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S.E.M. and cytofluorimetric characterization of *Dinocras cephalotes* haemocytes (Plecoptera, Perlidae)

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ABSTRACT. Haemocytes of the stonefly *Dinocras cephalotes* have been characterized by light and scanning electron microscopy (SEM), adherence to a plastic surface, and phagocytosis of latex particles. Haemocytes appear to consist of at least two cell populations: granulocytes and plasmatocytes, which can be distinguished by morphological and functional criteria. Morphologically, granulocytes are rounded and vesiculated, while plasmatocytes have an irregular shape, with many filopodia, and a slightly vesiculated content. Culturing haemocytes "in vitro" resulted in the spreading onto the surface of an adherent population (plasmatocytes), and in a loosely attached population (granulocytes). Phagocytosis assay showed the capacity of *D. cephalotes* plasmatocytes to engulf exogenous particles. Haemocytes of adults and nymphs of *D. cephalotes* were also analysed by flow cytometry and compared with those of *Perla grandis*, another species of Perlidae. We observed a slight difference in haemocyte morphology among nymphs and adults, and a shift of cell populations was also detected.

KEY WORDS: haemocytes, stoneflies, plasmatocytes, granulocytes, phagocytosis, flow cytometry.

INTRODUCTION

The insect circulatory system is responsible for moving fluids through body cavities and appendages, but, as is well known, is not involved in respiration. Insect haemolymph is composed of a liquid part, or plasma, and of a cellular fraction with cells having different morphologies, collectively called haemocytes (RATCLIFFE et al., 1985; GUPTA, 1986). Haemocytes display many important physiological functions, such as wound healing during repair of the exoskeleton, in transferring molecules and nutrients (GUPTA, 1986), and in the defence response against pathogens, parasites and foreign substances (ANDERSON & CHAIN 1986; HUNG et al., 1993). These defence functions performed by haemocytes are exerted by means of cellular and humoral activities (DRIF & BREHÉLIN, 1989; BULET et al., 1991; MARMARAS et al., 1994; CHARALAMBIDIS et al., 1995; SCAPIGLIATI et al., 1997; SCAPIGLIATI et al., 1998; BULET et al., 1999).

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In Plecoptera, studies on *Acroneuria* and *Diura* species (ARNOLD, 1966; PRICE & RATCLIFFE, 1974) showed that the haemolymph contains 20-40000 cells/mm^3 and that haemolymph coagulation is very fast. Also, it was pointed out that blood composition in the adults changed with age (ARNOLD, 1966), although this can be due to the cell fixation as well as to cytolysis processes (In: ZWICK, 1980). There is no general agreement in the literature on the existence of different haemocytes. GUPTA (1985) stressed the existence of five haemocyte types in Plecoptera: prohaemocyte, plasmatocyte, granulocyte, spherulocyte and coagulocyte. PRICE & RATCLIFFE (1974) also considered the adipohaemocytes as a kind of haemocyte.

SUTCLIFFE (1962) gave some information on the chemical composition of *Dinocras cephalotes* (Curtis 1827) and *Perla bipunctata* (Pictet, 1833) haemolymph (osmolarity, conductivity, free amino acids, etc.), however, this study did not give information on the haemocyte morphology.

In the Plecoptera, as in other minor insect orders, the knowledge about haemocytes is scarce and more studies

on the morphology and the function of these cells are needed. The aim of the present study was to add new insight about Plecoptera haemocytes by using light and scanning electron microscopy (SEM), phagocytosis test and, for the first time in this insect group, flow cytometry analysis.

MATERIAL AND METHODS

Insects

Dinocras cephalotes and *Perla grandis* (Panzer, 1799) were collected in the River Nera (Italy) in June and July 1998 and in April and May 2000. Nymphs were collected with a net from the river. Adults were collected directly from vegetation or from the river bank rocks with entomological tweezers or by an entomological net. They were transported alive to the laboratory in cooled containers and used for the morphological and functional studies.

Haemocyte collection

Haemocytes were collected from specimens by dissection of the abdomen. The haemolymph was collected by carefully washing each insect with 0.15 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8 mM Na_2HPO_4, pH 7.4 (PBS), opening the insect over a Petri dish in PBS containing 10 mM EDTA to prevent coagulation. Haemocytes were harvested by centrifuging at 450g for 5 min, washed in 500 µl of PBS, stained with 0.2% Trypan blue in PBS to assess cell viability and counted in a haemocytometer.

