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

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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.

2. Material and methods

2.1. Animals

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

2.2. Hemolymph collection

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 Between 9 and 13 ml of hemolymph was then withdrawn from the anterior mantle vein [46] using a syringe with 18-gauge needle.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

2.3. Chemicals

Sodium chloride (NaCl), anhydrous and hexahydrate magnesium chloride (MgCl_2 and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), calcium chloride (CaCl_2), bovin serum albumin (BSA), Bradford reagent,

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

2.4. Morphological characterization of *S. officinalis* Hcs

2.4.1. Hemolymph cell monolayer stainings

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

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

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

2.4.2. Electron microscopy

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

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

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

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

2.5. Biochemical analysis

2.5.1. Enzyme extraction

Hcs and plasma were separated in non diluted hemolymph by $500 \times g$ centrifugation for 10 min at 4°C. Plasma was then removed, checked for absence of Hc microscopically, and stored in aliquots at -80°C until analysis. Upon complete plasma removal, Hc pellets were gently rinsed in either Tris buffer pH 7 (0.1 M Tris-HCl, 0.45 M NaCl, 26 mM MgCl₂ and 10 mM CaCl₂) for phenoloxidase (PO) assays [51] or Tris buffer pH 8 (10 mM Tris-HCl and 150 mM NaCl) for phosphatase, lysozyme and protease inhibitor (PI) assays [52]. Cells were resuspended at 10×10^6 cells ml⁻¹ in same extraction buffer and sonicated at 60 W for 20 s. Cell extracts were then centrifuged at $10,000 \times g$ and their supernatant aliquoted and stored at -80°C until analysis.

2.5.2. Enzymatic assays

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

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

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

Lysozyme activity was quantified according to Malham et al. [55]. Fifty μl of HEW lysozyme ($85 \mu\text{g ml}^{-1}$ in Tris buffer pH 8 described in section 2.5.1.) standard was serially diluted in triplicate in 96-well flat bottom plates (BD, USA). Fifty μl of each sample was added in triplicate to the 96-well plates as well as 50 μl of Tris buffer pH 8, as blanks. One hundred and fifty μl of the substrate, freeze-dried *M. lysodeikticus* (75 mg/100 ml of 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 ($\text{C}_6\text{H}_8\text{O}_7$), 2.1 g/100 ml distilled H_2O ; NaCl, 0.09 g/100 ml buffer)), was added to each well. The reductions in turbidity in the wells were read on Mithras LB 940 luminometer (Berthold, Thoiry, France) at 25°C for 5 minutes at 10 second intervals at 450 nm using negative kinetics. Lysozyme concentrations were calculated from the standard curve (μg HEW lysozyme equivalent ml^{-1}). Final lysozyme-like activity was thus expressed as μg HEW lysozyme eq. mg protein^{-1} .

As described by Malham et al. [55] and Thompson et al. [56], PI activity was measured by transferring 20 μl of sample and 10 μl of trypsin TPCK ($100 \mu\text{g ml}^{-1}$ of 0.05 M Tris buffer pH 8) in 96-well flat bottom plates (BD, USA), and mixed at room temperature for 5 minutes. In parallel, intrinsic trypsin activity was measured by replacing 10 μl of trypsin by Tris buffer pH 8 described above (section 2.5.1.). A positive control was used by replacing the sample with Tris buffer pH 8. Two hundred μl of BAPNA substrate solution (5.2 mg BAPNA ml^{-1} DMSO) in 10 ml of 0.01 M trizma base buffer pH 7.4) was added to each well and incubated for 15 minutes at room temperature. Absorbance was read at 405 nm using Mithras LB 940 luminometer (Berthold, Thoiry, France), and PI activity was expressed as the percentage of 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

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

2.6. Flow cytometry (FCM) analysis

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

2.6.1. Freshly sampled Hc cytomorphology

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

2.6.2. Phagocytosis assays

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

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

2.6.2.1. *In vitro* impact of time and temperature

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

2.6.2.2. *In vitro* impact of plasma concentrations

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

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

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

2.7. Data analysis

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

3. Results and Discussion

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

3.1. Hc viability and concentration

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

3.2. Hc identification and characterization

3.2.1. Light microscopy

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

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

3.2.2. Electron microscopy

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

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

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

3.3. Enzymatic assays

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

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

3.4. FCM analysis

3.4.1. Fresh Hc cytogram

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

3.4.2. Phagocytosis experiments

3.4.2.1. Time and temperature

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

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

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

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

The quantity of ingested beads measured by FCM evolved in similar ways as the percentage of phagocytic Hcs. Mean quantity of beads in Hcs increased during the entire 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** after 180 min at 4, 15 and 25°C, respectively (Fig. 5B). Concurrent TEM observations confirmed the number of ingested beads registered by FCM (Fig. 6A) **and visually demonstrated** the engulfment process with (1) Hc-bead adhesion, (2) bead internalization by almost continuous close contact between membrane and particle, and (3) closing phagosome (Fig. 6B-C). Occasionally, we also observed lysosomal fusion with phagosome (Fig. 6D).

