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RESEARCH REPORT

Hemocyte types and some plasmatic properties of two edible crabs *Cancer borealis* and *Cancer pagurus*

D Parrinello[1], MA Sanfratello[1#], M Celi[2#], M Vazzana[1]

[1] *Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche, Sezione di Biologia Animale e Antropologia Biologica, Università degli Studi di Palermo, Italy*
[2]*Istituto per l'Ambiente Marino Costiero U.O. di Capo Granitola - Consiglio Nazionale delle Ricerche, Granitola, Trapani, Italy*

# Equal contribution

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Abstract

Cancer pagurus and *Cancer borealis* and are edible crabs produced by economically relevant aquaculture. In this study the hemocytes and some plasmatic parameters of *Cancer borealis* and *Cancer pagurus* were examined. The cell features of the hemocytes were observed using light and scanning electron microscopy (SEM). Granulocytes, semigranulocytes and hyalinocytes were mainly identified on the basis of size, presence/absence and quantity of the cytoplasmic granules and the nucleus-to-citoplasma (N/C) ratio. SEM observations were useful for disclosing the surface features of these cells, and the same characteristics were found in both crab species. A smooth surface distinguishes elongated hyalinocytes and a rough texture the irregular surface of spherical/ovoid granular cells. Total (THC) and differential hemocyte counts (DHC) were performed, and the differences between the two crab species were disclosed. Also we were valuated pH and osmolarity values, agglutinating activity and different protein contents of the hemolymph.

Key Words: crustacean; Jonah crab; brown crab; hemocyte classification

Introduction

Hemocytes circulating in hemolymph play a key role in the innate immune response of crustaceans, and are the first line of defense against internal pathogens such as viruses, bacteria and parasites (Bauchau, 1981). Hemocytes can lead to phagocytosis, encapsulation, and the lysis of foreign cells (Smith and Söderhäll, 1983; Ratcliffe *et al*., 1985; Söderhäll and Smith, 1986; Johansson and Söderhäll, 1989; Söderhäll and Cerenius, 1992). They also play an essential role in melanization (Johansson *et al*., 2000). In addition, they release the humoral factors (including agglutinins) that are involved in phagocytosis and cell communication, leading to the degranulation of hemocytes (Hose *et al.,* 1987; Johansson, 1995; Johansson *et al*., 2000). However, the classification and functional morphology of crustacean hemocytes is often controversial due to the methods and criteria used. Classification is generally based on the presence/absence of cytoplasmic granules, and three types of circulating hemocytes have usually been reported as hyalinocytes (cells without evident granules), semigranulocytes (containing small granules) and granulocytes (with abundant cytoplasmic granules) (Bauchau, 1981). However, granulocytes have also been called granular eosinophils or granular amebocytes, while semigranulocytes have been regarded as monocytes or intermediate cells, and hyalinocytes as phagocytes or pro-hemocytes (Toney, 1958; Stang-Voss, 1971; Ravindranath 1974; Bodammer 1978; Cornick *et al*., 1978; Smith and Ratcliffe 1978). Johnston *et al.* (1973) distinguished two hemocyte types (α-and β-cells) in *Carcinus maenas*, and these can be recognized on the basis of the presence/absence of glycogen-containing granules (William and Lutz, 1975). In *Callinectes sapidus*, Clare and Lumb (1994) identified three hemocyte types, hyaline cells, and small and large granulocytes. In *Panulirus homarus*, meanwhile, four types of hemocytes (pro-hyalocytes, hyalocytes, eosinophilic granulocytes and

Corresponding author:
Mirella Vazzana
Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche-Sez. di Biologia Animale e Antropologia Biologica
Università degli Studi di Palermo,
Via Archirafi 18, 90123 Palermo, Italy
E-mail: mirella.vazzana@unipa.it

chromophilic granulocytes) have been described (Manjula *et al*., 1997). Finally, 11 cell types that are morphologically distinguishable have been described in *Homarus americanus* (Battison *et al.,* 2003).

