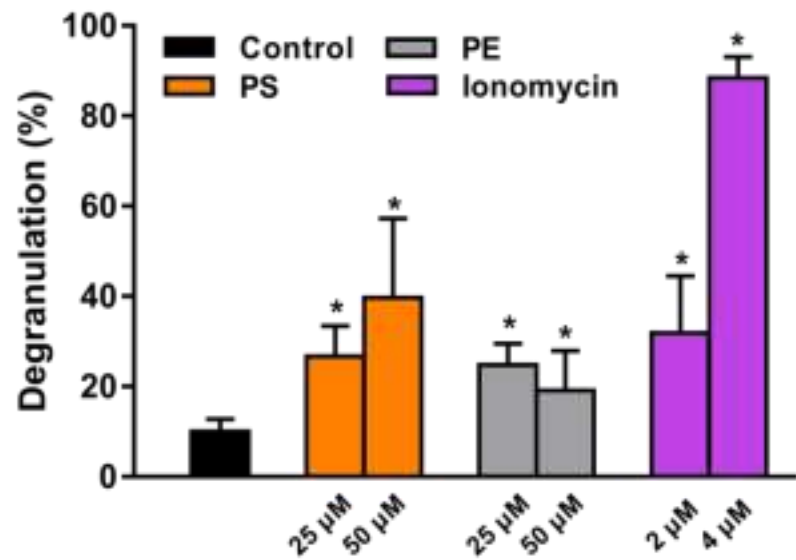
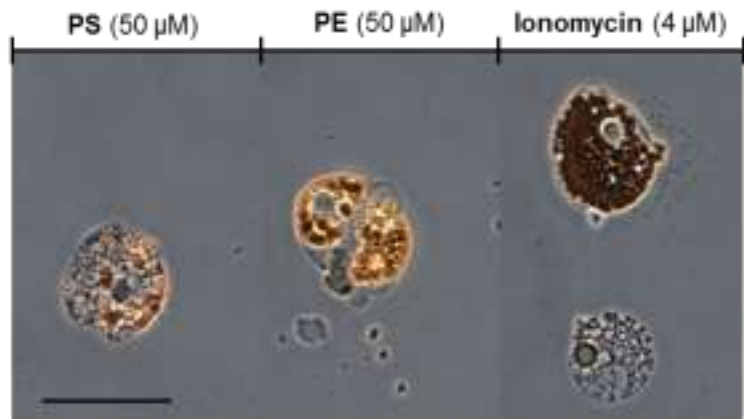
Figure 6 Antimicrobial activities of red spherule cell lysates from *Paracentrotus lividus*. Gram-positive (*B. megaterium*, *B. subtilis*) and Gram-negative (*E. coli*) bacteria as well as yeast (*S. cerevisiae*) were incubated in the presence/absence of the iron chelator EDTA, or, red spherule cell lysates (2.5×10^5) for 1 hour at room temperature. Following treatment, microbes were serially diluted in PBS, pH7.4 so that 200 colony forming units (CFUs) were plated onto standard agar medium (LB for bacteria and YEPD for yeast), or, agar that had been enriched with iron (Fe^{3+} , ferric ammonium citrate). The colour scale on the right indicates the mean number of viable microbes present on agar, i.e. CFUs ($n = 3$). An asterisk (*) indicates a significant difference ($p < 0.05$) between the control and treatment. **B)**

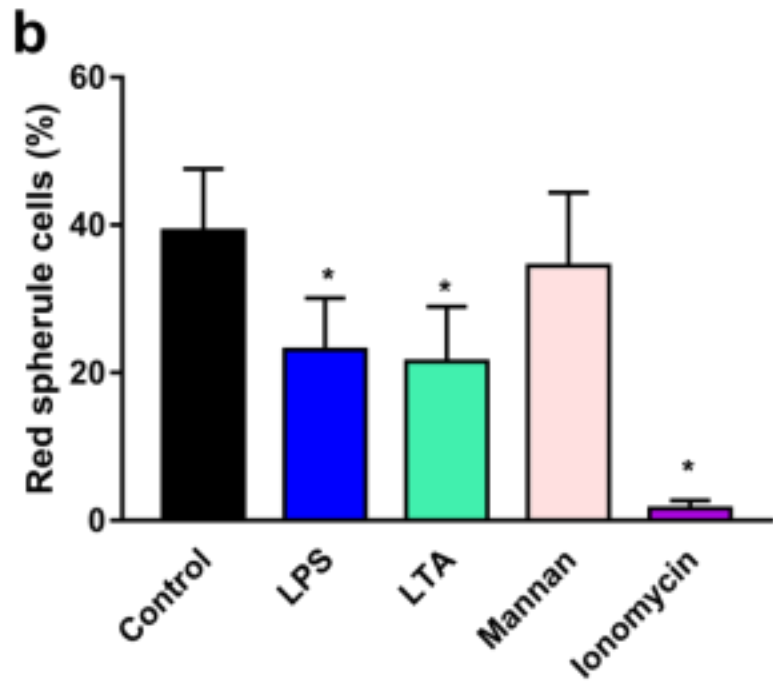
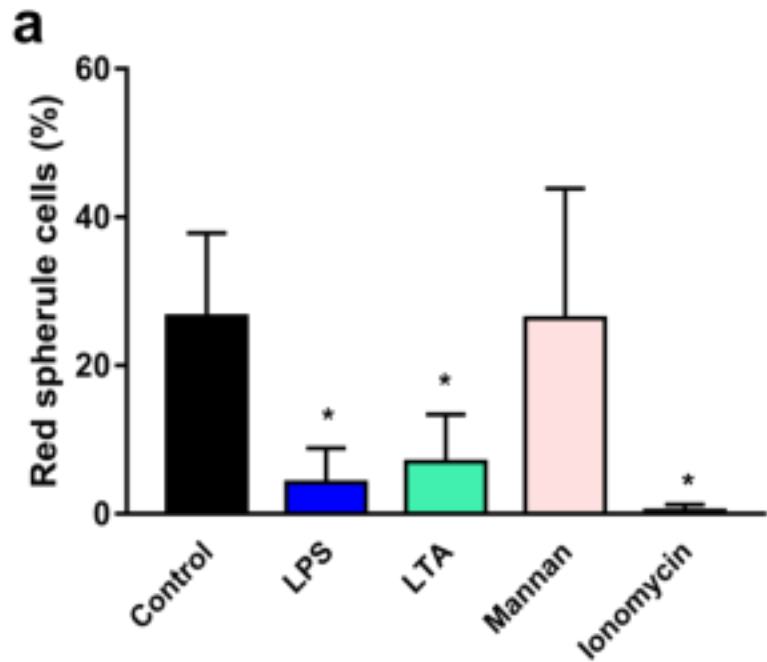