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

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

3.4.2.2. Opsonization assay

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

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

4. Conclusions

Our study demonstrates, using FCM, light- and electron-microscopy, that a single granulocyte population with variable internal complexity circulates in the hemolymph of *S. officinalis*, as in other cephalopods. Acid phosphatase, lysozyme, and for the first time in cephalopods proPO system enzymes were detected in Hcs, but not in plasma [31,116]. These cells were able to recognize and incorporate foreign material at high rate independently of temperature and without need for plasma. Concurrent TEM and FCM analysis suggested a role for Hcy in foreign particle coating probably associated with its hypothesized agglutinin function. These data provide important information to understand the Hc-mediated immunity 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 ± 4.24	-	[63]
Sepiidae				
<i>Sepia officinalis</i>	31	7.23 ± 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 ± 7.32	2.3 - 25.0	[40]
<i>O. vulgaris</i>	92	10.3 ± 7.77	0.49 - 32.0	[62]
Gastropod				
Ampullariidae				
<i>Pomacea canaliculata</i>	3	1.1 ± 0.1	-	[20]
Haliotididae				
<i>Haliotis discus discus</i>	38	2.24	-	[18]
Planorbidae				
<i>Biomphalaria tenagophila</i>	10	0.25 ± 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 ± 2.21	-	[67]
<i>Cerastoderma glaucum</i>	10	0.55 ± 0.22	-	[9]
Mytilidae				
<i>Mytilus edulis</i>	10	5.68 ± 3.63	-	[67]
<i>Perna perna</i>	60	3.41 ± 1.77	-	[68]
<i>Perna viridis</i>	20	1.30 ± 0.35	0.73 - 2.20	[12]
<i>P. viridis</i>	6	5.54 ± 1.30	-	[7]
Ostreidae				
<i>Crassostrea ariakensis</i>	15	0.71 ± 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 ± 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 ± 0.51	-	[72]
Veneridae				
<i>Chamelea gallina</i>	120	-	1.2 - 2.4	[73]
<i>Macrocallista nimbosa</i>	51	1.08 ± 0.47	0.12 - 2.06	[58]
<i>M. nimbosa</i>	40	0.99 ± 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 μ m. Inset: high magnification of mitochondria (m) and Golgi apparatus (G). Bar: 0.1 μ m; (D) Transmission electron micrograph of circulating Hcs presenting numerous vesicles; electron-dense lysosomal granules (arrow) and electron-lucent vesicles (arrowhead). Bar: 0.5 μ m. (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 μ m latex beads (b). Bar: 1 μ m. Sequential events of the internalization of beads (B-D); (B) Hc-bead adhesion. Bar: 0.1 μ m; (C) bead engulfment. Bar: 0.2 μ m; (D) and fusion between phagosomal and lysosomal compartments. Bar: 0.5 μ m.

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.