Each cell type is active in defence reactions, with the hyaline cells chiefly involved in phagocytosis, the semigranular cells in encapsulation, and the granular cells in the storage and release of the prophenoloxidase (proPO) system and cytotoxic factors (Giulianini *et al.*, 2007; Celi *et al.*, 2015). The proportion of hemocyte types in the hemolymph varies among species. Total hemocyte counts (THC) and differential hemocytes counts (DHC) have been reported as stress indicators (Jones, 1962; Le Moullac *et al.*, 1998; Lorenzon *et al.*, 2008), and may be valuable tools for monitoring the health status of crustacean species (Jussila *et al*., 1997; Mix and Sparks, 1980; Battison *et al.*, 2003).

The crustacean decapods *Cancer pagurus* and *Cancer borealis* are paired as sister species (Harrison and Crespi 1999), and are edible crabs produced by economically relevant aquaculture (Robichaud and Frail, 2006; Stentiford, 2008). The data reported in the literature on the ecology of the two species are limited and fragmentary.

The two species live in intertidal and subtidal habitats and consume a wide variety of prey (Lawton and Elner, 1985; Creswell and Marsden, 1990). The Jonah crab, *Cancer borealis* occurs along the east coast of North America, while *Cancer pagurus* is found in the Northeast Atlantic Ocean, along the coast of Europe (Galan and Eriksson 2009). Jonah crabs are commercially harvested in the U.S. and Canada. The crustacean *C. pagurus*, which is commonly known as either the European edible crab or the brown crab, is the most commercially important crab species in Western Europe. Both species are fished offshore using baited pots or other traps. The aim of this study is to provide further information on the cells and plasmatic aspects of the species described above. In particular, the paper examines the morphological characterization of circulating hemocytes under light and scanning electron microscopy (SEM), the basic profile of the THC and DHC, and some plasmatic properties of the hemolymph.

Materials and methods

Animals

Fifteen specimens of the Jonah crab *Cancer borealis* (catch area Northwest Atlantic) and the same number of *Cancer pagurus* (catch area Northeast Atlantic), weighing 500 ± 100 g each, were supplied by L.P.A PESCA srl (Rimini). The specimens were held in running seawater tanks at the premises of another local company, Prontomar srl (Palermo), until the time of sale. Prior to collection of the hemolymph, the crabs were maintained in a circulating seawater aquarium at the university premises (10 °C *Cancer borealis* and 15 °C *Cancer pagurus*) for about 48 h according to the work of Vogan and Rowley (2002).

Hemolymph sampling

According to the work of Söderhäll and Smith (1983), 15 crabs of both species were anesthetized on ice for 10 min and the hemolymph was then withdrawn from the unsclerotized membranes of the cheliped. Since extremely rapid coagulation occurred, a syringe (23-gauge needle) with an equal volume of an anticoagulant (glucose 100 mM, NaCl 450 mM, sodium citrate 30 mM, citric acid 26 mM, EDTA 10 mM, pH 4.6) was used to prevent clotting. Consequently, plasma, separated by centrifuging (400*g*) at 4 °C for 10 min, was used to evaluate some of the plasmatic parameters.

Total and differential hemocyte counts and cytological staining

The total hemocyte number per mL (THC) was determined using a Neubauer hemocytometer chamber. Differential counts were performed on slides prepared with 100 µl of a diluted cell suspension ($3x10^6$ cells). The hemocyte monolayer was fixed with 1 % glutaraldehyde in 3.2 % NaCl for 30 min at 4 °C. The hemocytes were stained with a May-Grünwald solution (3 min) followed by a Giemsa solution (1:10 dilution for 10 min), and dehydrated with ethanol. After immersion in xylene (6 min), the slides were closed with a Eukitt mounting medium (Fluka) (Celi *et al*., 2013). The cells were then counted in random areas, and the numbers and relative proportions of hemocyte types were calculated by counting at least 200 cells on each slide. The cells were observed under a Leica DMRE microscope, and the DHC was determined using the following equation:

$$\text{DHC (\%)} = \frac{\text{number of different hemocyte cell types}}{\text{total hemocyte cells counted}} \times 100$$

The cell sizes (length and width) and nuclear to cytoplasmic (N/C) ratios were calculated with the Image J software, which is an image processing program.