Absorbance spectrum of RSC lysate ($\sim 27 \mu$ M echinochrome A) in the absence and presence of 200 μ M ferric ammonium citrate. The concentration of echinochrome A in 1×10^5 RSCs was calculated by following the extraction method developed by Service and Wardlaw [16],

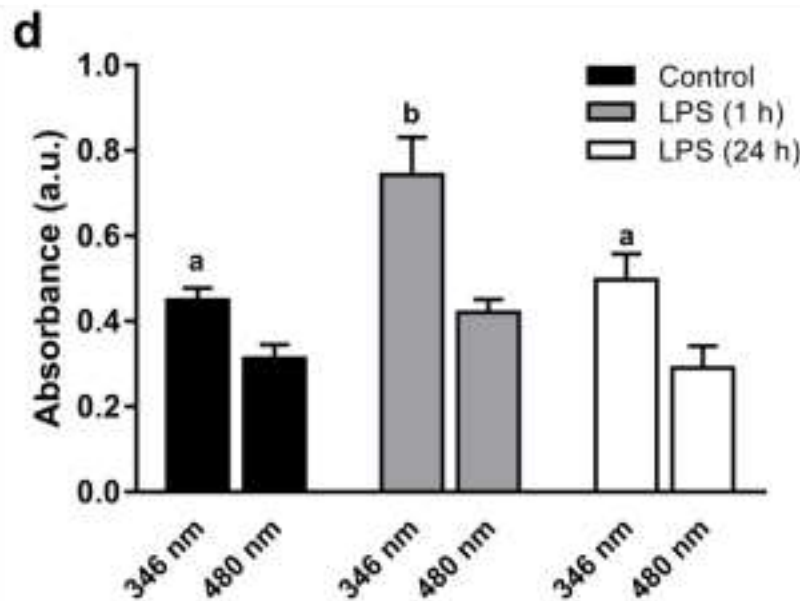
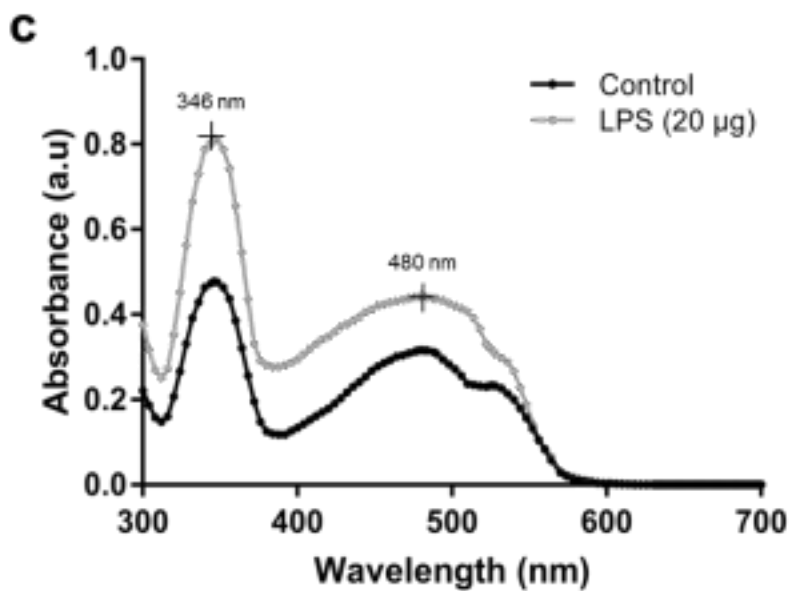
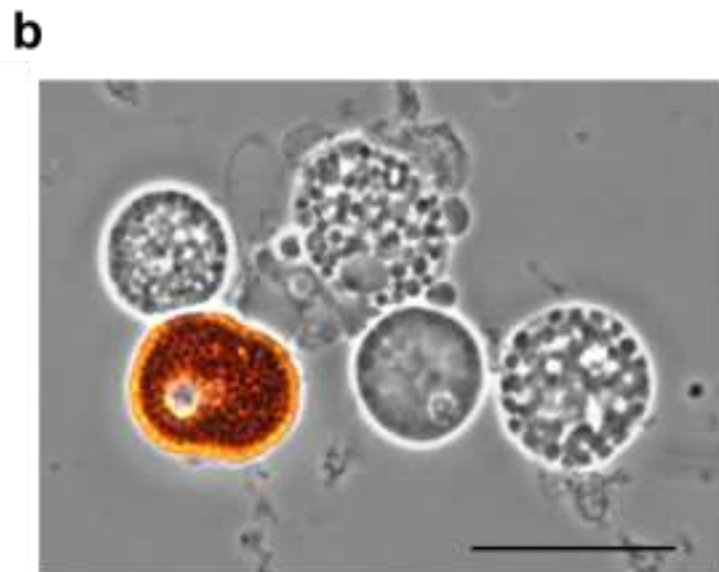
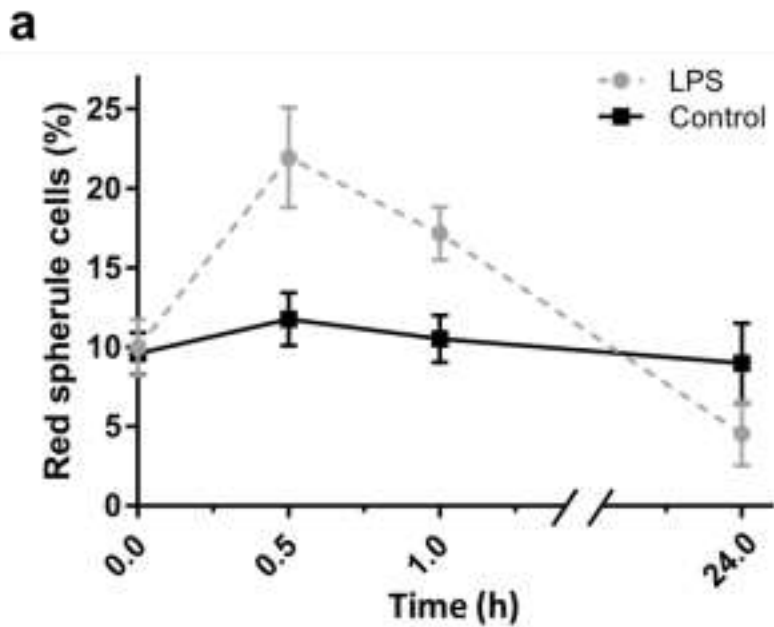
and taking into account that there $\sim 6.3 \times 10^5$ RSCs per mL of *P. lividus* coelomic fluid. **C)**
Molecular structure of the 1,4-naphthoquinone pigment, echinochrome A.

