Response of Sabella spallanzanii to multiple stressors. The combined effect of infection and copper sulphate

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Keywords: Polychaetes, Immunity response, Multiple stressors, Enzymatic activity, TLR, AIF-1.

Abstract

The aim of this work is to study the immune responses of the polychaete *Sabella spallanzanii* after exposure to copper sulphate, an immunomodulating agent in marine organisms, and the multiple stresses caused by inoculation with *Escherichia coli*, in order to validate the species as a model organism in marine-coastal biomonitoring programmes. Polychaetes were housed in our laboratory and divided into five experimental groups: 1. Control (Naive), 2. Filtered sea water + TBS injection, 3. Filtered sea water + *E. coli* injection, 4. $CuSO_4$ + TBS injection, 5. $CuSO_4$ + *E. coli* injection. The immune variables, esterase (EST) and alkaline phosphatase activity (ALP), cytotoxicity and detoxifying/antioxidant enzymes such as glutathione peroxidase (GPx) were evaluated in total body extracts of the animals. Moreover, toll-like receptor (TLR), allograft inflammatory factor-1 (AIF-1), lysozyme (LYS) and haemagglutinating activity were investigated to highlight possible interactions. Indeed, the results of this work demonstrate the immunomodulating effect of copper sulphate on *S. spallanzanii* total body extracts related to oxidative stress and inflammatory markers.

1. Introduction

Metals can affect systems in different organisms, but the mechanisms by which they act are complex and have not been fully explored. Their toxicity depends on many factors, such as the type and nature of the metal, its biological role, the organism exposed and the period of its life cycle (Briffa et al., 2020). Some metals are toxic only at high concentrations because they are essential elements and natural elements in the aquatic environment (Mesquita et al., 2019). Copper is one of the most dangerous elements from an ecotoxicological point of view (Tchounwou et al., 2012). At high concentrations, it causes damage to the reproductive system, produces oxidative stress given its nature as an enzyme cofactor which, together with oxidase and oxygenase acts as a catalyst for the formation of reactive oxygen species (SJ and D, 1995), alters enzyme activities and the immune system, modifies DNA and damages the normal survival mechanisms of many macroinvertebrates. Changes in pH and increasing water temperature are determinant factors of copper toxicity (Childs, 2013; Ferreira-Cravo et al., 2009; Stewart et al., 2021); indeed, low pH increases the concentration

of Cu^{2+} in the environment, a more bioavailable form which is more easily bioaccumulated in organisms, and recent evidence has predicted a 115% increase in free copper ions over the next 100 years in rich coastal waters (Richards et al., 2011). Antifouling paints, fertilisers, fungicides and algaecides used in both agriculture and aquaculture often include copper in the form of copper sulphate ($CuSO_4$) in varying concentrations, depending on the commercial formulation; pentahydrate ($CuSO_4*5H_2O$) is the formulation most commonly used in freshwater and marine aquaculture systems. $CuSO_4$ has also been used as a therapy to reduce parasitic infections in fish aquaculture. It is estimated that the copper concentration in ocean waters generally does not exceed values of 4 μg/l (Tavares-Dias, 2021), but higher concentrations can be found in coastal waters and estuaries which are more subjected to pollution (10-50 μg/l).

Stressors occur simultaneously in the natural environment and interact with each other. Individually, they can have a negative impact on organisms, but when they are combined, the effect can be amplified, affecting biodiversity and ecosystem functionality over the longer term. Indeed, multiple stressors can have synergistic (greater than the additive effects), antagonistic (less than the sum of single components) or, rarely, neutral effects on marine animal health (Parisi et al., 2021).

Sabella spallanzanii is a sessile benthic polychaete widely distributed throughout the Mediterranean, where it is commonly found in shallow sheltered areas and up to 30 m deep in the most exposed waters. It has recently been introduced in Australia and New Zealand, and it is considered an ecosystem engineer (Atalah et al., 2019). Studies have emphasized the qualities of the species both as a bioindicator for monitoring environmental quality (Dean, 2008) and as a biorestorer when inserted in marine environments near fish farm discharges due to its sessile and filtering behaviour (Bocchetti et al., 2004; Giangrande et al., 2005), and have further highlighted the fact that *S. spallanzanii* is a species capable of responding efficiently to heavy metal pollution with antioxidant defences. It is of fundamental importance for survival that sessile organisms have immune systems which are capable of responding to stressors in a fast and effective way. Among the humoral responses, coelomocytes in annelids play an important role as they are able to perform phagocytic functions useful for recognizing and eliminating pathogenic agents such as bacteria and viruses (Prochazkova et al., 2020).

In invertebrates in particular, "self-non self" recognition is a basic mechanism that activates the immune system. Lysozyme activity is a phylogenetically conserved humoral response and has been studied in many invertebrate species. It corresponds to the primary and rapid defence of organisms against pathogens and is a bactericidal hydrolytic enzyme which hydrolyses the β-1,4 glycosidic bonds of peptidoglycan (an important component of the cell wall of Gram-negative bacteria), resulting in the rupture of bacterial walls due to destabilization of the membrane (Li et al., 2008), and lysozymes are present both in the mucus and tissues, and in the annelids has been found in the coelomic liquid (Marcano et al., 1997; Stabili et al., 2009).

Lectins are another relevant family of proteins involved in different biological processes such as immune response, development, cell adhesion, egg-sperm recognition. Lectins can mediate the activation of immunity effectors due to their ability to bind carbohydrates present on the cellular surface. Galactose-binding lectins have been documented in marine invertebrates including polychaeta and oligochaeta and, in particular, in *S. spallanzanii* in which it has been demonstrated that, at high concentrations of arsenic, the recognition of pathogens mediated by lectins present in the mucus of the fan worm is inhibited (Cammarata et al., 2019; Dara et al., 2022). Toll-like