Plasma pH, osmolarity and protein assessment

The hemolymph's pH was measured with a glass microelectrode and a pH meter (Crison, Italy). Osmolarity was estimated with a freezing-point depression osmometer (Roebling, Berlin, Germany). The total protein concentration of the plasma was estimated using a Qubit fluorometer (Life Technologies) for sensitive fluorescence-based quantization assays in accordance with the manufacturer's instructions.

Scanning electron microscopy (SEM)

Fresh hemolymph from *C. borealis* and *C. pagurus* was dropped directly on to coverslips pre-treated with 0.1 % poly-L-lysine. After adhesion, the monolayer was fixed in a cacodylate buffer (0.1 M, pH 7.3) containing 2.5 % glutaraldehyde for 30 min at 4 °C. The cells, which were washed with a cacodylate buffer, were post-fixed with 1 % osmium

Table 1 Morphometric measures and differential count of hemocyte types from *Cancer borealis* and *Cancer pagurus* hemolymph

Cell types		*Cancer borealis*	*Cancer pagurus*
Hyaline	% (DHC)	47.6±2.2	30±1.4
	Cell length (µm)	19.3±1.66	19.4±2.2
	Cell width (µm)	4.8±0.9	3.3±0.6
	N/C (%)	25±4	36±9
Semigranulocytes	% (DHC)	45.2±3.3	60±6.2
	Cell length (µm)	9.7±0.46	11.4±1.1
	Cell width (µm)	8.7±0.6	5.5±1
	N/C (%)	23±4	26±5
Granulocytes	% (DHC)	7.2±2.4	9.4±0.3
	Cell length (µm)	13.0±1.7	11.3±1.5
	Cell width (µm)	8.8±1.33	6.6±0.9
	N/C (%)	16±1	23±2

DHC: differential hemocyte count; N/C: nucleus/cytoplasm ratio. Values are expressed as mean ± SD and range n = 15.

tetroxide for 30 min at 4 °C, dehydrated in graded alcohol and dried at the critical point. The preparations were mounted on stubs, gold coated in a sputter coater and examined under a LEO 420 SEM. Hemolymph from three distinct specimens was examined.

Hemagglutination assay

The hemagglutinating activity (HA) of two fold-diluted samples was assayed in a 96-well microtiter U-plate containing a suspension of 1 % rabbit red blood cells (RRBC) or sheep red blood cells (SRBC) in phosphate buffered saline (PBS-E: 6 mmol/l KH_2PO_4, 0.11 mmol/l Na_2HPO_4, 30 mmol/l NaCl, pH 7.4). Erythrocytes were supplied by the "Istituto Zooprofilattico della Sicilia" (Palermo, Italy) and maintained in a sterile Alsever's solution (27 mmol/l sodium citrate, 115 mmol/l D-glucose, 18 mmol/l EDTA and 336 mmol/l NaCl in distilled water, pH 7.2). Tris-buffered saline (TBS; see below) enriched with 1 % RRBC and SRBC with 0.1 % (w/v) gelatin was used as the reaction medium. Twenty-five microliters of plasma were serially diluted and mixed with an equal volume of erythrocyte suspension and incubated at 37 °C for 1 h. The titer of the hemagglutinating activity (HT) was expressed as the highest dilution showing a positive score for agglutination. Plasma from several specimens was separately assayed, and TBS was used in place of the plasma as a negative control. Each assay was performed in duplicate and the HA titer was expressed as the average of the recorded values.

Agglutination of yeast

A suspension of 100 mg of yeast in 10 ml of physiological solution was washed twice with physiological saline and centrifuged at 400*g* for 5 min. The yeasts were inactivated by an autoclave, washed twice in saline and divided into aliquots of 1 ml. An aliquot was centrifuged at 400*g* for 5 min and 25 µl of pellet was resuspended in 1 ml of TBS gel. The plate was prepared following the procedure described above and incubated for 1 h at 37 °C. The titer of the HT was calculated as set out above.

