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# **A single coelomic cell type is involved in both immune and respiratory functions of the coastal bioindicator annelid: *Capitella C-Channel1* from the English Channel**

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1 **A single coelomic cell type is involved in both immune and respiratory functions of the**  
2 **coastal bioindicator annelid: *Capitella* C-Channel1 from the English Channel**

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23 **Keywords:** *Capitella capitata*, erythrocytes, immune function, antimicrobial peptide, globin

24

25 **ABSTRACT**

26 The polychaete *Capitella* is a typical member of the 'thiobiome', and is commonly used as an  
27 eutrophication indicator species in environmental assessment studies. To deal with a sulfide-rich and  
28 poisonous surrounding, cells in close contact with the environment, and thus able to play a major role  
29 in detoxication and survival, are circulating cells. This work aimed to morpho-functionally describe  
30 the circulating coelomic cells of *Capitella* from the English Channel inhabiting the sulfide-rich mud in  
31 Roscoff Harbor. In general, worms have three types of circulating cells, granulocytes involved in  
32 bacterial clearance and defense against microorganisms, eleocytes with an essentially trophic role and  
33 elimination of cellular waste, and erythrocytes which play a role in detoxification and respiration *via*

34 their intracellular hemoglobin. By combining diverse microscopic and cellular approaches, we provide  
35 evidence that *Capitella* does not possess granulocytes and eleocytes, but rather a single abundant  
36 rounded cell type with the morphological characteristics of erythrocytes *i.e.* small size and production  
37 of intracellular hemoglobin. Surprisingly, our data show that in addition to their respiratory function,  
38 these red cells could exert phagocytic activities, and produce an antimicrobial peptide. This latter  
39 immune role is usually supported by granulocytes. Our data highlight that the erythrocytes of  
40 *Capitella* from the English Channel differ in morphology and bear more functions than the  
41 erythrocytes of other annelids. The simplicity of this multi-task (or polyvalent) single-cell type makes  
42 *Capitella* an interesting model for studies of the impact of the environment on the immunity of this  
43 bioindicator species.

44

## 45 **1. Introduction**

46 *Capitella* spp. are small (*ca.* 1 cm in length) benthic polychaetes belonging to the family Capitellidae.  
47 *Capitella* Fabricius, (1780) is generally considered to be a cosmopolitan genus, found from the coastal  
48 “thiobiome” (Boidin-Wichlacz et al., 2021) to deep-sea regions (Silva et al., 2016) that are enriched in  
49 heavy metals and sulfide, up to levels that are toxic for most metazoans. Reish & Gerlingher (1997)  
50 reported that *Ophryotrocha labronica* (polychaete) was the most sensitive more heavy metal  
51 contaminants (Reish and Gerlinger, 1997). Changes in the benthic macrofauna community observed  
52 after episodes of severe oxygen deficiency indicate differential tolerance to oxygen concentrations  
53 (Diaz and Rosenberg, 1995), with mollusks and polychaetes being typically more tolerant to oxygen  
54 deficiency than echinoderms, fishes, and crustaceans. Although these types of extreme habitats allow  
55 less competition-induced stress, specific genetic, physiological adaptations and peculiar symbiotic  
56 associations are needed for the survival and thriving of these constraining biomes (Hourdez et al.,  
57 2021). Several studies have now provided evidence that the immune system is an interesting marker to  
58 follow the adaptation of annelids to their environment. For instance, a phenotypic switch of the  
59 immune coelomic cells is observed in coastal *Hediste diversicolor* populations differentially exposed  
60 to heavy metals in their natural habitats (Cuvillier-Hot et al., 2014). The number of coelomic cells  
61 producing antimicrobial peptides (AMPs) of the Pompei worm *Alvinella pompejana* decreases as  
62 animals are exposed to lethal temperatures, thereby likely affecting the immunity of the animals  
63 (Ravaux et al., 2013).

64 The characterization of coelomocytes in invertebrate aquatic species is a first step in understanding  
65 immunological processes. In general, there are three main circulating cell types in the coelomic fluid:  
66 granulocytes, eleocytes and erythrocytes (Vetvicka and Sima, 2009). Granulocytes (also called  
67 amoebocytes) form the largest category, present in almost all polychaete species studied so far.  
68 Granulocytes exhibit a granulated cytoplasm and exert phagocytic, encapsulation and cytotoxic  
69 activities (Berlov and Maltseva, 2016; Porchet-Henneré and Vernet, 1992). A second type, called

70 eleocytes, has often been described as agranular lymphocytes or adipo-spherular cells (Liebmann,  
71 1942). Eleocytes may also be phagocytic and participate in disposing or disintegrating muscle fibers  
72 that result from developmental changes during metamorphosis (Baskin, 1974). These free coelomic  
73 cells are also considered principal distributors of metabolites during sexual maturation (Fischer and  
74 Hoeger, 1993). Eleocytes are not found in all polychaete families, as they are missing in Arenicolidae,  
75 Glyceridae, Nephtyidae, and Syllidae (Franchini et al., 2016; Vetvicka and Sima, 2009). The third cell  
76 type, erythrocytes (literally red blood cells with a nucleus), is found in a relatively small number of  
77 taxonomically diverse invertebrates (Glomski and Tamburlin, 1990). In marine annelids (polychaetes),  
78 they have been reported in Capitellidae, Cirratulidae, Glyceridae, Terebellida and Opheliidae  
79 (Goodrich, 1898; Jouin-Toulmond et al., 1996). According to Dhainaut and Porchet-Henneré (1988)  
80 erythrocytes in polychaetes form two lineages which differ in morphology and functional  
81 specialization (Dhainaut and Porchet-Henneré, 1988), such as regeneration or phagocytosis. In most  
82 annelid species, erythrocytes are specialized in the transport or storage of oxygen that reversibly binds  
83 to intracellular hemoglobin (Seamonds and Schumacher, 1972). However, in certain annelids species  
84 possess large extracellular hemoglobin that is capable to bind and transport oxygen, and ensures a  
85 significant form of oxygen storage (Hourdez et al., 2000; Weber and Vinogradov, 2001). An  
86 investigation comparing the respiratory properties of the erythrocytes between ten sibling species,  
87 morphologically similar to *Capitella capitata*, pointed to distinctive and non-overlapping differences  
88 in their diameter according to the species. More importantly, the respiratory properties of the  
89 erythrocytes are distinctive of most of the species examined, for unclear adaptive reasons (Mangum et  
90 al., 1992). Overall, in invertebrate species, however, respiratory pigment properties usually reflect  
91 environmental conditions (Weber and Vinogradov, 2001). Hemoglobins (Hb) contained in  
92 erythrocytes of a North American population of *Capitella* (likely corresponding to the recently  
93 described *C. telata*) have high to moderate oxygen affinities, little or no pH dependence, and no  
94 cooperativity. These properties are more typical of oxygen storage globins such as myoglobins. In  
95 members of the families Capitellidae, Cirratulidae, Glyceridae and Opheliidae, erythrocytes have been  
96 shown to also be capable of active phagocytosis. Unlike eleocytes, however, they primarily  
97 unselectively phagocyte foreign material (Stanovova, 2019; Vetvicka and Sima, 2009). However, to  
98 date, erythrocytes have not been shown to exert immune functions.

99 In polychaetes such as Capitellidae and Alvinellidae, inhabiting sulfide rich habitats, hemoglobins  
100 may also protect the annelid worms from the toxic action of sulfide by a detoxifying process (Jouin-  
101 Toulmond et al., 1996; Terwilliger, 1998; Wippler et al., 2016). Other authors have shown an  
102 emerging role for invertebrate intracellular hemoglobins in innate immunity, in bivalves. Purified Tg-  
103 HbI and Tg-HbII from the bivalve *Tegillarca granosa* showed antibacterial activities against Gram-  
104 negative and Gram-positive bacteria (Bao et al., 2016; Coates and Decker, 2016).

105 Coelomocytes, and notably granulocytes, are generally endowed with defensive capacities, namely  
106 phagocytic, encapsulating, and microbicidal activities that contribute to both cellular and humoral

107 responses (Cuvillier-Hot et al., 2014). Components that participate in innate immune responses  
108 include humoral factors such as antimicrobial peptides toxic for most (AMPs) and lysozyme-like  
109 proteins, which are secreted in body fluids. AMPs play an important role, as they are produced  
110 constitutively or induced upon infection in coelomocytes, as well as in the body-wall to defend against  
111 invading pathogens (Bruno et al., 2019; Wu et al., 2021). In marine polychaetes, such an induction has  
112 been shown in the lugworm *Arenicola marina* producing Arenicin-1 and 2, in *Hediste diversicolor*  
113 secreting Hedistin, and in the hydrothermal vent *Alvinella pompejana* producing Alvinellacin  
114 (Maltseva et al., 2014; Tasiemski et al., 2014, 2004). A putative AMP called Capitellacin has been  
115 predicted in *Capitella teleta* based on genomic analysis (Tasiemski et al., 2014). Capitellacin has been  
116 recently been shown to possess antibacterial and antibiofilm activities against *E. coli* (Safronova et al.,  
117 2022). A lysozyme activity has also been detected in coelomocytes and coelomic fluid of the  
118 polychaetes *Arenicola marina* and *Hediste diversicolor* (Cuvillier-Hot et al., 2014; Grossmann and  
119 Reichardt, 1991).

120 The North American species *C. teleta* has attracted attention because it has several typical annelid  
121 specificities and exhibits some advantages for developmental and regenerative studies (Boyle et al.,  
122 2014; Seaver, 2016; Seaver and Kaneshige, 2006). Genetic studies have revealed that several species  
123 of *Capitella* can be found on the shores of Brittany, including one hereafter referred to as *Capitella* C-  
124 Channel1 (Boidin-Wichlacz et al., 2021). So far, no study on the immune defense system of this  
125 species has been conducted, except studies showing that sequences of the genomes from *Capitella* sp.  
126 suggest the existence of Toll-like receptors (TLRs), which are pattern-recognition receptors (PRRs)  
127 (Davidson et al., 2008). In this context, the goal of this paper is to investigate the cellular and humoral  
128 response immunity of the bioindicator species C-Channel1 using an array of morpho-functional  
129 approaches.

130

## 131 **2. Materials and methods**

132

### 133 *2.1. Animals*

134

135 All described experiments were performed on C-Channel1 (corresponding to *Capitella* C-Channel1 in  
136 Boidin-Wichlacz et al. 2021) adults that were collected between 2015 and 2023  
137 in Roscoff (France) (48°42.743' N, 4°00.092' W). *Capitella* were collected at low tide. The surveyed  
138 sites (an average of 4 sampling points in a 1 m<sup>2</sup> zone) are enriched in silts, with a high concentration of  
139 organic carbon in the Roscoff Harbor (Hourdez et al., 2021). The sediment was sieved on a 500 µm  
140 mesh in the field and animals were brought back to the laboratory for sorting under a dissecting  
141 microscope. *Capitella* have been reared at Lille laboratory in aquaria containing a 2–3 cm layer of  
142 sediment and filled with artificial seawater (Instant Ocean® Sea Salt), equipped with an aeration

143 system and kept at room temperature (21 °C) as described earlier (Boidin-Wichlacz et al., 2021). To  
144 feed the worms, the sediment was regularly supplemented with a mixture of ground commercial dried  
145 baby cereal (HiPP Biologique, France).

146

## 147 2.2. Morphological description of the coelomic cells

148

### 149 2.2.1. Observation of the cell types *in situ*

150 Whole adults (n= 3) were fixed for 4 h in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH  
151 7.4) at 4 °C and progressively dehydrated with increasing concentrations of ethanol in water (55% to  
152 100%). They were embedded in a hydrophilic resin (Technovit® 7100, Electron Microscopy Sciences)  
153 following the manufacturer's instructions. Ten-µm thick sections were stained for 4 min with toluidine  
154 blue at 0.1% in water. Specimens were examined with a Zeiss Stemi 305 stereomicroscope. Images  
155 were taken with a camera and the Zen software.

156

### 157 2.2.2. Flow cytometry analysis of the coelomocyte population

158 Coelomic fluid from 10 animals (for a total volume of 10 µL) was collected using a glass capillary  
159 introduced into the coelomic cavity. Cellular samples were analyzed with an EPICS XL-MCL4;  
160 Beckman Coulter flow cytometer. During the analytical experiments, 10,000 threshold events per  
161 sample were collected, with side scatter for cell complexity/granularity, and forward scatter for cell  
162 size. The results were analyzed using the Expo 32 software (Cytometry systems).

163 Coelomic fluid from 4 heterogeneous batches of 10 individuals were analyzed in spectral experiments  
164 with a SONY SP6800 Spectral Cell Analyzer : 5,000 events per sample were collected, with the side  
165 scatter providing information about cell complexity/granularity, and the forward scatter for measuring  
166 cell size. The acquisition was done with 3 lasers with exciting wavelengths at 488 nm, collinear 405  
167 nm and 638 nm. The emission was recollected by 32 photomultiplier tubes (PMTs) from 500 to 800  
168 nm and 2 more from 420 to 480 nm.

169

### 170 2.2.3. Observation of the cell surface of the coelomocytes by scanning electron microscopy (SEM)

171 Coelomocytes from five animals (around 5 µL), were fixed with paraformaldehyde 4% in seawater for  
172 1 h, then were dehydrated through an ethanol series (55–100%), critical-point dried, and platinum-  
173 coated (20 nm) in a Balzers SCD-030 sputter-unit before observation using SEM (JEOL JSM-840A) at  
174 20 kV accelerating voltage.

