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1 **Hemocyte morphology and phagocytic activity in the common**
2 **cuttlefish (*Sepia officinalis*)**
3

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24 1. Introduction

25 Invertebrates resist pathogens despite their lack of adaptive immunity [1]. The
26 tremendous variety of invertebrate life histories and ecological niches suggests a great
27 diversity of immune strategies [2]. Among Mollusca – one of the most diverse groups of
28 animals [3], studies of immunity have **mostly** focused on bivalves (e.g. [4–14]) and
29 gastropods (e.g. [15–20]), while few studies have focused on cephalopods [21,22].
30 Cephalopods are an interesting model because of their (1) vertebrate-like high-pressure closed
31 circulatory system, (2) high sensitivity to environmental parameters, (3) short-life span and
32 (4) elevated metabolic rate [23,24]. Moreover, their economical importance has recently
33 **grown** in terms of **fisheries** and aquaculture (e.g. [25–29]).

34 As in other invertebrates, the cephalopod immune system relies on humoral factors
35 and cell-mediated mechanisms acting together to eliminate invading micro-organisms [30,31].
36 Humoral factors mainly include lectins (e.g. agglutinins, opsonins), antimicrobial factors (e.g.
37 peptides, acid phosphatases, lysozymes), and several **signaling** pathways including
38 prophenoloxidase (proPO) and proteolytic cascades [2,32]. In contrast, cell-mediated defense
39 mechanisms are primarily performed by hemocytes (Hcs) – cells synthesized in white bodies
40 and freely circulating in plasma and infiltrating in tissues [33,34]. Hcs are of central
41 importance to invertebrates because of their involvement in numerous physiological functions
42 [35–39], including their ability to **phagocytose**, encapsulate and destroy foreign particles
43 [18,30,40]. In cephalopods, Hcs have **mainly** been described as a one cell-type population¹

¹ **Abbreviations:** FCM: flow cytometry; Hc: hemocyte; Hcy: hemocyanin; MPS: molluscan physiological saline; NR: neutral red; PI: protease inhibitor; PO: phenoloxidase; proPO: prophenoloxidase; SEM: scanning electron microscopy; SD: standard deviation; TEM: transmission electron microscopy.

44 with large lobate nucleus and abundant granules, able to phagocytose [31,34,40–42].
45 However, these Hc descriptions were mainly performed in Octopodidae as well as in the
46 sepiolidae *Euprymna scolopes* and little is known about the immune cellular factors of other
47 cephalopod species such as the sepiidae (cuttlefish) *Sepia officinalis*. Because of the distinct
48 ecology of Sepiidae within Cephalopodia [27,43,44], they may also have distinct immune
49 requirements.

50 In this study, we performed cytological stainings, electron microscopy and flow
51 cytometry (FCM) analysis to morphologically characterize *S. officinalis* Hcs. In addition, we
52 investigated humoral factor localization between plasma and cells, and phagocytic reactions at
53 several incubation times, temperatures and plasma concentrations. Our results highlighted one
54 granulocyte population with various densities of acidophilic granules and unstained vesicles.
55 The Hcs, which contained acid phosphatase, lysozyme and proPO system enzymes, had high
56 phagocytic ability, modulated by plasma in our assay conditions.

57 2. Material and methods

58 2.1. Animals

59 Thirty-one adult cuttlefish *S. officinalis* (21.5 ± 3.1 cm mantle length) were obtained
60 from traps deployed during spring 2011 and 2012 along the Calvados coast (Normandy,
61 France). Cuttlefish were then conditioned at the Centre de Recherches en Environnement
62 Côtier (Luc-sur-Mer, Normandy, France) in 4500-L tanks in an open seawater circuit for at
63 least 2 days, fed with crustaceans *Crangon crangon* and *Carcinus maenas*, and starved for 1
64 day before experimentation. The sex of each individual was determined.

65

66 2.2. Hemolymph collection

67 Before hemolymph sampling and following ethical procedures (Directive
68 2010/63/EU), cuttlefish were anesthetized as described by Andrews et al. [45] through
69 placement for 10 min in seawater containing 2% ethanol. Between 9 and 13 ml of hemolymph
70 was then withdrawn from the anterior mantle vein [46] using a syringe with 18-gauge needle.
71 The sample was transferred into a sterile tube, diluted or not with one volume of cooled sterile
72 antiaggregative modified Alsever solution (115 mM glucose; 27 mM sodium citrate; 11.5 mM
73 EDTA; 382 mM NaCl pH 7.5) [47], depending on the procedure (see below), and kept on ice
74 to minimize cell clumping. Hc viability was checked by mixing one volume of Alsever-
75 diluted hemolymph with one volume of trypan blue solution (0.4%) and Hc concentration was
76 determined with non diluted hemolymph using a Thoma cell. Once sampling was completed,
77 animals were euthanized by increasing ethanol concentration to 10% [48].

78

79 2.3. Chemicals

80 Sodium chloride (NaCl), anhydrous and hexahydrate magnesium chloride ($MgCl_2$ and
81 $MgCl_2 \cdot 6H_2O$), calcium chloride ($CaCl_2$), bovin serum albumin (BSA), Bradford reagent,

82 trypsin TPCK (N-Tosyl-L-phenylalanine chloromethyl ketone), N α -benzoyl-L-arginine 4-
83 nitroanilide hydrochloride (BAPNA), *p*-nitrophenyl-phosphate, dimethyl sulfoxide (DMSO),
84 trizma hydrochloride (Tris-HCl), trizma base, 3,4-dihydroxy-L-phenylalanine (L-DOPA),
85 tropolone, hen egg white (HEW) lysozyme, freeze-dried *Micrococcus lysodeikticus*, sodium
86 phosphate dibasic dihydrate (Na₂HPO₄·2H₂O), citric acid (C₆H₈O₇), Wright stain, neutral red,
87 L-15 medium (Leibovitz), potassium chloride (KCl), magnesium sulphate heptahydrate
88 (MgSO₄·7H₂O), formaldehyde solution, L-glutamine, streptomycin, sodium hydroxide
89 (NaOH), ethylenediaminetetraacetic acid (EDTA), trypan blue solution, methanol and HEPES
90 were obtained from Sigma-Aldrich (France). Halt Protease Inhibitor Cocktail, EDTA-Free
91 (100X) was obtained from Thermo Fisher Scientific (Waltham, USA). Ethanol was obtained
92 from Carlo erba (Milan, Italy). Hemacolor® staining kit was obtained from Merck Millipore
93 (Darmstadt, Germany). Low melting point agar was obtained from Carl Roth (Lauterbourg,
94 France). All chemicals used for electron microscopy i.e. glucose, sodium citrate,
95 glutaraldehyde, sodium cacodylate, sucrose, osmium tetroxide (OsO₄), propylene oxide,
96 araldite resin, uranyl acetate and lead citrate were obtained from Electron Microscopy
97 Sciences (Hatfield, PA, USA).

98

99 *2.4. Morphological characterization of S. officinalis Hcs*

100 *2.4.1. Hemolymph cell monolayer stainings*

101 For **Hc** staining, one drop of hemolymph was placed on a Thermanox™ coverslip
102 (Thermo Fisher Scientific, Waltham, USA) and allowed to adhere for 30 min at 15°C. Before
103 staining, coverslips were rinsed in Molluscan Physiological Saline (MPS; 0.4 M NaCl, 0.1 M
104 MgSO₄, 20 mM HEPES, 10 mM CaCl₂ and 10 mM KCl modified after [49]) to remove
105 plasma. Hemacolor® staining was performed according to the manufacturer's
106 recommendations. Wright staining was performed after 1 min air-drying by 1 min

107 dehydration in absolute methanol, following by 1 min in Wright solution (0.66% in
108 methanol), then diluted (1:4) in distilled water during 4 min and rinsed. To highlight the cell
109 lysosomal system, neutral red (NR) uptake was performed by incubating cells for 30 min in
110 NR solution (1:500; NR stock solution (20 mg NR/ml DMSO):MPS) in a moist chamber at
111 15°C, before observation.

112 Freshly spread Hcs were observed using an inverted binocular microscope (Leica®
113 DM IRB, Leica Microsystems, Wetzlar, Germany). Stained Hc observations were carried out
114 with a Nikon Eclipse 80i light microscope with computer-assisted microscopic image analysis
115 system, NIS-elements D 2.30 software (Nikon®, Champigny-sur-Marne, France).

116

117 2.4.2. Electron microscopy

118 After 10 min $300 \times g$ centrifugation, Hc pellets ($N = 5$) were rinsed with MPS and
119 fixed in 3.2% glutaraldehyde in 0.31 M sodium cacodylate buffer, with 0.25 M sucrose (pH
120 7.4) during for 90 min at 4°C. The cells were washed 3 times in rinsing solution (0.4 M
121 sodium cacodylate, 0.3 M sucrose, pH 7.4). Then, cells were post-fixed 1 h with 1% OsO₄ in
122 cacodylate buffer 0.2 M, with 0.36 M sucrose (pH 7.4) at 4°C (protected from light), and
123 washed in rinsing solution.

