Vol. 49: 227–234, 2002 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published June 3

Morphological and cytoenzymatic characterization of haemocytes of the venus clam *Chamelea gallina*

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ABSTRACT: A morphological and enzymatic characterization of *Chamelea gallina* haemocytes was carried out as a prerequisite for further studies on venus clam immunobiology. Two main types of circulating haemocytes were identified (1) hyalinocytes (79.2%), agranular cells with a central nucleus surrounded by a little cytoplasm, and (2) granulocytes (16.5%), smaller granular cells with smaller nuclei. Small cells with a strongly basophilic nucleus and a thin layer of peripheral cytoplasm, probably undifferentiated blast cells (4.3%), were also observed. Both granulocytes and hyalinocytes can assume a spreading or round morphology. The enzymatic activities of haemocytes were also investigated. Some of the granulocytes and hyalinocytes were positive for hydrolytic enzymes, suggesting a role for these cells in phagocytosis; no oxidative enzymes were detected in *C. gallina* haemocytes. Granulocytes and hyalinocytes can easily adhere to the substratum and exhibit a low phagocytosis activity towards foreign particles (6.3%), whereas the fraction of cells containing ingested material significantly increased after pre-incubation of test particles with cell-free haemolymph, which suggests the presence of opsonin(s) in the haemolymph.

KEY WORDS: Haemocytes · *Chamelea gallina* · Cytoenzymology · Phagocytosis

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INTRODUCTION

Venerid clams represent an important economic resource throughout the world. In Italy, major fisheries are located along the central and northern Adriatic coastlines, and *Chamelea gallina* is by far the most exploited species, with peaks of total catch (using hydraulic dredges) of more than 100000 t yr^{-1} in the 1980s (Froglia 1989, Del Piero 1998). *C. gallina* is a common infaunal filter-feeding organism in the Mediterranean Sea, living in habitats characterized by limited variations in temperature and salinity. Intense fishing efforts on this economically relevant species (Froglia 1975,1989, Barillari et al. 1978, del Piero 1998) have compromised the natural resource because of the higher risk of mortality in populations stressed by overfishing. *C. gallina* fisheries experienced significant mortalities in 1991, 1993, 1996, 1998.

One effect of stress may be the suppression of immune responses, making the clams more vulnerable towards invading organisms. Haemocytes play an important role in mollusc immunity, as they are involved in the recognition of invading foreign organisms and their subsequent ingestion, encapsulation or lysis (Adema et al. 1991, Vetvicka and Sima 1998). Lytic enzymes play a key role in these processes. They are produced in the granulocytes and released into phagosomes (Cheng & Cali 1974, Cheng & Rodrick 1975, Cheng 1983, Yoshino 1988, Pipe 1990, Pipe et al. 1995, Cajaraville et al. 1996, Carballal et al. 1997c). Lysosomal hydrolases are compartmentalized within electron-dense specific granules (Yoshino & Cheng 1976, Rodrick & Ulrich 1984, Pipe 1990). Degranulation has been associated with the release of lysosomal enzymes into serum during active phagocytosis (Cheng 1981, Mohandas et al. 1985, Cheng & Dougherty 1989).

Most studies on bivalve haemocytes have been carried out on oysters and mussels, and few studies have investigated species belonging to the Veneridae

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(Cheney 1971, Foley & Cheng 1974, Moore & Eble 1977, Huffman & Tripp 1982, Lopez et al. 1997a,b). Since no data are available concerning *Chamelea gallina* haemocytes and their role in immunity, we carried out a morpho-functional characterization of *C. gallina* haemocytes as a prerequisite for further studies on the immunobiology of the venus clam in order to define a series of immuno-biomarkers to be used in the evaluation of stress conditions for this species.

Our results indicate the presence of 2 main haemocyte types, namely granulocytes and hyalinocytes, both exhibiting lysosomal enzymes and phagocytosis activity.

