Circulating hemocytes from larvae of the paper wasp *Polistes dominulus* (Hymenoptera, Vespidae)

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Abstract

Circulating hemocytes from larval stages of the paper wasp *Polistes dominulus* were characterized by light and transmission electron microscopy. Three types were identified: prohemocytes, plasmatocytes and granulocytes. The first two are agranular cells while the latter present typical cytoplasmic inclusions called granules. Plasmatocytes differ from prohemocytes being larger, showing lower nucleus/cytoplasm ratio and they possess many phagolysosomes. The substantial uniformity of most subcellular features and the presence of "intermediate forms" support the "single-cell theory" i.e., there is only one cell line that originates from the prohemocyte and leads to the granular cell passing through the plasmatocyte. This hypothesis seems to be confirmed by functional tests. Indeed, most part of cells adheres to the glass and is able to phagocytize fluorescent microspheres.
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1. Introduction

In an attempt to characterize the hemocytes of the paper wasp *Polistes dominulus* larvae it might be suitable to remember that anyone venturing into the study of insect hemocytes knows only too well how discouraging it is in the beginning to have to contend with a bewildering array of terminologies that exists in the literature (Gupta, 1985). More than 20 years have passed and the situation has even worsened because of the large amount of newly published data, mainly inherent to the ultrastructural and functional analysis of these cells. Instead of simplifying and clarifying previous observations, this modern approach has uncovered new possible parameters to be evaluated and consequently it has further on complicated a nomenclature that was itself controversial and misleading.

In this work we present a description of hemocytes from larvae of *P. dominulus* (3rd and 4th instars), in the aim of filling in the gap present in the literature about this topic:

there are scanty and outdated published data on hemocytes from wasps or other Hymenoptera (Chauvin, 1968; Ahmad, 1988) and this is in great contrast with the large amount of information about other insect orders like Dictyoptera, Diptera, Lepidoptera, Orthoptera (Giulianini et al., 2003). Our study is based on light microscopy, transmission electron microscopy and foremost functional tests i.e., phagocytosis of fluorescent beads and adhesion on glass slides. We know today that a proper methodological approach requires the combination of different analysis techniques when studying insect hemocytes. This may include analysis of thin sections at an ultrastructural level, examination of semithin section, using the light microscopy for hemocytes samples fixed at blood removal; observation of cells behavior in monolayers by means of phase contrast microscopy or SEM; employ of antibody and genetic markers (Lavine and Strand, 2002; Ribeiro and Brehélin, 2006). We concentrate on few specific developmental stages in order to reduce the large variability due to the physiological state of both the cell and the organism which may condition the morphology of hemocytes (mainly at an ultrastructural level), which is another

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aspect often neglected by hematologists. For example, the pattern of the cellular elements in the hemolymph of a larva may be quite different from that present in an adult, mostly in holometabolous insects (Meylaers et al., 2007). In addiction to this, we should not forget that experimental conditions always represent a stress source for cells, therefore *in vitro* dynamics can quite differ from *in vivo* behavior.

2. Materials and methods

2.1. Animal rearing and hemolymph collection

Polistes dominulus Christ came from Asciano, in the surroundings of Siena (Tuscany, Italy). Hibernating groups were collected at the end of winter and arranged in $20\,cm \times 20\,cm \times 20\,cm$ Plexiglas cages in order to let them found new colonies. In the field, nests were picked up in summer and housed in a similar way. Both groups were maintained at 15L/9D and $28 \pm 2C°$; they were fed with sugar, water and *Sarcophaga* sp. larvae *ad libitum*.

Hemolymph was collected from larvae of *P. dominulus* by operating a small incision on the cuticle of chilled larvae and collecting the oozing hemolymph drop with a precision microliter pipette. Samples were kept in ice and immediately resuspended in Grace's Insect Medium (Invitrogen–Gibco) to prevent hemolymph coagulation and cell impairment.

2.2. Light microscopy

Hemolymph-solution samples derived from wasp larvae (50–100 µl for each individual) were cytocentrifuged onto slides with a Shandon Instrument Cytospin II running at 400 rpm for 2 min. Hemocytes were then stained with May Grünwald–Giemsa for morphological examination and observed with a Leitz Dialux 22 light microscope.