Statistical analysis

All values were from five samples in triplicate. Data were given as arithmetic means ± standard deviations. We used analysis of the t test for a comparison of the N/C ratio between species.

Results

Light microscopy observations

The *C. borealis* and *C. pagurus* hemocyte monolayers showed flattened and well-spread cells, and three morphologically distinguishable cell types were found. May-Grünwald-Giemsa cytological staining allowed us to recognize: 1. hyalinocytes (*C. borealis*: 19.3 ± 1.66 µm in length; *C. pagurus*: 19.4 ± 2.2 µm in length) that had an elongated shape and missing cytoplasmic granules; 2. semigranulocytes (*C. borealis*: 9.7 ± 0.46 µm in length; *C. pagurus*: 11.4 ± 1.1 µm in length) with a few granules; and 3. granulocytes (*C. borealis*: 13.0 ± 1.7 µm in diameter; *C. pagurus*: 11.3 ± 1.5 µm in diameter) (Table 1) with a great number of evident cytoplasmic granules.

The hyalinocytes had a central nucleus, a high nucleus-cytoplasm *ratio*. In *C. borealis* N/C ratio was 25 ± 4 %, significantly ($p< 0.05$) lower than in *C. pagurus* with N/C ratio of 36 ± 9 % and basophilic cytoplasm (Figs 1A, D, G, L). In both species, the semigranulocytes had a central or an eccentric nucleus (*C. borealis* 26 ± 5 %; *C. pagurus* 23 ± 4 % N/C) (Figs 1B, E, H, J), whereas the granulocytes had an eccentric nucleus. In *C. borealis* N/C ratio was 23 ± 2 %, significantly ($p< 0.01$) lower than in *C. pagurus* 16 ± 1 %, (Figs 1C, F, I, K). The granulocytes mainly contained eosinophilic granules (eosin staining), which were large and few in number in the *C. borealis* hemolymph, and small and numerous in the *C. pagurus* hemolymph (Figs 1I, K). Only a few basophilic granulations were observed in granulocytes from both crab species. The semigranulocytes of *C. borealis* contained small basophilic granules, whereas the semigranulocytes in the cytoplasm of *C. pagurus* contained both basophil and eosinophilic small granules.

When examined by an SEM, hemocytes from both crab species had the same morphological features and similar measures as observed with light microscopy (Figs 1D, E, F, L, J, K). The elongated hyalinocytes had a smooth surface, whereas an irregular surface characterized ovoid or spherical semigranular cells, and a rough texture of the cell surface distinguished spherical granulocytes.

THC and DHC

The THC was 4.7 ± 0.4x10^6 cell/ml in *C. borealis* and 4.4 ± 0.6x10^7 cell/ml in *C. pagurus*. In the first species of crab, the hyalinocytes and semigranulocytes were numerous and similar in number (45.2 ± 3.3 % and 47.6 ± 2.2 % respectively). In contrast, in the second species, the hyalinocytes were present in a lower proportion (30 ± 1.4 %) and the semigranulocytes a higher proportion (60 ± 6.2 %). Finally, the granulocyte proportions were similar in each species (7.2 ± 2.4 % and 9.4 ± 0.3 %, respectively).

Hemolymph parameters and hemagglutinating activity

To check for plasmatic parameters, the pH, osmolarity and protein content of 15 specimens of each crab species were recorded by examining the plasma samples prepared in the presence of an anticoagulant (1:3 plasma/anticoagulant *ratio*). The pH values were 7.2 ± 0.08 in *C. borealis* and 7.4 ± 0.04 in *C. pagurus*. The osmolarity was 958 ± 28 mOsm/kg (*C. borealis*) and 762 ± 19 mOsm/kg (*C. pagurus*), while the protein content ranged from 27.3 ± 5.1 µg/µl (*C. borealis*) to 38.8 ± 6.2 µg/µl (*C. pagurus*).