MATERIALS AND METHODS

Clams (shell length 20 to 30 mm) were collected with a hydraulic dredge at Venice Lido, on the west coast of the Northern Adriatic Sea, in front of the Venice lagoon. They were kept in the laboratory for 2 to 3 d before experiments in 70 l tanks filled with aerated seawater that was renewed every other day (salinity 35 ± 1‰; temperature 17 ± 1°C) and fed with microalgae (*Isochrysis galbana* and *Phaeodactylum tricornutum*).

Heamolymph collection. Haemolymph was collected from the anterior adductor muscle of clams with a syringe containing 10 mM of L-cysteine in filtered seawater (FSW), pH 7.5, to a final dilution of 1:1 haemolymph/L-cysteine. Haemolymph was also collected in the absence of L-cysteine and centrifuged at 780 × *g* for 10 min to pellet cells and obtain cell-free haemolymph (CFH).

Haemocyte cultures. Haemolymph was centrifuged at 780 × *g* for 10 min and the pellet was resuspended in FSW to achieve a final concentration of 10^6 cells ml^{-1}. Sixty µl of haemocyte suspension were placed in the centre of a culture chamber, which was fitted with coverslips and incubated for 30 min at room temperature to allow the cells to adhere to the coverslips.

Haemocyte morphology. Haemocyte monolayers were fixed for 30 min at 4°C in a solution of 1% glutaraldehyde in FSW containing 1% saccharose. They were then washed in phosphate-buffered solution (PBS: 1.37 M NaCl; 0.03 M KCl; 0.015 M KH_2PO_4; 0.065 M Na_2HPO_4), pH 7.2, and stained for 4 min in 10% Giemsa solution. Finally, haemocytes were mounted in Acquovitrex (Carlo Erba). Observation was carried out with a Leica DMLB light microscope at 1250×. Haemocytes were incubated in Neutral Red (Merck) solution (8 mg l^{-1} in FSW) and observed immediately to identify lysosomal compartments.

Adhesion assay. The number of haemocytes in 30 fields were evaluated and compared with that of monolayers on poly-L-cysteine-treated coverslips to assess the adhesion index, i.e. the percentage of adhering cells on untreated coverslips. The haemocytes were stained with Giemsa, mounted and observed with image-analysis equipment (Casti Imaging).

Phagocytosis assay. Haemocyte monolayers were incubated for 60 min at room temperature with 60 µl of either a yeast (*Saccharomyces cerevisiae*) suspension in FSW (yeast/hemocyte ratio = 10:1) or 1% carmine in FSW and incubated. Carmine was also pre-incubated for 30 min in CFH to examine opsonic activity. After incubation, uningested particles were washed out by dipping in FSW. Haemocytes were finally fixed, stained with Giemsa solution, mounted and observed under a light microscope. Attached foreign particles were enumerated.

Cytoenzymology. Haemolymph was collected in Alsever's solution (4.2 g NaCl; 8 gr sodium citrate; 0.55 g citric acid; 20.5 glucose), pH 7.5, and 300 µl of haemocyte suspension (10^5 cells ml^{-1}) were spun at 18 × *g* for 3 min (Cytospin 3, Shandon), air-dried for 20 min and fixed in glutaraldehyde. Specific cytochemical reactions were employed to demonstrate the presence of several hydrolytic and oxidative enzymes on haemocytes. Substrates were omitted from control slides. For β-glucuronidase and acid phophatase assays, cells were also pre-incubated for 60 min with 3 µm latex beads.

Acid esterase (Lojda 1977): Haemocytes were washed with 0.1 M phosphate-citric acid, pH 5.5, and incubated for 16 h at 4°C in the following reaction mixture: 3 mg naphthol acetate (Sigma) dissolved in 500 µl acetone, 250 µl Solution A (0.4 g new fuchsin [Sigma], 2 ml HCl 36%, 8 ml distilled water), 250 µl Solution B (4% $NaNO_2$ in distilled water), and 19 ml phosphate-citric acid buffer. Haemocytes were then washed with distilled water and mounted. Enzyme-reactive sites stained pink-brown.