124 For scanning electron microscopy (SEM), cells were sedimented for 7 days on
125 Thermanox™ coverslip coated with poly-l-lysine (Thermo Fisher Scientific, Waltham, USA).
126 They were then dehydrated in progressive bath of ethanol (70-100%) and critical point dried
127 (Leica® EM CPD030). Finally, cells were sputtered with platinum and observed with
128 scanning electron microscope JEOL 6400F. Freshly sampled cell diameters were determined
129 by measuring 100 cells per cuttlefish ($N = 5$).

130 For transmission electron microscopy (TEM), cells were pellet in 2% low melting
131 point agar at 40°C, and then dehydrated through increasing concentrations of ethanol (70-

132 100%) and propylene oxide 100%, embedded in araldite resin and allowed to polymerise for
133 48 h at 60°C. Ultrathin sections were done and contrasted with 2.5% uranyl acetate diluted in
134 50% ethanol for 30 min and contrasted for 5 min in Reynold's lead citrate [50]. Finally, cells
135 were observed by transmission electron microscope JEOL 1011 and images were obtained
136 with Camera Megaview 3 and Analysis Five software.

137

138 2.5. Biochemical analysis

139 2.5.1. Enzyme extraction

140 Hcs and plasma were separated in non diluted hemolymph by 500 × g centrifugation
141 for 10 min at 4°C. Plasma was then removed, checked for absence of Hc microscopically, and
142 stored in aliquots at -80°C until analysis. Upon complete plasma removal, Hc pellets were
143 gently rinsed in either Tris buffer pH 7 (0.1 M Tris-HCl, 0.45 M NaCl, 26 mM MgCl₂ and 10
144 mM CaCl₂) for phenoloxidase (PO) assays [51] or Tris buffer pH 8 (10 mM Tris-HCl and 150
145 mM NaCl) for phosphatase, lysozyme and protease inhibitor (PI) assays [52]. Cells were
146 resuspended at 10 × 10⁶ cells ml⁻¹ in same extraction buffer and sonicated at 60 W for 20 s.
147 Cell extracts were then centrifuged at 10,000 × g and their supernatant aliquoted and stored at
148 -80°C until analysis.

149

150 2.5.2. Enzymatic assays

151 All activities were expressed in relation to protein concentration measured by the
152 Bradford method [53] using BSA as standard.

153 Total acid phosphatase activity was determined according to Moyano et al. [54] using
154 *p*-nitrophenyl-phosphate 2% as substrate in a 1 M Tris buffer at pH 3. Then, 10 µl of
155 supernatant was added to 10 µl of substrate in 96-well flat bottom plates (BD, USA). After 30
156 min incubation at 25°C, 100 µl of NaOH 1 M were added to stop the reaction. The

157 absorbance was measured at 405 nm using a Mithras LB 940 luminometer (Berthold, Thoiry,
158 France). Total acid phosphatase activity was expressed as specific activity (U mg^{-1} protein)
159 where one enzymatic unit corresponded to the absorbance recorded after incubation.

160 Lysozyme activity was quantified according to Malham et al. [55]. Fifty μl of HEW
161 lysozyme ($85 \mu\text{g ml}^{-1}$ in Tris buffer pH 8 described in section 2.5.I.) standard was serially
162 diluted in triplicate in 96-well flat bottom plates (BD, USA). Fifty μl of each sample was
163 added in triplicate to the 96-well plates as well as 50 μl of Tris buffer pH 8, as blanks. One
164 hundred and fifty μl of the substrate, freeze-dried *M. lysodeikticus* (75 mg/100 ml of
165 phosphate/citrate buffer pH 5.8 ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 4.45 g/250 ml distilled H_2O ; citric acid
166 ($\text{C}_6\text{H}_8\text{O}_7$), 2.1 g/100 ml distilled H_2O ; NaCl, 0.09 g/100 ml buffer)), was added to each well.
167 The reductions in turbidity in the wells were read on Mithras LB 940 luminometer (Berthold,
168 Thoiry, France) at 25°C for 5 minutes at 10 second intervals at 450 nm using negative
169 kinetics. Lysozyme concentrations were calculated from the standard curve ($\mu\text{g HEW}$
170 lysozyme equivalent ml^{-1}). Final lysozyme-like activity was thus expressed as $\mu\text{g HEW}$
171 lysozyme eq. mg protein^{-1} .

172 As described by Malham et al. [55] and Thompson et al. [56], PI activity was measured
173 by transferring 20 μl of sample and 10 μl of trypsin TPCK ($100 \mu\text{g ml}^{-1}$ of 0.05 M Tris buffer
174 pH 8) in 96-well flat bottom plates (BD, USA), and mixed at room temperature for 5 minutes.
175 In parallel, intrinsic trypsin activity was measured by replacing 10 μl of trypsin by Tris buffer
176 pH 8 described above (section 2.5.I.). A positive control was used by replacing the sample
177 with Tris buffer pH 8. Two hundred μl of BAPNA substrate solution (5.2 mg BAPNA ml^{-1}
178 DMSO) in 10 ml of 0.01 M trizma base buffer pH 7.4) was added to each well and incubated
179 for 15 minutes at room temperature. Absorbance was read at 405 nm using Mithras LB 940
180 luminometer (Berthold, Thoiry, France), and PI activity was expressed as the percentage of
181 trypsin sample inhibition (TI) compared to the positive control.

182 In order to partly discriminate PO synthesis and activation site, special care was taken to
183 avoid unwanted activation of proPO during each step of the experiment. PO-like activity was
184 measured spectrophotometrically by recording the formation of *o*-quinones, as described by
185 Luna-Acosta [51] with slight modifications to distinguish artificially activated PO (APO)
186 (corresponding to PO-like activity resulting from zymogenic PO (proPO) activation plus
187 already 'active' form) and *in vivo* 'active' PO form [57]. PO assays were conducted in 96-
188 well flat bottom plates (BD, USA). L-DOPA was used as substrate, at a final concentration of
189 10 mM [51] and prepared extemporaneously in Tris buffer pH 7 described above (section
190 2.5.1.). Tropolone (10 mM) and trypsin TPCK (1 g l⁻¹) were used respectively as PO inhibitor
191 and elicitor as previously described in *S. officinalis* [57,58]. To avoid uncontrolled proPO
192 activation by intrinsic proteases, Halt Protease Inhibitor Cocktail, EDTA-Free (1X) wide
193 spectrum PI was used. For each sample, autoxidation of sample, 'basal', 'inhibited' and
194 'activated' PO-like activities were measured. For non-enzymatic sample autoxidation, 10 µl
195 of sample was mixed with Tris buffer pH 7. For 'basal' PO-like activity, 10 µl of sample was
196 firstly mixed during 10 min with 1.4 µl Halt Protease Inhibitor Cocktail (100X), followed by
197 adapted volume of Tris buffer pH 7 and 80 µl L-DOPA. Similarly, for inhibited or APO-like
198 activity, 10 µl of sample was mixed with 10 µl of tropolone (140 mM) or trypsin TPCK (14 g
199 l⁻¹), Tris buffer pH 7 and 80 µl L-DOPA. Each measurement was systematically controlled by
200 replacing sample by buffer, always in a final volume reaction of 140 µl. Immediately after L-
201 DOPA addition, PO-like activities were monitored at 25°C for 5 h using Mithras LB 940
202 luminometer (Berthold, Thoiry, France) at 490 nm [51]. When an inhibited PO-like activity
203 was measured, this value was subtracted from APO and PO-like activities. Tropolone, with its
204 copper chelator and peroxidase substrate properties, ensured that PO-like activity alone was
205 detected (and not peroxidase). Results were also systematically corrected for non-enzymatic

206 autoxidation of the substrate and were expressed in enzyme unit (1 U) per mg of total protein.
207 One U corresponded to an increase of 0.001 in the absorbance per min at 25°C [57].

208

209 2.6. Flow cytometry (FCM) analysis

210 FCM analyses were performed using a Gallios flow cytometer (Beckman Coulter). In
211 our study, excitation light was provided by a 22 mW blue diode (488 nm), and fluorescence
212 was collected in the FL1 channel with a 525 nm bandpass filter. For each sample, about
213 20,000 events were acquired. Data were analysed using Kaluza software (Beckman Coulter).

214

215 2.6.1. Freshly sampled Hc cytomorphology

216 Hc morphology was based upon relative flow-cytometric parameters, Forward Scatter
217 (FSC) and Side Scatter (SSC). FSC and SSC commonly measure particle size and internal
218 complexity, respectively. Internal complexity, also reported as granularity, depends upon
219 various inner components of the cells including shape of the nucleus, amount and types of
220 cytoplasmic granules, cytoplasmic inclusions and membrane roughness [59]. Freshly
221 withdrawn Hc was pelleted by $300 \times g$ centrifugation for 5 min at 4°C, rinsed in MPS, before
222 to be fixed in 3.7% formaldehyde in MPS and kept in dark at 4°C until FCM analysis.

223

224 2.6.2. Phagocytosis assays

225 Evaluation of phagocytosis was based on the ingestion of carboxylate-modified
226 FluoSpheres® beads (1.0 µm, yellow-green carboxylate-modified FluoSpheres®, Molecular
227 Probes) by Hcs. Phagocytosis was expressed as (i) the percentage of cells that had ingested
228 three or more microbeads [60,61], and (ii) the average number of microbeads per phagocytic
229 Hcs [18].