SEM analysis

Haemocytes were placed on coverslips in a Petri dish containing a culture medium (Grace's insect medium - GIBCO Europe, Paisley, Scotland) at 25-28°C for one hour, washed three times in PBS and fixed in 1ml/well of 5% glutaraldehyde and 4% paraformaldehyde buffered with 0.1M sodium cacodylate (pH 7.2) for 2 hr at 4°C (Karnovsky, 1965). After rinsing overnight in the same buffer, the samples were post-fixed in 1% OsO_4 in the same buffer for 1 hr at 4°C and dehydrated in a graded ethanol series (50% to 100%). Afterwards, the coverslips were dried by the critical point method with liquid CO_2 in a Balzer CPD 020 apparatus, attached to specimen stubs, coated with gold in a Balzer Union MED 010 evaporator, and observed with a 5200 Jeol JMS scanning electron microscope. Pictures were taken on Kodak T-max film exposed at 200 ISO.

Phagocytosis test

Haemocytes were placed on coverslips in a Petri dish containing culture medium at 25–28°C for one hour. After that, they were incubated in 2 ml of culture medium containing 5 µl of polystyrene latex particles of 0.8 µm diameter, for half an hour. Then they were washed in PBS and prepared for SEM observation as described above.

Cell culture and flow cytometry

Where necessary, cells were cultured for 2 hours at 25°C in 3-cm Petri dishes containing 3 ml of Grace's insect medium to allow adherent cells to attach and spread. Non-adherent cells were removed by washing the plate with PBS, and collected by centrifugation.

Cells were washed in PBS and fixed with 0.5 ml of 2% paraformaldehyde in PBS for 15 min at 4°C. Flow cytometry of non adherent cells was performed on an FACScalibur flow cytometer, and between 3,000 to 10,000 cells were counted for each experiment without the selection of a particular population during acquisition, unless indicated. Cells were analysed by the following parameters: side scatter (SSC), forward scatter (FSC), and spontaneous fluorescence channel (FITC) (Tierno de Figueroa et al., 2001).

RESULTS AND DISCUSSION

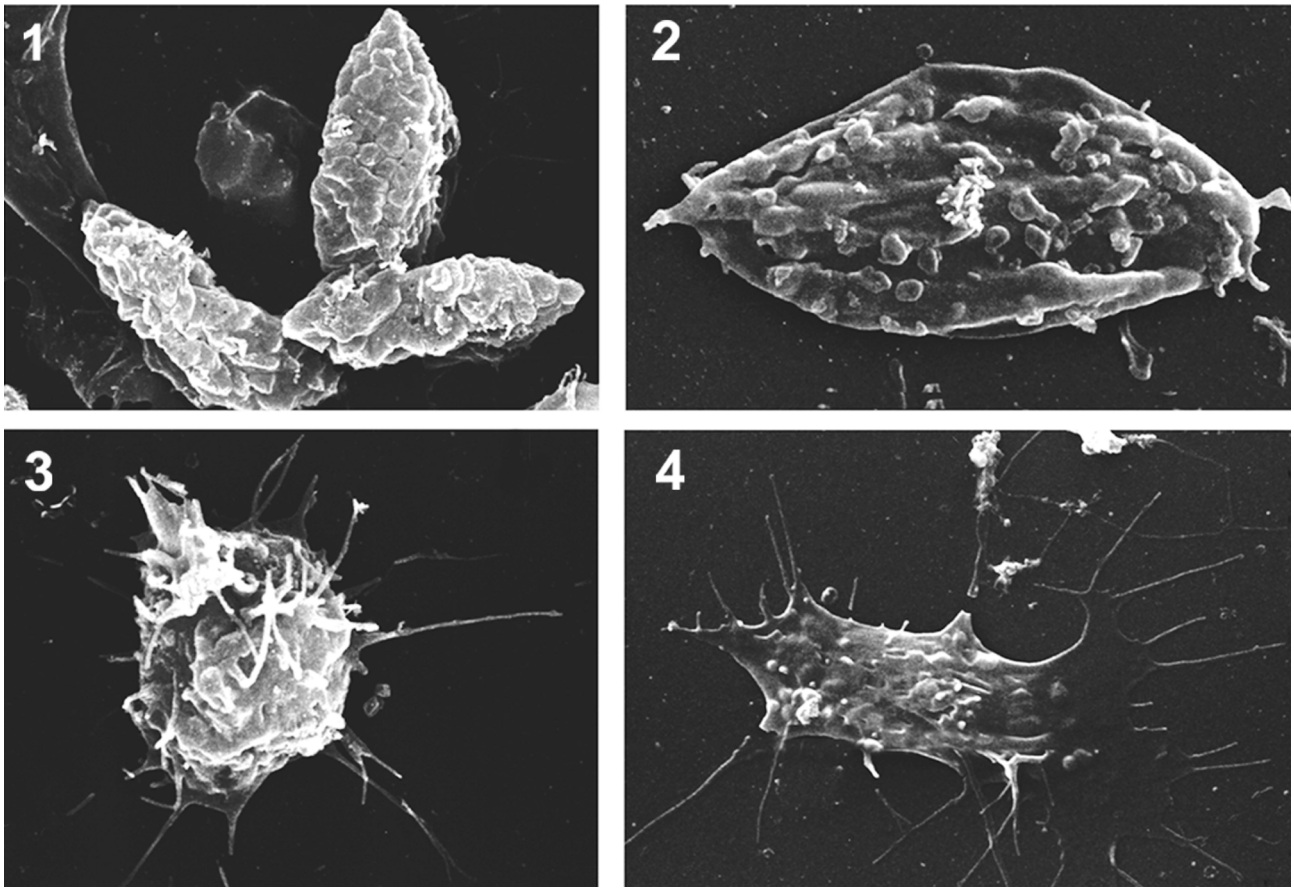
In this study we investigated cell composition of the haemolymph of the stonefly *Dinocras cephalotes*. SEM observations showed the presence of at least two different haemocyte populations: plasmatocytes and granulocytes (Figs 1-6), probably belonging to the two more widespread cell types found in the haemolymph of studied insect species (Gupta, 1985, Scapigliati & Mazzini, 1992).