Acid phosphatase (Lojda et al. 1979): Haemocytes were washed in 0.1 M sodium acetate, pH 5.2, for 10 min and incubated for 3 h at 37°C in a reaction mixture made by dissolving 10 mg naphthol AS-B phosphate (Sigma) in 400 µl dimethylformamide (DMF), 400 µl Solution A, 400 µl Solution B, and 20 ml of sodium acetate buffer (0.1 M, pH 5.2). After incubation, haemocytes were washed in the buffer solution and mounted. Enzyme-reactive sites stained red.

Alkaline phosphatase (Burstone 1962): Haemocytes were washed with 0.1 M Tris-HCl, pH 9, for 10 min and incubated for 2 h at 37°C in a reaction mixture similar to that used for acid phosphatase detection, but containing 20 ml of Tris-HCl buffer instead of sodium acetate. Haemocytes were then washed for 10 min in Tris-HCl and mounted. Enzyme-reactive sites stained red.

Arylsulphatase (Goldfisher 1965): Haemocytes were washed for 10 min in 0.1 M sodium acetate, pH 5.2, and incubated for 2 h at 37°C in 0.16 g of *p*-nitrocatechol-sulphate (Sigma) in 4 ml distilled water, 12 ml sodium acetate buffer, and 4 ml 8% $PbNO_3$. After incubation, haemocytes were washed with distilled water and then with ammonium sulphide solution for 2 min. Finally, they were washed with distilled water and mounted. Enzyme-reactive sites stained brownish-black.

Chloroacetyl esterase (Moloney et al. 1960): Haemocytes were washed in PBS, pH 6.5, for 10 min and incubated for 1 h at room temperature in a reaction mixture made by dissolving 6 mg naphtol chloroacetate (Sigma) in 1 ml DMF, and finally added to 19 ml PBS containing 20 mg Fast Blue B (Fluka). They were then washed in PBS and mounted. Enzyme-reactive sites stained blue.

β-glucuronidase (Hayashi et al. 1964): Haemocytes were washed in 0.1 M sodium acetate, pH 5.2, for 10 min and incubated for 3 h at 37°C in the following reaction mixture: 4 mg naphtol AS-BI β-glucuronide (Sigma) dissolved in 250 µl DMF, 400 µl Solution A, 400 µl Solution B and 20 ml of sodium acetate buffer. After incubation, haemocytes were washed in sodium acetate buffer and mounted. Enzyme-reactive sites stained red.

Nonspecific esterase (Gomori 1948): Haemocytes were washed with PBS for 10 min and incubated for 3 h at 4°C in the following reaction mixture: 10 mg naphtol AS-D acetate (Sigma) dissolved in 1 ml acetone, added to 49 ml PBS containing 80 mg Fast Blue B (Sigma). Haemocytes were then washed in PBS and mounted. Enzyme-reactive sites stained blue.

5′-nucleotidase (Wachstein & Meisel 1957): Haemocytes were washed for 10 min in 0.2 M Tris-maleate, pH 7.2, and incubated for 2 h at 37°C in 20 mg adenosine-5′-monophosphate (AMP, Sigma) dissolved in 22 ml of distilled water, 20 ml Tris-maleate buffer and 3 ml of 2% aqueous solution of $PbNO_3$ containing 5 ml $MgSO_4$. Haemocytes were then washed with distilled water and immersed in 1% ammonium sulphide solution for 2 min. Finally, they were washed with distilled water and mounted. Enzyme-reactive sites stained black.

Peroxidase (Graham & Karnovsky 1966): Haemocytes were washed in PBS for 10 min and incubated for 2 h at 37°C in 0.5 mg ml^{-1} of 3-3′ diaminobenzidine tetrahydrochloride (DAB, Sigma) in distilled water containing 0.02% H_2O_2. Then haemocytes were washed in distilled water and mounted. Enzyme-reactive sites stained brown.

Phenoloxidase (Hose et al. 1987): Haemocytes were washed with PBS for 10 min and incubated for 30 min at 37°C in a saturated solution of L-dihydroxyphenyl-L-alanine (L-DOPA). They were then washed with PBS and mounted. Enzyme-reactive sites stained black.