230 Hemolymph-diluted Alsever solution was used to plate Hcs at 1.0×10^6 cells per well
231 in 6-well plates, into which three volumes of sterile artificial seawater (25.5 g l⁻¹ NaCl, 6.4 g l⁻¹
232 MgSO₄, 5.2 g l⁻¹ Hepes, 1.1 g l⁻¹ CaCl₂, 0.75 g l⁻¹ KCl pH 7.4) were added to allow cell
233 adhesion. After 60 min of incubation in dark at 15°C, supernatant was removed and cells were
234 covered with 1 ml modified sterile Leibovitz L-15 medium pH 7.6 (20.2 g l⁻¹ NaCl, 0.54 g l⁻¹
235 KCl, 0.6 g l⁻¹ CaCl₂, 1 g l⁻¹ MgSO₄·7H₂O and 3.9 g l⁻¹ MgCl₂·6H₂O) [62], supplemented with
236 2 mM L-glutamine, 100 µg ml⁻¹ streptomycin and 60 µg ml⁻¹ penicillin G into which
237 FluoSpheres® were added at a ratio of 1:100 (Hc:beads). Hcs from same individual cuttlefish
238 were analyzed at each time.

239

240 2.6.2.1. *In vitro* impact of time and temperature

241 Hcs were collected from 5 cuttlefish as described in section 2.2. To determine
242 temperature-dependence of *S. officinalis* phagocytosis, three different temperatures were
243 investigated; one physiological temperature (15°C) and two temperatures (4 and 25°C) out of
244 the thermal window determined by Melzner et al. [23] with 15°C-acclimated cuttlefish. In this
245 context, media with FluoSpheres® were acclimated to different working temperatures before
246 addition to Hcs. Then, 6-well plates were placed in dark at 4, 15 and 25°C CO₂-free
247 incubators, during 30, 60, 120 and 180 min incubation. Afterwards wells were rinsed with
248 MPS to remove bead excess, following by gentle scrapping and centrifugation at 300 × g for 5
249 min at 4°C. Resulting supernatants were removed, pellets resuspended in 3.7% formaldehyde
250 solution in MPS and stored in the dark at 4°C until FCM analysis.

251

252 2.6.2.2. *In vitro* impact of plasma concentrations

253 To study the plasma opsonization process, FluoSpheres® were added to modified L-
254 15 medium mixed with cell-free plasma at 0 (as control), 1, 10 and 50%. These mixtures were

255 incubated at 15°C during 1 h before homogenization and addition to cells. After 2 h
256 incubation at 15°C, cells were treated as previously described (cf 2.6.2.1.) for FCM analysis.

257 In parallel, bead phagocytosis was performed in modified L-15 medium or 100%
258 plasma, and Hcs analysed by TEM as described in section 2.4.2.

259

260 2.7. Data analysis

261 Residual distributions were tested for normality (Shapiro test) as well as homogeneity of
262 variances (Levene test). Student's *t*-test was used to compare Hc concentrations between male
263 and female individuals, specific activities between Hc and plasma compartments, PO- and
264 APO-like activities in each compartment, and phagocytic parameters between control and
265 several plasma treatments. One-way analysis of variance (ANOVA) followed by non-
266 parametric pairwise permutational *t*-tests ($N < 30$) was used to determine the impact of
267 temperature on phagocytic parameters at each time. Analysis of covariance (ANCOVA) was
268 used to compare phagocytic parameter time-evolution as function of the temperature. All
269 results are expressed and displayed as mean \pm standard deviation (SD). The statistical
270 significance was designed as being at the level of $p < 0.05$. R software was used for statistics
271 and graphics.

272

273 3. Results and Discussion

274 Little is known about cephalopod Hcs, while Hc populations have been extensively
275 studied in other mollusks (bivalves and gastropods). Our study provides the most
276 comprehensive description of the circulating Hc population in the cuttlefish *S. officinalis* by
277 using FCM, light and electron microscopy. We also report biochemical- and phagocytosis
278 analysis of Hc interaction with plasma.

279

280 3.1. Hc viability and concentration

281 Hc viability in all cuttlefish used in this study was higher than 99% (data not shown),
282 and no Hc concentration difference was observed between males ($7.20 \pm 5.70 \times 10^6$ cells ml⁻¹;
283 $N=18$) and females ($7.27 \pm 5.75 \times 10^6$ cells ml⁻¹; $N=13$). Consequently, we obtained a mean
284 Hc concentration in hemolymph of *S. officinalis* of $7.23 \pm 5.62 \times 10^6$ cells ml⁻¹ ranging from
285 0.92 to 20.31×10^6 cells ml⁻¹. This mean concentration and concentration variability are
286 consistent with reports on cephalopod Hcs (Table 1). Although highly variable, the Hc
287 concentration of *S. officinalis* appeared slightly higher than that of the loliginidae *Sepioteuthis*
288 *lessoniana*, similar to that of the sepiolidae *Euprymna scolopes*, and lower than that of
289 Octopodidae, where the highest Hc concentrations within cephalopods are found (10×10^6
290 cells ml⁻¹) [40,42,63–67]. Generally, Hcs of gastropod and bivalve mollusks appear less
291 concentrated with most values ranging between 1 and 5×10^6 cells ml⁻¹ (Table 1)
292 [6,7,9,11,12,18–20,59,68–75], albeit with population-dependent variation [7,12,76].

293

294 3.2. Hc identification and characterization

295 3.2.1. Light microscopy

296 After adhesion, fresh cuttlefish Hcs rapidly displayed many thin pseudopodia, and
297 most cells contained refringent and non-refringent granules of various densities in their

298 cytoplasm (Fig. 1). Among different stains used, Wright's differential staining allowed the
299 best **observation** of spontaneously adhering Hcs. A single cell type with large, lobate nucleus,
300 slightly basophilic cytoplasm and **acidophilic** granules was identified (Fig. 2A). Lucent
301 vesicles were also **observed** in the cytoplasm of some cells. NR uptake revealed dense
302 lysosomal system in most cells (Fig. 2B). According to the classification of bivalve Hcs, **2**
303 main classes are usually accepted: granulocytes with cytoplasmic granules and hyalinocytes
304 with few or no granules [77]. Based on the high granule density found in most Hcs, we
305 classify *S. officinalis* Hcs as granulocytes. These observations are consistent with the majority
306 of studies performed in **O**ctopodidae [31,34,41,78,79], **S**epiidae [33] and **S**epiolidae [42,**80**],
307 which reported one granular cell population circulating in **the** hemolymph **and** also called
308 macrophage-like Hc [34,81].

309

310 3.2.2. Electron microscopy

311 SEM observations of circulating cuttlefish Hcs showed a single cell type of 10.3 ± 0.8
312 μm **in** diameter **and** able to form numerous pseudopodia (Fig. 3A-B), consistent with **the**
313 **above described light microscopy observations**. This cell size is consistent with the 8-10 μm
314 cell diameter measured on non-circulating mature Hcs in the cuttlefish white bodies
315 (leukopoietic tissues) [33] and appeared slightly lower than octopod *O. vulgaris* and *E.*
316 *cirrrosa* Hcs: 11.6 ± 1.2 and 12-15 μm , respectively [63,82].

317 TEM observations of circulating Hcs highlighted a large lobate nucleus without nucleoli
318 but highly condensed chromatin mainly localized along the inner surface of the nuclear
319 membrane (Fig. 3C). The cytoplasm of most cells contained **various sizes and densities of** two
320 distinct inclusion **types** (Fig. 3D): electron-dense granules corresponding to lysosomes [33],
321 and electron-lucent membrane-surrounded vesicles, containing various densities of **molecules**
322 **with similar size and circular shape as Hcy molecules** (Fig. 3E-F; [83–87]). These molecules

323 are referred to as Hcy-like hereafter. In contrast to *E. cirrhosa* and *O. vulgaris* Hcs, neither
324 different electron-dense granules nor lipid droplets were observed [31,34,63], suggesting
325 different granule contents between these cephalopods. Mitochondria, Golgi apparatus and
326 rough endoplasmic reticulum that often surrounded the nucleus were also observed in these
327 cells (Fig. 3C inset).

328

329 3.3. Enzymatic assays

330 Hydrolytic enzyme lysozymes and acid phosphatases were detected in Hc extract alone
331 (Table 2), consistent with their known lysosomal distribution [88,89]. These data are in
332 agreement with carbohydrates and acid phosphatases detected by stainings in *E. cirrhosa* Hcs
333 [66] and suggest their synthesis in *S. officinalis* Hcs or earlier during their maturation [66,90].
334 PI activities were exclusively found in plasma (Table 2), corresponding at least in part to the
335 already described α_2 -macroglobulin activity – the second most abundant protein in *S.*
336 *officinalis* plasma [91–93]. Interestingly, PIs were recently reported in *E. cirrhosa* and *E.*
337 *scolopes* Hcs [31,42], underlying the need for future investigations.