receptors (TLRs) are membrane glycoproteins characterized by three domains: (1) the extracellular N-terminal domains with leucine-rich repeats (LRRs) that recognize antigens, (2) the transmembrane domain, and (3) the intracellular domain Toll/IL-1 receptor (TIR), necessary for the recruitment of molecules that activate the signal pathway downstream (Akira and Takeda, 2004; Prochazkova et al., 2019). They are classified into two clusters based on the number of cysteines on the C-terminal end of LRRs: single cysteine cluster TLRs (sccTLRs) that include vertebrates and some insects, and multiple cysteine cluster TLRs (mccTLRs) (Leulier and Lemaitre, 2008). The first earthworm TLR (from the sccTLR cluster) was isolated from the annelid *Eisenia andrei* (EaTLR). This receptor has very large intraspecies variability and is expressed in all its body tissues. Phylogenetic analysis has shown the similarity of EaTLR to a TLR from the polychaete annelid *Capitella teleta*, and with TLRs of molluscs and echinoderms. In addition, its expression in coelomocytes can be upregulated by the bacterial challenge (Škanta et al., 2013). When immune cells engage a foreign agent, they produce cytokines which are intercepted by cellular receptors that activate specific immunity genes such as the Allograft inflammatory factor-1 (AIF-1), which plays a fundamental role in regulating host responses to inflammatory stimuli and the modulation of immune response (Baranzini et al., 2019). AIF-1 is a calcium-binding protein with molecular weight of about 17 KDa that is ubiquitously expressed and has been well preserved phylogenetically from sponges to humans (Müller et al., 2002). In annelids, the first evidence of AIF-1 occurred in the leech *Hirudo medicinalis*, where it was observed that it contributes to the recruitment of immune cells (granulocytes and macrophages) after lipopolysaccharide (LPS) infection or in wound healing, leading to the activation of an effective response against infection with pathogens (Vizioli et al., 2020). The objective of this study was to further validate the use of the macroinvertebrate *S. spallanzanii* as a bioindicator of marine-coastal ecosystem quality due to the need to provide new data on immune responses following exposure to multiple stressors, such as copper sulphate and bacterial inoculum, which we examined using immunological biomarkers.

2. Materials and Methods

2.1 Sampling and experimental plan

Animal collection took place in the months of April-May in the port area of the Cala di Palermo, a site subject to anthropogenic impact and from which samples were previously taken for studies conducted by the research group. The organisms (N=15) were then transported to the Department of Earth and Marine Sciences, University of Palermo, and housed in the Marine Immunobiology laboratories.

Copper sulphate solutions were prepared at a concentration of 0.4 mg/l (Harland and Nganro, 1990); TBS solution (150 mM NaCl, 10 mM Tris- HCl, pH 7.4) was prepared for the control of bacterial injection; bacterial suspension of the *Escherichia coli* strain was prepared at a concentration of $1x10^7$ bacteria.

For the experimental plan, three "naive" controls were considered, animals which had not been treated and from which the tissues were directly stored. After being cleaned of epiphytes, the remaining 12 organisms were subjected to the following treatments: 6 were placed in tanks containing sea water, while the other 6 were placed in tanks containing sea water and copper sulphate (for a total of 4 tanks with 3 organisms each, at a fixed temperature of 18° C). These

conditions were maintained for 4 days. Subsequently, 3 animals were taken from seawater only and 3 animals which had been exposed to the pollutant, after being cleaned with filtered seawater, were inoculated with *E. coli* LPS, while the other 6 were injected with TBS only. Injections of *E. coli* and TBS were made just below the base of the fan to avoid causing autotomy of the fan. Three hours after the bacterial injection, the animals were sacrificed in order to collect the tissues for subsequent analysis (Figure 1).

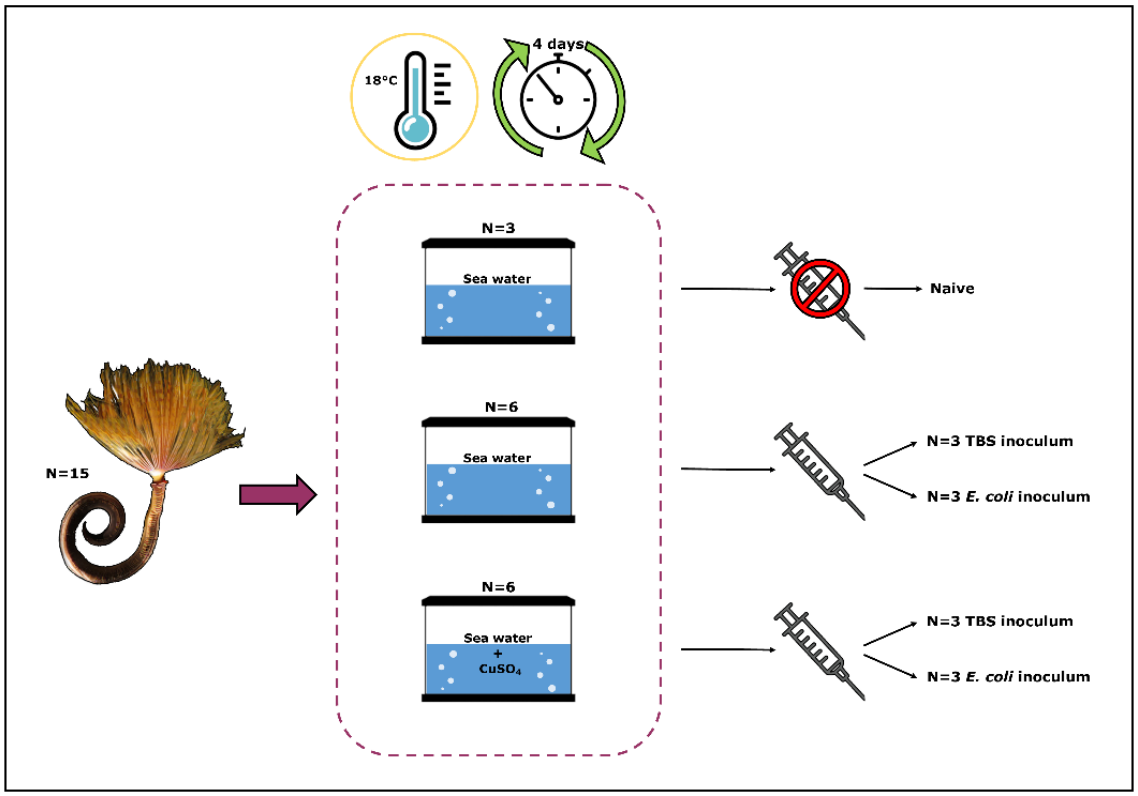


Fig. 1

2.2 Chemicals, molecular biology reagents

Chemicals and reagents were from Sigma-Aldrich (USA). *E. coli* (ATCC 25922) was obtained from Chrisope Technologies (Louisiana, USA). *Micrococcus lysodeikticus* (ATCC 4698) was supplied by Sigma-Aldrich (USA).

2.3 Protein concentration

Protein concentration measurement was conducted on tissue extracts according to the method found in (Bradford, 1976). TBS was used for blank (NaCl 150 mM, Tris-HCl 10 mM, pH 7.4). Absorbance was read at 595nm (RAYTO RT-2100C), and a calibration curve defined through bovine serum albumin (BSA) was used to obtain protein concentration expressed in mg/ml.

2.4 Sample treatment

Tissues were homogenized in polycarbonate tubes with 10 mL TBS buffer (150 mM NaCl, 10 mM Tris- HCl, pH 7.4) and centrifuged (12,000 rpm for 30 min at 4° C). The supernatant was collected

and stored in Eppendorf tubes at −20° C. Extracts were adjusted at 0.5mg/mL before performing enzymatic assays.