Morphologically, granulocytes contain many granules and do not seem to have evident filopodia (Fig. 1), whereas plasmatocytes have an irregular shape, few or many filopodia, and little granular content (Fig. 1-6). These morphological differences could be related to different physiological properties, with plasmatocytes mainly involved in cellular reactions (phagocytosis and encapsulation), and granulocytes in humoral reactions (secretion), as previously shown in stick insects (Scapigliati et al., 1997).

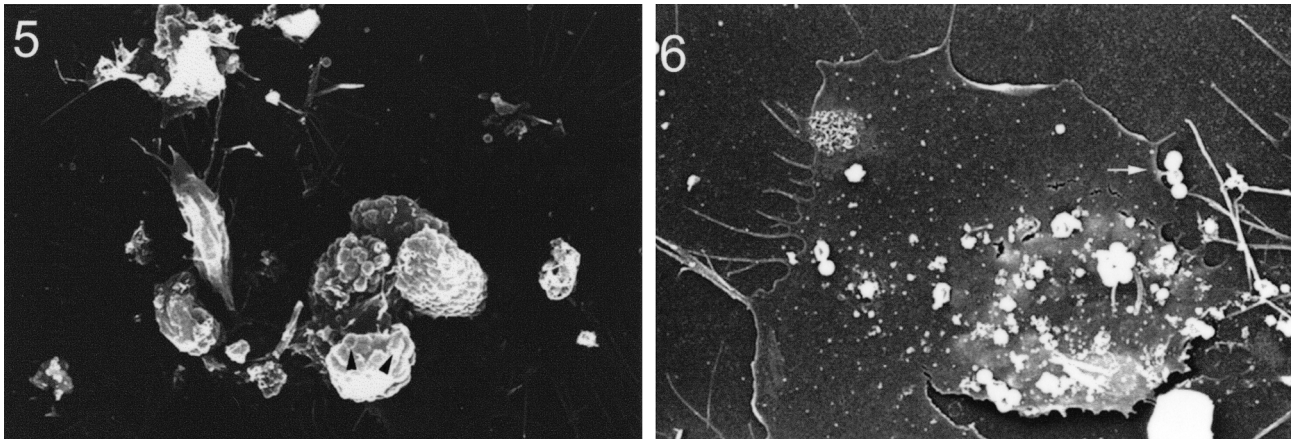
In relation to adherence capacity, during "in vitro" culturing, both cell types showed some adherence to a glass surface, but washing of culture wells with PBS showed that plasmatocytes were more strongly attached.

When adherent, insect plasmatocytes can have different morphologies, and *D. cephalotes* "spindle shaped" adherent plasmatocytes displayed high similarity with those of *Bacillus rossius* previously described (Scapigliati et al., 1993).

The phagocytosis of foreign particles was employed to distinguish between plasmatocytes and granulocytes (Scapigliati & Mazzini, 1994). In *D. cephalotes,* adherent haemocytes were able to phagocytose exogenous particles (Figs 5-6). These preliminary observations seem to



Figs 1-4. – SEM images of haemocytes of *Dinocras cephalotes*. 1. Granulocytes (x3000). 2. A "Spindle shaped" plasmatocyte (x5000). 3. A "sea urchin-like" plasmatocyte (x4500). 4. A completely adherent plasmatocyte (x3000).