338 PO-like activities were measured in both hemolymph compartments (Table 2). Siddiqui
339 et al. [94] showed in *S. officinalis* that plasma-associated PO-activity resulted from Hcy,
340 which molecule shares a structurally and functionally equivalent active site with POs [95].
341 Therefore, PO-like activities found in Hc extracts probably result, at least in part, from Hcy
342 presence in electron-lucent vesicles (TEM analysis). We note that PO activity was not
343 previously detected in cephalopod Hcs. In contrast, proPO activation – the difference between
344 PO- and APO-like activity, occurred in Hc extract alone ($p=0.01$).

345

346 3.4. FCM analysis

347 3.4.1. Fresh Hc cytogram

348 According to the criteria of cell size (FSC) and cell complexity (SSC), FCM supported
349 the presence of a single Hc population in cuttlefish (Fig. 4A), albeit with variable internal
350 complexity in some individuals (Fig. 4B), consistent with the above described TEM
351 observations. Notably, recent *O. vulgaris* Hc characterization using TEM and FCM reported 2
352 circulating granulocyte morphs based on size, which were interpreted as different maturing
353 stages of the same cell type [63]. Consistently, a study of *S. officinalis* Hc synthesis suggested
354 a Hc maturation model with a single cell precursor [33]. This cephalopod organization is
355 distinct from that of bivalves and gastropods, where most Hc studies reported several
356 circulating Hc types derived from one or more cell precursors (e.g.
357 [5,8,9,11,15,17,18,20,32,96,97]). Our results further support the presence of a single mature
358 circulating cell type in the Hc population of cephalopods.

359

360 3.4.2. Phagocytosis experiments

361 3.4.2.1. Time and temperature

362 Both phagocytosis parameters (i.e. percentage of phagocytic Hcs and quantity of
363 ingested beads) appeared time-dependent at all temperatures tested (Fig. 5).

364 After 30 min, 35.5, 40.1, and 41.7% of cells were phagocytic at 4, 15 and 25°C,
365 respectively, subsequently reaching 63.3, 70.6 and 69.8% after 180 min (Fig. 5A). These
366 phagocytic percentages are consistent with previous observations of Hcs in *O. vulgaris* and *E.*
367 *cirrrosa* where 50 and 70% of phagocytic cells were reported, respectively [40,98]. In
368 contrast, a lower phagocytic percentage ($\leq 13\%$) was recently reported for *O. vulgaris* Hcs
369 after 120 min incubation at 15°C [63]. The filtered seawater used as media during this *in vitro*
370 experiment may account for this lower rate. Overall, our results are among the highest
371 phagocytic percentages reported among mollusks, regardless of experimental conditions (e.g.
372 [5–7,11,12,15,18,72,75,96,99–104]).

373 The time-dependent evolution of phagocytosis measured in our study is consistent with
374 previous findings on Hcs of mollusk bivalves [5,12,99,102,105] and gastropods [15,18,106],
375 but also crustacean [107] and fish blood cells [108]. Within cephalopods, Malham et al. [98]
376 showed similar evolution on *E. cirrhosa* Hcs, whereas Rodriguez-Dominguez et al. [40]
377 reported constant *O. vulgaris* Hc phagocytic percentages from 45 to 120 min incubation at
378 22°C.

379 Notably, temperature sensitivity appears species-specific in cephalopods, as no effects
380 were observed in our study, whereas negative effects of low temperatures (4-10°C) were
381 reported on Hc phagocytosis **rate** in *O. vulgaris* [40], and *E. cirrhosa* [98]. Although
382 temperatures used were extremes and out of the thermal window of 15°C-acclimated
383 cuttlefish [23] (25°C - inducing anaerobic metabolism, and 4°C - a lethal temperature [109]),
384 no significant effects on phagocytosis parameters were **found** in our study. This low effect of
385 temperature on Hc phagocytosis ability is consistent with the eurythermy of *S. officinalis*.

386 The quantity of ingested beads measured by FCM evolved in similar ways as the
387 percentage of phagocytic Hcs. Mean quantity of beads in Hcs increased during the entire
388 experiment from 5.9, 7.0 and 6.8 beads **cell⁻¹** after 30 min to 16.4, 18.7 and 16.9 beads **cell⁻¹**
389 after 180 min at 4, 15 and 25°C, respectively (Fig. 5B). Concurrent TEM observations
390 confirmed the number of ingested beads registered by FCM (Fig. 6A) **and visually**
391 **demonstrated** the engulfment process with (1) Hc-bead adhesion, (2) bead internalization by
392 almost continuous close contact between membrane and particle, and (3) closing phagosome
393 (Fig. 6B-C). Occasionally, we also observed lysosomal fusion with phagosome (Fig. 6D).

394 Our phagocytosis assay revealed similar rates of bead engulfment as those found in *O.*
395 *vulgaris* [40], but higher rates than in the mollusk bivalve *Perna viridis* and gastropods
396 *Lymnaea stagnalis*, *Haliotis discus discus* and *Turbo cornutus* [12,18,106], highlighting
397 higher engulfment capacity of cephalopod Hcs. Nevertheless, such differences could be

398 explained by the high number of beads per cell used in our study (1:100) compared to those of
399 previous ones ($\leq 1:10$).

400

401 3.4.2.2. Opsonization assay

402 As observed under FCM, opsonization experiments differently affected Hc phagocytosis
403 percentage and engulfed bead number. While phagocytosis occurred without incubation of
404 Hcs or beads in plasma, an increase in ingested bead number occurred after incubation in 1 or
405 10% plasma ($p=0.008$ and 0.045 , respectively) (Fig. 7). In contrast, incubation of beads and
406 Hcs in 50% plasma led to a decrease of phagocytosis percentage ($p=0.04$). TEM observations
407 of phagocytosis conducted with plasma revealed a bead-coating by Hcy-like molecules (Fig.
408 8A-B) as previously suggested in cephalopods [82,98,110]. This observation could explain
409 Hcy presence in Hc electron-lucent vesicles. Moreover, Hcy-like molecule presence in
410 bridges between beads (Fig. 8C) suggested an agglutinating function, consistent with the
411 increasing engulfed bead number observed at 1 and 10% plasma (Fig. 7). Such findings are
412 consistent with a recent *Octopus maya* plasma agglutinin (OmA) characterization, showing
413 homology between OmA subunits and one functional unit of Hcy from *Octopus dolfeini* [111].
414 Moreover, bead engulfment increased after as low as 1% plasma addition, underlying the high
415 concentration of the molecule in charge of this process, and Hcy is known to represent more
416 than 90% of cephalopod plasma proteins [98,112–114]. Such agglutinating function could
417 also explain the phagocytic percentage decrease occurring with 50% plasma. Indeed,
418 important bead agglutination might limit the availability of these particles for phagocytic
419 process by increasing the size of particles to engulf. Ballarin et al. [115] described a similar
420 phagocytic decrease resulting from agglutinins in the ascidian *Botryllus schlosseri* after yeast
421 pre-incubation in high plasma concentrations ($\geq 50\%$). In cephalopods, this Hcy property
422 could explain the decrease of phagocytosis by *O. vulgaris* Hcs measured after pre-incubation

423 of zymosan in 100% plasma [40], because of zymosan particle (about 3 μm) aggregation.
424 Malham et al. [98] and Ballarin et al. [115] highlighted also the importance of phagocytose
425 duration in this type of study, as phagocytosis enhancement after plasma incubation mainly
426 occurred after 30 min. This is consistent with our study and that of Rodriguez-Dominguez et
427 al. [40] which report plasma-dependent phagocytosis decrease after 120 and 90 min of
428 phagocytosis duration, respectively. Our results demonstrate the presence of a Hcy-like
429 coating molecule with agglutinin function in *S. officinalis* plasma, which can modulate
430 phagocytosis according to (1) pre-incubation plasma concentration and (2) phagocytosis
431 duration.

432 433 **4. Conclusions**

434 Our study demonstrates, using FCM, light- and electron-microscopy, that a single
435 granulocyte population with variable internal complexity circulates in the hemolymph of *S.*
436 *officinalis*, as in other cephalopods. Acid phosphatase, lysozyme, and for the first time in
437 cephalopods proPO system enzymes were detected in Hcs, but not in plasma [31,116]. These
438 cells were able to recognize and incorporate foreign material at high rate independently of
439 temperature and without need for plasma. Concurrent TEM and FCM analysis suggested a
440 role for Hcy in foreign particle coating probably associated with its hypothesized agglutinin
441 function. These data provide important information to understand the Hc-mediated immunity
442 in the common cuttlefish, and a useful background for future studies of cephalopod Hcs.