Statistical analysis. All experiments were repeated 4 times, using pools of haemolymph from 10 clams each. The percentage of positive haemocytes and the phagocytic index (percentage of phagocytic haemocytes) were evaluated on 1500 cells per pool. Statistical analysis of haematocrit values was performed using a Student's *t*-test.

RESULTS

Two main types of circulating haemocytes were identified: hyalinocytes and granulocytes. Hyalinocytes (79.2%) were generally devoid of granulations and had a large, dark blue basophilic nucleus surrounded by light blue cytoplasm. Granulocytes (16.5%) were smaller than hyalinocytes, with smaller, dark blue nuclei and blue-stained basophilic granules in their cytoplasm (Fig. 1B). Haemocytes with a strongly basophilic nucleus and a thin, light blue peripheral cytoplasm were also found (4.3%); they probably represent blast cells or undifferentiated haemocytes (Fig. 1A). Both granulocytes and hyalinocytes can assume a spreading (irregular shape with pseudopodia of varying lengths) or a round morphology (Table 1). Among granulocytes, 81.1% were spreading cells, whereas 18.9% were round cells, and 53.2% of hyalinocytes were spreading cells and 46.8% were round cells (Fig. 1A). Table 2 shows the mean values of cell (C) and nucleus (N) sizes (longest axis excluding pseudopodia and longest nuclear diameter, respectively), measured by image analysis, and the N/C ratio. Hyalinocytes (C = 11.51 µm, N = 4.74 µm) were bigger than granulocytes (C = 9.42 µm; N = 4.48 µm), but had a smaller N/C ratio. Blasts were the smallest cell type and had the highest N/C ratio. Spreading cell dimensions were more variable. A low number (<1%) of binucleated circulating cells was also observed. A haematocrit value (concentration of circulating cells) of 1.2 to 2.4 × 10^6 cells ml^{-1}, evaluated on pools of 10 clams, shows seasonal variations, which were significantly higher in spring and summer (Fig. 2).

Table 1. *Chamelea gallina*. Morphological characterization of haemocytes obtained on Giemsa-stained slides. Values are mean (±SD, n = 4) percentage of various cell types

% granulocytes		% hyalinocytes		% blast cells
16.5 ± 1.3		79.2 ± 1.3		4.3 ± 1.1
% spreading	% round	% spreading	% round	
81.1 ± 2.5	18.9 ± 2.5	53.2 ± 2.6	46.8 ± 2.6	

Table 2. *Chamelea gallina*. Mean (±SD) nuclear and cell diameters and their ratio (N/C) in haemocytes. n = sample size

Parameter	Granulocytes (n = 100)	Hyalinocytes (n = 100)	Blast cells (n = 50)	Spreading cells (n = 100)	Round cells (n = 100)
Cell diam. (µm)	9.42 ± 4.05	11.51 ± 3.92	5.97 ± 0.72	10.73 ± 4.27	7.71 ± 1.80
Nuclear diam. (µm)	4.48 ± 0.73	4.74 ± 0.90	4.26 ± 0.55	4.60 ± 0.84	3.86 ± 0.64
N/C	0.48 ± 0.15	0.41 ± 0.10	0.71 ± 0.11	0.43 ± 0.12	0.50 ± 0.11