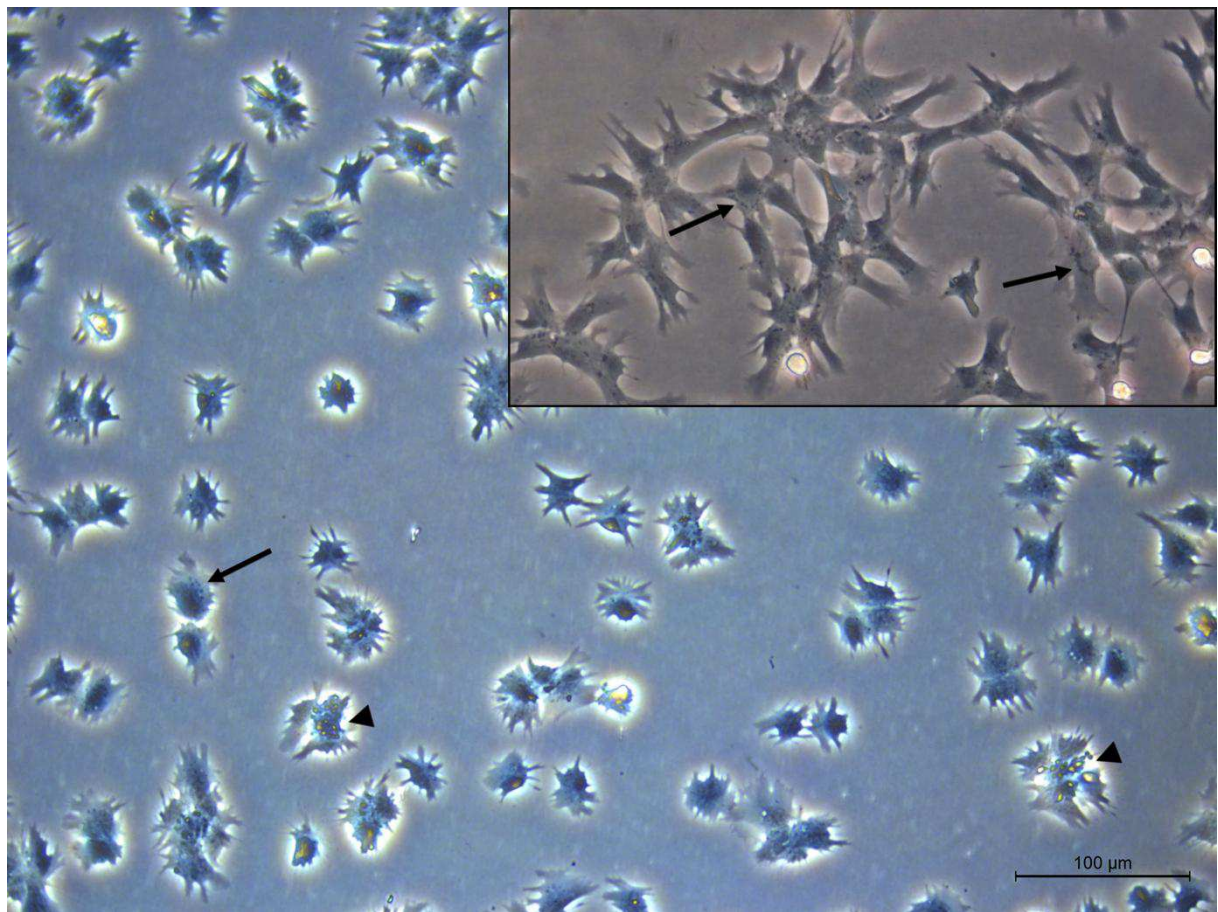


Figure 1