2.5 Bacterial culture

E. coli and *M. lysodeikticus* were grown in Luria-Broth (LB) to log phase at 37° C. The relationship between cell number by plate count and cultures and absorbance evaluation (600 nm) was determined to standardize the number of cells during all the experiments. Bacteria were killed by heat (120° C, 1 atm for 20 min). After centrifugation at 15,000 rpm for 15 min at 4° C, the heat-killed bacteria were washed three times with sterile TBS. *E. coli* was resuspended in TBS, while *M. lysodeikticus* was suspended in sodium phosphate buffer (NaH_2PO_4 0.02M e Na_2HPO_4 0.02M, pH 6.2), both were used to obtain 1×10^9 cells/ml and stored at 4° C until use.

2.6 Glutathione peroxidase activity (GPx)

Enzymatic activity was measured in triplicate on *S. spallanzanii* extracts according to (Ross et al., 2000). In 96-well flat-bottomed plates, 50 µl of sample at standard concentration (0.5 mg/ml) was incubated with 100µl TMB (3,3' 5,5'-tetramethylbenzidine). The reaction was stopped after 30 minutes of dark incubation with sulfuric acid (H_2SO_4) 2M. The absorbance was read spectrophotometrically at 450nm in a microplate reader (RT-2100C) and the GPx produced was expressed in U/mg of protein according to the equation: U/mg = Abs * V_f/CP where V_f is the final volume of the well and CP is the protein concentration of the sample.

2.7 Alkaline phosphatase activity (ALP)

Samples were incubated in an equal volume of 4 mM p-nitrophenyl phosphate liquid in 100 mM ammonium bicarbonate containing 1 mM $MgCl_2$ (pH 7.8). The enzymatic kinetic was evaluated according to (Ross et al., 2000) at regular intervals of 5 min to 1 h at 405 nm with a microplate reader (RAYTO RT-2100C). One unit (U) of activity was defined as the amount of enzyme required to release 1 micromole of p-nitrophenol produced in 1 min.

2.8 Esterase activity (EST)

According to the method of (Ross et al., 2000), the same volume of sample was incubated with 0.4 mM p-nitrophenylmyristate substrate in 100 mM ammonium bicarbonate containing 0.5% of Triton X-100 (pH 7.8, 30 °C). The increase of the optical density and the determination of the activity was evaluated as for ALP.

2.9 Lysozyme activity (LYS)

To evaluate LYS in *S. spallanzanii* extracts according to (Parry et al., 1965), 30µl of each sample were place in a 96-well flat-bottomed plate and incubated with 270µl of bacterial suspension (A 540 = 0.5-0.7) in triplicate. 30µl TBS buffer was replaced in the control sample. The reaction was carried out at 25° C, and absorbance (450nm) was measured every 30 seconds for 10 min. A unit of LYS was defined as the amount of sample causing a decrease in absorbance of 0.001/min (U min^{-1}), and U/ml was calculated in accordance with the formula: U/ml = (Δ abs/min^{-1} * dilution factor *1000)/enzyme volume buffer.

2.10 Western blot

Compatibility with the antibodies used, Polyclonal TLR-4 synthetized from rabbit (Santa Cruz sc-10741) and Polyclonal AIF-1 produced in the leech *H. medicinalis*, was verified on the NCBI (National Center for Biotechnology Information) database of the protein sequences present in the same species or in species phylogenetically close to our model (see Supplementary Material). TLR-4 and AIF-1 expressed in a cell lysate of *Mytilus galloprovincialis*, with which a positive cross-reaction had previously occurred, was used for the positive control. Western blot analysis was performed to determine the antibody specificity against selected target proteins in *S. spallanzanii*. SDS-PAGE was carried out according to the method of (Laemmli, 1970) using a 4% (stacking) and a 12% (separating) polyacrylamide gel for 60 min at 180 V using a Bio-Rad mini gel kit. Tissue extracts were run in parallel lines, following antibody suppliers' recommendations. Proteins separated by SDS-PAGE were electroblotted into a nitrocellulose membrane. The gels were prepared in blotting buffer (20 mM Tris base, 192mM glycine, methanol 10%), and a semi-dry blotting bath (Bio-Rad Laboratories) was used (10V for 75 min). The filter membrane was soaked in blocking buffer (PBS containing 3% BSA and 1% Tween-20), incubated with Anti Hm-AIF1 diluted 1:5000 in TBS-Tween20 with BSA 0.1% left overnight, washed with blocking buffer and incubated with antirabbit IgG-alkaline phosphatase conjugate (1:15 000 in washing buffer, 0.1% BSA) for 1 h. Antibody binding was detected by chromogen substrate BCIP/NBT.

The expression of molecules in the different kinds of treatments was investigated through densitometric analysis carried out using the open-source software Image J software (http://rsbweb.nih.gov/ij/download.html) (accessed on 20 July 2021).

2.11 Agglutinating activity

Rabbit red blood cells (RaRBC), supplied by “Istituto Zooprofilattico Sperimentale della Sicilia”, were washed three times in phosphate buffered saline (PBS), centrifuged at 500 x g for 10 min at 4 °C and suspended at 1% in TBS containing 0.1% (w/v) gelatine. A volume (25 µl) of *S. spallanzanii* extract was serially (2-fold) diluted in TBS-gelatine containing 3mM $CaCl_2$ in 96-well round-bottomed microtitre plates (Denmark), and an equal volume of erythrocyte suspension was added. The hemagglutinating titre (HT) was measured after 1 h incubation at 37° C and expressed as the reciprocal of the highest dilution showing clear agglutination (Ballarin et al., 2008).

2.12 Carbohydrate specificity

Inhibition agglutinating activity assays were carried out using decreasing concentrations (starting from 130 mM in TBS pH 7.4, 3 mM $CaCl_2$, 1% gelatine) of monosaccharides (D- galactose, D-glucose) and the disaccharide D-sucrose. The assay was also performed on the agglutinating activity of erythrocyte suspension, in the presence of 25µl of sugar in the erythrocyte suspension.