175

### 176 2.2.4. Differential staining for light microscopic discrimination of the coelomocyte population

177 Nuclear staining of the cells with DAPI (4,6-diamidino-2-phenylindole): the cells from 5 animals fixed  
178 with paraformaldehyde at 4% in seawater for 1 h were centrifuged on coated slides using a  
179 cytocentrifuge at 4,000 rpm for 8 min at 4 °C, and then washed three times with phosphate-buffered

180 saline (PBS at 0.1 M, pH 7.4) for 10 min and incubated with a DAPI (0.5 µg/mL in PBS) (ROTH)  
181 solution in the dark for 20 min at room temperature.

182 Differentiation between acidic and alkaline vesicles in the cytoplasm of the coelomocytes: 20 mg of  
183 neutral red was dissolved in 1 mL DMSO (Sigma), then filtered through Whatman filter paper and  
184 diluted 1/5 in PBS. The coelomic fluid from four worms was removed (around 20 µL) and then added  
185 to the diluted neutral red solution for 5 min at room temperature. Neutral red stains vacuoles from red  
186 (for acidic vacuoles) to yellow (for alkaline vacuoles). The coelomic fluid from four worms was  
187 removed for this purpose.

188 Iron detection related to the presence of hemoglobin in the coelomocytes: Coelomic fluid of the  
189 animals (around 5 µL, n= 5 individuals) deposited on a Superfrost glass slide (cytocentrifuged at 4,000  
190 rpm for 8 min at 4 °C) was immersed with Perls reagent (2% potassium ferrocyanide and 2%  
191 hydrochloric acid) for 30 min. Ferric ions then produce Prussian blue, an insoluble blue compound.  
192 The slides were thoroughly rinsed in distilled water for 5 min. The counterstain was carried out with a  
193 modified Harris hematoxylin solution according to Mayer (Sigma) for 5 min.  
194 Coelomocyte populations were examined using optical microscopy (Zeiss Axio Imager M2) and  
195 images were taken with a camera and using the Zen Microscopy software (Zeiss). Using Image-J  
196 software on microscopic pictures, we measured length, width and surface area of stained and unstained  
197 cells in suspensions, placed on glass slides and covered with a 24 x 50 mm coverslip.

198

199 2.2.5. Ultrastructural study of the coelomocytes by transmission electron microscopy (TEM)

200 The cells of five animals were collected as indicated in section 2.2.2, and immediately fixed according  
201 to the protocol previously described (Boidin-Wichlacz et al., 2012). Cells were dehydrated with  
202 ethanol and embedded in Epon (polymerization at 60 °C for 48 h). Ultrathin sections (80–90 nm) were  
203 made from the Epon blocks, placed on 200-mesh copper grids, and stained with uranyl acetate and  
204 lead citrate with a standard protocol. Specimens were observed with a Hitachi H 600 electron  
205 microscope.

206

207 *2.3. Molecular identification of hemoglobin from Capitella C-Channel1*

208

209 2.3.1. RNA extraction

210 The total RNAs of each group, with 10 individuals per condition were extracted with the TRI-Reagent  
211 solution (Sigma), following the manufacturer's protocol. The concentration of extracted RNA was  
212 measured with the Qubit® RNA HS kit and the Qubit® 3.0 Fluorometer.

213

214 2.3.2. cDNA synthesis

215 Complementary DNA (cDNA) synthesis using the RT-PCR System protocol (Fermentas, Thermo  
216 Fisher Scientific Inc) was performed according to the manufacturer's instructions with 1.2 µg of RNA

217 per sample. Controls RT Amplification was carried out with GoTaq® G2 DNA Polymerase  
218 (Promega). Reaction mixtures contained 10 μmol.L<sup>-1</sup> of each primer, 10 μmol.L<sup>-1</sup> of each  
219 deoxynucleotide triphosphate, 5X Go Taq® Flexi green buffer (Promega), and 5 U of GoTaq G2 Flexi  
220 DNA polymerase (Promega). The final volume was adjusted to 50 μL with sterile water. The PCR  
221 program involved an initial denaturation step at 95 °C for 3 min, followed by 39 cycles of 95 °C for 30  
222 sec, 60 °C for 30 sec, and 72 °C for 30 sec, with a final elongation step at 72 °C for 5 min. Primers  
223 used for the semi-quantitative PCR were designed with the Primer3 Input software  
224 (<http://primer3.ut.ee/>). Hemoglobin, F5'-GAAACCGGTCGTCTAAATCA-3', R 5'-  
225 CCATGATGATTGTTGAAGTT- 3'.

226

### 227 2.3.3. Cloning and sequencing

228 The PCR products were purified with the Nucleospin Extract Kit (Macherey-Nagel) and cloned in the  
229 TA Cloning kit (Promega), according to the protocol provided by the manufacturer. DNA plasmids  
230 were Sanger-sequenced with the FM13/RM13 universal primer using the BigDye Terminator kit  
231 (Applied Biosystems, Foster City, CA).

232

### 233 2.3.4. Sequence analysis

234 The nucleotide sequences were translated into amino acid sequences using the universal genetic code  
235 with the Expert Protein Analysis System (<http://www.expasy.org/>). The resulting sequences were used  
236 for BLASTP searches on the NCBI website (<http://www.ncbi.nlm.nih.gov/blast>) to recover similar  
237 sequences for phylogenetic studies. The sequences resulting from amplification were deposited in the  
238 GenBank database under the accession numbers MT577032 for hemoglobin. The deduced amino acid  
239 sequences were aligned with the corresponding sequences from various animals using the ClustalW  
240 software. Based on this alignment, an UPGMA tree was constructed using the Mega-X software  
241 package (<http://www.megasoftware.net>). The analyzed globin sequences were retrieved from  
242 GenBank along with the 17 intracellular globin sequences from the genome of *Capitella teleta*, four  
243 sequences from the genome of *Lumbricus terrestris* and three sequences from the genome of  
244 *Platynereis dumerilii* identified by Song et al. (Song et al., 2020). All accession numbers are listed in  
245 Table 1.

246

## 247 2.4. Investigation of phagocytic activity of the coelomocytes

248

### 249 2.4.1. Phagocytic activity

250 For each individual (n=10), toluidine blue-stained *Saccharomyces cerevisiae* (Baker's yeast type II),  
251 were used as target cells for phagocytosis. Two hundred and fifty milligrams of *S. cerevisiae* were  
252 suspended in 5 mL of PBS with 8% glucose (PROLABO) containing 1% toluidine blue (ROTH). The  
253 suspensions were then washed twice by centrifugation at 3000 g, 4 °C (for 5 min per wash). After



254 gently mixing, the tube was incubated for 4 h at RT. Phagocytosis was quantified and qualitatively  
255 analyzed through morphological examination. All the samples were counted under a microscope (Axio  
256 lab A1, Zeiss) with a camera and the Zen 2011 module (Zeiss) for image analysis. Results are  
257 expressed as the percentage of phagocytic cells.

258 For each experimental individual (n=10), a volume (about 10  $\mu$ L) of cells was mixed at a ratio of 50  
259 bacteria per cell with *Vibrio alginolyticus* (Urakawa and Rivera, 2006) and *Bacillus hwajinpoensis*  
260 (Cuvillier-Hot et al., 2018) are bacteria typical of the habitat where the annelid worm species is living.  
261 Bacteria were labeled with Fluorescein-5-isothiocyanate (FITC; Interchim, France) by incubating 10<sup>9</sup>  
262 bacteria per ml in a solution of 0.01 mg of FITC per mL, 0.1 M sodium carbonate at pH9.6, for 1 h at  
263 25°C and then washing the bacteria three times with phosphate-buffered saline (PBS). After gently  
264 mixing, the tube was incubated for 1.3 h at RT. After incubation, each sample was fixed in  
265 paraformaldehyde 4% and stored at 4 °C. Part of the samples were further processed by FACS  
266 (Fluorescence Activated Cell Sorting). The other part of the samples was used for confocal  
267 microscopy analysis. To distinguish phagocytotic cells, we used the double-fluorescence technique of  
268 labeling the bacteria with FITC and the SYTO 60 red (Invitrogen) fluorescent nucleic acid stain after  
269 cell fixation. Fixed cells are diluted with 200  $\mu$ L of PBS. Samples are placed in the cytospin (Thermo  
270 Scientific® Cytospin® 4 Cytocentrifuge) for 4min at 2000 rpm. The smears on the slides are covered  
271 with a solution of SYTO 60 diluted 1/3000 for 15 min at room temperature. After two 10 min rinses  
272 with PBS, the slides were observed under a confocal microscope. Confocal images were acquired with  
273 a line scanner microscope (LSM880, Zeiss) through a 63x objective lens (Plan-Apochromat 63x/NA  
274 1.4 oil immersion). A z-stack image was recorded with a voxel size of 0.085  $\mu$ m\*0.085  $\mu$ m\*0.369  $\mu$ m.  
275 SYTO 60 was excited with a 633 nm laser, and its fluorescence was measured between 643 and 720  
276 nm. The FITC signal coming from the bacteria was excited with an argon laser at a wavelength of 488  
277 nm, and the fluorescence was collected between 500 and 544 nm. 3D rendering and orthogonal views  
278 were obtained with the Imaris software (Oxford Instruments, 10.0.1 version). A phagocytosis  
279 experiment with yellow latex beads of 1  $\mu$ m in diameter, at a ratio of 5 beads per cell (Fluoresbrite  
280 Plain YG 1.0 Micro Microsphere, Polysciences) are presented in supplementary data. Phagocytic  
281 activity of the coelomocytes at 1.3 h after adding the beads was estimated as the percentage of  
282 fluorescent events detected by the cytometer in the gates corresponding to the cell populations.

283

#### 284 2.4.2 Analysis of flow Cytometry Phagocytosis Data

285 The data were extracted from the SP6800 instrument, without any spectral unmixing, to keep the  
286 autofluorescence of the cells. The acquisition data were analyzed with the Kaluza software  
287 (BeckmanCoulter v2.2). The strategy was to draw a region in FSC/ SSC which excluded the bacteria;  
288 this exclusion was checked with the tube with labelled bacteria. In this gating region we observed the  
289 fluorescence corresponding to cells labelled by phagocytosis with FITC. Red fluorescence emission  
290 helped to identify cellular autofluorescence from the coelomic fluid.

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2.5. Identification and function of immune effectors: lysozyme and other antimicrobial peptides

2.5.1 Lysozyme expression

The total RNAs of each group, with 10 individuals per condition were extracted with the TRI-Reagent solution (Sigma), following the manufacturer’s protocol. The concentration of extracted RNA was measured with the Qubit® RNA HS kit and the Qubit® 3.0 Fluorometer. The ADNc synthesis was carried out as described in paragraph 2.3.2. Primers used for the semi-quantitative PCR were designed with the Primer3 Input software (<http://primer3.ut.ee/>). The nucleotide sequence encoding Lysozyme was identified in the *C. teleta* Joint Genome Institute (JGI) database. The specific primers used: Lysozyme, F5-‘CCTCTGCGAGCTTCACCTAC-3’, R5 -ATGGAAGGTACTGCACAGGG-3’. The PCR production, loaded on an agarose gel, revealed a single band of the expected size, which was purified with the Nucleospin Extraction kit (Macherey-Nagel), and cloned in TA Cloning kit (Promega) according to the protocol provided by the manufacturer. DNA plasmids were Sanger-sequenced with FM13/RM13 universal primers using BigDye Terminator (Applied Biosystems, Foster City, CA). The resulting sequences were used for BLASTP searches on the NCBI website (<http://www.ncbi.nlm.nih.gov/blast>) to recover similar sequences for phylogenetic studies. The sequences resulting from amplification were deposited in the GenBank database under the accession numbers MT577031 for the lysozyme. The deduced amino acid sequences were aligned with the corresponding sequences from various animals using the ClustalW software. Based on this alignment, an UPGMA tree was constructed using the Mega-X software package (<http://www.megasoftware.net>). Since lysozyme activity has been demonstrated for i-type proteins belonging to both deuterostomes (echinoderms) and protostomes (mollusks and annelids), all the analyzed sequences of different types of lysozymes-i were retrieved from GenBank. All accession numbers are listed in Table 1.

2.5.2 Lyso-plate assays

Enzymatic activity was measured using the *M. luteus* lytic assay (*Micrococcus lysodeikticus*, Sigma) as described by Osserman and Lawlor (Osserman and Lawlor, 1966). Assays were performed on pools of whole three animals and of coelomic fluid collected from three animals. In this assay, the diameter of the cleared zones is proportional to the concentration of lysozyme. The diameters can be measured directly and compared to the diameters obtained with standard solutions of lysozyme for quantification (expressed in mg.mL<sup>-1</sup>).

2.5.3 Microbial challenge

Forty adults were sorted into two groups, and each of these groups processed into two batches: the control individuals and the microbial-challenged individuals. For the first group, whole animals were used. For the second group, only coelomocytes were used. The four groups (n= 10 each) were

328 transferred into boxes containing 2 mL seawater enriched with a cocktail of live bacteria (*Vibrio*  
329 *alginolyticus* and *Bacillus hwajinpoensis* at  $1 \times 10^5$  CFU/mL) for 16 h at room temperature. *Vibrio*  
330 *alginolyticus* and *Bacillus hwajinpoensis* are bacteria typical of the habitat where worm species is  
331 living. A control experiment under the same conditions but without the addition of the cocktail of live  
332 bacteria was also carried out. All these experiments were carried out in triplicate.