The plasma from the two species exerted *in vitro* agglutinating activity in the absence of calcium cations, as revealed with rabbit and sheep erythrocytes and yeast (*S. cerevisiae*). The *C. borealis* plasma agglutinated both erythrocyte types up to a serial dilution of 1:64, whereas lower titers were found by assaying the *C. pagurus* plasma with rabbit (titer: 1:4) and sheep (titer: 1:2) erythrocytes, respectively. The agglutinin titers of the plasma samples from both species, assayed with yeast, were 1:16 and 1:2, respectively.

Discussion

It is known that live crabs are generally very delicate animals that should always be handled with great care. In fact, different research reports the negative effects of transport on the physiology of crustaceans (Lorenzon *et al.*, 2008; Woll *et al.*, 2010). In this study, before starting our observations, we determined that the animals showed no physiological changes after 48 h of acclimatization following a short journey (about 20 min) compared to samples taken directly at the commercial point.

Morphological characterization, cell type identification, differential numbering of hemolymph cell populations of reared crab hemocytes, some plasmatic parameters and agglutinating titers of the plasma hemolymph may contribute to examinations of the physiological role of reared crabs and the monitoring of their health (Celi *et al.*, 2013).

The cell features of circulating hemocytes from *C. borealis* and *C. pagurus* were examined with May-Grünwald-Giemsa staining, and using light and SEM observations. According to the morphological criterion proposed by Bauchau and Plaquet (1973), Hose *et al.* (1990), and Sung and Sun (2002), granulocytes, semigranulocytes and hyalinocytes in the hemolymph from both crab species were identified on the basis of the presence/absence, quantity and cytochemical features of the granules, size and N/C ratios of the cells. A similar classification has been adopted for hemocytes from other crustacean species (Jussila *et al*., 1997; Gargioni and Barracco, 1998; Hammond and Smith, 2002; Yavuzcan and Atar, 2002, Matozzo and Marin 2010). Fusiform hyalinocytes with a basophilic cytoplasm and a central nucleus were smaller than granule-containing cells, which had a higher nucleus/cytoplasm *ratio*. The cytoplasm of the semigranulocytes contained a central or eccentric nucleus, whereas the granulocytes always had an