2.13 Statistical analysis

After performing a normality test on the dataset to verify the distribution of the data and the homoscedasticity assumption, one-way ANOVA was conducted. The analysis of multiple comparisons was performed using the Tukey post-hoc test to highlight differences between treatments. The analyses were carried out using GraphPad Prism Version 8.0.0. for Windows (www.graphpad.com) (accessed on 22 August 2021) and R studio environment (https://www.R-project.org/) for the Principal Component Analysis (PCA). All experiments were performed in triplicate, and the values used were the mean of the three assays ± SEM. Differences between means were considered significant for * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3. Results

3.1 Macroscopic observations

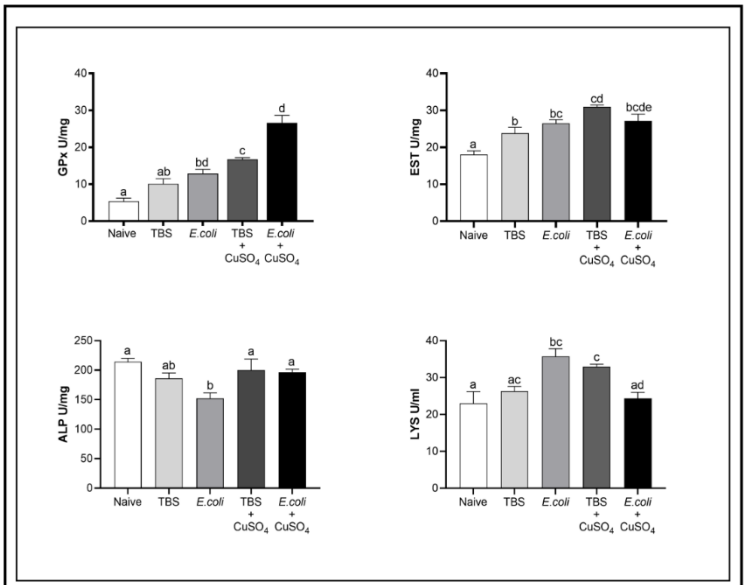
No mortality was recorded in any of the groups, whether exposed to copper sulphate or not.
After gently extracting the animal from the tube in which it had remained for the entire duration of the experimental plan, the morphological alterations resulting from the four treatments were evaluated. Polychaetes exposed to copper sulphate showed a slight darkening (brownish to grey) of the ventral part near the ventral sac. Following bacterial inoculation after exposure to $CuSO_4$, a slight swelling was found near the injection area, always in the ventral part, as indicated in Figure 2. None of these anomalies were present in naïve polychaetes or in organisms subjected to TBS inoculation only.

Fig. 2

3.2 Enzymatic response

Enzymatic responses are shown in Figure 3. GPx activity measured in *S. spallanzanii* whole body extracts increased in samples subjected to treatments with respect to control organisms (naïve), even more notably in samples exposed to the pollutant and injected with the *E. coli* suspension. Multiple comparison analysis showed significant results for animals injected with bacteria, exposed to $CuSO_4$, and for the group exposed to $CuSO_4$ + *E. coli* compared to naïve, and also as shown in the graph between treatments. The ordinary one-way ANOVA analysis showed a p-value <0.0001 for the variables "treatment" (T). The EST trend was similar to that of GPx compared to the control group, and the combined effect of the bacteria inoculum with the presence of copper sulphate seemed to determine a reduction of enzyme activity. Also in this case, the one-way ANOVA evidenced a p-value of <0.0001 between columns (T). A different behaviour was assessed for ALP kinetic activity (one-way ANOVA p value=0.0013). Generally, the activity was always around 200 U/mg of protein, but a slight inhibition of the activity was observed for the groups inoculated with *E. coli* compared to controls (p=0.0006). The post-hoc Tukey test highlighted, moreover, differences between *E. coli* vs $CuSO_4$ (p=0.0183) and between treatments *E. coli* vs $CuSO_4$ + *E. coli* (p=0.0264).

As regards LYS, the statistical analysis showed a significant difference between treatment groups equal to a p value of 0.0005. The greatest amount of LYS produced was measured in samples injected with the bacteria suspension. In general, the activity was higher in organisms exposed to stressors compared to the naïve group. Moreover, the Tukey test underlined significant differences between organisms inoculated with TBS and *E. coli* (p =0.0240), in the *E. coli* vs $CuSO_4$ groups, and between samples exposed only to $CuSO_4$ vs $CuSO_4$ + *E. coli* (p=0.0417).



Ordinary one-way ANOVA	F	P value	R square
GPx	40.63	<0.0001	0.8659
EST	12.61	<0.0001	0.6776
ALP	5.632	0.0013	0.3916
LYS	7.891	0.0005	0.6121

Fig .3

3.3 Western blot

The expression of TLR-4 and AIF-1 were investigated using the Western blot method for the various treatments (Figures 4-5). Respectively, higher integrated density values (IDV) were found for treated groups compared to controls, in particular for organisms inoculated with *E. coli* and for the group subjected to the combined effect. Statistical significance was highlighted by one-way ANOVA and Tukey post-hoc test, returning a p-value <0.0001 for both groups with respect to naïve (Fig. 6a). Moreover, IDV measurement for the expression of AIF-1 showed an increase for groups inoculated with *E. coli* compared to naïve for the treatment TBS + $CuSO_4$. The expression was inhibited for the combined treatment (Fig. 6b). Tukey post-hoc test emphasized differences between *E. coli* vs $CuSO_4$ (p-value = 0.0003) and only $CuSO_4$ vs combined treatment (p-value=0.0062).