333

334 2.5.4 Gene expression of lysozyme and other AMPs on whole animals and coelomocytes  
335 Lumbricin (GenBank : ELU06641.1) and Capitellacin (GenBank : AMQN01012506.1), both AMPs  
336 previously described, seem to be interesting candidates for AMPs search. The nucleotide sequence  
337 encoding Lumbricin was identified in the *C. teleta* Joint Genome Institute (JGI) database. The total  
338 RNAs of each group, with 10 individuals per condition were extracted with the TRI-Reagent solution  
339 (Sigma), following the manufacturer's protocol. mRNA of cells from 10 animals, was isolated with  
340 the Dynabeads® mRNA DIRECT™ Micro Kit (Ambion, Life technology). RT reactions were  
341 performed as described in the previous section (section 2.3.2). Primers used for the semi-quantitative  
342 PCR were designed with the Primer3 Input software (<http://primer3.ut.ee/>). Lumbricin: F5'-  
343 GCTAAGAGGATTCGGGCTTG-3', R 5'-CTCATCTGCGGCATTAGCTG-3'; expected product size  
344 130 bp. Lysozyme: F5'-CCTCTGCGAGCTTCACCTAC-3', R5'-ATGGAAGGTACTGCACAGGG-  
345 3'; expected product size 243 bp. Capitellacin: F 5'-ATCATGGTTACTGGCGTCGT-3', R 5'-  
346 CGTCAAATGAGGCGGAAGTA -3'; expected product size 490 bp. Amplification for sequencing  
347 used the same PCR conditions as described in the previous section, but the 5X Go Taq® Flexi green  
348 buffer (Promega) was replaced by an unstained buffer to prevent interference with cloning. Success of  
349 amplification was evaluated by electrophoresis in a 1.5% agarose gel. The cDNA obtained was used to  
350 quantify the relative quantity of lysozyme-like, Lumbricin, and Capitellacin mRNA compared to  
351 GAPDH (GlycerAldehyde-3-Phosphate DeHydrogenase) (protein ID :159122 from the JGI Genome  
352 site) mRNA, used as reference (F 5'-GATGGCAAGATGAAGGGATTC and R 5'-  
353 AGAGCAATGCCTGCCTTG-3').

354

355 2.5.5 Immuno-localization of Cc-Lumbricin and Cc-Capitellacin by immunohistochemistry  
356 Bacterially challenged and non-challenged *Capitella* worms were fixed in paraformaldehyde (4% in  
357 filtered seawater at 4 °C) for 4 h. A region of Capitellacin (LGAWLAGKVAGTVATYAWNRYV)  
358 was chemically synthesized, coupled to Ovalbumin and used for the immunization procedure of two  
359 New Zealand White rabbits (Saprophyte Pathogen free) according to the protocol of Covalab™  
360 (France). The polyclonal anti-Lumbricin antibody is the one described in (Schikorski et al., 2008).  
361 Immunohistochemistry on 7-µm thick paraffin sections on adult tissues was performed with the rabbit  
362 Capitellacin antiserum (1:400) and the rabbit Lumbricin antiserum (1:400) and the FITC-conjugated  
363 anti-rabbit secondary antibody (1:100; Jackson Immunoresearch Laboratories). Nuclear staining of the  
364 cells using DAPI was also carried out, as described above. Samples were examined using a

365 fluorescence microscope equipped with the ZEISS Axio Imager 2 software, allowing us to merge  
366 images captured under different wavelengths.

367

### 368 2.5.6 Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophores (SDS-PAGE)

369 Mucus from challenged and unchallenged *Capitella* (as described in the section "Microbial  
370 challenge") was recovered using a sterile swab. The protein concentrations of the mucus extract and of  
371 the whole animal were determined using a protein–dye binding assay kit (Bio-Rad Laboratories, CA,  
372 USA) based on the Bradford protein analysis method (Bradford, 1976). Bovine gamma globulin was  
373 used as a standard. Proteins were separated by denaturing SDS-PAGE electrophoresis. The running  
374 gel was composed of 12% acrylamide (12% acrylamide; Tris-HCL 1.5 M, pH 8.8; 0.1% SDS; 0.1%  
375 ammonium persulfate; 0.01% TEMED) and the stacking gel was composed of 4% acrylamide (4%  
376 acrylamide; Tris-HCL 0.5 M pH 6.8; 0.1% SDS; 0.1% ammonium persulfate; 0.01% TEMED). A total  
377 of 57 µg of protein was loaded in Laemmli buffer (Tris 125 mM pH 6.8; 20% glycerol; 4% SDS and  
378 5% β-mercapto-ethanol). Gels were run at 70 V for 15 min and then at 130 V for 60 min.

379

### 380 2.5.7 Western blot

381 The proteins of the SDS-PAGE gel were transferred to a 0.2 µm nitrocellulose membrane (Bio-Rad)  
382 by semi-dry electroblotting (0.8-1.2 mA/cm<sup>2</sup>). After transfer, the gel was stained by Coomassie  
383 Brilliant Blue R-250 (Bio-Rad), to check that protein transfer to the membrane was complete. The  
384 membrane was blocked for 1 h in PBS at 0.1 M containing 0.05% Tween 20 and 5% casein and was  
385 then probed with the rabbit polyclonal anti-Lumbricin and anti-Capitellacin antibodies (1:1000  
386 dilution) in the blocking solution (PBS at 0.1 M with 5% w/v nonfat dry milk) overnight at 4 °C. After  
387 three washings with PBS / 0.05%-Tween 20, the membrane was incubated for 1 h in the blocking  
388 solution at room temperature with the peroxidase-conjugated anti-rabbit secondary antibody Abcam  
389 (1:5000 in PBS at 0.1 M containing 0.05% Tween 20; at 1 h). A Clarity™ Western ECL Substrate  
390 (Bio-Rad) was used to visualize the chemiluminescence of the immunolabelling with a Kodak Bio  
391 Max light film.

392

## 393 3 Results

394

### 395 3.1 A single small-sized cell population in the coelomic fluid of *Capitella C-Channel1*

396

397 Analysis by flow cytometry (Fig. 1) shows a cloud of red dots, each of which represents a single cell  
398 that has passed through the instrument (Fig. 1A, Gate1). Based on differences in their physical  
399 parameters (Forward Scatter (FSC) and Side Scatter (SCC)), a single population was detected. The  
400 existence of a single cell type was also confirmed by the morphological observations of these cells.  
401 First on sections of whole animals stained with toluidine blue (Fig. 1B), a non-specific, basic dye with

402 a good affinity for acidic structures such as nucleic acids (ribosomes, nucleus and endoplasmic  
403 reticulum).  
404 Zooming in on these cells showed they had a round or oval shape with an eccentric nucleus  
405 surrounded by a wide cytoplasm with acidic affinity (Fig. 1C). Observed with no fixative on a glass  
406 slide, the coelomic cells naturally appeared all red in color (Fig. 1D), numerous ( $1.3 \cdot 10^7$  cells.mL<sup>-1</sup>)  
407 and small. Measurements made on cells stained with various non-fluorescent stains had a Ferret's  
408 diameters of 9.12 to 9.31  $\mu$ m, whereas DAPI fluorescence and unstained cells showed a fuzzier limit,  
409 which resulted in Ferret's diameters of 11.69 and 12.61  $\mu$ m, respectively (see Table S1 in  
410 Supplementary material).  
411 Scanning electron microscopy showed the coelomocytes have a relatively smooth surface (Fig. 1E).  
412 The structural analysis showed a nucleus that was regular in outline (Fig. 2A). A residual body with  
413 electron-dense contents was also present in the cytoplasm, well-rounded in appearance, and with a  
414 diameter of 1  $\mu$ m (black arrow on Fig. 2A and white arrow on Fig. 2B). The perinuclear cytoplasm  
415 contained mitochondria and a few vacuoles (Fig. 2C). Staining by neutral red was observed in the cell  
416 as either an orange-yellow coloration (indicating a rather acidic affinity) within small vesicular  
417 compartments (white arrow on Fig. 2D). The alkaline residual body exclusively stained bright pink  
418 (black arrow on Fig. 2D).

419

### 420 3.2 *The coelomocytes are erythrocytes containing intracellular hemoglobin*

421

422 Perls' stain of the cells showed an iron deposit visible as a bright blue granular staining (black arrow,  
423 Fig. 3A). In addition to accumulating iron metal, all coelomocytes were naturally colored by a red  
424 pigment present inside, presumably a respiratory pigment (Fig. 3C). RT-PCR using hemoglobin primers  
425 combined with nucleotide sequencing allowed the molecular identification of a hemoglobin-like protein  
426 transcript from the cells of *Capitella* (Fig. 3B). The spectra with 488nm excitation are relatively similar  
427 (Fig. 3D). But with the others excitations, 405 and 638 nm, we have spectra with different intensities of  
428 red fluorescence, which could better correspond to different amounts of hemoglobin. This allows us to  
429 assume that in the mixture there are populations with different amounts of hemoglobin. This difference  
430 is very clear in figure 3E in the form of 2 spectra; the red arrow indicates the spectrum with a higher  
431 intensity, thus a greater content of hemoglobin, than the spectra indicated by the black arrow. This  
432 difference varies with different animal batches (Supplementary material, Fig S1). The animals are  
433 therefore not homogeneous in their quantity of hemoglobin.

434 To better understand the origin of the globin found in the coelomocytes of C-Channel1, we carried out  
435 a phylogenetic analysis using representatives of the diversity of globins found in annelids. This dataset  
436 includes representatives of the typical intracellular globins, extracellular globins, as well as  
437 myoglobins, neuroglobins, and the unusual polynoid extracellular hemoglobins (Belato et al., 2020;  
438 Projecto-Garcia et al., 2017; Weber and Vinogradov, 2001) present in various annelid species. We

439 produced a maximum-likelihood phylogenetic tree with this sampling of 51 protein sequences (Fig. 4).  
440 The globins fall into two well-supported (100% bootstrap) main groups: (1) the typical annelid  
441 extracellular globins and (2) the intracellular globins (myoglobins, neuroglobins and erythrocytic  
442 globins), along with the unusual polynoid extracellular globins. All the *Capitella* sequences fall well  
443 within the intracellular globins lineage, and the C-Channel1 globin clusters with other intracellular  
444 globins from *Capitella teleta* (sequences extracted from the genomic data), with very high confidence  
445 (99% bootstrap). Because of the presence of circulating intracellular globin, the coelomic cells of C-  
446 Channel1 can be designated as erythrocytes.

447

### 448 3.3 *Erythrocytes with phagocytic activity*

449

450 Erythrocytes incubated with Toluidine-blue-colored yeast cells for 4 h showed an ability to phagocyte  
451 up to 10% of yeast cells (Fig. 5A). Flow cytometry analysis was also performed (Fig. 5B, 5C and 5D)  
452 on cells incubated for 1.3 h with FITC labeled bacteria or yellow latex beads (Supplementary material,  
453 Fig. S2). In figure 5B, the left SSC plot shows non-labelled bacteria with 6% of background  
454 autofluorescence (in the frame B). Compared to this, the right-hand plot with the FITC-labeled  
455 bacteria are positive at 98% (in the frame B). On figure 5C and D in the left columns, the FSC/SSC  
456 plot allows us to frame a region A within the cell-size range which excludes the smaller debris and the  
457 majority of the bacteria. When the cells have been in contact with labeled bacteria (Fig. 5D) the cells  
458 region A shows a slight modification of the SSC and thus of the complexity or granularity of the cells  
459 (SSC). Gated on this Region A, the right columns of figures 5C and D shows the FITC / red emission  
460 plot as function of FITC, to contrasts the non-labeled cells (Fig 5C right) and the cells in contact with  
461 FITC bacteria (Fig. 5D right). The cells in region C-- correspond to the part of biggest bacteria which  
462 could be in region A (on the FSC/SSC plot, Fig 5C, left column) but they are completely negative for  
463 the red emission. The autofluorescence of bacteria is lower than the coelomic fluid cells in the red  
464 emission. So the animals cells of interest are in the upper part of the plot : in the C+ region the cells  
465 are positive for the red emission but negative for the FITC fluorescence. In the C++ region the cells  
466 are positive for the red emission and for FITC fluorescence : it represents 0.64% for the non-labeled  
467 cells and 4.38% for the cells in contact with the FITC bacteria : cells that have phagocytosed the  
468 bacteria . The test of phagocytic activity revealed that the erythrocytes had ingested up to 3.74% of the  
469 labelled bacteria (Fig. 5D) and the phagocytosis is mainly present in cells with low hemoglobin levels  
470 (red square). Similar percentage of ingestion was obtained with yellow latex beads (Fig S2). Figure 5E  
471 shows the engulfment of labelled bacteria by an erythrocyte. Image of confocal optical sections of a  
472 phagocytic erythrocyte in xyz and xz planes (Fig. 5F) shows marked bacteria within the cell  
473 cytoplasm.