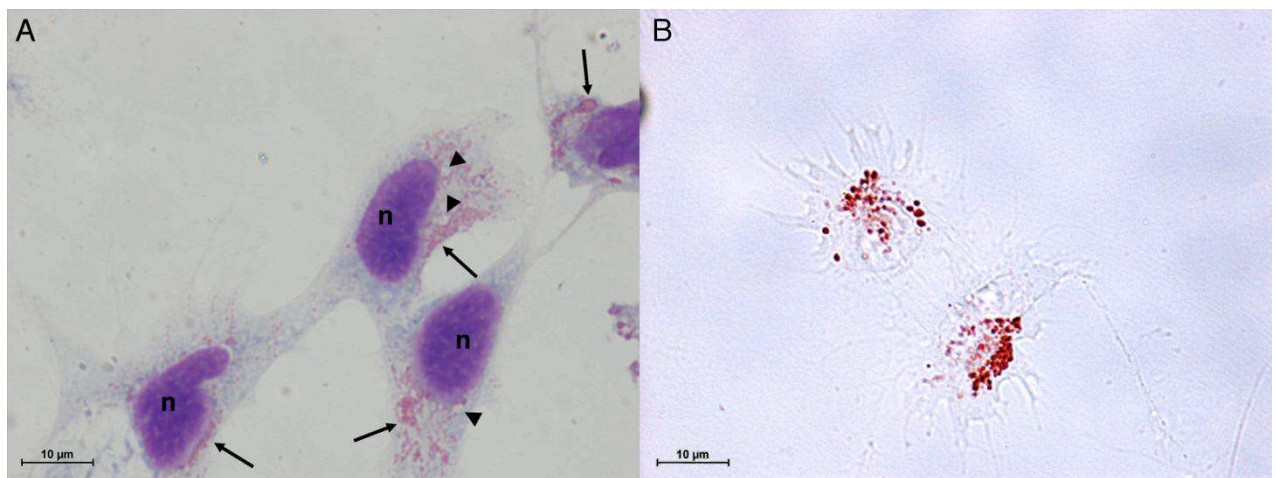
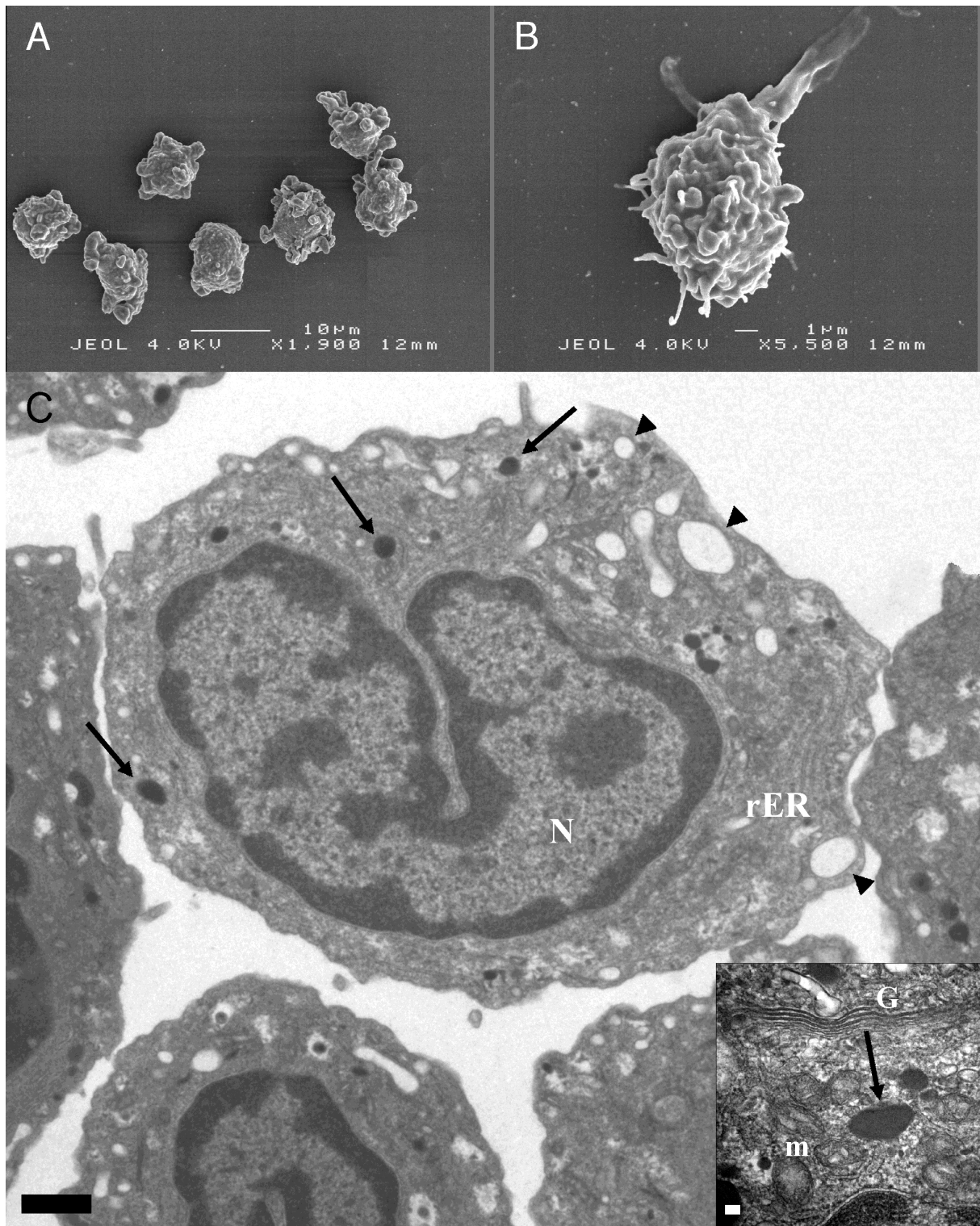