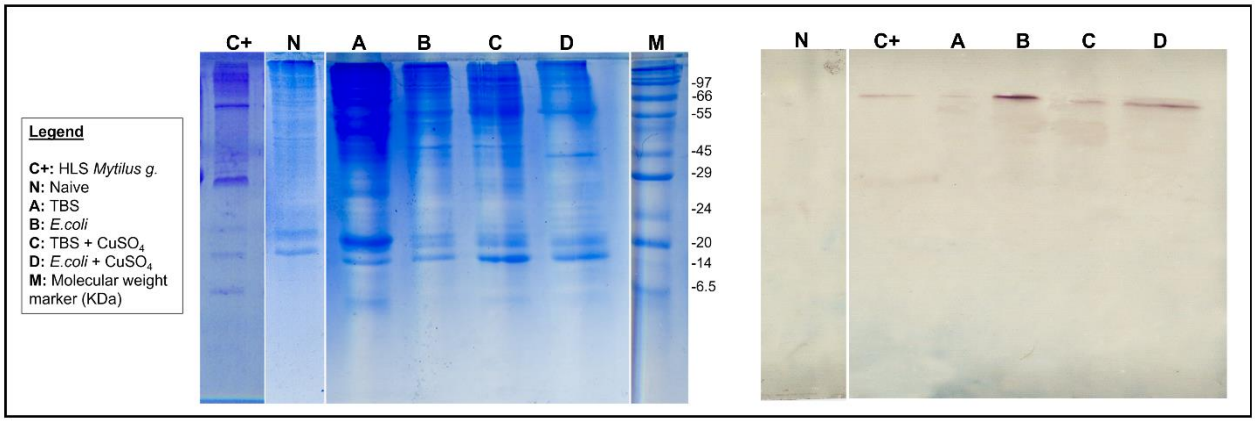


Fig. 4

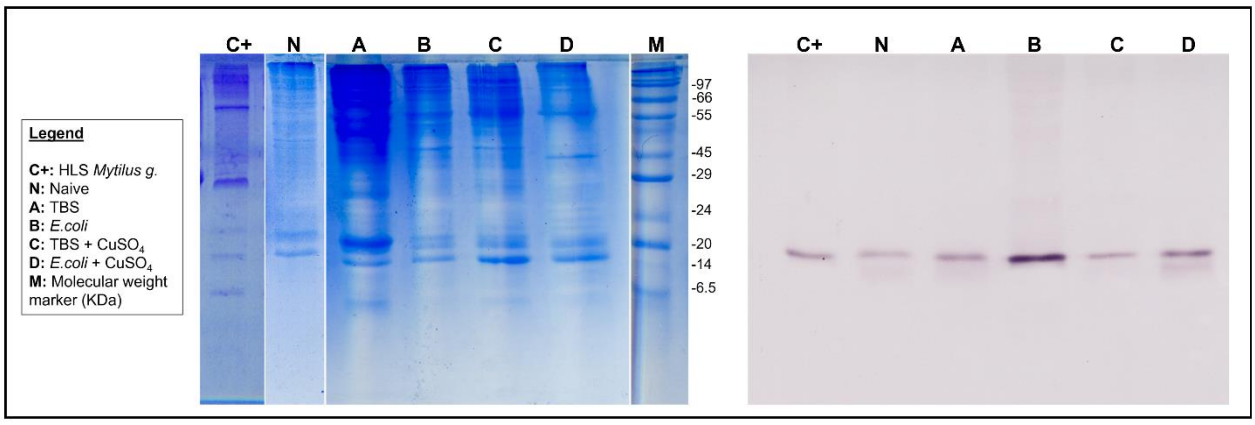
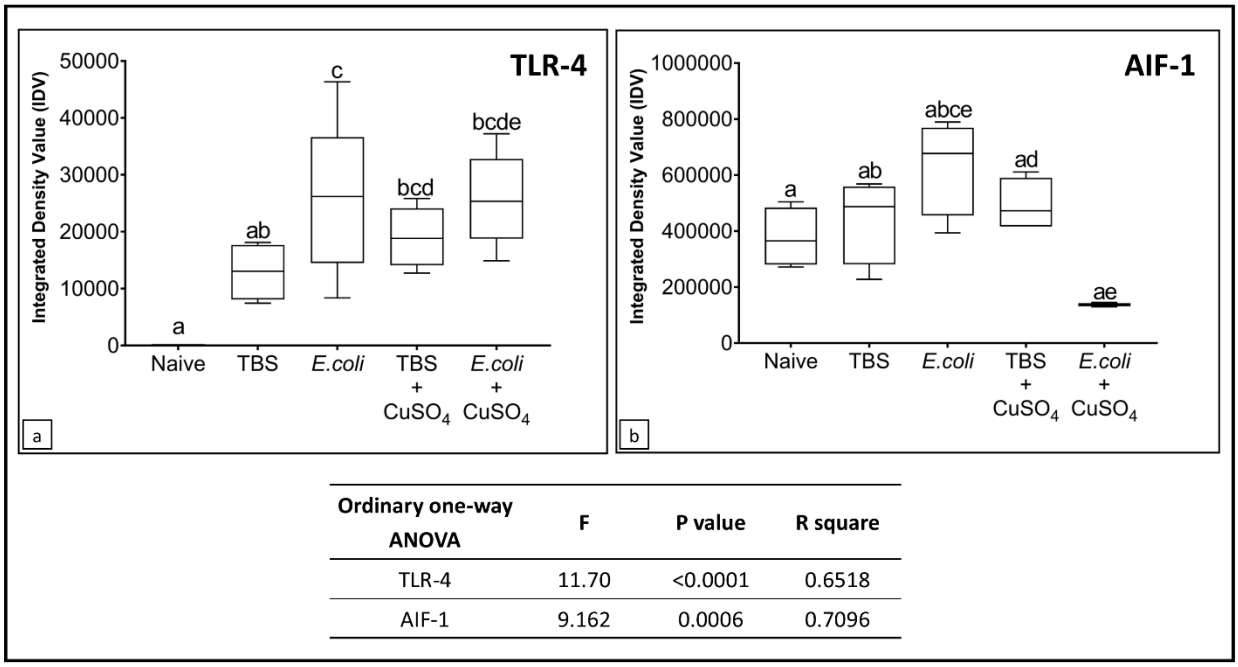


Fig. 5



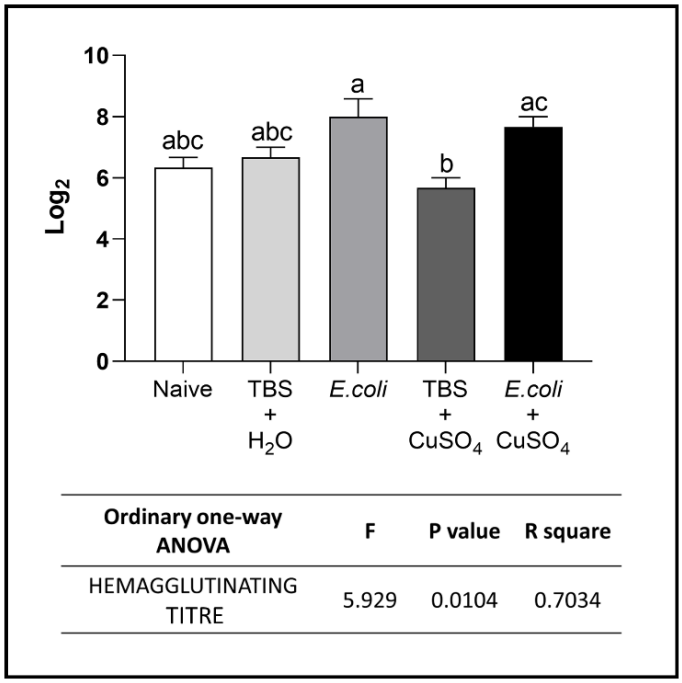
Ordinary one-way ANOVA	F	P value	R square
TLR-4	11.70	<0.0001	0.6518
AIF-1	9.162	0.0006	0.7096

Fig. 6

3.5 Agglutination and inhibition activities

No strong differences among the hemagglutinating titres of the different treatments were evidenced, but both groups inoculated with the *E. coli* suspension showed a slight increase of activity compared to naïve and TBS-injected groups. On the contrary, the measured biological activity decreased in the TBS inoculated group exposed to copper sulphate (Fig. 7). No statistical differences were evidenced between naïve and the internal control of the experiment (TBS inoculated + sea water). The multiple comparison analysis underlined statistically significant differences between the two treatments injected with *E. coli* and the group exposed to the contaminant and injected with buffer. In particular, the Tukey test evidenced a p-value of 0.0126 for the *E. coli* vs TBS + $CuSO_4$ treatments and a p-value of 0.0316 for *E. coli* vs $CuSO_4$.