### 474 3.4 *Capitella C-Channel1 erythrocytes produce an AMP but no Lysozyme*

475

476 At first, lysozyme (GenBank ID MT577031) from C-Channel1 (*Cc*) was molecularly identified by  
477 RT-PCR combined with cloning and nucleotide sequencing. A phylogenetic analysis on lysozyme  
478 comparison with other deuterostome and protostome sequences from seventeen species (Fig 6A) was  
479 carried out. The distance tree obtained showed that the analyzed annelid sequences form a distinct  
480 lineage including destabilase I from *Hirudo medicinalis*, with the exception of *Caenorhabditis elegans*  
481 sequence lysozyme-i. Lysozyme-like C-Channel1 seems to be closely related to the lysozyme of the  
482 North American species *Capitella teleta*. An activity able to lyse *M. lysodeikticus* cell walls was  
483 detected in whole *Capitella* worms, after an overnight incubation at 37°C. A clear ring of lysis, 12 mm  
484 in diameter was observed in animals stimulated (ab) by bacteria, for a diameter of 0.8 mm only in  
485 unstimulated animals (a). No lytic activity was observed in the coelomic cells (Fig. 6B). After adults  
486 had been exposed to a high load of live environmental bacteria (*V. alginolyticus* and *Bacillus*  
487 *hwajinpoensisat*), the transcripts of lysozyme were detected by semi-quantitative RT PCR in the whole  
488 animals (Fig. 6C, lane a), but not in their coelomocytes (Fig. 6C, lane c) confirming that the  
489 erythrocytes do not express the lysozyme gene. When the *Capitella* worms were microbially  
490 challenged, they expressed the lysozyme more intensely (Fig. 6C, lane ab), while the challenged  
491 coelomocytes still did not express it (Fig. 6C, lane cb).

492 The gene expression patterns of the lysozyme-like, *Cc*-Lumbricin, and also *Cc*-Capitellacin were then  
493 investigated by semi-quantitative RT-PCRs first in the whole individuals challenged and unchallenged  
494 by bacteria, and secondly in the erythrocytes collected from bacterially challenged or unchallenged  
495 *Capitella*. As presented in figure 7A, only *Cc*-Capitellacin was produced by the coelomocytes. The  
496 cellular gene expression of this AMP was even increased when the worms had been exposed to  
497 bacteria. The other AMP (*Cc*-Lumbricin) (Fig. 7A) was not produced by the erythrocytes, and as  
498 observed for the lysozyme, the transcripts were detected in the bacterially challenged animals. Western  
499 blot analyses were performed on the same tissues from the same whole individuals and their mucus.  
500 The known molecular mass of Lumbricin I is 7.2 kDa, and 6.7 kDa for *Hm*-Lumbricin. The Western  
501 blot showed a 10 kDa band in the *Capitella* worm's mucus, which likely corresponds to the *Cc*  
502 Lumbricin (Fig.7B). The Western blot analysis (Fig. 7C and 7D) confirmed the synthesis of the  
503 peptide, Capitellacin, but also showed a band of slightly greater molecular weight than expected. The  
504 band detected at less than 17 kDa could be the precursor of Capitellacin.

505 Immunohistochemistry using the anti-*Hm*-Lumbricin (Fig. 8A to 8D) and the anti-Capitellacin (Fig.  
506 8F to 8G) were performed and also supported the RT-PCR and Western Blot results. Controls were  
507 performed with pre-immune serum (Fig. 8C, C1 and H, H1). *Cc*-Lumbricin was detected under basal  
508 (no exposure) conditions (Fig. 8A), the peptide was immunodetected in the digestive tract (Fig. 7A)  
509 and integument (Fig. 8A1). Interestingly, the immune staining for *Cc*-Lumbricin was most strongly  
510 observed in the tegument after bacterial challenge (Fig. 8B1). We observed an immunodetection of  
511 bacteria trapped within the mucus of challenged animals (Fig. 8D), confirming the observation of *Cc*-

512 Lumbricin produced in mucus secretion by Western blot (Fig. 7C and 7D). The Capitellacin-like  
513 peptide (*Cc*-Capitellacin) was immunodetected under basal conditions in the digestive tract (Fig. 8F),  
514 that immune staining for *Cc*-Capitellacin was strongest in the tegument after bacterial challenge  
515 (Fig.8G). The *Cc*-Capitellacin peptide was also detected in the erythrocytes (Fig. 8F1), but after  
516 bacterial challenge only (Fig. 8G1).

517

#### 518 **4. Discussion**

519

520 Coelomocytes present in the coelomic fluid of all polychaetes are usually heterogeneous in morphology,  
521 size, relative abundance, and function, which makes a single standard classification a difficult task.  
522 Nonetheless, it is rare to describe a single type of cell in the coelomic fluid with various functions. In  
523 the polychaete *Glycera dibranchiata* the only population described in the coelomic fluid was mainly  
524 studied for its intracellular hemoglobin (Seamonds and Schumacher, 1972). Dales (1964) described a  
525 single type of coelomocytes in *Amphitrite johnstoni*, while earlier writers described 3 types of cells in  
526 the coelomic fluid of that species (Dales, 1964). There are three types of coelomocytes described for  
527 *Arenicola marina*, but these cells are likely to represent the early stages of differentiation of granular  
528 integrations: with a change in physiological state, granules can appear and accumulate. The distribution  
529 of granular cells depends on the age and sex of the animal and is also correlated with the stage of  
530 maturation of sex products (Persinina and Chaga, 1994). Based on our study, we conclude that a single  
531 cell-type is present in the coelomic fluid of adults C-Channel1.

532 Furthermore, the detection of intracellular hemoglobin within these coelomocytes makes it possible to  
533 classify them as erythrocytes. The size of C-Channel1 erythrocytes is similar to that of *Alvinella*  
534 *pompejana* erythrocytes ( $10.78 \pm 2.15 \mu\text{m}$ , n=98) (Jouin-Toulmond et al., 1996), however in the  
535 Terebellidae family erythrocytes are larger, measuring about  $20 \mu\text{m}$  (Terwilliger et al., 1985). The  
536 erythrocytes of C-Channel1 have a regular shape, which contrasts with erythrocytes from *Amphitrite*  
537 *ornata* and *Enoplobranchus sanguineus* (Glomski and Tamburlin, 1990).

538 A study on erythrocytes oxygen binding capacities in capitellid polychaetes shows that it is generally  
539 species-specific, and the authors suggest that the differences may prove to be adaptations to thermal  
540 properties of their environment, to their body size, or both (Mangum et al., 1992). The presence of  
541 high-affinity hemoglobin and oxygen-combining capacities has been shown in the coelomic fluid of  
542 *Capitella capitata* of the Yeahn Estuary in Devon (Wells and Warren, 1975). The ultrastructure of the  
543 large ( $20 \mu\text{m}$  diameter) erythrocytes from *Pista pacifica* observed with transmission electron  
544 microscopy shows a homogenous gray electron-dense cytoplasm that is believed to be hemoglobin  
545 (Glomski and Tamburlin, 1990). Compared to *P. pacifica*, the small erythrocytes of C-Channel1 do  
546 not contain lipid granules, and the electron-dense material is much finer and homogeneous. Song et al.  
547 (2020) have identified 17 intracellular globin paralogues, all closely related, in the genome of  
548 *Capitella* spI (*C. teleta*) and no extracellular globin. They suggest that the ancestral annelid



549 extracellular hemoglobins were secondarily lost and replaced by red blood cell globins. Our BLAST  
550 analysis revealed that the intracellular hemoglobin amino acid sequences of C-Channel1 matched  
551 those of intracellular hemoglobin from *Capitella teleta*. Spectral scans of erythrocytes studied in the  
552 wavelength range of 500-650 nm, where the fluorescence spectra are mainly emitted by the  
553 hemoglobin, show variability, perhaps due to exposure to hypoxia, age, size or sexual maturity of the  
554 individuals. We have demonstrated the presence of lighter-colored animals in C-Channel 1 population  
555 (Boidin-Wichlacz et al., 2021). The hemoglobin from the annelid polychaete *Glycera dibranchiata*,  
556 present in coelomic erythrocytes, was demonstrated to be of a heterogeneous (Seamonds et al., 1971).  
557 It is possible that the electron-dense body observed in C-Channel 1 erythrocytes also contained  
558 hematin, as shown for the erythrocytes from *Urechis* and those from *Alvinella pompejana* (Jouin-  
559 Toulmond et al., 1996). Jouin-Toulmond et al. describe the large dense bodies *Paralvinella* and  
560 *Alvinella* as by-products of hemoglobin synthesis combined with iron. *Alvinella pompejana*, is  
561 exposed to strong hypoxia and high sulfide concentrations. Under these conditions, the oxidation of  
562 hydrogen sulfide could occur everywhere in the coelom. Hematin was present in the granules, it can  
563 catalyze oxidation of sulfide and may thus protect the animals from sulfide poisoning. This feature  
564 could contribute to the success of *Capitella* spp. in sediment of eutrophicated areas that are often  
565 anoxic and highly sulfidic, due to anaerobic bacterial degradation of organic matter. Hemoglobin and  
566 sulfide affect one another in several ways. Hemoglobin derivatives are also probably present,  
567 explaining the variations in hemoglobin spectra. The formation of sulfhemoglobin has been observed  
568 in annelids from sulfide-rich environments (Abele-Oeschger and Oeschger, 1995; Zal et al., 1998).  
569 Methemoglobin has been clearly demonstrated in the annelid *Arenicola marina* (Abele-Oeschger and  
570 Oeschger, 1995; Bailly and Vinogradov, 2005; Hourdez et al., 1999), which showed that  
571 methemoglobin formation occurred with a concomitant liberation of hydrogen peroxide, a potent  
572 oxidizing agent which can oxidize sulfide. Nonetheless, other strategies have also been suggested  
573 exposed to high sulfide concentrations, including an epibiosis with the giant bacteria *Thiomargarita*  
574 sp. which has been shown to be advantageous for the survival of *Capitella* spp. facing seasonal  
575 increases of free sulfide in the sediments (Hourdez et al., 2021).  
576 In the morphological examination of the erythrocytes, we observed the presence of vacuoles. Although  
577 some small vacuoles and vesicles may be involved in pinocytosis, we have no direct evidence for this  
578 or other functions of these organelles. However, the small number of polyribosomes seen in many of  
579 the erythrocytes examined, suggests that protein synthesis is occurring at a low level as described for  
580 *Glycera dibranchiata* and *Glycera abbranchiata* erythrocytes (Glomski and Tamburlin, 1990;  
581 Seamonds and Schumacher, 1972). Patterns of cytoplasmic organelle development and distribution  
582 observed in certain invertebrate erythrocytes are diverse. The holothurian *Eupentacta quinquesemita*  
583 retains a considerable quantity of cytoplasmic organelles throughout their existence in these  
584 hemocytes (Fontaine and Hall, 1981). But other patterns have been observed, in some invertebrate  
585 erythrocytes which reach a maximal complement of cytoplasmic structures at the peak of synthetic

586 activity followed by its reduction to a lower level which persists throughout the life-span of the cell  
587 (Fontaine and Lambert, 1977). One wonders whether this phenomenon also occurs at C-Channel1  
588 erythrocytes with partial or total removal of cytoplasmic structures such as lysosomes, rough  
589 endoplasmic reticulum, polyribosomes and Golgi apparatus.

590 In most coelomate invertebrates (e.g., molluscs, annelids, arthropods, echinoderms, and tunicates),  
591 some of the blood cells are phagocytic. In polychaetes, active phagocytosis has often been  
592 experimentally examined by injecting small particles such as ferritin or Indian ink, which are taken up  
593 by endocytosis and stored inside vacuoles of the coelomic cells. In C-Channel1 we have shown 10%  
594 phagocytic for yeast and 3.7% for labeling bacteria. The same percentage was evaluated for  
595 phagocytosis of latex beads (supplementary data). Erythrocytes described in *Glycera siphonostoma* by  
596 Goodrich (1898) also ingest foreign particles, and would therefore be actively phagocytic (Goodrich,  
597 1898). *Amphitrite johnstoni* has also been cited as having erythrocytes that appear phagocytic  
598 (Glomski and Tamburlin, 1990). The erythrocytes found in C-Channel1, as in *S. alveolata*, would  
599 exhibit endocytic activity (Meyer et al., 2021). Capitellid appear monophyletics and phylogenetically  
600 as a sister taxa to the *Echiurian spoonworms* (Bleidorn et al., 2003; Kobayashi et al., 2022). Their  
601 basal position in the annelid tree might account for the pluripotency of their erythrocytes, rather than  
602 having a great diversity of specialized cell types, as more evolved annelids.

603 Lysozyme activity is a phylogenetically conservative humoral mechanism that has been studied in  
604 several classes of invertebrates. In most cases lysozyme activity was detected in the coelomic fluid of  
605 the studied species of annelids, rarely exclusively in coelomocytes. We were not able to detect lysozyme  
606 activity at the erythrocyte level, but we have demonstrated lysozyme activity and humoral defense  
607 process by protein synthesis in response to *V. alginolyticus* and *B. hwajinpoensisat* in the whole animal.  
608 BLAST analysis revealed that the lysozyme amino acid sequences of C-Channel1 matched those of  
609 Lysozyme protein of *Capitella teleta* (whose lysozyme activity has not been evaluated), with 89%  
610 identities, but also 62% amino acid similarities with Destabilase 1 of *Hirudo medicinalis* (Zavalova et  
611 al., 2012). This lysozyme may turn out to be a destabilase-lysozyme with both lysozyme and peptidase  
612 activity, which has yet to be demonstrated.