Figure 2



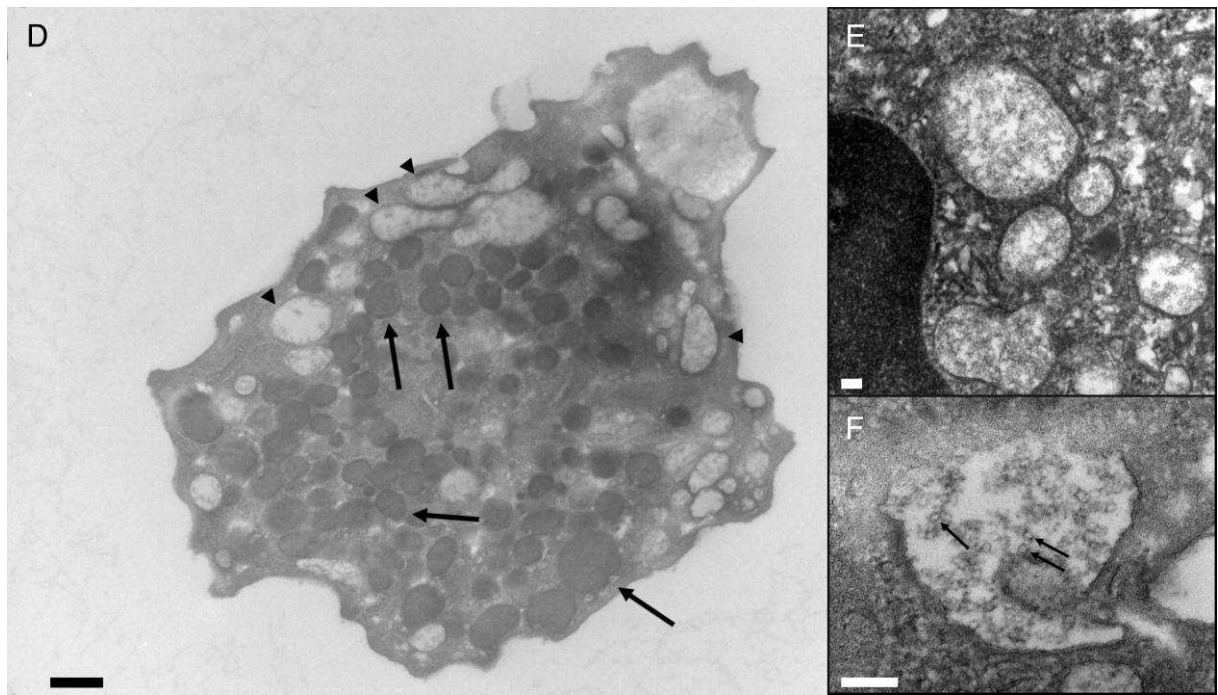


Figure 3