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765

Table 1: Reported circulant Hc concentrations (mean \pm SD and range, $\times 10^6$ cell ml^{-1}) in cephalopod, gastropod and bivalve mollusks

Species	N	Concentration	Range	Authors
Cephalopod				
Loliginidae				
<i>Sepioteuthis lessoniana</i>	18	2.80 \pm 4.24	-	[63]
Sepiidae				
<i>Sepia officinalis</i>	31	7.23 \pm 5.62	0.92 - 20.31	Present study
Sepiolidae				
<i>Euprymna scolopes</i>	-	5.0	-	[42,64]
Octopodidae				
<i>Eledone cirrhosa</i>	≥ 5	> 10.0	-	[65,66]
<i>Octopus vulgaris</i>	28	10.67 \pm 7.32	2.3 - 25.0	[40]
<i>O. vulgaris</i>	92	10.3 \pm 7.77	0.49 - 32.0	[62]
Gastropod				
Ampullariidae				
<i>Pomacea canaliculata</i>	3	1.1 \pm 0.1	-	[20]
Haliotididae				
<i>Haliotis discus discus</i>	38	2.24	-	[18]
Planorbidae				
<i>Biomphalaria tenagophila</i>	10	0.25 \pm 0.13	0.11 - 0.55	[19]
Turbinidae				
<i>Turbo cornutus</i>	35	1.50	-	[18]
Bivalve				
Cardiidae				
<i>Cerastoderma edule</i>	10	4.54 \pm 2.21	-	[67]
<i>Cerastoderma glaucum</i>	10	0.55 \pm 0.22	-	[9]
Mytilidae				
<i>Mytilus edulis</i>	10	5.68 \pm 3.63	-	[67]
<i>Perna perna</i>	60	3.41 \pm 1.77	-	[68]
<i>Perna viridis</i>	20	1.30 \pm 0.35	0.73 - 2.20	[12]
<i>P. viridis</i>	6	5.54 \pm 1.30	-	[7]
Ostreidae				
<i>Crassostrea ariakensis</i>	15	0.71 \pm 0.22	0.33 - 1.23	[11]
<i>Crassostrea virginica</i>	-	-	0.65 - 2.80	[69]
Pectinidae				
<i>Argopecten irradians</i>	≥ 20	37.9	-	[70]
Pharidae				
<i>Ensis siliqua</i>	10	6.48 \pm 2.50	-	[67]
Semelidae				
<i>Scrobicularia plana</i>	≥ 10	2.99	-	[6]
Tridacnidae				
<i>Tridacna crocea</i>	6	-	0.3 - 2.6	[71]
Unionidae				
<i>Cristaria plicata</i>	30	2.37 \pm 0.51	-	[72]
Veneridae				
<i>Chamelea gallina</i>	120	-	1.2 - 2.4	[73]
<i>Macrocallista nimbosa</i>	51	1.08 \pm 0.47	0.12 - 2.06	[58]
<i>M. nimbosa</i>	40	0.99 \pm 0.52	0.26 - 2.2	[74]

In italic: data calculated from standard-error or 95% confident interval.

Table 2: Specific activity repartition in hemolymph compartments: Hcs (10×10^6 cell ml^{-1}) and plasma. Asterisk (*) indicates significance between PO- and APO-like activities in each compartment ($p < 0.05$)

Specific activity	<i>N</i>	Hemolymph compartment	
		Hc	Plasma
Acid phosphatases (U mg prot ⁻¹)	9	23.5 ± 10.6	0.1 ± 0.1
Lysozymes (μg HEW lysozyme eq. mg prot ⁻¹)	10	21.9 ± 9.0	0.1 ± 0.1
PIs (trypsin inhibition %age)	9	0.0	31.8 ± 14.6
PO-like (U mg prot ⁻¹)	10	20.3 ± 9.5	2.6 ± 1.0
APO-like (U mg prot ⁻¹)		30.5 ± 7.4*	2.9 ± 0.9

Figure 1: Freshly adhesive *Sepia officinalis* Hcs presenting refringent (arrowhead) and non-refringent (arrow) granules. Inset: Hcs completely spread

Figure 2: Stained *S. officinalis* freshly adhesive Hcs; (A) Wright staining highlighting large nucleus (n), slightly basophilic cytoplasm, acidophilic granules (arrow) and lucent granules (arrowhead) in spread hemocytes; (B) Neutral red uptake staining of two Hcs highlighting lysosomal system.

Figure 3: Electron micrographs of *S. officinalis* circulating Hcs. (A) SEM micrograph of several Hcs showing similar aspect; (B) SEM micrograph of one hemocyte producing many pseudopodia; (C) Transmission electron micrograph of circulating Hcs presenting characteristic lobate-nucleus (N) with highly condensed chromatin along the inner surface of the nuclear membrane, well-developed rough endoplasmic reticulum (rER), electron-dense lysosomal vesicles (arrow) and electron-lucent vesicles (arrowhead). Bar: 1 μm . Inset: high magnification of mitochondria (m) and Golgi apparatus (G). Bar: 0.1 μm ; (D) Transmission electron micrograph of circulating Hcs presenting numerous vesicles; electron-dense lysosomal granules (arrow) and electron-lucent vesicles (arrowhead). Bar: 0.5 μm . (E-F) Electron-lucent vesicles showing inner Hcy-like molecules (small arrow). Bars: 100 nm.

Figure 4: Flow cytometer bivariate plots showing distributions of particle size (FSC) vs internal complexity (SSC) of fresh Hcs of *S. officinalis*. Insets representing histogram of both variables. (A) Typical cuttlefish dot-plot; (B) Dot-plot showing wide internal complexity (SSC) distribution sometimes observed.

Figure 5: Graphs representing evolution of phagocytic parameters function of time (30, 60, 120 and 180 min) at three temperatures (4, 15 and 25°C) ($N = 5$); (A) Phagocytic Hc percentages and (B) engulfed bead number.

Figure 6: TEM micrographs of *S. officinalis* Hc. (A) Phagocytic Hc presenting several engulfed 1 μm latex beads (b). Bar: 1 μm . Sequential events of the internalization of beads (B-D); (B) Hc-bead adhesion. Bar: 0.1 μm ; (C) bead engulfment. Bar: 0.2 μm ; (D) and fusion between phagosomal and lysosomal compartments. Bar: 0.5 μm .

Figure 7: Graph representing phagocytic Hc percentage and mean engulfed bead number of *S. officinalis* Hcs ($N \geq 4$; 2h incubation at 15°C) function of plasma concentration (%) treatments. Statistically significant differences were made against control (0% plasma concentration) at each plasma concentration for phagocytic cell percentage (°) and engulfed bead number (*); * and ° $p < 0.05$; ** $p < 0.01$.

Figure 8: TEM micrographs of 1 μm latex beads (b). (A) One bead after incubation in medium without plasma add; (B) one bead after incubation in plasma presenting important Hcy-coating (arrow). Bars: 100 nm. (C) Apparent Hcy-like molecule implication in bead aggregation (arrow). Bar: 200 nm.