613 By now, several Lumbricin homologs have been identified and described from other polychaetes (Bodó  
614 et al., 2019; Cho et al., 1998; Li et al., 2011) and leech species (Schikorski et al., 2008). In *Eisenia*  
615 *andrei* earthworms, the highest expression of *Ea-Lumbricin* mRNA appears in the proximal part of the  
616 intestine, while other tested organs, such as coelomocytes, have moderate or low levels (Bodó et al.,  
617 2019). We localized the expression of the Cc-Lumbricin mainly in the body wall of C-Channel1 by RT-  
618 PCR analysis and by immunohistochemistry. Lumbricin-like antimicrobial peptides named Lumbricin-  
619 PG and PP-1 were isolated from skin secretions and coats the epithelial surface of earthworms (Li et al.,  
620 2011; Wang et al., 2003). Based on the environments of life of *Capitella* sp., it is rational to think that  
621 there are effective anti-infective agents in its skin secretions and epidermis, similar to the earthworms.  
622 The results of RT-PCR analysis and Western blot, these findings demonstrate the idea that Cc-Lumbricin

623 coats the epithelial surface of C-Channel1 whole body, where it might contribute to its first line  
624 protective barrier.

625 AMPs, such as Arenicin from *Arenicola marina* (Ovchinnikova et al., 2004), Perinerin from *Perinereis*  
626 *aibuhitensis* (Weidong et al., 2003), Hedistin from *Hediste diversicolor* (Tasiemski et al., 2007), and  
627 Alvinellacin from *Alvinella pompejana* (Tasiemski et al., 2014) are predominantly expressed in  
628 coelomocytes. To date, coelomocytes containing AMPs were identified as granulocytes or hyaline cells,  
629 but no AMP has been identified in erythrocytes. The highest expression levels of *Cc*-Capitellacin were  
630 detected in stimulated *Capitella* worms and their coelomocytes. In C-Channel1 the erythrocytes are  
631 likely to produce and secrete *Cc*-Capitellacin into the coelomic fluid, where it exerts its antibacterial  
632 activities, thus activating the second line of defense when microorganisms penetrate the body.

633 Some authors have suggested that the internal heterogeneity of eleocytes, granulocytes, or red-blood  
634 cells is related to the presence of different stages of differentiation of a single cell lineage in the whole  
635 coelomic fluid. Modern integrated annelid research focuses primarily on the structure and functions of  
636 specific proteins, especially antimicrobial peptides. In these studies, little attention is paid to the  
637 morphology and classification of whole cells.

638

## 639 **5. Conclusions**

640 Based on their morphology and their shared functions, the coelomocytes of *Capitella* C-Channel 1  
641 appear to belong to a single cell type, the erythrocytes. This cell type is primarily involved in  
642 respiration. Our study shows they also have a role in phagocytosis and production/secretion of AMP.  
643 A Lysozyme-like activity has been detected in whole animals, but not at the erythrocyte level. The  
644 presence of Lumbricin analog and Capitellacin analog (*Cc*-Lumbricin and *Cc*-Capitellacin) in the  
645 mucus covering the epidermis of the whole animal or in coelomocytes, demonstrates that the  
646 distribution and functioning of AMPs in C-Channel1 share some features with AMPs from other  
647 annelids.

648

## 649 **Author contributions**

650 Céline Boidin-Wichlacz and Aurélie Tasiemski designed the experiments. Céline Boidin-Wichlacz  
651 performed most of the experiments. Séphane Hourdez contributed to phylogenetic analyses. Ann  
652 Andersen contributed to the Transmission electron microscopy analysis. Céline Boidin-Wichlacz  
653 wrote the manuscript. Nathalie Jouy performed the research and analyzed the data in flow cytometry.  
654 Ann Andersen, Séphane Hourdez and Aurélie Tasiemski critically revised the manuscript.

655

## 656 **Declarations of interest**

657 The authors declare no potential conflicts of interests with respect to the research, authorship or  
 658 publication of this article

### 659 **Data Availability Statement**

660 The dataset analyzed during the current study is available from the corresponding author on reasonable  
 661 request.

### 662 **Ethics approval**

663 Not applicable

664

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666

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672

### 673 **Table**

674 Table 1. Sequence accession numbers for the phylogenetic approach.

<b>Species</b>	<b>Protein</b>	<b>GenBank ID</b>
<i>Capitella teleta</i>	Lysozyme	ELT93524
<i>Hirudo medicinalis</i>	Lysozyme i	AAA96144
<i>Eisenia fetida</i>	Lysozyme	AGJ83864
<i>Eisenia andrei</i>	Lysozyme	DQ339138
<i>Mytilus edulis</i>	Lysozyme i	ABB76765
<i>Crassostrea virginica</i>	Lysozyme i	BAE47520
<i>Ruditapes philippinarum</i>	Lysozyme i	BAC15553
<i>Asterias rubens</i>	Lysozyme i	AAR29291
<i>Apostichopus japonicus</i>	Lysozyme i	ABK34500
<i>Bathymodiolus azoricus</i>	Lysozyme i	AAN16208
<i>Bathymodiolus thermophilus</i>	Lysozyme i	AAN16209
<i>Calyptogena sp.</i>	Lysozyme i	AAN16211
<i>Mytilus galloprovincialis</i>	Lysozyme i	AJQ21515
	Lysozyme	OPL33781
<i>Lamellibrachia satsuma</i>	Lysozyme	KAI0215534
<i>Dimorphilus gyrocolliatus</i>	DgyrCDS4060	CAD5115039
<i>Urechis unicinctus</i>	Invertebrate-type lysozyme	AWA82039
<i>Platynereis dumerilii</i>	Extracellular, globin Egb_A1a	QQO51916
	Globin Ngb	QQO51934
<i>Arenicola marina</i>	Haemoglobin B2	CAI56309
	Haemoglobin B1	CAJ32742
	Haemoglobin A2	CAI56308
	Haemoglobin A2b	CAJ32740
	Haemoglobin A2c	CAJ32741
<i>Alvinella pompejana</i>	Intracellular haemoglobin	Q53I62

<i>Glycera dibranchiata</i>	Extracellular globin	QAT81156
	Extracellular globin	QAT81157
	Extracellular globin	QAT81159
	Extracellular globin	QAT81158
	Globin P3	AAA29161
	Globin P2	AAA29160
	Globin P1	CAA37995
<i>Lumbricus terrestris</i>	Chain D, hemoglobin chain d1	56967019
	Extracellular, Chain B, Globin II	56967017
<i>Lepidonotus semilectus</i>	Extracellular, Chain A, Globin IV	56967016
	Extracellular, Chain C, Globin III, Extracellular globin	56967018
<i>Halosydna brevisetosa</i>	Extracellular globin	QAT81218
<i>Aphrodita japonica</i>	Neuroglobin	QAT81169
<i>Aphrodita aculeata</i>	Neuroglobin	QKD76907
<i>Branchipolynoe seepensis</i>	Nerve myoglobin	QKD76908
<i>Branchipolynoe symmytilida</i>	Nerve myoglobin	AAC47259
	Extracellular single-domain globin	ACU21605
	Extracellular single-domain globin	ACU21604
	Extracellular tetra-domain globin	ACU21599
	Extracellular tetra-domain globin	ACU21598
	Extracellular tetra-domain globin	ACU21597
	Extracellular tetra-domain globin	ACU21596
<i>Eulagiscinae</i> sp.	Intracellular single-domain globin	ALH21945

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676

## 677 **Figures**

678 **Figure 1: Coelomocyte population:** A) Flow cytometry analysis of *Capitella* C-Channel 1 cells. Gate  
679 1 represents a homogeneous population of coelomocytes. The y-axis represents the dot plots of  
680 forward scatter-height (FSC-Height), versus side scatter-height (SSC-Height) on the x-axis. Each red  
681 dot represents a single cell that has passed through the instrument and debris are marked in gray. The  
682 yellow dots result from red dots overlapping each other. B) On whole-body longitudinal sections  
683 stained with toluidine blue, coelomocytes are visible inside the coelom throughout the middle part of  
684 the body. The red rectangle focuses on some of them in figure. C) Zoomed in view of the red rectangle  
685 in image B, showing that the coelomocytes appear of similar size and homogeneously stained around  
686 an eccentric nucleus. D) Microscopic observation of the natural red color of the coelomocytes,  
687 indicating they are erythrocytes. E) Observation of the smooth outer surface of the coelomocytes by  
688 scanning electron microscopy. Abbreviations: c. Cytoplasm, Cc. Coelomocyte, Cu. Cuticle, Dt.  
689 digestive tract, Lm. longitudinal muscles, n. nucleus.

690

691 **Figure 2: Cell description.** A) Staining of coelomocytes by labeling nuclear DNA with DAPI.  
692 Presence of a single dense body (arrow) in the cytoplasm. B) Electron microscopy images showing  
693 circulating cells. White arrows show electron dense bodies. C) Transmission electron microscopy of a  
694 circulating cell in the coelomic cavity. The black arrow shows a polyribosome. D) Neutral red stain

695 with emphasis on a bright pink-tinted alkaline structure (black arrow) and yellow/orange colored  
696 acidic vacuoles (white arrow). Abbreviations: c. cytoplasm, m. mitochondria, n. nucleus, v. vacuoles.  
697

698 **Figure 3: Respiratory pigment.** A) Perls' stain was used to reveal iron stores. Iron deposit appears as  
699 a bright blue granular staining (black arrows) all around the periphery of the cytoplasm. B) Live whole  
700 body observed under binocular microscope showing the natural red color of coelomocytes. The white  
701 dotted lines delimit the setigerous segments. C) Detection of intracellular hemoglobin transcript by  
702 RT-PCR in coelomocytes. D) Spectral data of all cells in the mixture obtained under a 488 nm laser.  
703 E) Spectral data of all cells in the mixture obtained under 405 and 638nm laser excitation spectrum  
704 charts. The red arrow indicates the cells spectrum with a high content of hemoglobin. The black arrow  
705 indicates the cells spectrum with a low content of hemoglobin. Abbreviations: Cc. Coelomic cells, He.  
706 Hemoglobin, Dt. Digestive tract, M. DNA ladder (100 bp).

707 .

708

709 **Figure 4 : The origin of the globin found in the coelomocytes of C-Channel1.** Maximum likelihood  
710 phylogenetic tree of representatives of the typical intracellular or extracellular hemoglobins, as well as  
711 myoglobins (Mb), neuroglobins (Ngb), and the unusual polynoid extracellular hemoglobins. The tree  
712 was produced with PhyML based on an alignment of 51 proteins (192 amino acid positions) using a  
713 Dayhoff matrix. The nodes' confidence was evaluated with a bootstrap approach for 100 replicates  
714 (only values greater than 70 are indicated). Species names and accession numbers are indicated in the  
715 node labels. The blue rectangle shows the extracellular globin lineage, the orange rectangle indicates  
716 the unusual polynoid extracellular globins. All other globins are intracellular (neuroglobin, myoglobin,  
717 and erythrocytic gobins). The green rectangle indicates the known *Capitella teleta* (Cte) globins  
718 extracted from the genome, and this lineage includes the *Capitella* C-Channel 1 globin (large arrow).  
719 *Lte= Lumbricus terrestris*

720

721

722 **Figure 5: Phagocytic activity.** A) Light microphotograph of coelomocytes with toluidine blue-stained  
723 *Saccharomyces cerevisiae* (arrows point at Baker's yeast type II). B) Flow cytometry analysis to  
724 verify the labelling of bacteria mixed with a 50% ratio of *Vibrio alginolyticus* and *Bacillus*  
725 *hwajinpoensis*. The left column shows the Side Scatter Channel/FITC plot of non-stained bacteria i.e.  
726 their level of autofluorescence. The right column corresponds to Side Scatter /FITC plot of bacteria  
727 labeled with green fluorescence (FITC) showing their high level of fluorescence. In both figures, the  
728 box framed B corresponds to the positive region of labelled bacteria. C) The scatter plots for un-  
729 labelled *Capitella* cells: in left column, the FSC/ SSC plot and in the right column their FITC/ red  
730 emission plot. D) The scatter plots for *Capitella* cells in contact with FITC-labelled bacteria : in the  
731 left column the FSC/ SSC emission plot. On each figure, the red boxes frame the cells with low

732 hemoglobin level. These experiments were performed three times (n=3). E) Cellular phagocytosis  
733 analyzed by confocal microscopy. Cell samples were acquired in 3D using the Imaris software. The  
734 double staining allowed to clearly establish whether or not a green FITC-labeled bacteria was  
735 internalized by an erythrocyte stained in red with SYTO 60. F) Optical sections of the previous figure  
736 with orthogonal views, showing successively a single plane in the three directions; xy, xz or yz as  
737 indicated (by the white lines). This enabled to check the exact location of the green fluorescing bacteria  
738 within the red cytoplasm of cell. Scale bar for all panels is 3  $\mu$ m.