2.3. Transmission electron microscopy (TEM)

For ultrastructural analysis hemolymph samples were drawn from 3rd to 4th instar larvae (20 µl × larva), pooled and directly transferred in 1.5 ml test tubes (5 larvae × tube) containing 0.1 M, pH 7.2 phosphate buffer (PB) to which 3% sucrose had been added. The material was centrifuged at 1100 rpm for 5 min in order to obtain a pellet of hemocytes and this operation was repeated after each passage. Fixation occurred in 2.5% glutaraldehyde in PB at 4 °C for 5 h and then the material was rinsed in PB several times, post-fixed in 1% osmium tetroxide in PB at 4 °C for 1 h and rinsed again in PB. After dehydration in a graded series of ethanol to 100% ethanol and a step in propylene oxide, embedding in an Epon-Araldite mixture followed. Ultrathin sections, obtained with a Reichert Ultracut II E and a LKB Ultratome III, were routinely stained (1% uranyl acetate followed by 1% lead citrate) and observed with a TEM Philips CM 10 at 80 kV. All measurements are means ± S.D.

2.4. Adhesion test

Drops of hemolymph (∼20 µl each) from *P. dominulus* larvae were collected and placed on glass slides where a Vaseline ring was delimitated. The slides were set in a wet chamber successively covered and hemocytes were made settle for 20–30 min in presence of Grace's Insect Medium. Thereafter the slide was removed from the chamber and observed in phase contrast with a Leitz Dialux 22 light microscope.

2.5. Phagocytosis assay

For each *P. dominulus* larva 10 µl hemolymph were sampled and added to 100 µl Grace's Insect Medium in a 0.2 ml test tube previously covered. The material was then incubated with 0.1 µl of a fluorescent beads suspension for 30 min in soft oscillation. After that, samples were placed on glass slides and observed under the fluorescence microscope.

3. Results

3.1. Morphological observations

3.1.1. Light microscopy

The examination of the stained hemocytes from *P. dominulus* larvae (Fig. 1A) indicates the presence of three cell types. The first (type a) (Fig. 1B) is a small cell, with the nucleus occupying most part of the cellular body and a thin basophile cytoplasm which stains up blue after May Grünwald–Giemsa method. The nuclear chromatin appears as deep red–violet and the cytoplasm keeps substantially round in shape. The second (type b) (Fig. 1B) is a larger cell, with abundant cytoplasm softly stained in a range from pink to light grey. Also the nucleus appears paler than "type a" and the cell shape varies from round to irregular or elongated. In four cases we noticed a circulating hemocyte dividing by mitosis: generally it was an infrequent process (Fig. 1C–F). A round cell with the characteristics of a prohemocyte was also observed.