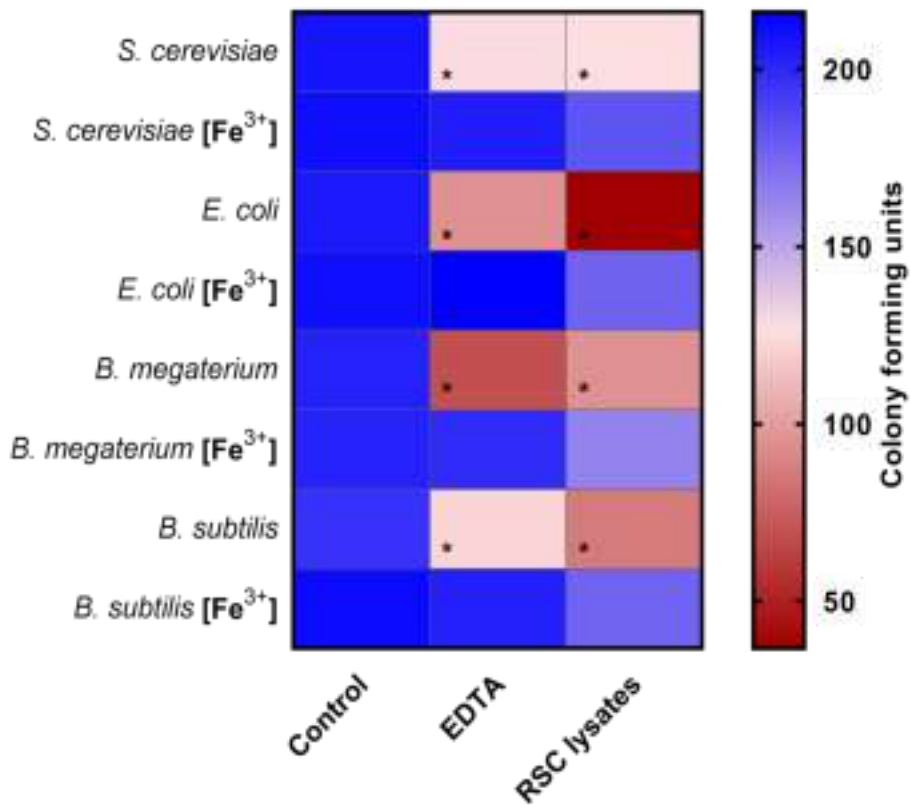
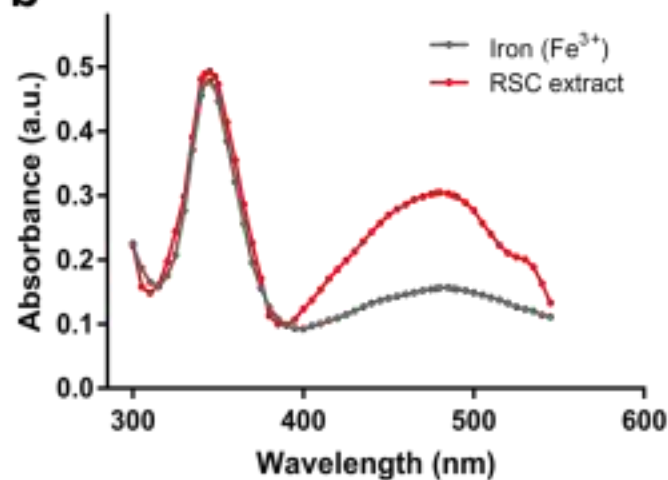
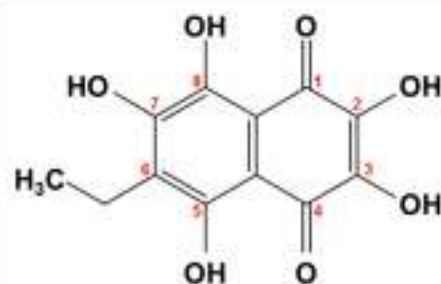


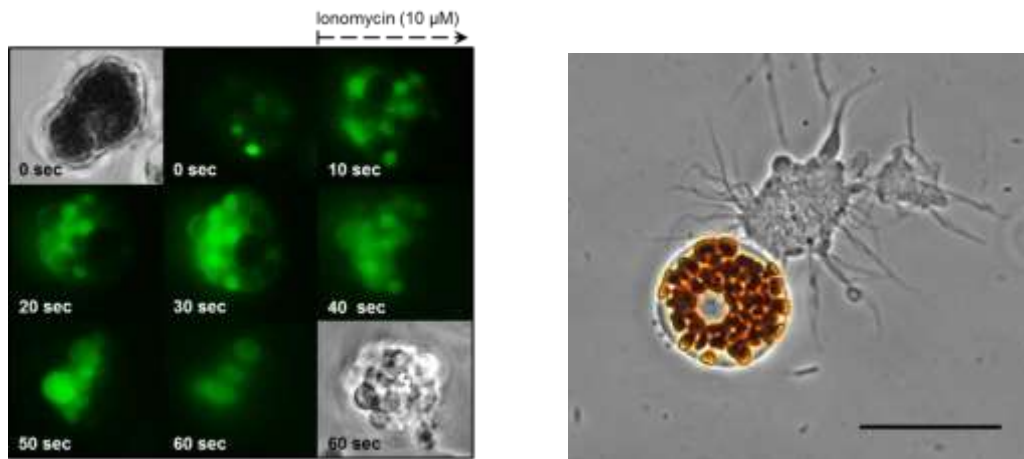


a**b**



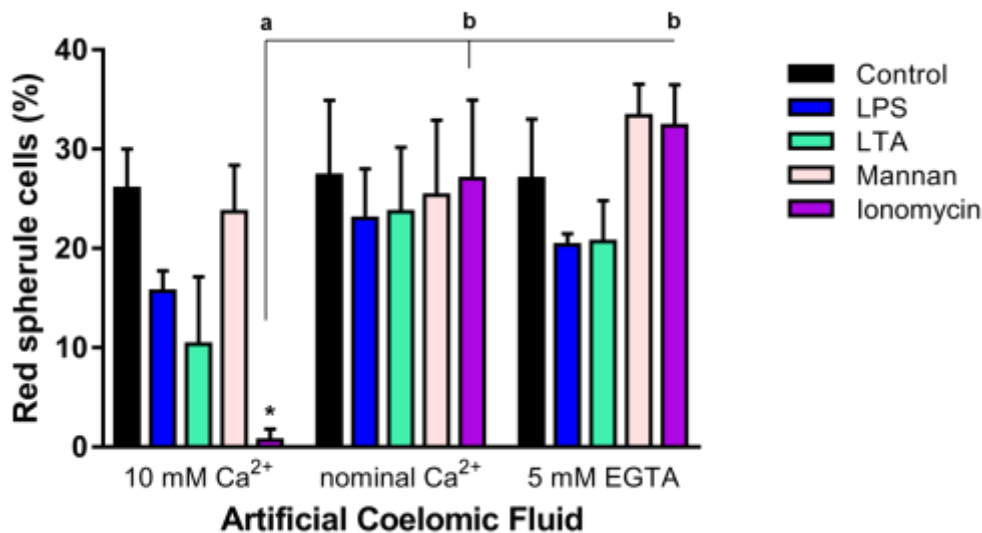


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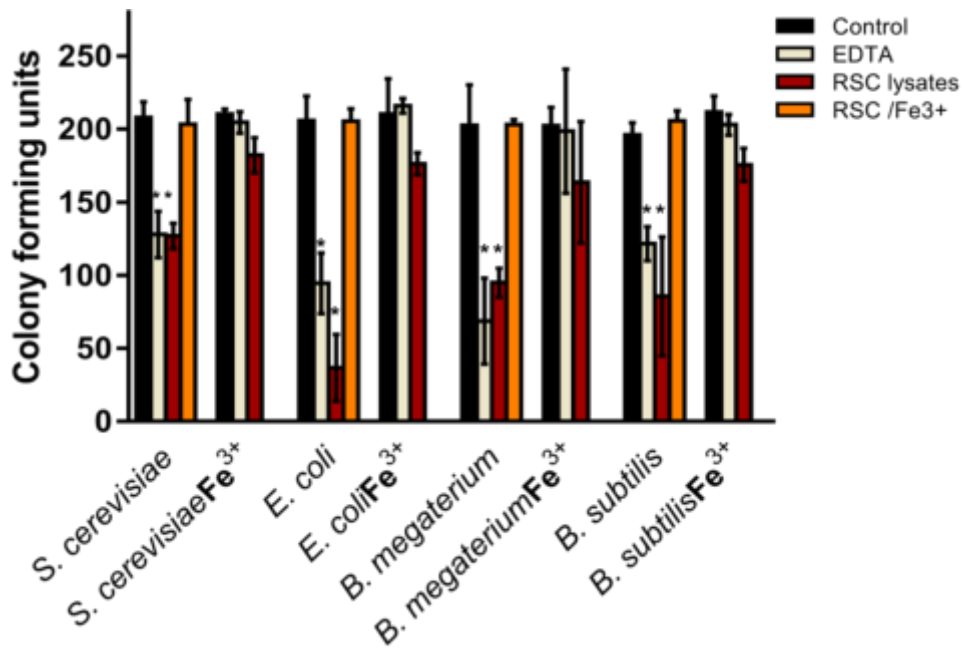


Supplementary Figure 1 [Left image] To visualise intracellular levels of calcium [Ca^{2+}]; pre- and post- activation, the calcium indicator Fluo-3 AM was used. Coelomic fluid from *Psammochinus miliaris* was extracted into an equal volume of anti-coagulant (20mM Tris-HCl, 0.5M NaCl, 70mM EDTA, pH 7.5) and incubated with 5 μM Fluo-3 AM and 0.1% PluronicTM F68 for 30 min at 4°C, followed by centrifugation at 170 x g for 5 min and re-suspended in ACF to a final cell number of $1 \times 10^6 \text{ mL}^{-1}$. The non-ionic detergent, PluronicTM F68, facilitates dispersion of AM esters in the medium^[1], and, incubating the coelomocytes at 4°C helps to prevent non-specific labelling of intracellular organelles. Labelled coelomocytes (200 μL) were placed into chambers of an Ibidi μ -slide and allowed to settle for 20 min at room temperature. Ionomycin (10 μM) was perfused into the chamber and calcium was visualised as an increased green fluorescence signal that results from it binding to Fluo-3. [Right image] Living coelomocytes removed from PBS-injected *Paracentrotus lividus*. An intact red spherule cell can be seen alongside a small phagocyte. Scale bar represents 10 μm . As the cell flattens the individual granules become more distinct.