739

740 **Figure 6: Antimicrobial protection.** A) Phylogenetic tree of lysozyme sequences from various  
741 invertebrate species. The tree was constructed with a maximum likelihood (ML) approach on a 79  
742 amino-acid alignment. The Dayhoff matrix was used for substitutions. Values at nodes are bootstrap  
743 values out of 100 replicates (only values greater than 70 are shown). The sequences from the  
744 echinoderms *Apostichopus japonicus* and *Asterias rubens* were used as outgroups. The blue rectangle  
745 indicates annelid sequences and the sequence from C-Channel 1 is indicated by the black arrow. B)  
746 Lysozyme activity was measured using the *M. luteus* lytic assay on a 90 mm diameter gel-coated box.  
747 The diameter of the cleared zones is represented by a red dotted circle. Animal (a), coelomocytes (c),  
748 microbially challenged animals (ab), microbially challenged cells (cb). C) PCR-Expression of  
749 lysozymes in different fractions, whole animal (a), coelomocytes (c) and microbially challenged whole  
750 animal (ab) or cells (cb). PCR control (Co) and marker (M) corresponds to the DNA ladder (100 bp).

751

752 **Figure 7: *Cc*-Lumbricin and *Cc*-Capitellacin distribution.** A) RT-PCR using primers for GAPDH  
753 (GlycerAldehyde-3-Phosphate DeHydrogenase), *Cc*-Capitellacin and *Cc*-Lumbricin. Expression  
754 in different fractions, whole animal (a), coelomocytes (c) and microbially challenged whole animal  
755 (ab) or cells (cb). GAPDH was used as constitutive control. Marker (M) represents a 100 bp DNA  
756 ladder is shown on the left and the PCR negative control with primers is indicated (CN). B) Western  
757 blot analysis of peptide extracts with anti-Lumbricin. The framed box highlights a slight peptide band  
758 with a molecular weight between 4.3 and 10 kDa present in the mucus of *Capitella*, but not in the  
759 whole animal. C) Western blot analysis of peptide extracts with anti-Capitellacin. The framed box  
760 highlights the presence of a massive band of peptides sizing between 4.6 and 10 kDa in the whole  
761 animal, particularly when bacterially challenged, but not detectable within its mucus. D) SDS-  
762 polyacrylamide gel of Coomassie blue-stained fractions. Animal (a), the microbially challenged whole  
763 animal (ab), mucus (m), mucus of microbially challenged animal (mb).

764

765 **Figure 8: Localization of *Cc*-Lumbricin and *Cc*-Capitellacin in *Capitella* C-Channel 1, after**  
766 **bacterial challenge with *V. alginolyticus* and *Bacillus hwajinpoensis*.** *Cc*-Lumbricin was labeled by  
767 anti-*Hm*-Lumbricin and Alexa 488 (green) in figures A to E. *Cc*-Capitellacin was labeled by anti-  
768 Capitellacin and Alexa 488 (green) in figures F to H. DNA was labeled by DAPI stain (blue) and

769 merged images are shown with ZEISS Axio Imager 2. A) Longitudinal body section of a C-Channel 1  
770 control. B) Section of C-Channel1 stimulated by bacteria for 16 hours. C) Controls, performed with  
771 pre-immune serum. A1-B1-C1) Zoomed in section of the integument from the previous image. D)  
772 Anti-Lumbricin antibody green labeling of bacteria trapped inside the mucus of *Capitella* stimulated  
773 with bacteria. E) Pre-immune labeling of bacteria trapped inside the mucus of C-Channel1 stimulated  
774 bacteria. F) Transversal section of a C-Channel1 control. G) Section of C-Channel1 stimulated by  
775 bacteria for 16 hours. H) Controls performed with pre-immune serum. F1-G1-H1) Zoomed in section  
776 of the integument from the previous image. Abbreviations: Cc. coelomocyte, Co. coelom, Cu. Cuticle,  
777 Dt. digestive tract, e. epidermis.

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992

Figure 1

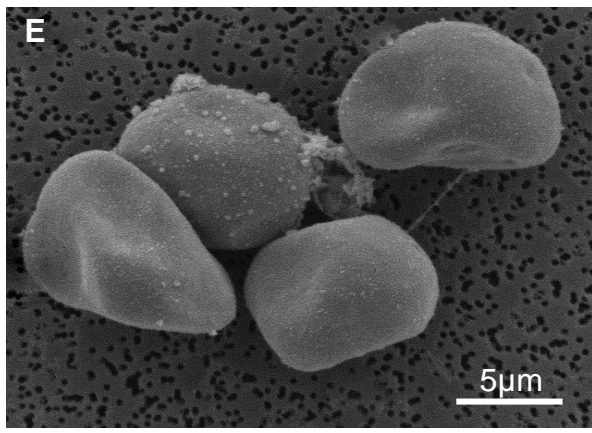
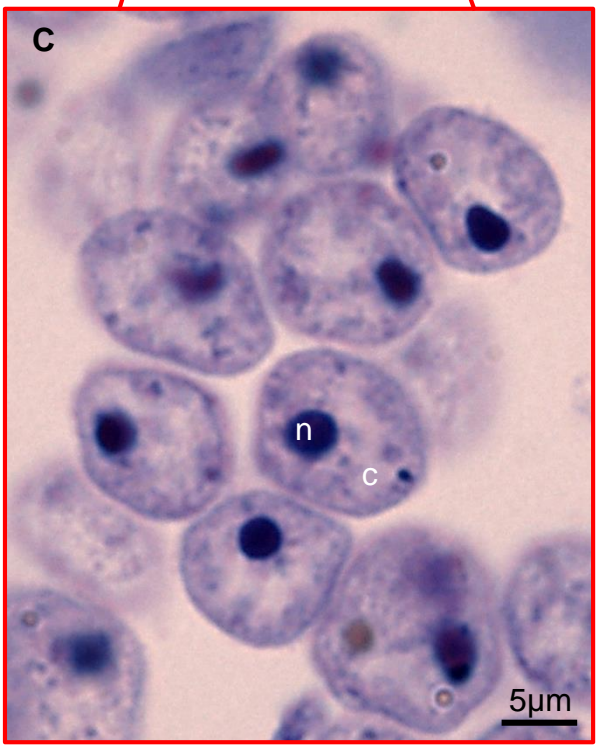
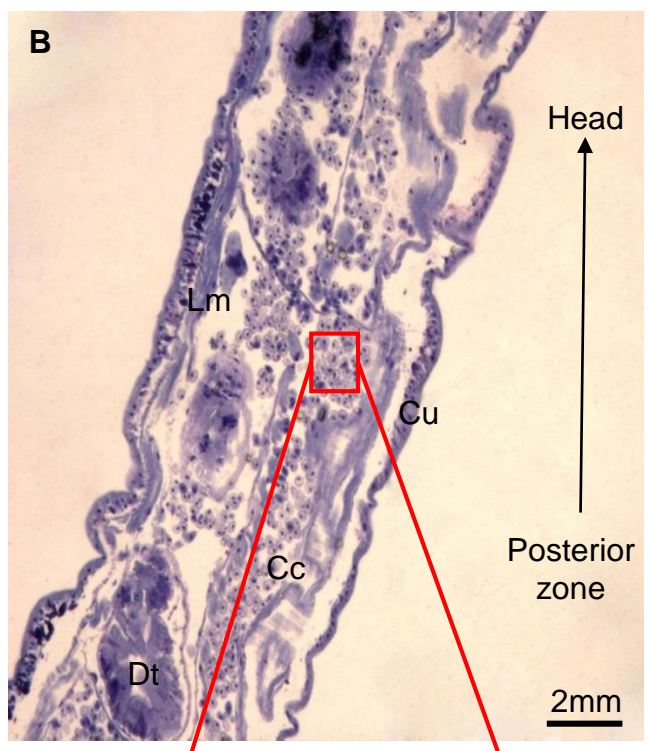
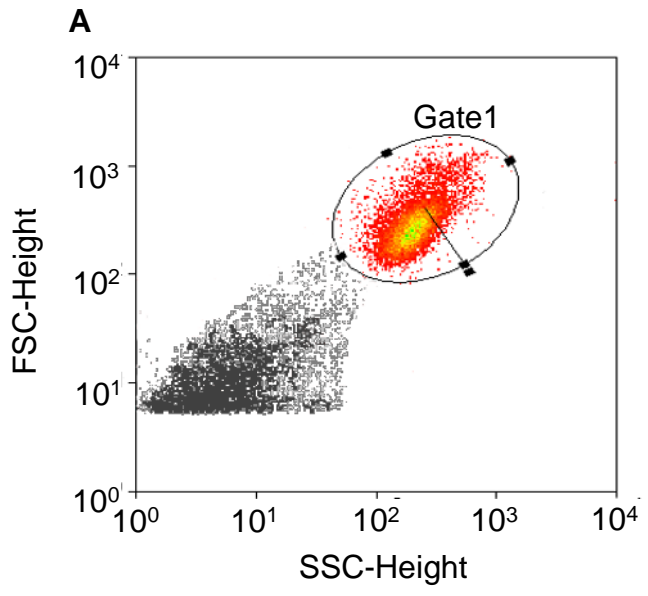


Figure 2

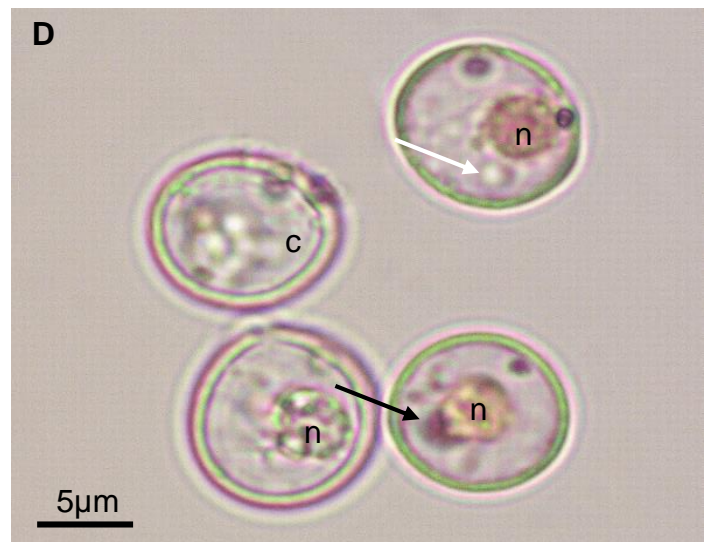
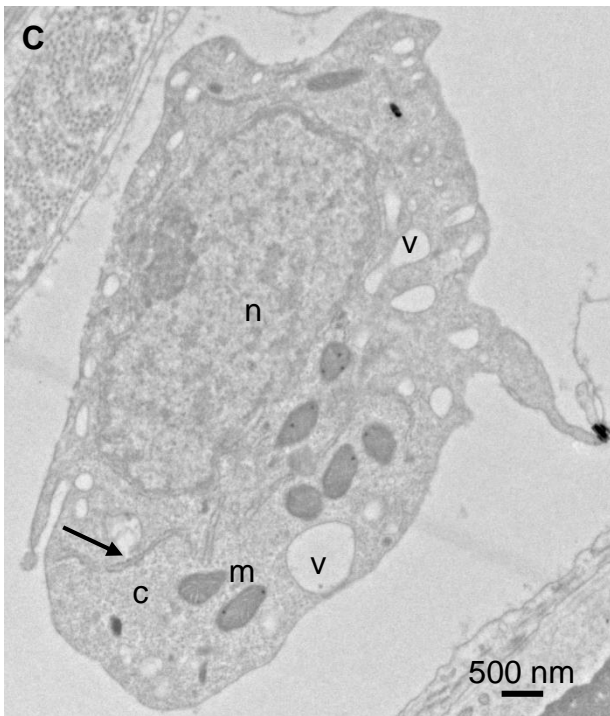
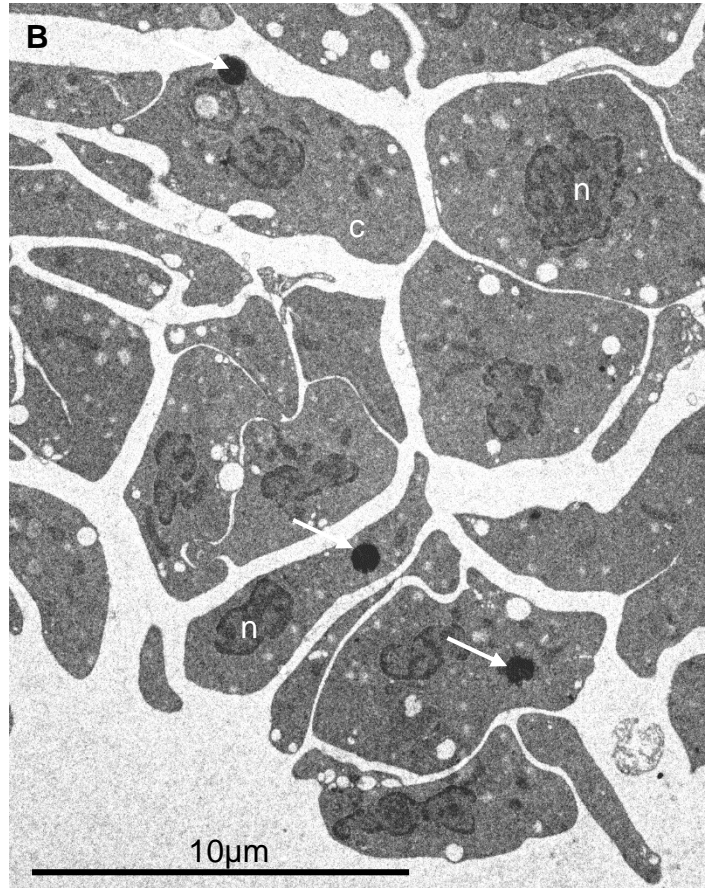
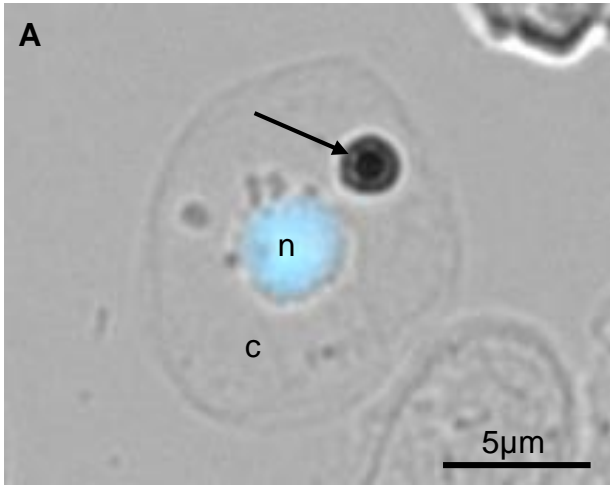