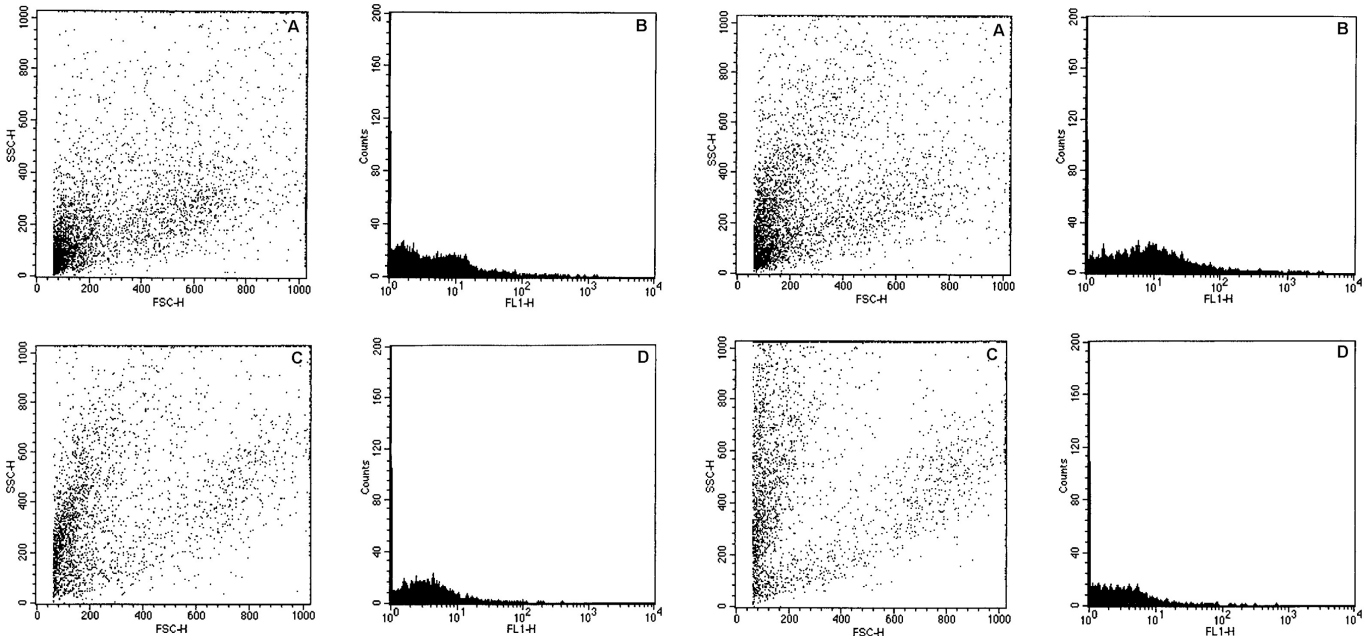
Figs 5-6. – Phagocytosis test with latex particles in *Dinocras cephalotes* (Fig. 5 x2000, arrowheads indicate latex particles engulfed; Fig. 6 x 2500, arrow indicates latex particles).

confirm that also in Plecoptera the phagocytic activity is restricted to plasmatocytes, as it has been pointed in other insects (Scapigliati & Mazzini, 1994).