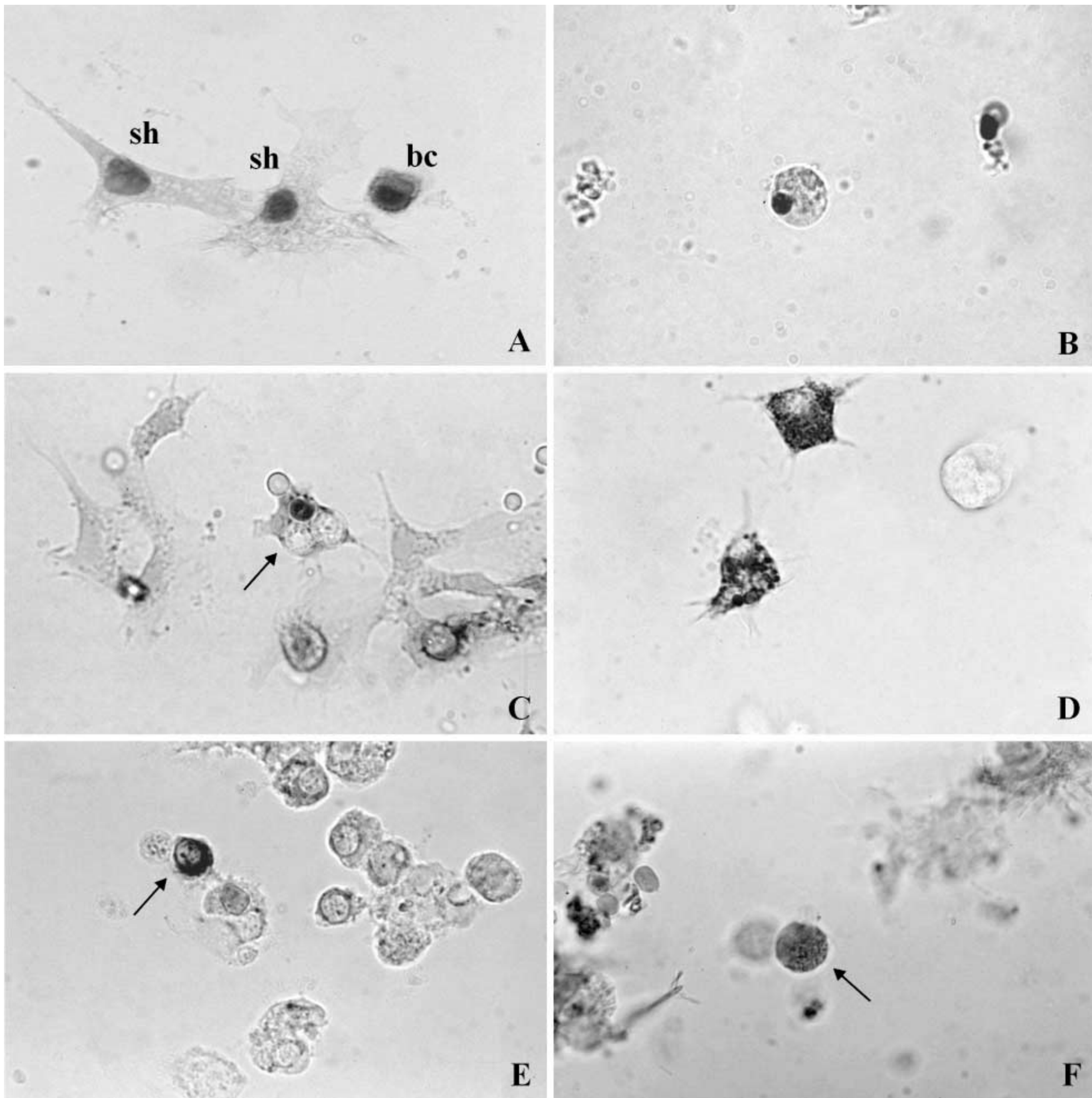

Fig. 1. *Chamelea gallina* haemocytes. (A) Spreading haemocytes (sh) and stem cell (sc) after Giemsa staining; (B) round granulocyte after Giemsa staining; (C) haemocyte containing 2 ingested yeast cells (arrowed); (D) cytoplasmic granules stained with Neutral Red dye; (E) haemocyte positive for β-glucuronidase activity (arrowed); (F) haemocyte positive for chloroacetyl esterase activity (arrowed). Light microscopy 1250×