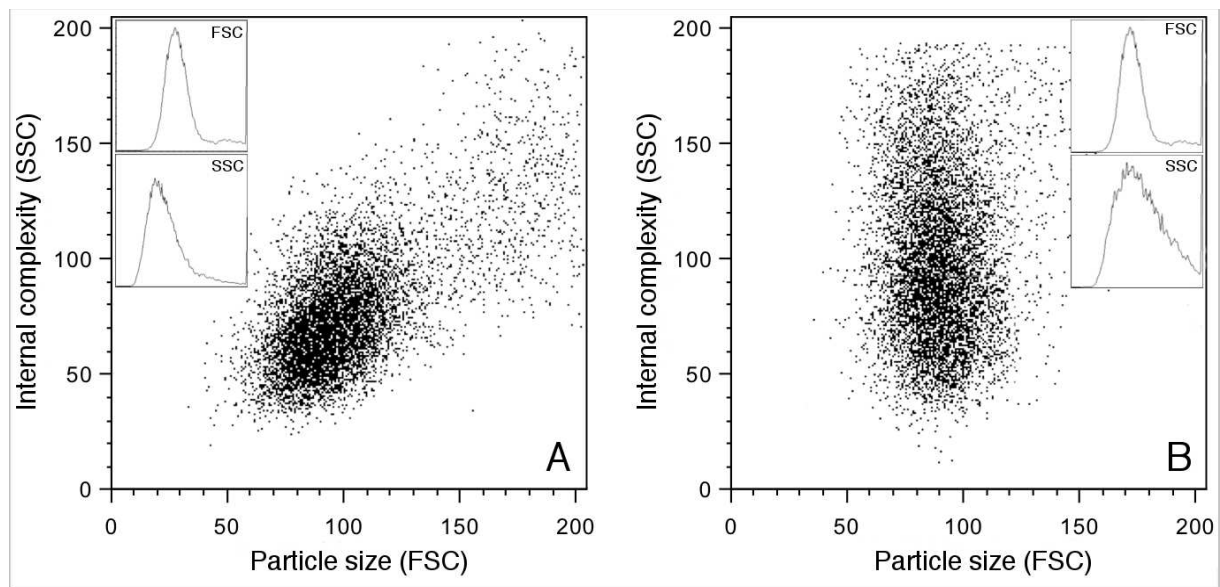


Figure 4

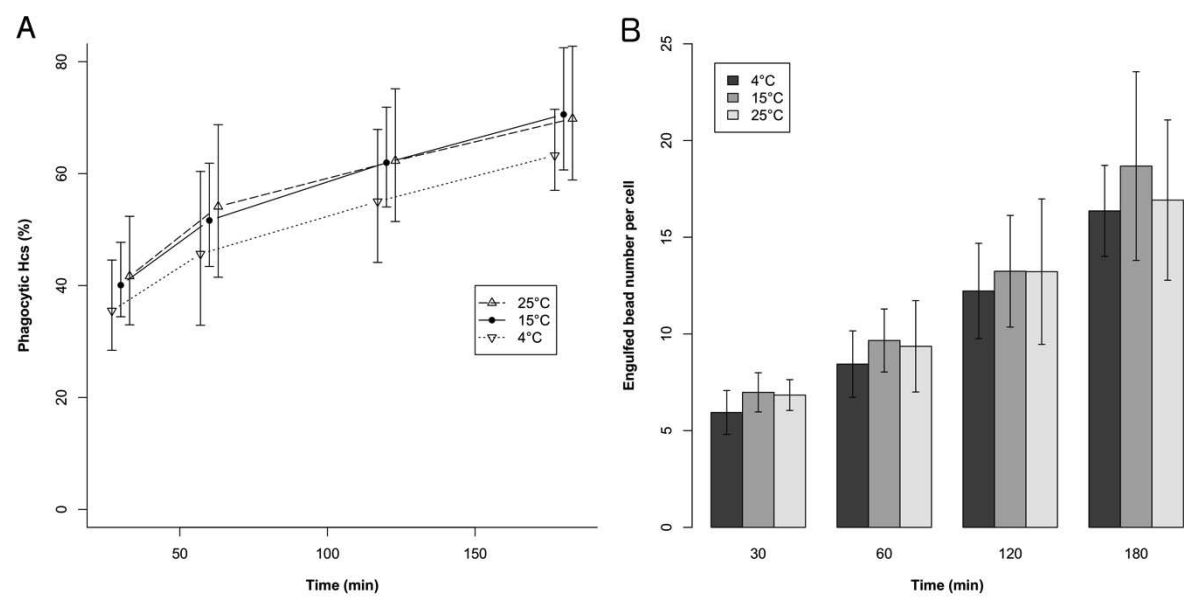