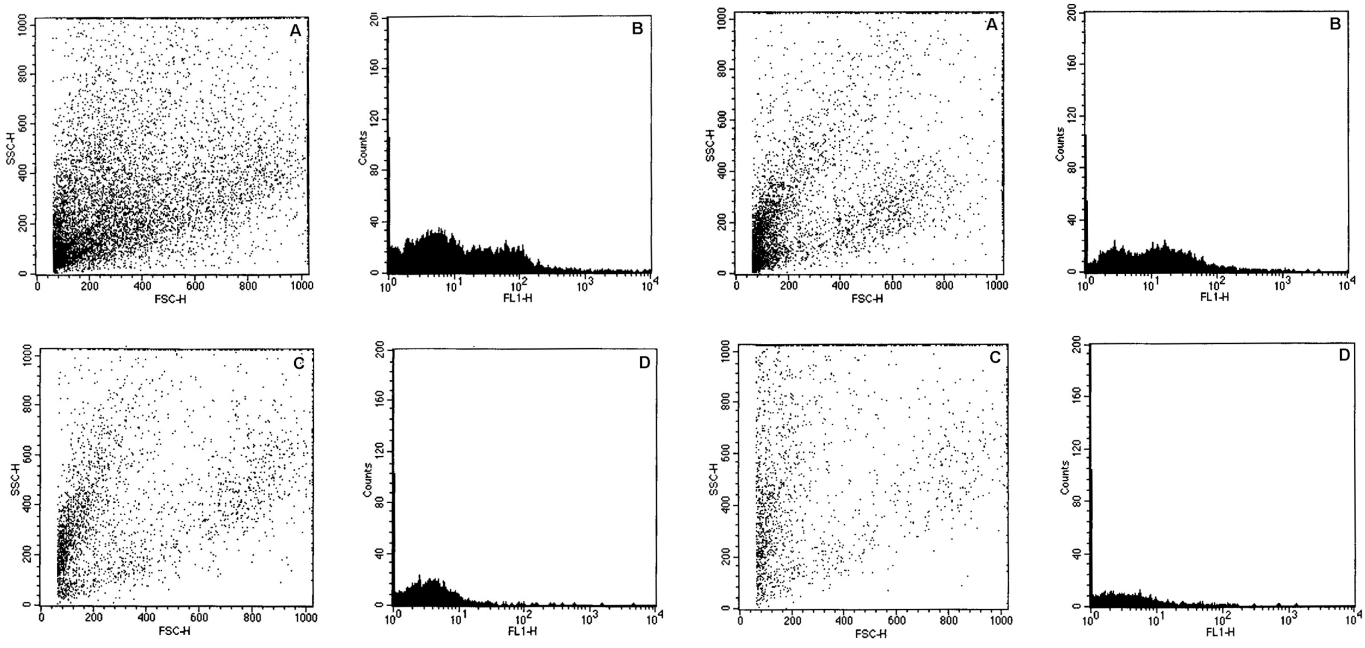
Other haemocyte types, such as prohaemocyte, spherulocyte or coagulocyte, present in other Plecoptera species and in other insect orders (Gupta, 1985), have not been detected in our study on *D. cephalotes* haemolymph.

Flow cytometry is a powerful technique to analyse the overall morphology of a great number of cells. FSC parameters describe the size of cells, increasing from low to high values, and SSC the "granularity", or the intracellular organ content. Analysing a mixed cell population will result in grouping cells having similar morphology in a same group with similar FSC and SSC coordinates. The flow cytometric analysis of haemolymph of *D. cephalotes* and *P. grandis* (the latter is a related species belonging to the same family Perlidae), showed certain homogeneity between the cell populations of the nymphs (young and mature) and the

adults (also when male and females are studied separately). Nevertheless, in the adult there is a decrease of cells having higher values of SSC and FSC. This decrease is observed in total haemocytes as well as in the adherent populations, and in both species (Figs 7-10, a, c), and it can be also appreciated by spontaneous fluorescence of cell populations (FL1 values) (Figs 7-10, b, d). These observations may be related to the species life cycle, because the adults do not feed and have a short life, in which they only mate and oviposit. It can be speculated that the change of relative number of cell types circulating in haemolymph during the imaginal life could be due to a progressive loss of some physiological functions related to the circulatory activity. Thus, a cytolysis process could be involved in this cell shift, confirming previous hypotheses by ZWICK (1980). Nevertheless, ARNOLD (1966) pointed out that in the Pteronarcyidae *Acroneuria arenosa* there is a progressive decrease in relative numbers of circulating plasmatocytes with age (in adults), due to their adherence to the walls of wing veins and presumably to others tissues within the body. In fact, JONES (1962) showed that in some cases in insects there is a decrease of the total haemocyte count due to adhesion of



Figs 7-8. – Flow cytometric analysis of *Dinocras cephalotes* haemocytes. Total (7) and non adherent (8) haemocytes were analysed for their FSC and SSC parameters (A, C), or for their fluorescence (B, D). Nymphs (A, B) and adults (C, D).



Figs 9-10. – Flow cytometric analysis of *Perla grandis* haemocytes. Total (9) and non adherent (10) haemocytes were analysed for their FSC and SSC parameters (A, C), or for their fluorescence (B, D). Nymphs (A, B) and adults (C, D).

haemocytes to the tissue surfaces, and that this change could be accompanied by significant changes in types of haemocytes. Taken together, our results and previous observations confirm that insect haemocyte cell populations are morphologically rather homogeneous. Also, it is possible that an active apoptotic process is involved in the shift of haemocyte populations during insect metamorphosis.

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