As regards the carbohydrate specificity test, no inhibitory activity was detected in presence of glucose or sucrose versus erythrocytes used as a target, but a very strong inhibition of agglutinative activity occurred in the presence of galactose, up to sugar dilutions of more than 1:2048 irrespective of the type of treatment (Table 1).



Ordinary one-way ANOVA	F	P value	R square
HEMAGGLUTINATING TITRE	5.929	0.0104	0.7034

Fig. 7

Table 1 Inhibition of hemagglutination activity of *S. spallanzanii* extracts against RRBC by different carbohydrate inhibitors.

(NI = No inhibition)

Inhibitor sugars	**Minimum inhibiting concentration (mM)**
D-galactose	0.098 - 0.049
D-glucose	NI
D-sucrose	NI

3.6 Principal component analysis of immunity markers

PCA analysis was performed on seven immunological variables, according to the different treatments, to highlight possible correlations and identify the main factors affecting *S. spallanzanii*'s health status in a multiple stress condition. The first and second principal components explain most of the variance. The PC1 explains 38.71 ± 1.65% of the data variance. Together with the second main component (PC2), the data variance is explained for 64.02 ± 1.33%. The graph of the PCA with the first two principal components and the superposed biplot indicates that, for the first component, all variables are positively correlated except for alkaline phosphatase. In contrast, for the second component, only the LYS variable and AIF-1 are positively related. In

general, the treatments are well clustered together, apart from some spurious data (Fig. 8a). For PC1, the major contribution is given by TLR-4 and EST (Fig.8a), for PC2, AIF-1 and GPx (Fig.8b).

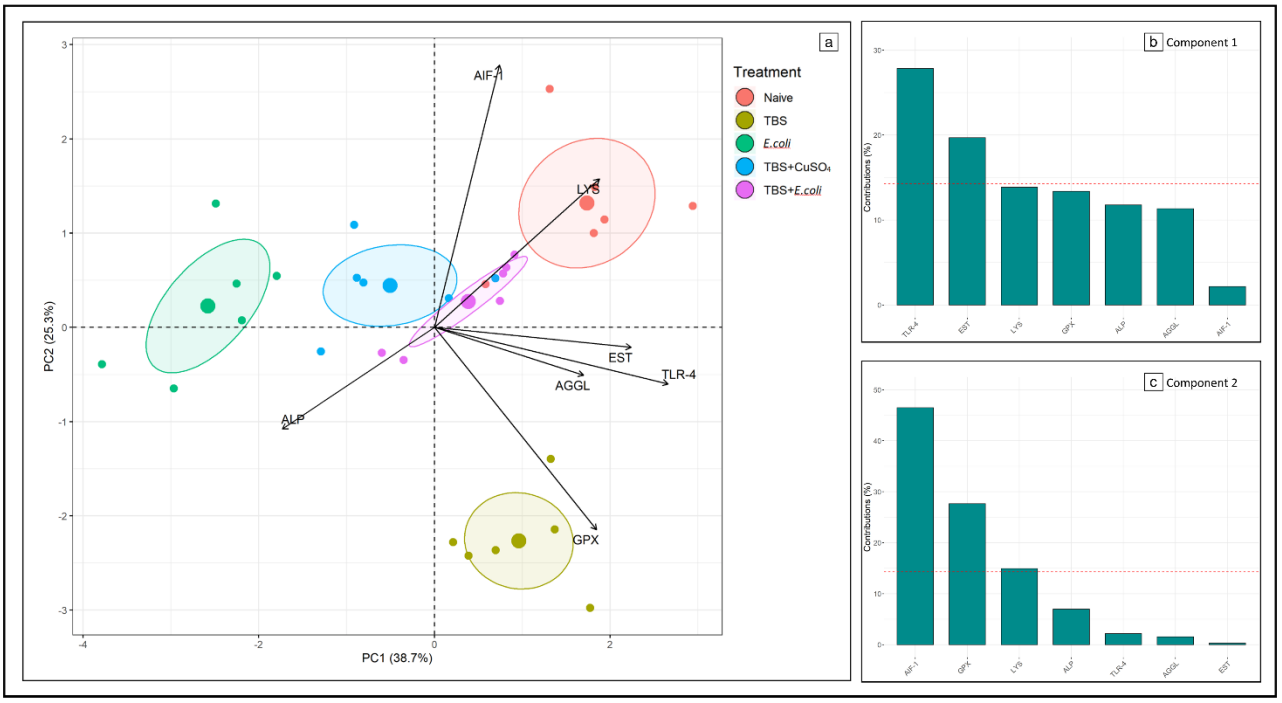


Fig. 8

4. Discussion

Recent years have seen a growing interest in the health and protection of aquatic environments and a greater awareness of the problem of marine pollution and its repercussions for all ecosystems. Heavy metals and their toxicity can be investigated in the aquatic matrix and in biological tissues using certain species of marine organisms as biomonitoring tools for the marine-coastal environment, and among these, *S. spallanzanii* stands as a reliable bioindicator for the monitoring of heavy metal contamination. The present study, in particular, analyses the immune responses of a Mediterranean polychaete, considering the entire organism. The animals were subjected to four different treatments consisting of two different stressors (chemical agent and bacterial inoculum) which were taken into consideration singularly and in combination, and the effects were analysed at the immune level using biomarkers that evaluate oxidative stress and inflammation. In the literature, many studies have shown that metals at high concentrations can displace the organism's internal homeostasis, causing an alteration of its immune system. Specifically, copper sulphate causes immunosuppression and immunomodulation effects, inhibiting some innate defences of the organism and thus decreasing its vital performance (Breton and Prentiss, 2019; Crupi et al., 2019; Gautam et al., 2018; Kumar et al., 2017). Recent studies have shown a slight change in colour from brownish to grey in the ventral thoracic area of *Eurythoe complanata* (Méndez et al., 2009) and *Perinereis aibuhitensis* (Tian et al., 2014) when exposed to copper. In our case, the fact that it did not have a firm response could be due to the short exposure to $CuSO_4$. Most of the toxicological studies performed in the laboratory with annelids, however, focus on the quantitative verification of dose-response relationships at physiological and biochemical levels and rarely describe external abnormalities produced by toxic substances. The visible swelling in the ventral area attributable to the specimens subjected to the combined treatment, in fact, coincides with a rapid response to the

infection (Stein and Cooper, 1983; Trapani et al., 2016) and may also have been augmented by metal exposure.