Figure 3

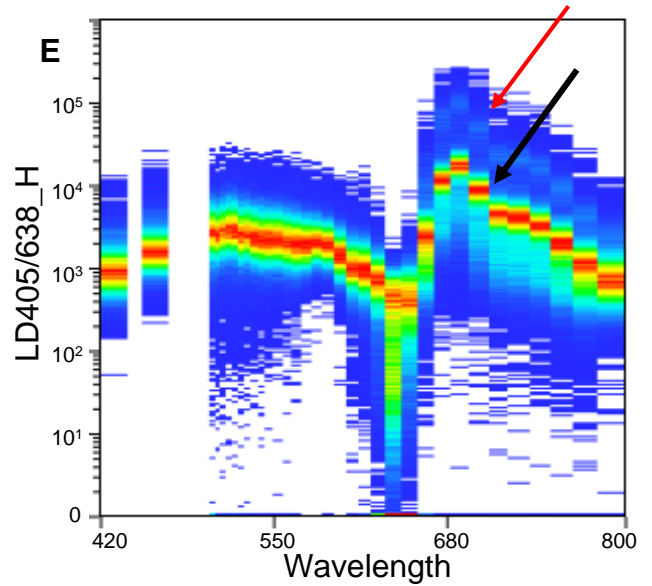
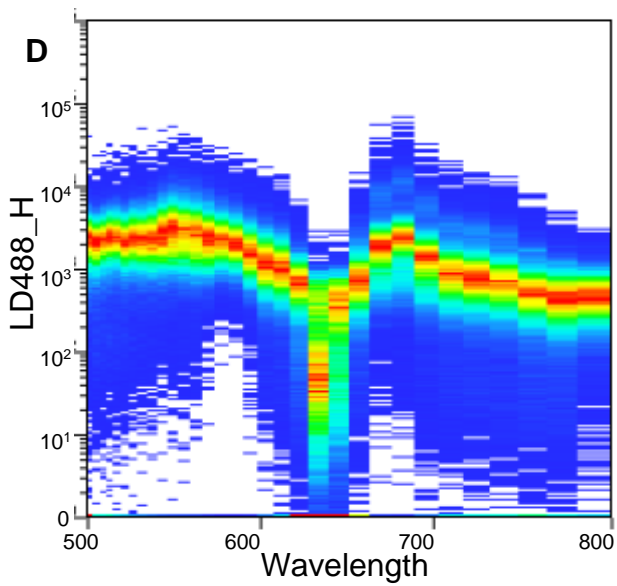
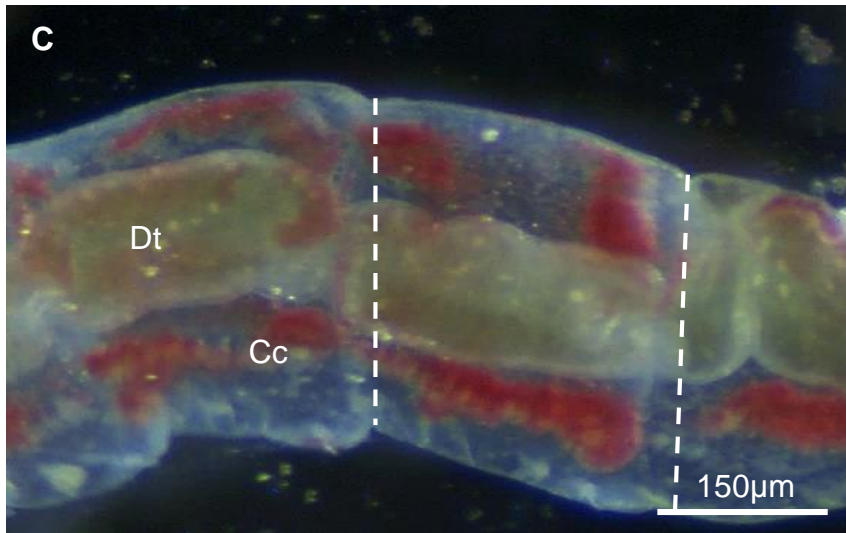
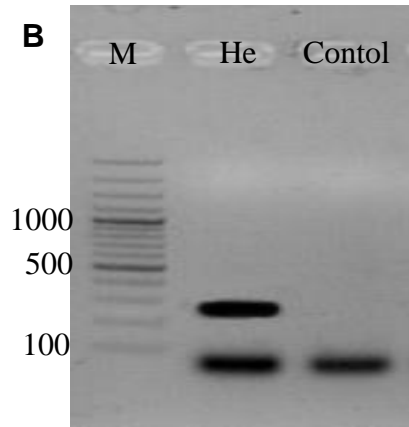
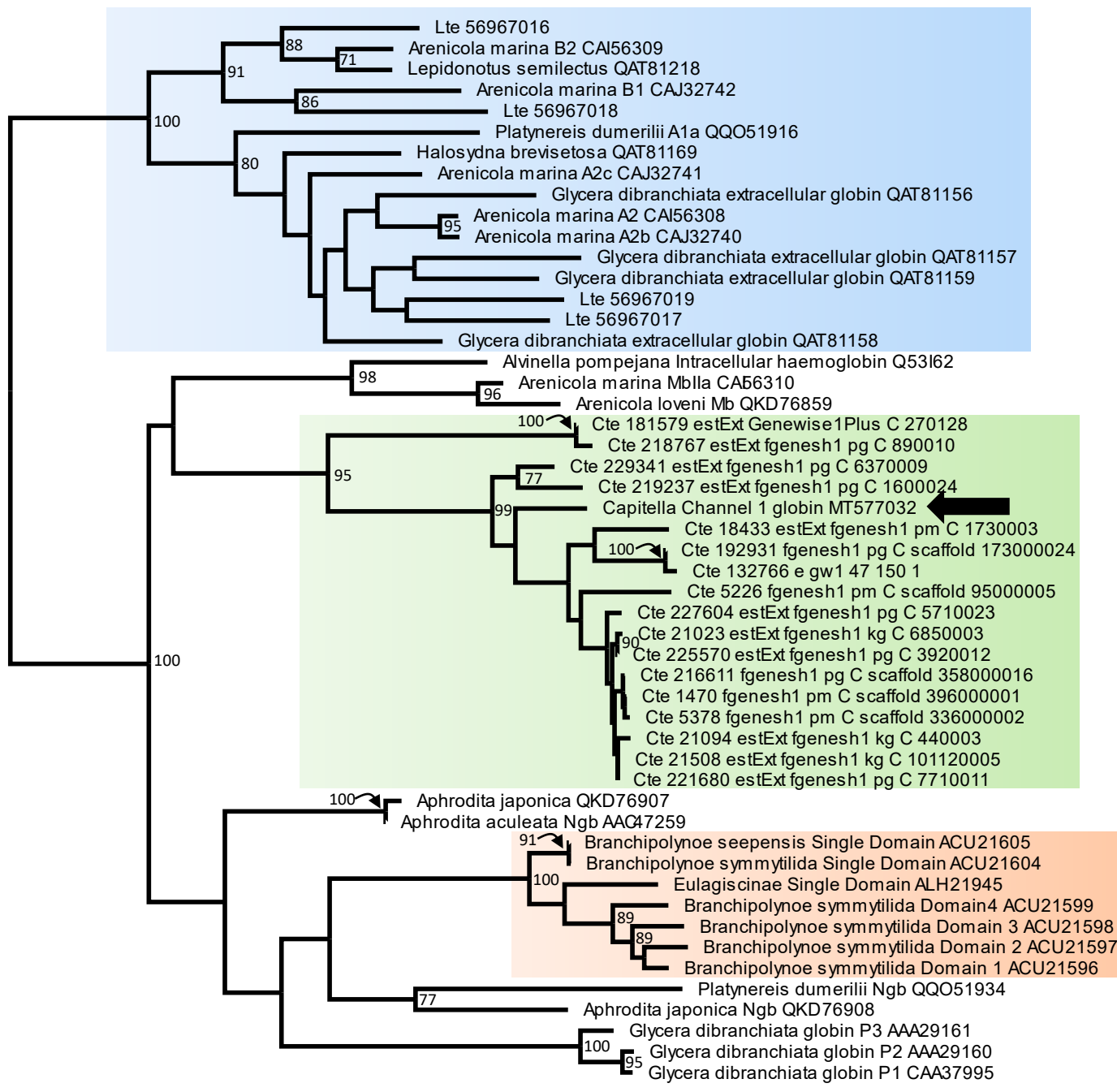
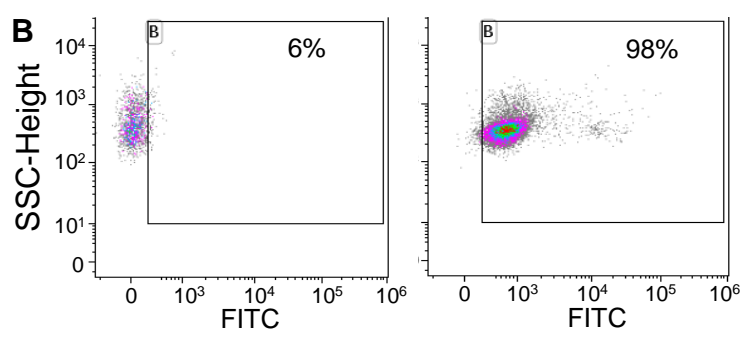
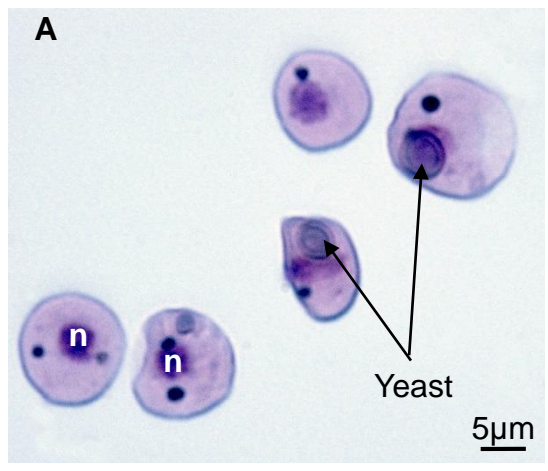