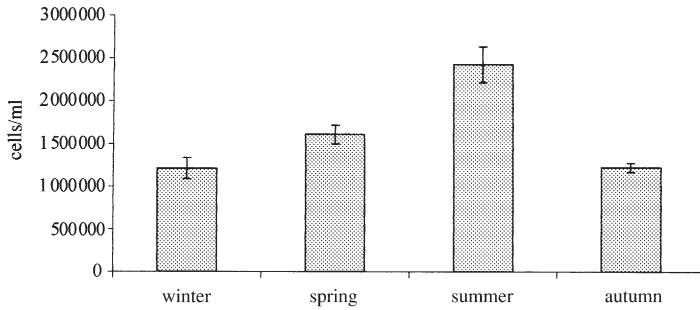

Fig. 2. *Chamelea gallina*. Seasonal variations in haematocrit values of hemolymph (mean ± SD, n = 3)

A low percentage (6.3 ± 1.3%) of haemocytes were able to phagocytize yeast cells (Fig. 1C), whereas most of them (92.7 ± 5.4%) adhered to glass slides. The adhesion assay proved that all cell types adhere to, and spread on glass coverslips. In the phagocytic assay in the presence of carmine particles: 2.8% (±1.01) of granulocytes and 9.8% (±0.2) of hyalinocytes showed phagocytic ability. After pre-incubation with CFH, these percentages increased (phagocytic granulocytes: 10 ± 1.7%, phagocytic hyalinocytes 12.7 ± 1.5%). Most haemocytes contain lysosomes inside their cytoplasm, as revealed by Neutral Red accumulation (Fig. 1D). Both granulocytes and hyalinocytes were positive for hydrolytic enzymes (Table 3); 80% of haemocytes were positive for chloroacetyl esterase (Fig. 1F), 67% were positive for arylsulphatase and 65% were positive for non-specific esterase. Acid phosphatase activity occurred in 38% of cells; the lowest frequency (6%) was observed for β-glucuronidase activity (Fig. 1E). None of the oxidative enzymes assayed were detected in clam haemocytes. Phagocytic activity changed enzyme levels (Table 4). The percentage of cells positive for β-glucuronidase significantly increased ($p < 0.001$) after the activation of haemocytes by pre-incubation with foreign particles (latex beads, 3 µm diameter).

Table 3. *Chamelea gallina*. Enzymatic activity of haemocytes. Values are mean (±SD, n = 4) percentage of positive cells. –: no activity

Enzimatic activity	% granulocytes (mean ± SD)	% hyalinocytes (mean ± SD)
Hydrolase		
Arylsulphatase	25.3 ± 3.1	41.2 ± 2.2
Chloroacetyl esterase	27.9 ± 1.2	51.3 ± 0.7
Acid esterase	–	–
Non-specific esterases	36.4 ± 2.5	28.2 ± 1.8
5'-nucleotidase	–	–
Acid phosphatase	12.3 ± 1.0	25.6 ± 0.6
Alkaline phosphatase	–	–
β-glucuronidase	2.8 ± 1.2	3.6 ± 0.5
Oxidase		
Phenoloxidase	–	–
Peroxidase	–	–

Table 4. *Chamelea gallina*. Enzymatic activity of haemocytes. Values are mean (±SD, n = 4) percentage of positive granulocytes and hyalinocytes with and without pre-incubation with latex. Statistical comparison was performed by Student's *t*-test. ***$p < 0.001$; ns: not significant at $p > 0.05$

Enzymatic activity	Normal condition		Pre-incubated (30 min) with latex (3 µm diam.)	
	% granulocytes	% hyalinocytes	% granulocytes	% hyalinocytes
β-glucuronidase	2.8 ± 1.2	3.6 ± 0.5	9.5 ± 1.8***	5.8 ± 0.6***
Acid phosphatase	12.3 ± 1.0	25.6 ± 0.6	13.1 ± 2.1ns	23.9 ± 3.2ns