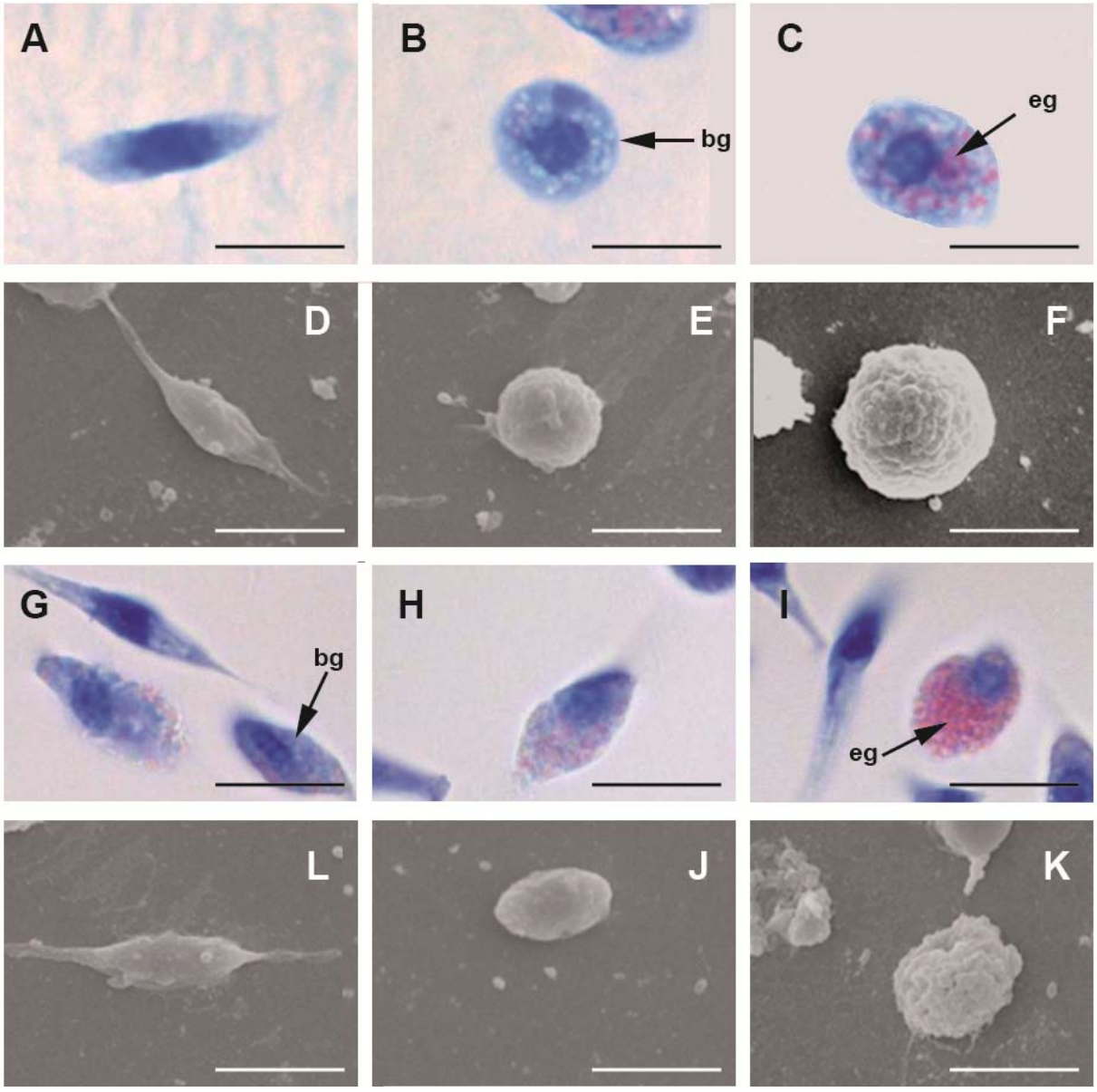

Fig. 1 Features of the hemocyte types from *Cancer borealis* and *Cancer pagurus* hemolymph. *C. borealis*: A - C: MayGrunwald-Giemsa stain; D-F: SEM observations. A, D: hyalinocytes, B, E: semigranulocytes, C, F: granulocytes. *C. pagurus*: G - I: May-Grünwald-Giemsa stain; L - K: SEM observations. G, L: hyalinocytes, H, J: semigranulocytes, I, K: granulocytes. Bars: 10 µm. The arrows indicate the eosinophilic granules (eg) and basophilic granules (bg).

eccentric nucleus. The cytoplasm of the granulocytes was packed with large and round granules, whereas the semigranulocytes contained a lesser amount of smaller granules. In both cases, the May-Grünwald-Giemsa staining disclosed that a large part of the granules were eosinophilic and differed between the two crab species; they were few in number and large in the *C. borealis* granulocytes, and small and numerous in the *C. pagurus* granulocytes. Finally, fine eosinophilic granules were also contained in the cytoplasm of *C. pagurus* semigranulocytes. SEM observations were useful for disclosing the surface morphology of these cells from the hemolymph, which showed the same main features in both crab species. The elongated hyalinocytes had a smooth surface, the ovoid or spherical semigranular cells an irregular surface, and the spherical granular cells a cell surface with a rough texture.