Figure 5

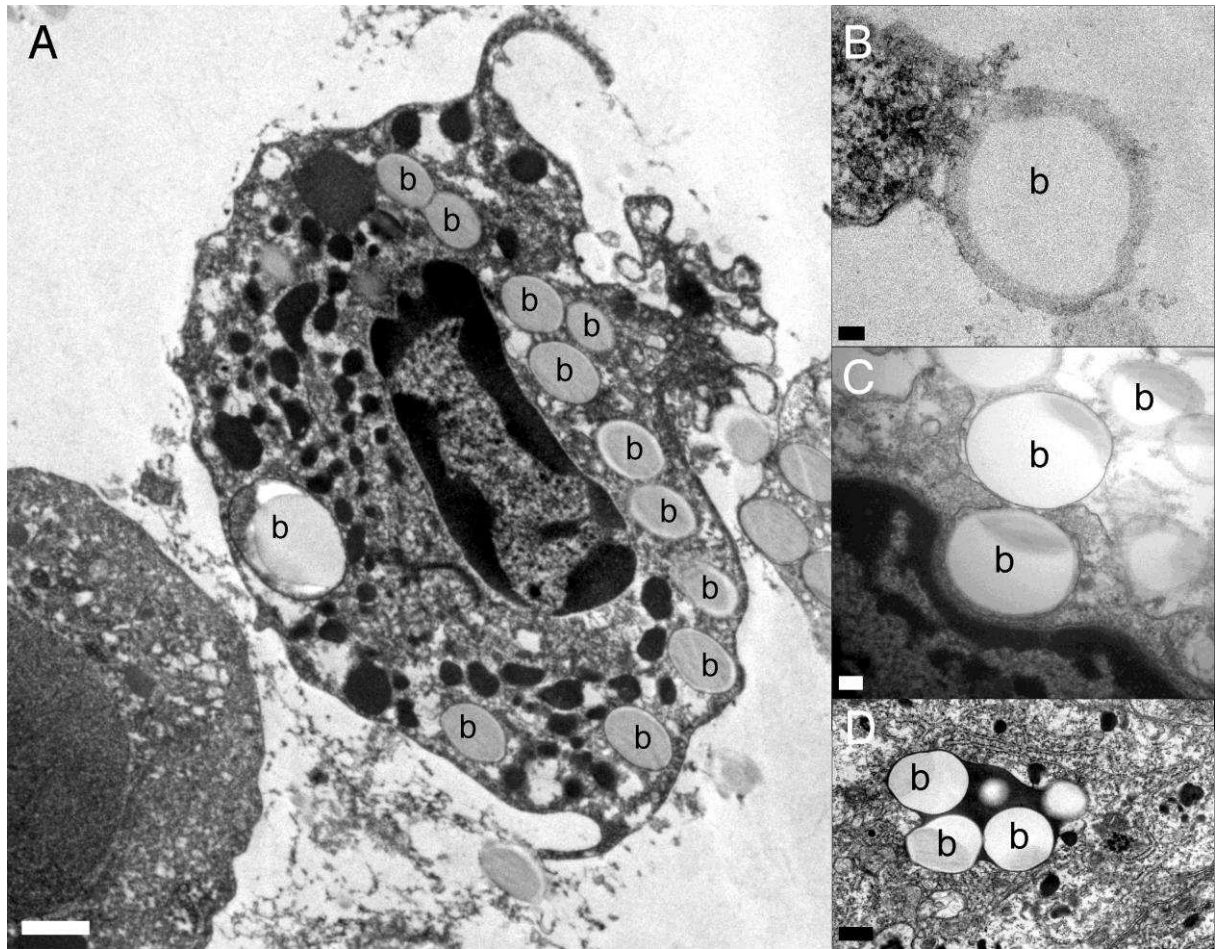


Figure 6

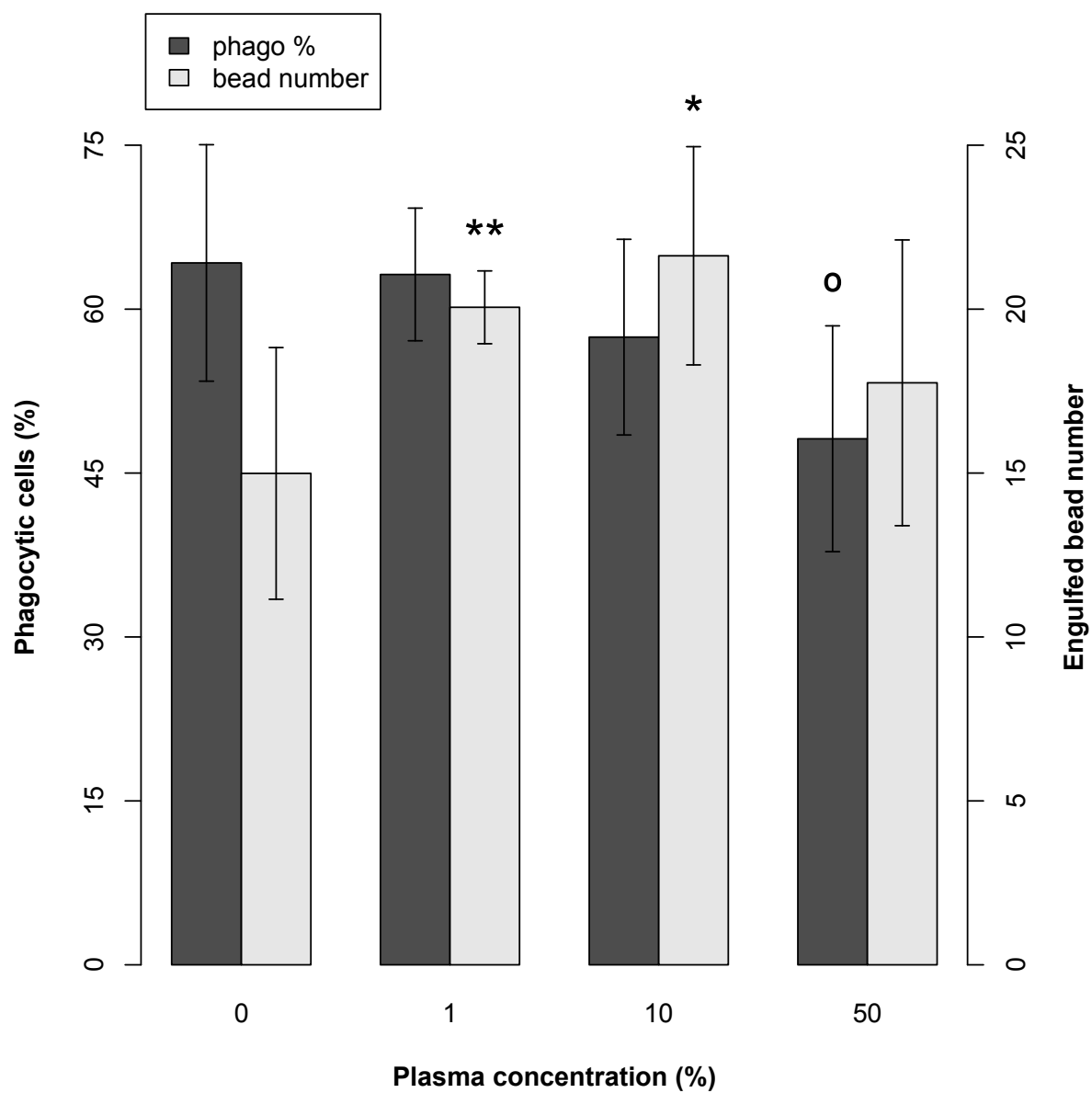