DISCUSSION

In accordance with Cheng (1981) and numerous other authors (see the review of Hine 1999), the haemocytes of *Chamelea gallina* can be grouped into 2 main categories, namely granulocytes (cells containing many granules) and hyalinocytes (with few or no cytoplasmatic granules). Earlier studies on venerid haemocytes indicated the presence of only 1 cell type, the granulocyte (Cuenot 1891, Zack 1955: both cited in Cheng 1981). Nevertheless, the existence of 2 haemocyte types was proposed by Huffman & Tripp (1982) in *Mya arenaria* and by Auffret (1985) in *Ruditapes philippinarum*. In the present work, we determined a percentage of 16.5% for granulocytes and 79.2% for hyalinocytes; these data are not in agreement with those of other authors. Renwrantz et al. (1979) found about 70% granulocytes and 30% agranulocytes in *Crassostrea virginica*, confirming a previous study by Galtsoff (1964); these percentages are typical for bivalves (Huffman & Tripp 1982, Carballal et al. 1997b). In our species, hyalinocytes were more abundant than granulocytes. The cytoplasm of some haemocytes exhibited yellow-orange inclusions larger than granules, most likely lipofuscin-containing phagosomes (Carballal et al. 1997b).

Although most of the haemocytes contained hydrolytic enzymes, only 6% ingested yeast *in vitro*. Such low activity was also reported for the mussels *Perna perna* (Barracco et al. 1999) and *Mytilus galloprovincialis* (Cajaraville & Pal 1995) and might be attributable to the lack, in experimental conditions, of humoral opsonins, which

are otherwise normally present in the haemolymph (Hardy et al. 1977). A higher percentage of phagocytic cells occurs after pre-incubation of latex beads with CFH; this suggests the presence of opsonin(s) in the haemolymph, in agreement with the report of Renwrantz and Stahmer (1983) for *M. edulis*.

The presence of binucleated circulating cells (both granulocytes and hyalinocytes) suggests that differentiated haemocytes may retain the ability to divide, and supports the hypothesis that granulocytes and hyalinocytes represent 2 different cell lines, in agreement with the results reported by Moore & Lowe (1977) for *Mytilus edulis* and by Carballal et al. (1997b) for *M. galloprovincialis*, although hyalinocytes are considered by some authors (Ottaviani et al. 1998) as a young differentiation stage of haemocytes, leading to granulocytes. Moreover, other studies have confirmed various stages of granulocyte differentiation with parallel growth of their specific granules (Cajaraville & Pal 1995, Carballal et al. 1997b). *Chamelea gallina* haemocytes display a good adhering ability, unlike the results reported for other venerid species (Foley & Cheng 1974, Lopez et al. 1997b).

In winter, the haematocrit value was 1.2×10^6; it significantly increased in spring (1.6×10^6, $p < 0.05$) and summer (2.4×10^6, $p < 0.001$), but decreased to winter levels in autumn. This variation may reflect the biological characteristics of the individuals, their physiological state and the environmental conditions (Auffret & Oubella 1995), and may be related to the haemopoietic activity of the bivalves (Anderson 1990, Pipe et al. 1995). The number of haemocytes were influenced by seasonal factors (such as temperature, reproductive cycle, food), as reported by Santarèm et al. (1994) for *Mytilus galloprovincialis*. Feng (1965) reported that the number of circulating haemocytes in *Crassostrea virginica* is influenced by the amount of food and the temperature. In our study, the highest value was found in summer, when the highest temperature and abundant food availability occurred in the Adriatic Sea.

In the present work, we also evaluated the presence of various enzyme activities in *Chamelea gallina* haemocytes by using cytoenzymatic techniques. Both hyalinocytes and granulocytes show positive evidence for certain hydrolytic enzymes inside small granules, which probably correspond to lysosomes (Hayashi 1971). As in other bivalves, not all granules in haemocytes are reactive to lysosomal enzymes (Yoshino & Cheng 1976, Huffman & Tripp 1982, Auffret 1989, Pipe 1990).