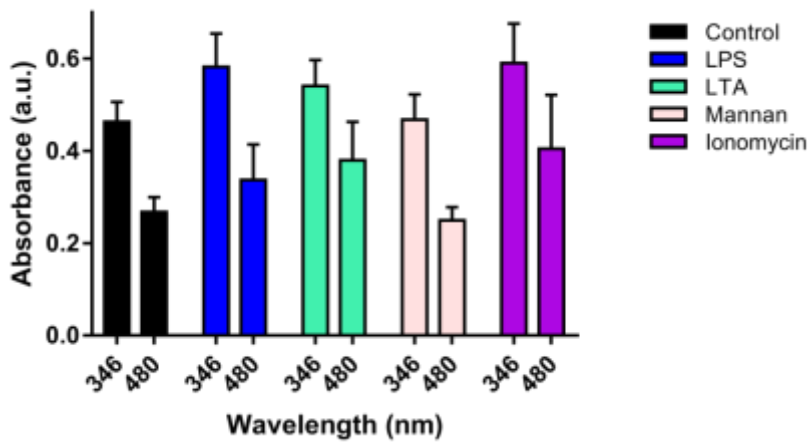
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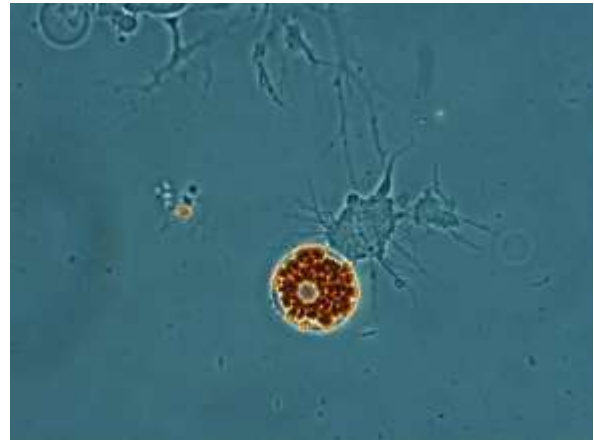
Supplementary Figure 2 Degranulation response of red spherule cells from *Psammochinus miliaris* ($n = 3$) following treatment with microbial ligands (25 μM) and ionomycin (10 μM) *in vitro* under different calcium regimes. Following removal and enumeration of coelomocytes (see methods) samples were re-suspended in regular artificial coelomic fluid (ACF) containing 10 mM Ca^{2+} , ACF without Ca^{2+} added (i.e. nominal) and ACF with 5 mM EGTA and no Ca^{2+} . Data are expressed as mean values + 95% CI. Unshared letters and an asterisk (control vs. ionomycin) indicate significant differences ($p < 0.05$).



Supplementary Figure 3 Antimicrobial activities of red spherule cell lysates in the presence/absence of ferric iron (Fe³⁺). Data from the main text (Figure 6) is expanded to include values where RSC lysate and ferric ammonium citrate (0.05% w/v) are exposed to microbes prior to plating on agar (orange bars). In this instance, the presence of iron appeared to prevent/inhibit the putative antimicrobial properties of echinochrome. Bars represent mean values with 95% CI, $n = 3$. An asterisk indicates a significant difference when compared to the control ($p < 0.05$).



Supplementary Figure 4 Echinochrome A assay. Absorbance of *Paracentrotus lividus* coelomocyte samples ($n = 5$) following treatment with microbial ligands (25 μ M) and ionomycin (2 μ M) *in vitro*. These two wavelengths (346 and 480 nm) correspond to the peak absorbance values of echinochrome a (derived from red spherule cells). Data are expressed as mean values + 95% CI.



Original images used for Figure 1C, Figure 5B, and Supp. Figure 1