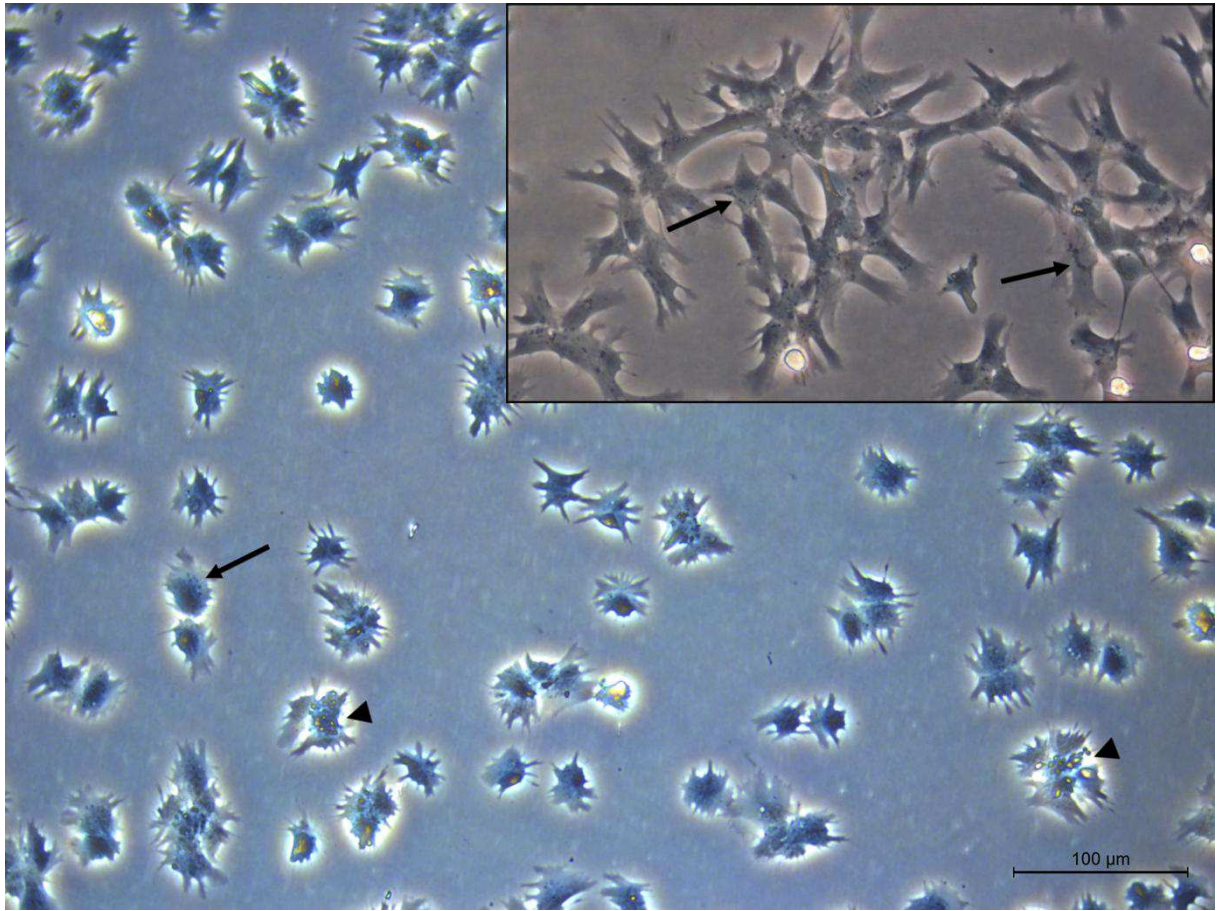


Figure 1

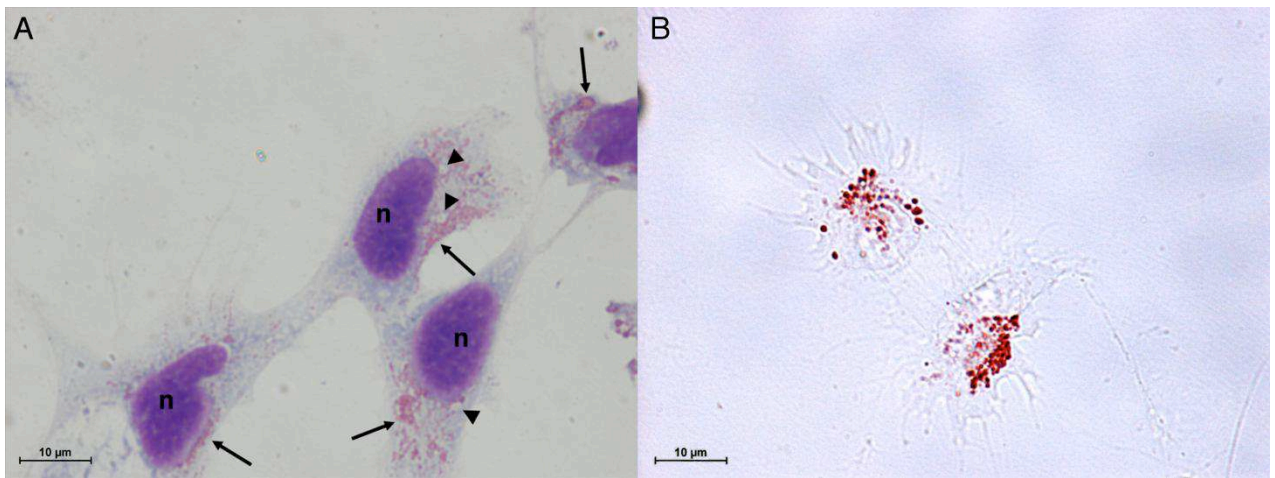
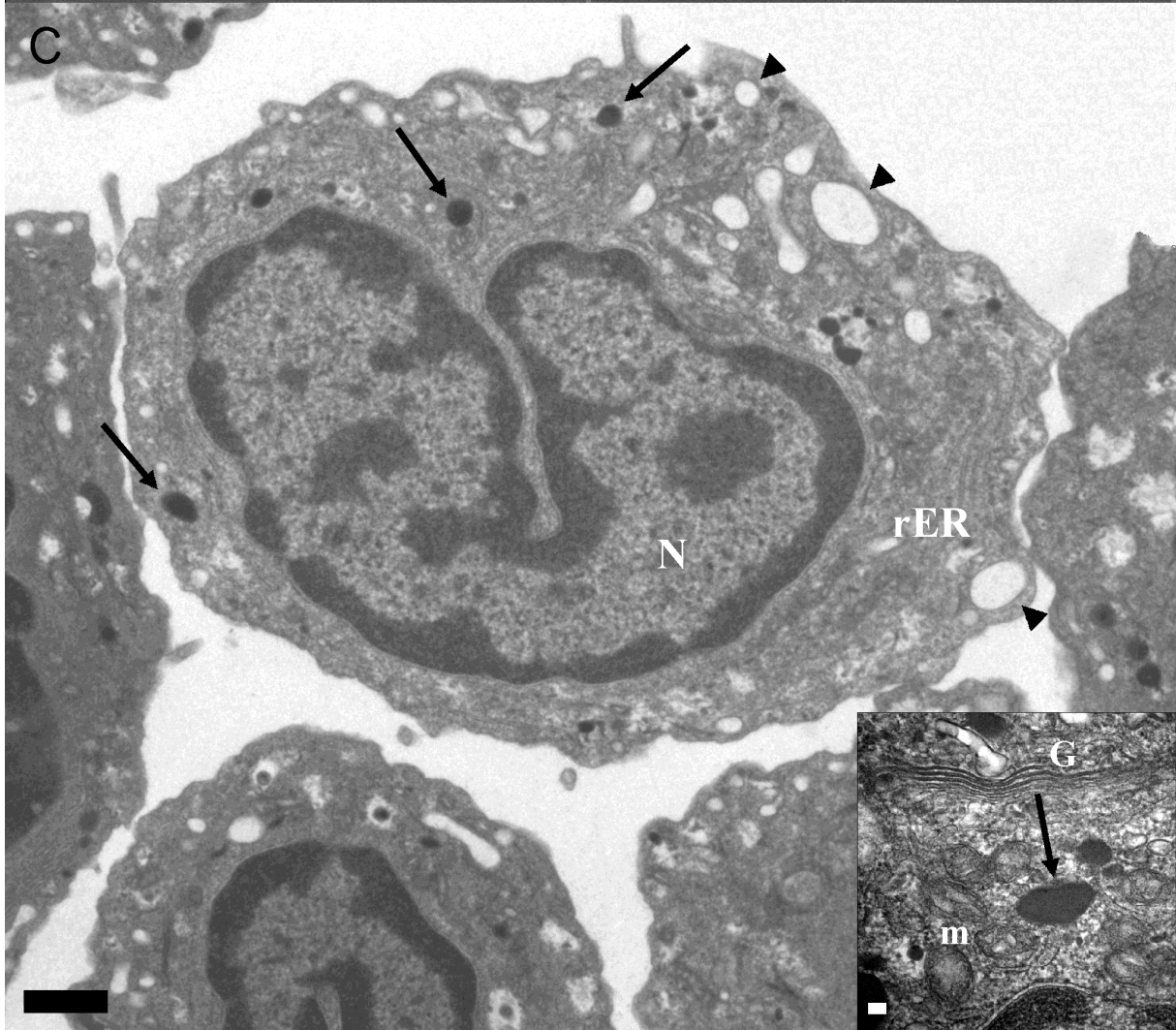
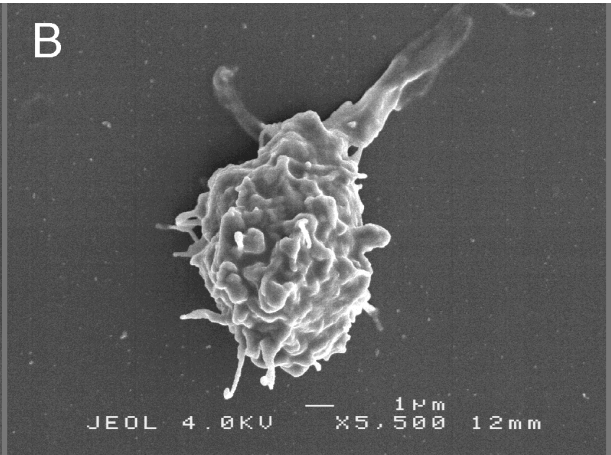
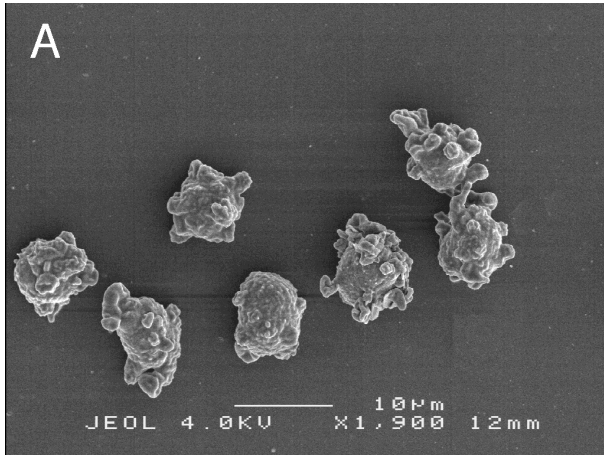