Hemocytes play a central role in the immune defenses of crustaceans (Söderhäll and Cerenius, 1992; Zhang *et al.*, 2006); in fact, the total and differential hemocyte counts provide a useful way of assessing the physiological state of an animal (Battison *et al.*, 2003). Unfortunately, although a wide range of values are available, differences in

the classification schemes used have prevented the comparison of hemocyte profiles among different crustaceans. (Rodriguez and Le Moullac, 2000). The THC for *C. pagurus* reported in this study is in accordance with the findings of Vogan and Rowley (2002) and Lorenzon *et al.* (2008), but no dates are reported in the research for *C. borealis*. The THC of *C. borealis* is lower than for *C. pagurus*, but is in accordance with the range presented for other crustaceans (Celi *et al.*, 2013; Filiciotto *et al.*, 2014). Probably, this difference is due to the different ecology and geographic distribution of the two species.

The DHC have provided varying results between crustacean species. Hyalinocytes and semigranulocytes were more represented in the circulating hemolymph of *C. borealis* and *C. pagurus*. Conversely, in *M. rosenbergii,* hyalinocytes comprised 70 % of the total hemocytes and no semigranulocytes were found (Vázquez *et al*., 1997). In *Sicyonia ingentis*, 50 - 60 % of the circulating hemocytes were hyalinocytes, whereas the semigranulocytes and granulocytes comprised 30 % and 10 % of the total, respectively (Hose and Martin, 1989). High percentages of hyalinocytes (five to eight times more abundant than granulocytes) have been observed in the crab *Eriocheir sinensis* (Bauchau and Plaquet, 1973), the lobster *Panulirus interruptus* (about 56 %) (Hose *et al.,* 1990) and the crayfish *Procambarus clarkii* (more than 70 %) (Lanz *et al.,* 1993). Conversely, in the lobster *H. americanus* and the crab *Loxorhynchus grandis*, the semigranulocytes reached more than 60 % of the total cell numbers (Hose *et al.,* 1990). Although the significance of this marked variability in the relative proportions of each hemocyte type among crustacean species remains unclear, it does appear to be useful when it comes to characterizing the hemocytes of every reared crab species. In addition, preliminary morphological observations of hemocytes under a light microscope and SEM could be useful for further fine structural studies, and may contribute to determining the functional activity of the cell types that may be involved in immunity.

Furthermore, although the plasmatic parameters were checked by using 1:3 diluted plasma in the presence of an anticoagulant, they could be health indicators in reared crustaceans (Chang *et al.*, 2007).

Several authors have reported the pH values of *C. pagurus*, but these were not identical (Lorenzon *et al.*, 2008; Woll *et al.*, 2010; Barrento *et al.*, 2011). We found similar pH values for both species that are also in according to the same authors above mentioned.

Many studies on crustaceans describe the osmolarity values in stress conditions (Charmantier *et al.*, 1989; Charmantier and Soyez, 1994; Lignot *et al.*, 2000). In this study also, different values of osmolarity for the two species examined were reported, with *C. borealis* having higher values. Conversely, the protein content was lower in the plasma of *C. borealis* than *C. pagurus*. Although hemolymph proteins are another important physiological parameter in crustaceans, the literature reveals wide variations in hemolymph protein concentrations (Depledge and Bjerregaard, 1989; Lorenzon *et al.,* 2011).

The agglutinins that play a role in protecting against bacterial infections are another important component of the hemolymph in terms of an immune defense role (Sahoo *et al.,* 2007). The agglutinins are synthesized and discharged in the hemolymph by hemocytes (Rodríguez *et al.*, 1995). The presence of agglutinins in the hemolymph of both examined crab species was revealed by using mammalian erythrocytes and yeast as targets. The results with respect to the two species were different, with more agglutinating activity being revealed for *C. borealis*. Since plasma samples were prepared with a cation-chelating agent, the possibility exists that agglutinins may have properties of calcium-independent lectins (Basil-Rose *et al*., 2014). In addition, due to the hemolymph sample dilution (1:3 with anticoagulant), the agglutinin titers may be higher than those reported here.

Finally, it is of interest to disclose the hematological and plasmatic parameters of reared crabs, because the DHC, plasmatic parameters and agglutinin titer could be influenced by the moult cycle, diet, harvesting, diseases and environmental contaminants (Lorenzon *et al.,* 2007; Matozzo *et al*., 2011).

Acknowledgments

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