The highest percentage of positive cells was observed for the 2 esterase types (acetylchloroesterase and non-specific esterase) and for arylsulphatase. It was reported that 95% of haemocytes were positive for acetylchloroesterase in *Mytilus galloprovincialis* (Carballal et al. 1997a). Non-specific esterase activity was found in *Ostrea edulis* and *Crassostrea gigas* haemocytes by Chagot (1989), in *Mya arenaria* by Huffman & Tripp (1982), in *Tapes philippinarum* by Cima et al. (2002), in *M. galloprovincialis* by Carballal et al. (1997a). Mollusc arylsulphatase has been located in the lysosomes, and less consistently in the Golgi bodies and endoplasmic reticulum, of many different tissues (Pipe & Moore 1985, Pipe 1987, 1990, Cajaraville et al. 1995, Pipe et al. 1997). Unlike esterases, β-glucuronidase activity was infrequent in *Chamelea gallina* haemocytes, in agreement with results reported for other bivalves (Bayne et al. 1979, Pipe 1990, Carballal et al. 1997a, Lopez et al. 1997a, Ottaviani et al. 1998). β-glucuronidase is considered a non-specific humoral defence molecule, owing to the fact that it can hydrolyse acid mucopolysaccharides, which are constituents of bacterial cell walls (Cheng 1976). Acid phosphatase is a typical marker for lysosomes (de Duve 1963), even though extralysosomal localisations of these enzymes have been described in several kinds of cells (Borgers & Verheyan 1985). Particularly in bivalves, a high activity of acid phosphatase was found in *Ruditapes decussatus* (Lopez et al. 1997a), *M. galloprovincialis* (Ottaviani et al. 1998), *Mercenaria mercenaria* (Yoshino & Cheng 1976), *Mya arenaria* (Huffman & Tripp 1982), *Crassostrea virginica* (Cheng & Downs 1988), *O. edulis* and *C. gigas* (Auffret 1989), *Perna perna* (Barracco et al. 1999), *M. galloprovincialis* (Bayne et al. 1979, Cajaraville et al. 1995, Carballal et al. 1997a), and *M. edulis* (Moore & Lowe 1977). Its presence was not reported in *T. philippinarum* (Cima et al. 2002). β-glucuronidase activity, but not acid phosphatase activity, increases after cell activation upon the recognition of foreign particles; this indicates a certain degree of regulation of the enzyme activity, in agreement with data obtained under anoxic conditions (Pampanin et al. 2002).

The presence of hydrolytic enzymes (phosphatases, sulphatases, glycosidases) inside cytoplasm granules strongly suggests that they are lysosomes, in agreement with data of Pipe (1990) and Carballal et al. (1997a). This is also confirmed by the neutral red uptake. The cellular uptake of neutral red, a cationic dye accumulated in the lysosomes in a biphasic manner, consists of an initial rapid flowing through the plasma membrane and a subsequent slow accumulation inside lysosomes (Seglen 1983). The amount of neutral red accumulated in cells can give an estimate of the volume of the lysosomal compartment (Pipe et al. 1997).

No oxidative enzymes were detected in *Chamelea gallina* haemocytes. The absence of phenoloxidase was also reported for *Scapharca inaequivalvis* (Holden et al. 1994), whereas this enzyme was present in the

haemocytes of 2 species of Mytilidae: *Mytilus edulis* (Waite & Anderse 1980, Coles & Pipe 1994, Pipe et al. 1997) and *M. galloprovincialis*; for these species, 100% haemocytes were positive for peroxidase and 73% for phenoloxidase (Carballal et al. 1997a). Cima et al. (2002) have demonstrated the presence of peroxidase activity in *Tapes philippinarum*, a member of the Veneridae family. Further research efforts will be directed towards a better comprehension of the role of haemocytes in *C. gallina* immunobiology.

Acknowledgements. This research was supported by a grant from the Commission of the European Communities, Agriculture and Fisheries (FAIR) specific RTD programme, CT98-4465, Evaluation and Improvement of Shellfish Dredge Design and Fishing Effort in Relation to Technical Conservation Measures and Environmental Impact (ECODREDGE). The authors thank Dr. A. Cirelli for technical support.

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Editorial responsibility: Albert Sparks, Seattle, Washington, USA

Submitted: May 31, 2000; Accepted: November 23, 2001
Proofs received from author(s): May 21, 2002