Figure 2



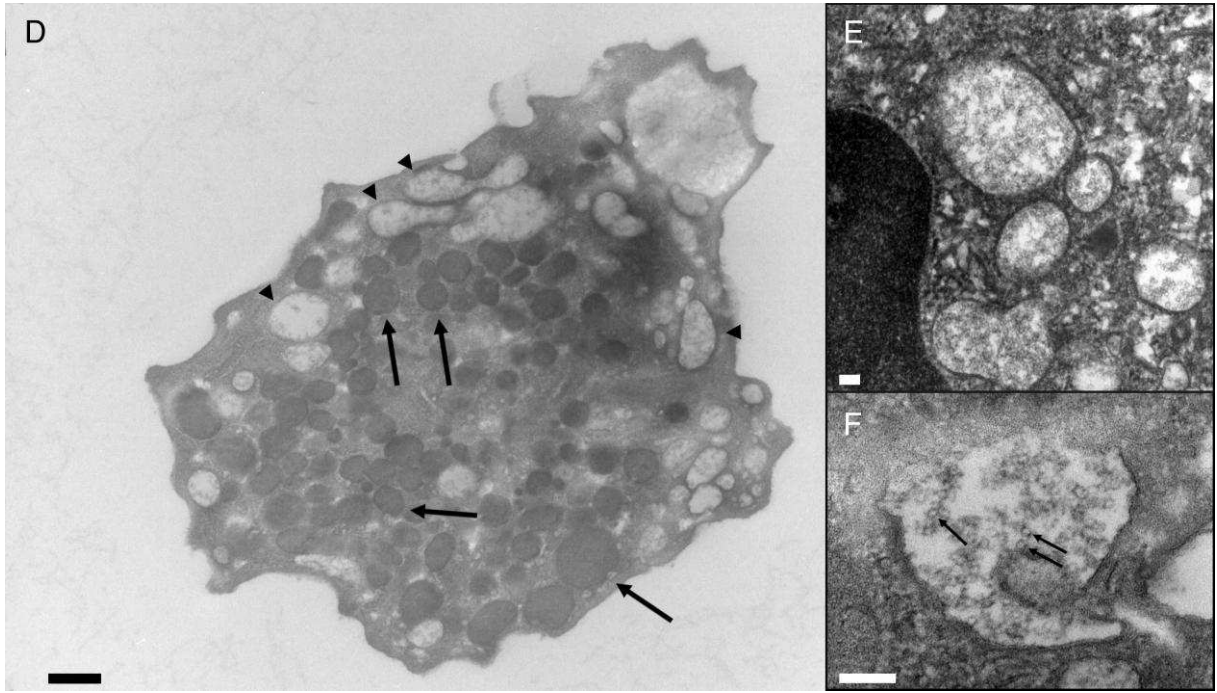


Figure 3

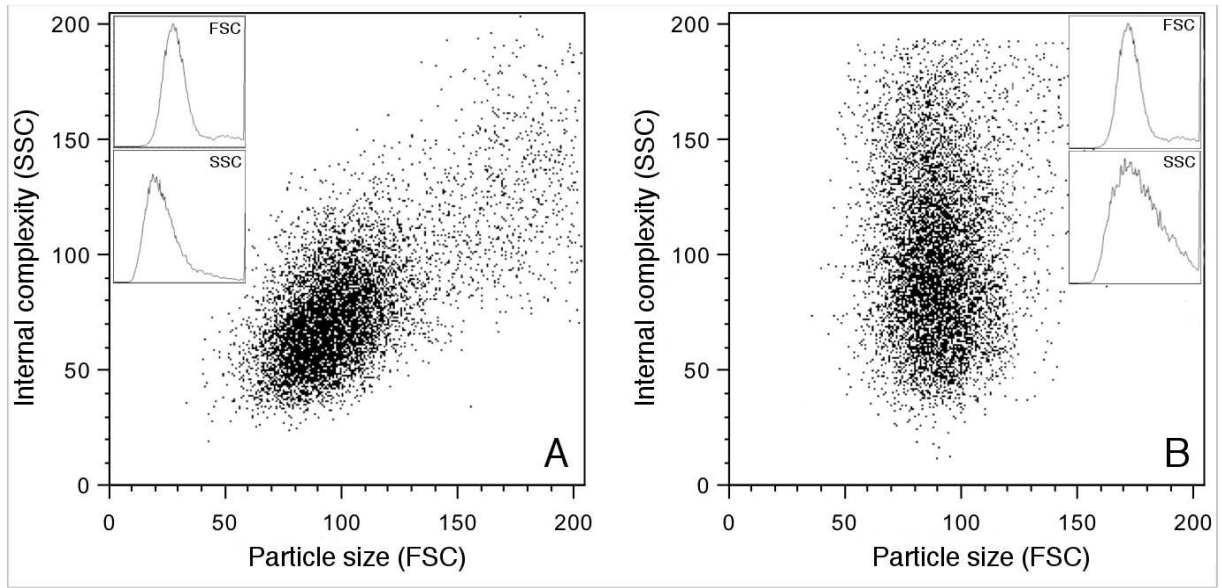


Figure 4

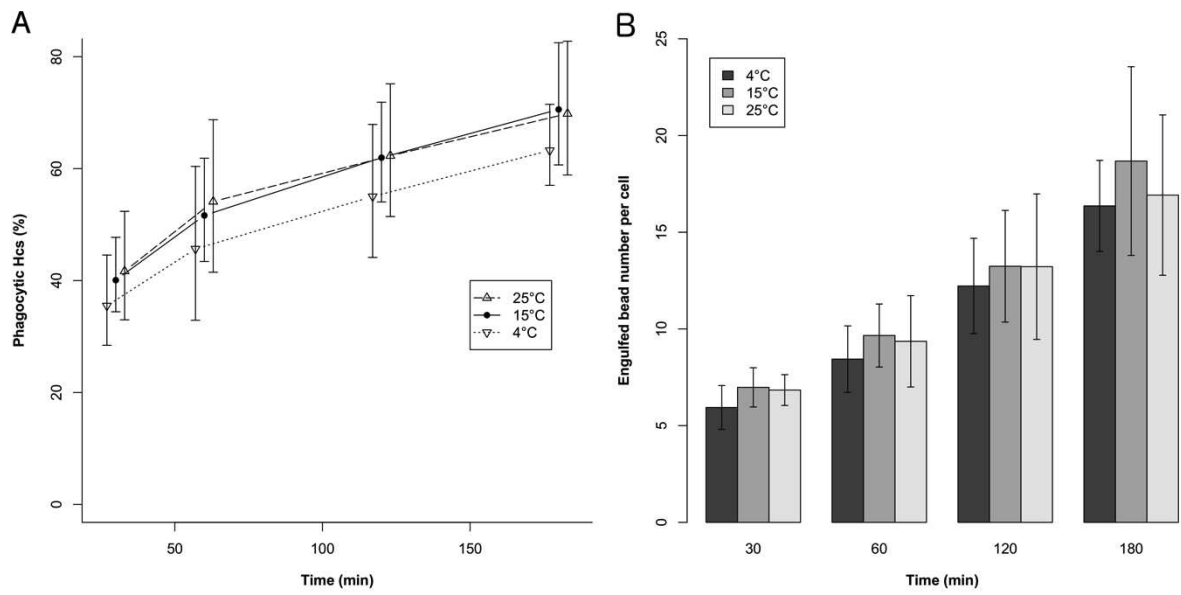