Figure 4



0.4

Figure 5



Gate	% Gated	Gate	% Gated
All	100.00	All	100.00
B	6.93	B	98.41

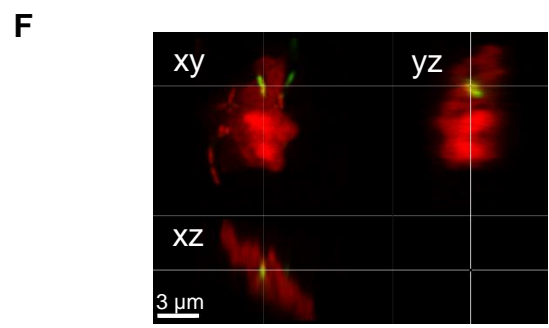
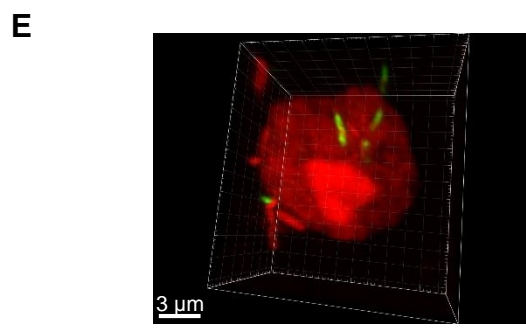
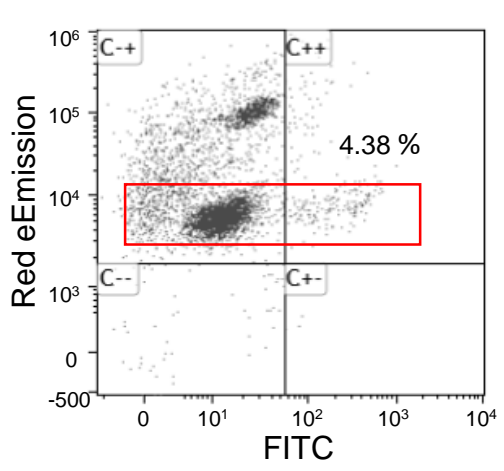
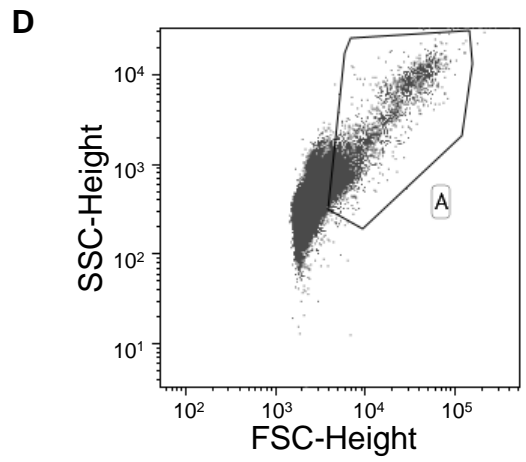
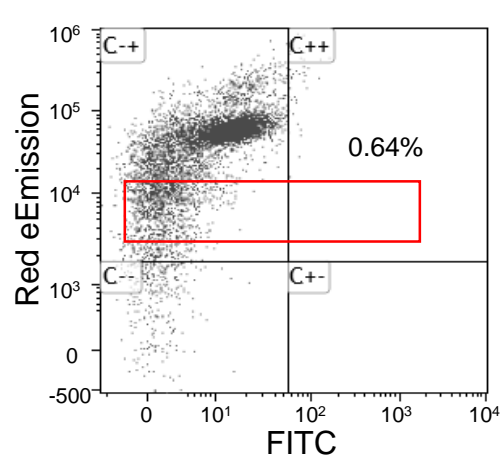
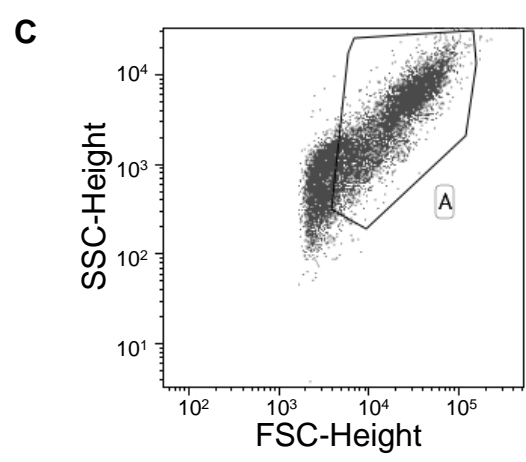


Figure 6

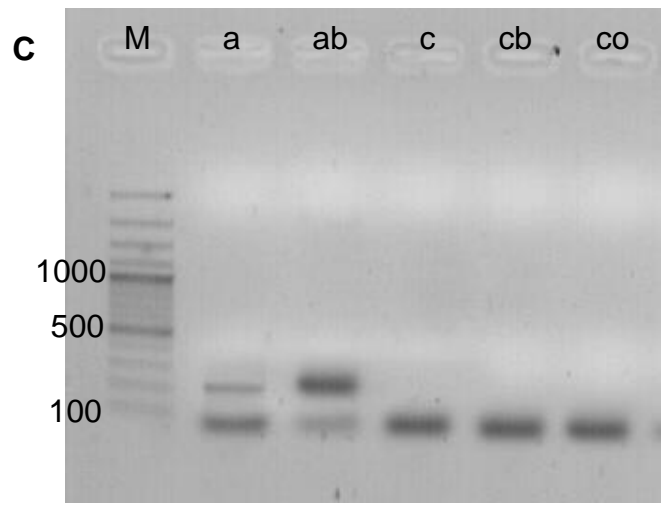
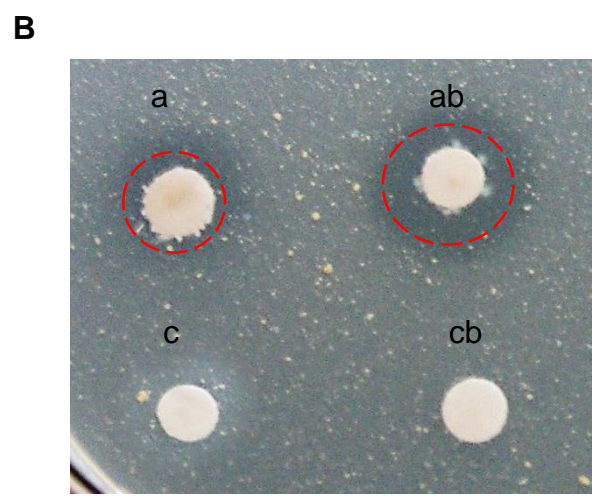
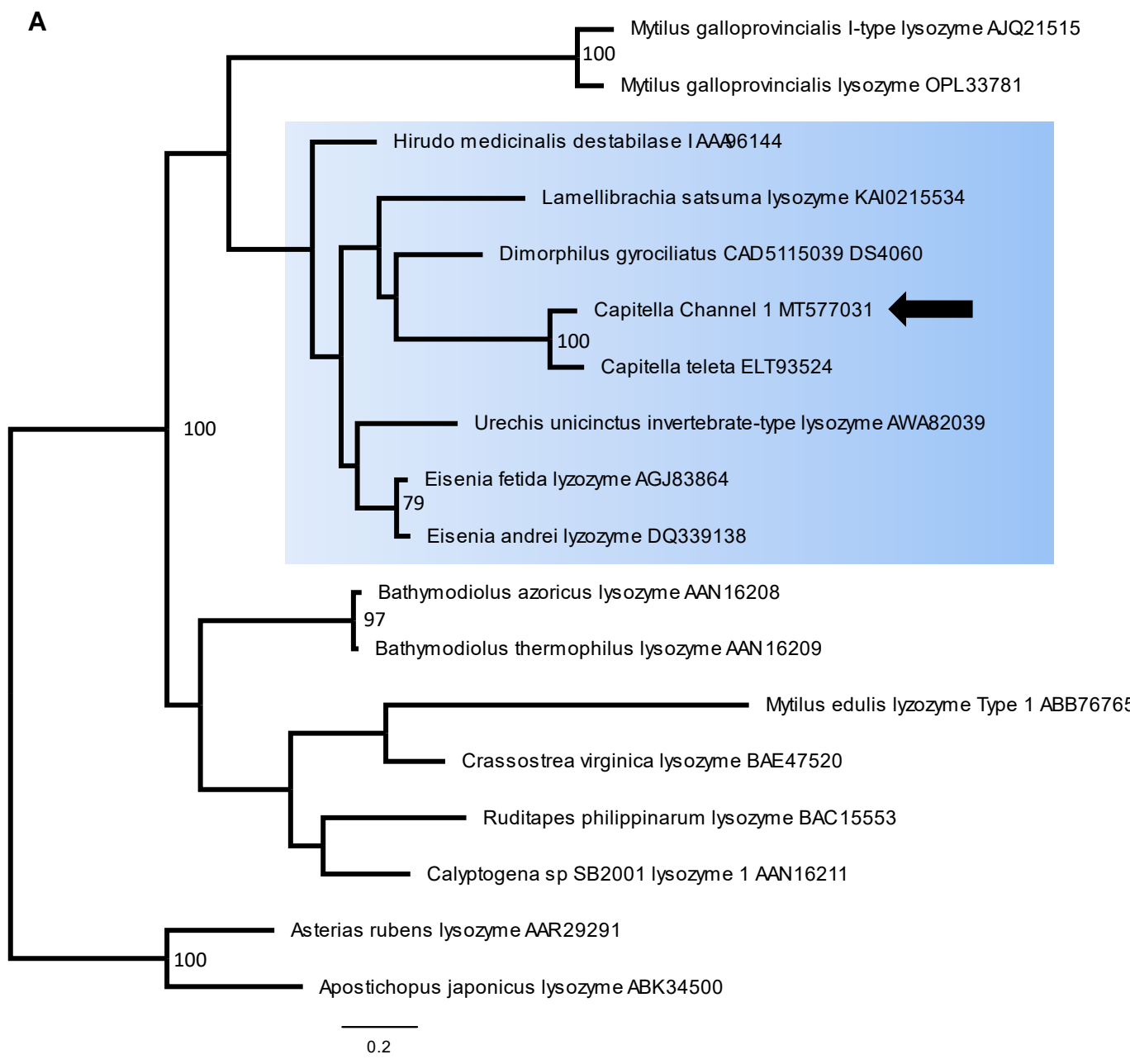


Figure 7

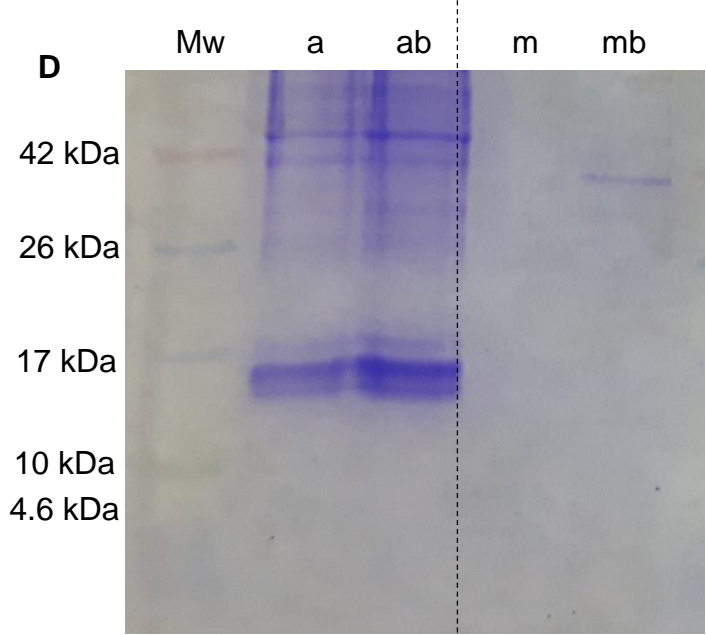
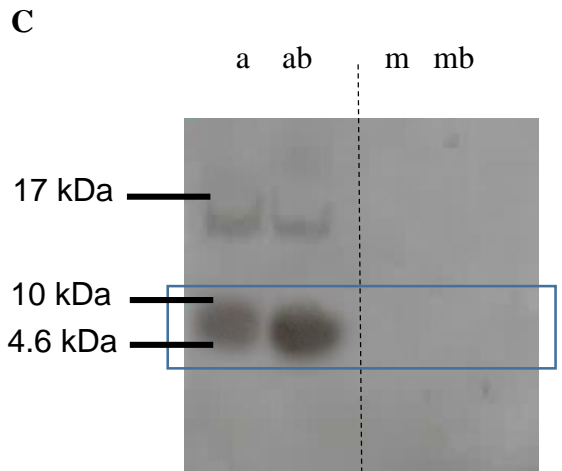
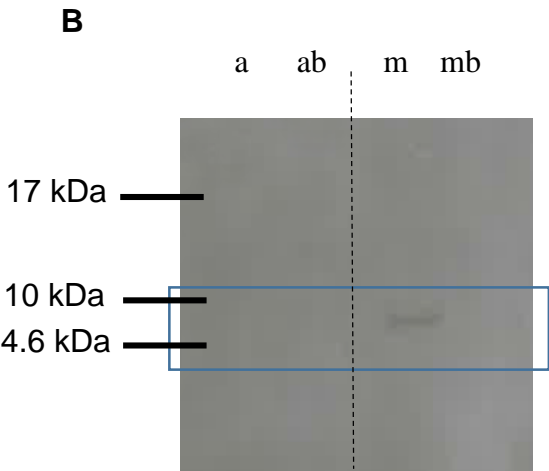
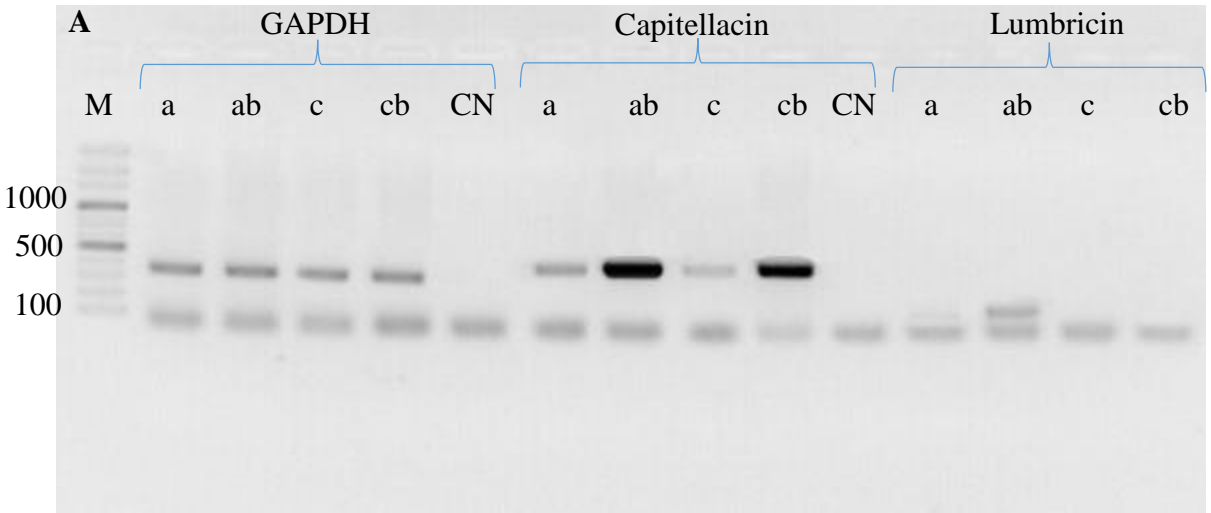


Figure 8