The TLR and Toll pathways are involved in the activation of several pathways in invertebrates (Azumi et al., 2003). For example, in the melanin cascade, in the transcription of antimicrobial peptides and in the activation of other signalling molecules. Curiously, TLRs (Kawai and Akira, 2011) are detectors of external particles, but they do not act as phagocytic receptors; nevertheless, they frequently cooperate with other nonopsonic receptors.

Here, we show the activation and modulation of TLR-4 in the extracts of *S. spallanzanii* under stress conditions. Indeed, TLR is clearly activated when the inflammation process is in progress. The greatest IDV values (corresponding to a high expression of protein) have been found in extracts of samples that were only inoculated with bacteria and in those that underwent the combined treatment, confirming the fundamental role of this receptor in immune response. The AIF-1 expression and modulation survey showed a positive cross-reaction between the Hm-AIF-1 antibody and the *S. spallanzanii* polychaete samples. The high presence of the molecule quantified as IDV in samples injected with bacterial suspension suggests that the main surface components of the bacteria are able to activate the mRNA expression of AIF-1, suggesting that this factor is significantly involved in defence mechanisms against Gram-negative bacteria. Indeed, in a study carried out on *Crassostea ariakensis,* AIF-1 was identified as a modulator of the immune response during the activation of macrophages, with a key role in the immune defence reaction of the host and in inflammatory response (Xu et al., 2014). Moreover, its involvement in stress-response mechanisms against environmental and immune challenges and the ability of heavy metals to inhibit their expression was also highlighted (Cuttitta et al., 2017).

Marine organisms have developed strategies against several pathogens living in their same environment, including a non-specific immunity molecule and a digestive enzyme, the lysozyme. It is well-studied in many aquatic organisms, and investigations of its expression and regulation at both transcriptional and protein levels have also been conducted on polychaetes (Ruane et al., 2000). LYS can act directly by damaging the bacterial cell wall or through its stimulatory effect on phagocytosis. As shown by (Fang et al., 2013) for mRNA expression in *Mactra veneriformis* after exposure to heavy metals for more than three days, they can cause an overexpression of LYS.

As demonstrated by (Dhainaut and Scaps, 2001), an increase of LYS activity is related to a higher release of protein into the body, causing the innate immune system to respond, and the modulation of protein biosynthesis plays a key role in the control of the humoral bacteriolytic activity in annelids.

In this work, the presence of copper induced the modulation of the enzyme. Indeed, treatments showed a general upregulation of the enzyme compared to naïve organisms, in particular, the highest activity was registered for samples injected with *E. coli* and for organisms exposed to $CuSO_4$ and inoculated with TBS, suggesting an attempt to respond to stress. The combined effect (copper + bacterial injection) showed an inhibition of the activity compared to xenobiotic exposition only. Contrary to what has been suggested by (Gagnaire et al., 2007), who reported that some genes involved in defence were up-regulated in *Crassostrea gigas* following combined exposure to pesticides and bacteria, the slight decrease in activity in the group with combined treatment of $CuSO_4$ and *E. coli* is due to the immunosuppressive action of the metal, as (Zapata-

Vívenes et al., 2005) observed in acute copper exposure; an excessive accumulation of copper in the body can trigger molecular and/or cellular changes, such as oxidative damage (Nusetti et al., 2001), when an infection occurs, resulting in negative effects on LYS function. As previously indicated by (Martinez, 2012) for polluted areas of the Gulf of Cariaco, these alterations can occur in polychaetes subjected to bacterial stress inhabiting copper contaminated environments.

Differently, (Goven et al., 1994) individuated an inhibition of LYS in *Lumbricus terrestris* correlated to the bioaccumulation of copper sulphate after five days of xenobiotic exposure, indicating that the metal altered the structural function of the molecule. Remarkably, other studies on annelids have detected different responses of LYS activity to short term of copper contamination (activation in *Amynthas hawayanus* and inhibition in *Eisenia foetida)* (Nusetti et al., 1999).

Copper appears to be potentially immunotoxic to bacteria-sensitized polychaetes, as it likely produces discomfort that disturbs the normal induction of LYS as a protective factor against bacterial infection. In other words, copper may have inhibited the ability of haemocytes to respond to bacterial stress, as highlighted in the study of (Castro et al., 2018).

LYS activity has also been extensively studied in earthworms, and it has been seen that it can be modulated by xenobiotics such as pesticides and herbicides (Fiołka et al., 2012). *S. spallanzanii* appear to be suitable for use in assessing risks of exposure of aquatic organisms to chemical contamination. According to the results of a work carried out on *Perinereis aibuhitensis*, which showed that peroxidase activity is induced by the presence of metal and that it decreases after ten days (Tian et al., 2014), an increase in enzyme activity was observed in relation to the type of treatment compared to the control group. The multiple stresses induced by copper and bacterial inoculum led to a clear increase in enzyme activity. These values could be attributed to the oxidative stress caused by copper that is worsened by the inoculum of *E. coli*, thus leading to a greater expression of this enzyme because interactions between pathogens and pollutants can be synergistic, antagonistic or additive, depending on the nature of the pollutant and the host-pathogen system (Fang et al., 2013). As regards agglutinating activity, at low dilutions, the lithic activity partly covered agglutination, probably attributable to the high protein concentration of the sample. The test further confirmed the presence of haemolysis, with fetidine and lysine present in the coelomic fluid of annelids (Stein and Cooper, 1983). Some molecules of the coelomic fluid, such as the fetidine produced by coelomocytes, inhibit bacterial growth and have the ability to lyse mammalian red blood cells (Engelmann et al., 2004). The agglutinating activity may be due to the presence of lectins, immune mediators in vertebrates and invertebrates that, among their many functions, also determine cellular agglutination and, specifically in *S. spallanzanii*, a galectin from the mucus (43 kDa, SsGBL) was characterized and purified which was capable of agglutinating bacteria (Cammarata et al., 2019) also in the presence of arsenic.

Because of their ability to bind carbohydrates implicated in the identification of possible host pathogens, lectins stop the spread of pathogens (Cammarata et al., 2014). As a result of these properties, among Annellida, *S. spallanzanii* is one of the most famous and abundant Mediterranean Sabellidae. In particular, several studies conducted on mucus secreted by this organism identified lysozyme-like activity and *in vitro* antimicrobial activity towards some Gram-negative bacteria in different stress conditions (Stabili et al., 2009). These previous results and the data shown here, confirm the relevant role of lectins and lysozyme-like activity in internal defence, particularly against potential pathogens; indeed, haemagglutinating activity here was higher in the groups

injected with *E. coli* suspension both in the presence and absence of copper, even if its presence seems to negatively affect agglutination. Moreover, the clear inhibition of agglutination in the presence of D-galactose suggests the presence of SsGBL not only in mucus but also in the body tissues, even though no specific modulation was observed. It is central to consider that *S. spallanzanii* lives in eutrophic habitats such as harbours where bacteria, including human pathogens, are plentiful. PCA results support the evidence of the several variables individually. The first component explains the high percentage of data variability (38.7%) and includes, in particular, detoxification markers. The major data variability contribution of the second component is given by AIF-1 and LYS molecules particularly involved in pathogen recognition. In this difficult context of a study of the combined effects of a pollutant-bacterial challenge, the PCA approach could be useful for determining the most relevant variables affecting the alteration of an animal's homeostasis. Even though our knowledge of the immune responses of various marine invertebrates remains partial, this work contributes to understanding the properties and roles of some markers investigated in the Mediterranean polychaete *S. spallanzanii*.