Figure 5

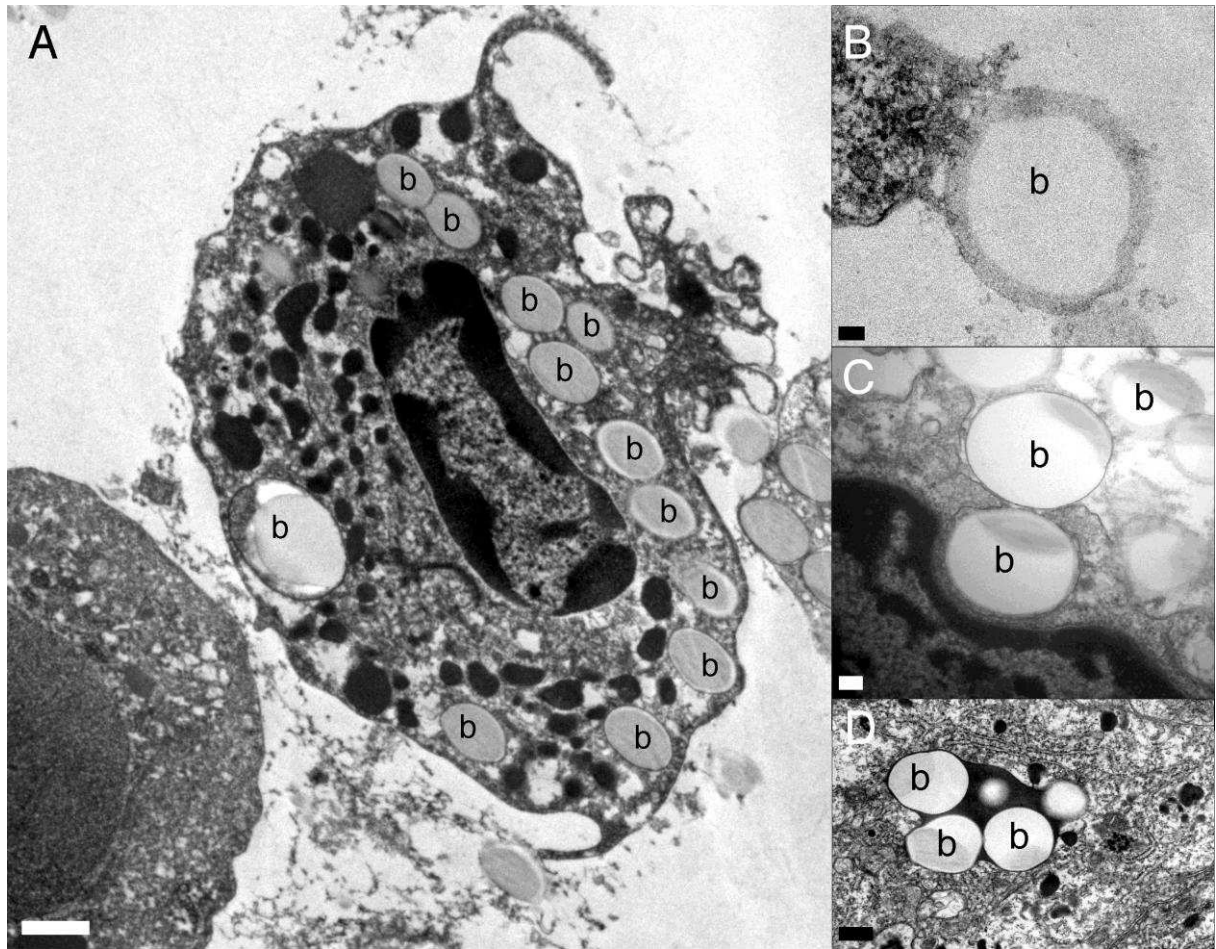


Figure 6

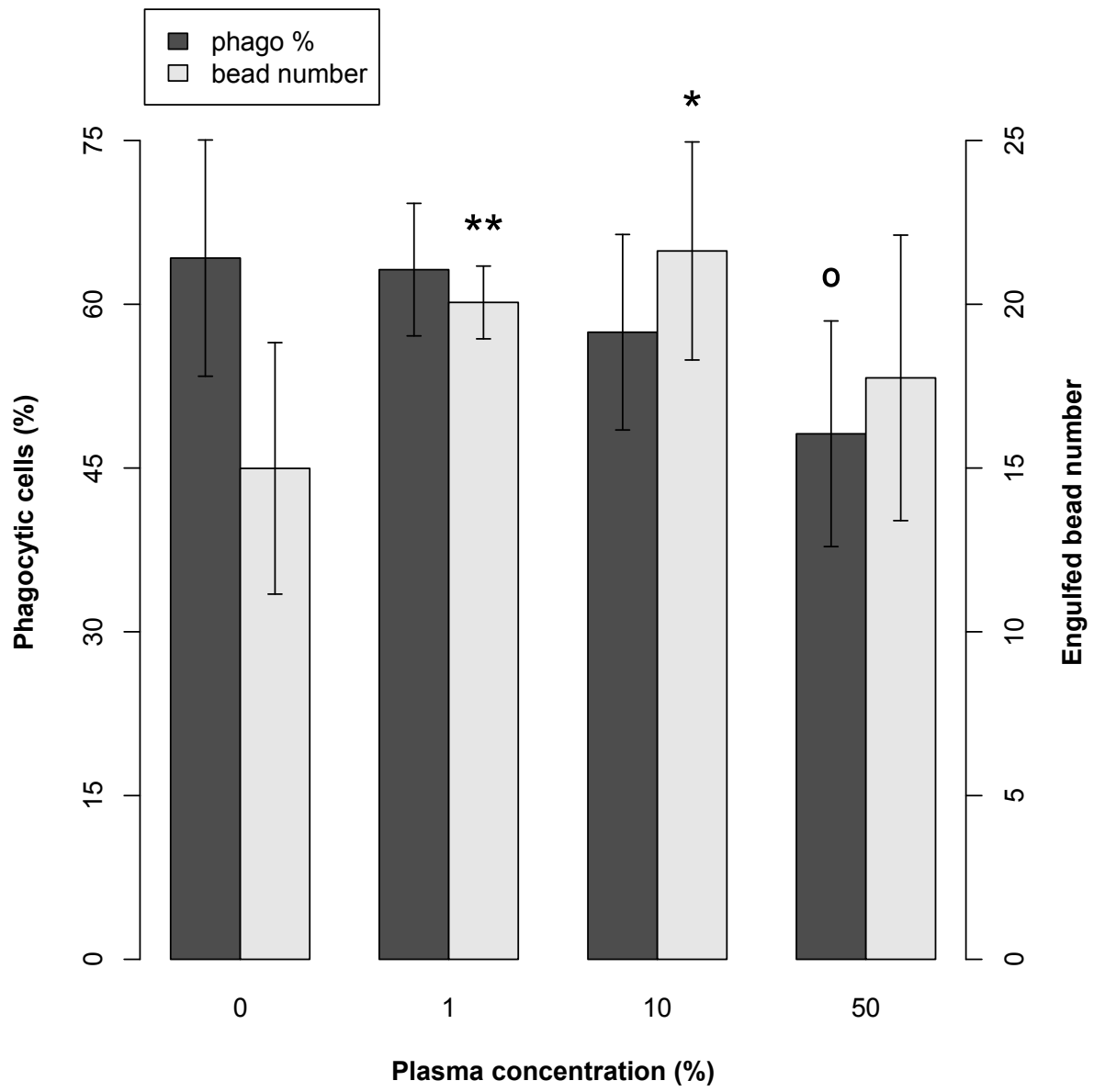


Figure 7

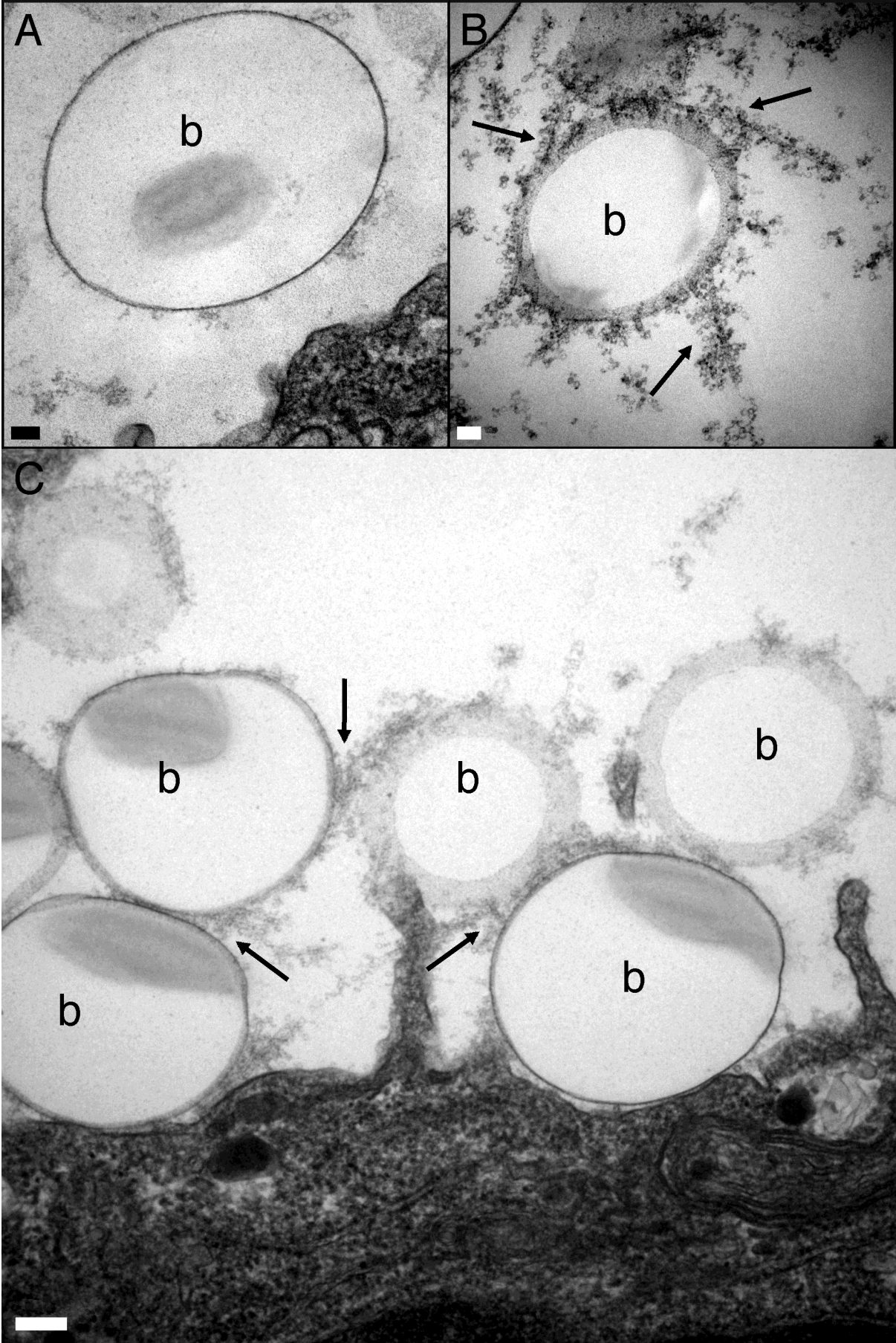


Figure 8