Figure 7

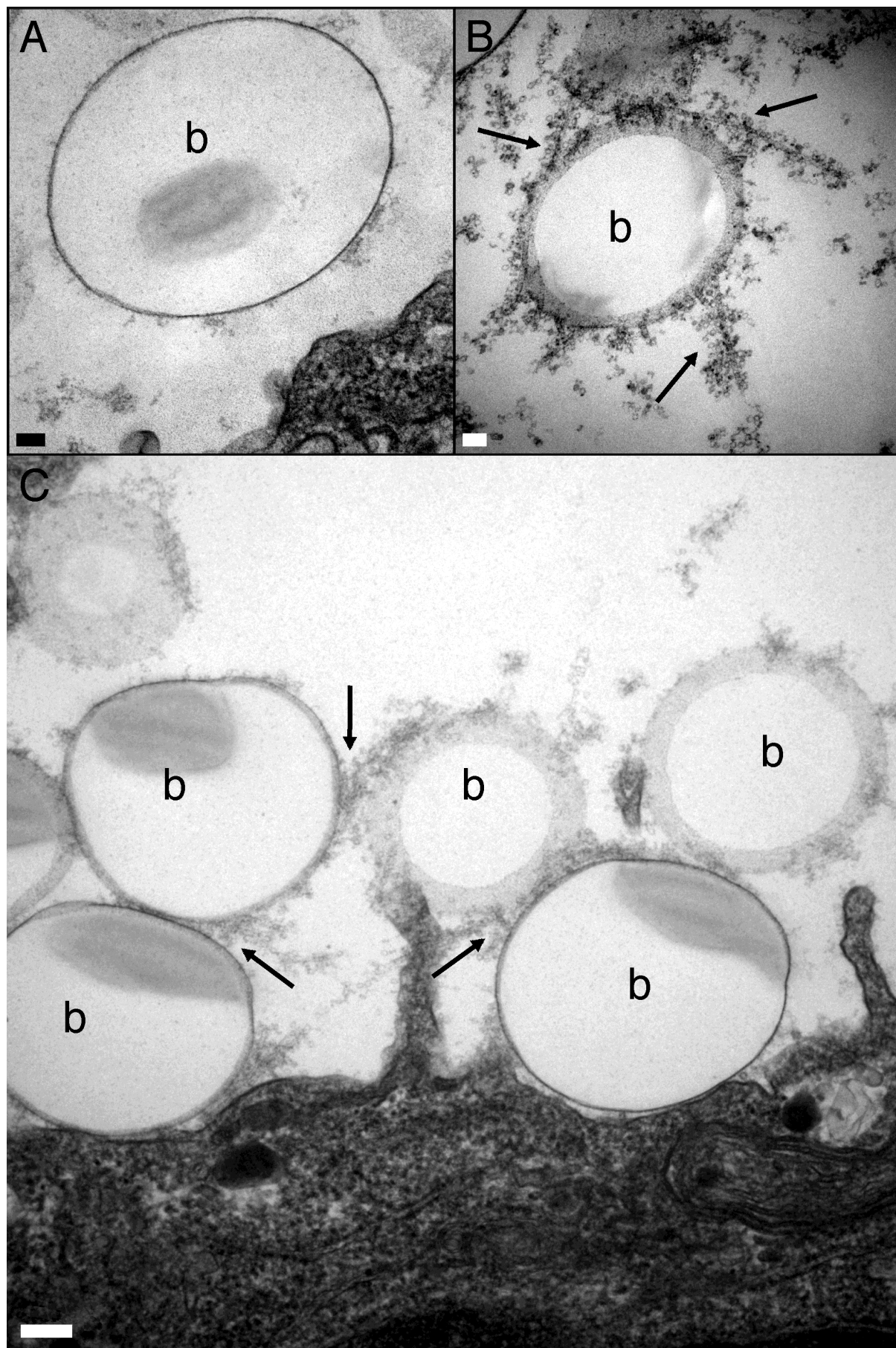


Figure 8