5. Conclusion

Environmental contamination has increased dramatically due to an exponential use of heavy metals (especially copper) in various industrial, agricultural, domestic and technological applications that particularly impact estuaries and coastal areas.

This study highlights the effects of four different treatments (TBS inoculum, bacterial inoculum, exposure to copper sulphate and combined treatment of bacterial inoculum and exposure to copper sulphate) on the whole body of the benthic macroinvertebrate model *S. spallanzanii*, an organism previously used by the research group that has once again proven efficient in ecotoxicological, immunological studies having, moreover, all the characteristics of ideal bioindicators. The markers used were also good biomarkers for investigating multiple stressors: metals and bacterial inoculum.

The activity of AIF-1 was clearly linked to events of inflammation and immune response. However, the spectrum of action and the involvement of this factor in the invertebrate's immune response need to be further investigated. Future studies on AIF-1 in these organisms will bring new knowledge about its biological functions, opening up unexpected insights for the understanding of the functions of AIF-1 in the immune response of mammals and in the control of human inflammatory diseases. The modulation of LYS and peroxidase enzyme responses, known stress markers, and the investigation of haemagglutination activity, have well highlighted the differences between the different treatments.

In line with other studies, copper sulphate had an immunomodulating effect on the internal defence system of the species under investigation, varying the immune response especially after bacterial inoculum.

The special feature of this work was the validation of the Mediterranean macroinvertebrate polychaete subjected to four different treatments, including a combined treatment with stressogenic effects detectable on several structural levels, so as to increase knowledge about the immune mechanisms that are activated and on how animals are able to manage the energies intended for the

maintenance of internal homeostasis. Studies assessing the sub-lethal effects of copper sulphate on organisms are important for encouraging earlier actions in situations of exposure to pollutants.

CRediT roles

La Corte Claudia: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Roles/Writing - original draft, Writing - review & editing; **Dara Mariano:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Roles/Writing - original draft, Writing - review & editing; **Bertini Federica:** Formal analysis, Investigation, Roles/Writing - original draft, Writing - review & editing; **Daniela Parrinello:** Funding acquisition, Writing - review & editing; **Daniela Piazzese:** Writing - review & editing; **Maria Giovanna Parisi:** Data curation, Funding acquisition, Project administration, Supervision, Validation, Roles/Writing - original draft, Writing - review & editing

Acknowledgements

The authors are grateful to Marta Lucchese for the *Sabella spallanzanii* illustration in the graphical abstract.

Funding

FFR Parisi (PJ_RIC_FFABR_2017_161753 MGP), and FFR Parrinello (PJ_RIC_FFABR_2017_130077 DP) supported this research.

Declaration of conflicts of interest: none

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Figure captions

Fig.1 Schematic drawing summarizing the experimental design

Fig. 2 For the injection, animals were gently pulled outside the tube until just below the crown, to avoid autotomy (boxed photo top left). Animals inoculated with *E. coli* showed swelling in the area (circled in yellow) 3h post injection. Specimens exposed to $CuSO_4$ also presented a darker ventral area than the rest of the body (evidenced with arrow).

Fig. 3 Graphics show the enzymatic response of GPx, EST, ALP and LYS in *S. spallanzanii*. Data were tested for normality, and differences between groups were shown by one-way ANOVA as mean ± standard error. The letters indicate statistically significant differences between treatments. Differences between means were considered significant for $p < 0.05$. The table shows the ANOVA summary of enzymes results.

Fig. 4 SDS-PAGE (12%) under non-reducing conditions and Western blot of *S. spallanzani* extracts. Molecular weights (kDa) of markers are on the right (lane M); on the left haemocytes lysate supernatant (HLS) of *M. galloprovincialis* used as positive control for Western blotting given previous findings in laboratory. The gel was stained with Coomassie blue. The nitrocellulose sheet was treated with anti-TLR4 primary antibody and alkaline phosphatase-conjugated anti rabbit IgG.; cross reaction after incubation with pre-immune serum is not shown.

Fig. 5 SDS-PAGE (12%) under non-reducing conditions and Western blot of *S. spallanzani* extracts. Molecular weights (kDa) of markers are on the right (lane M); on the left haemocytes lysate supernatant (HLS) of *M. galloprovincialis* used as positive control for Western blotting given previous findings in laboratory. The gel was stained with Coomassie blue. The nitrocellulose sheet was treated with anti-HmAIF-1 primary antibody and alkaline phosphatase-conjugated anti rabbit IgG.; cross reaction after incubation with pre-immune serum is not shown.

Fig. 6 Graphs show densitometric analysis values resulting from a nitrocellulose sheet analysed with Image J software. Different expressions of both TLR-4 (Fig. 8a) and AIF-1 (Fig. 8b) between treatments were highlighted by one-way ANOVA and show mean ± standard error. The letters indicate statistically significant differences between treatments. Differences between means were considered significant for $p < 0.05$. The tables show the ANOVA summary of TLR-4 and AIF-1 integrated density values results.

Fig. 7 Hemagglutination titre of the specimens toward rabbit erythrocytes. Differences between treatments were evidenced by one-way ANOVA. The letters indicate statistically significant

differences between treatments. Differences between means were considered significant for $p < 0.05$. The table shows the ANOVA summary of the hemagglutinating titre.

Fig. 8 Principal component analysis. (a) Scatter plot of the principal component analysis, including immunological markers and biological activities investigated on *S. spallanzanii*. TLR-4 (IDV), AIF-1 (IDV), GPx (U/mg), ALP (U/mg), EST(U/mg), LYS (U/ml), agglutination (AGGL) expressed in Log_2 according to treatments. Confidence ellipses were centred on the categorical variable "Treatment" with a confidence level of 0.95; (b) Contribution of the seven variables to the first component of the PCA expressed in percentage. Red dashed line on the graph indicates the expected average contribution; (c) Contribution of the seven variables to the second component of the PCA expressed in percentage. Red dashed line on the graph indicates the expected average contribution. Variables larger than the cut-off can be considered as important in contributing to the components.