3.1.2. Ultrastructural analysis

The investigation on the fine structure of hemocytes from *P. dominulus* larvae revealed a substantial uniformity in the presence and morphology of the principal subcellular components. Apparently, this might contrast with their complex structure which shows many peculiar organelles as required by the numerous different tasks performed by these cells. Actually, we did not perceive striking ultrastructural diversity among hemocytes, even when samples came from specimen belonging to different developmental stages; at the most, a variability in size and shape was often observed for some organelles, correlated with the activity of the cell at the moment of fixation. All these considerations seem to justify a generalized description of these cells.
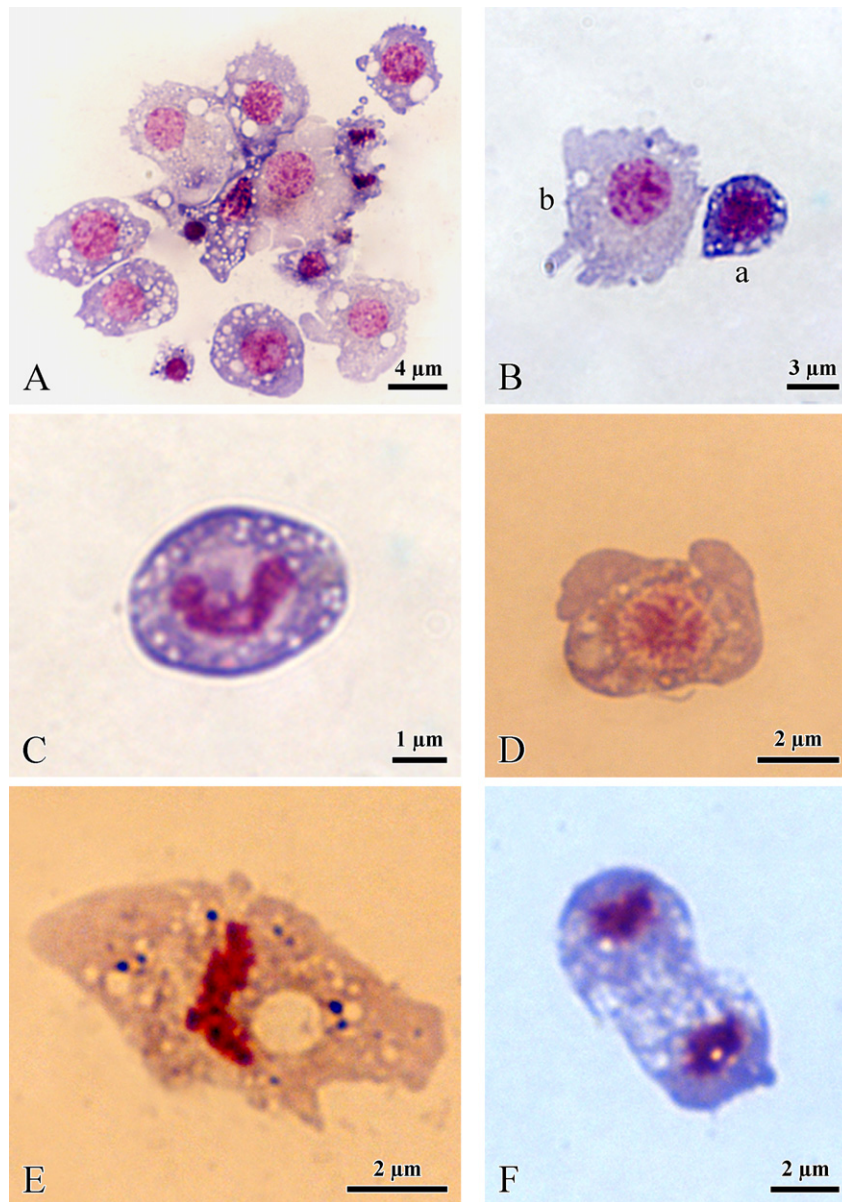
Fig. 1. (A) Circulating hemocytes from *Polistes dominulus* larvae stained for light microscopy observations. (B) The two cell types most frequently observed (a and b). (C–F) Circulating hemocytes dividing by mitosis at different steps of the process.

3.1.2.1. The nucleus. Nuclei show round, ovoid, sometimes irregular profiles. The chromatin is generally uniformly distributed and often few to many patches of electron-opaque chromatin are evident (Fig. 2A and B): the size of these regions is extremely variable (from 0.06 to 0.7 μm diameter) and they can be clumped in the middle of the nucleus as well as adherent to the nuclear envelope. Nucleoli are frequently visible (Fig. 2A and B). In some cases, perinuclear cisternae are considerably distended and may show small groups of vesicles in addition to finely dispersed material (Fig. 2B). The nucleus/cytoplasm ratio is variable: in general, hemocytes may have a high value or a low value for this ratio.

3.1.2.2. The cytoplasm. The abundance of functional cytoplasmic organelles observed in *P. dominulus* hemocytes is an evidence for the intense activity performed by these cells. They consist of standard organelles which are widely diffused among hemocytes and also in other cell lines because fundamental for the cell survival; phagolysosomes, whose size and quantity varies from cell to cell; granules i.e., peculiar inclusions which characterize some hemocytes that we call therefore "granular cells" or "granulocytes".

3.1.2.3. Standard organelles. Mitochondria are generally numerous, variable in size (0.55 ± 0.35 μm) and with well developed cristae; they are moderately electron-dense but
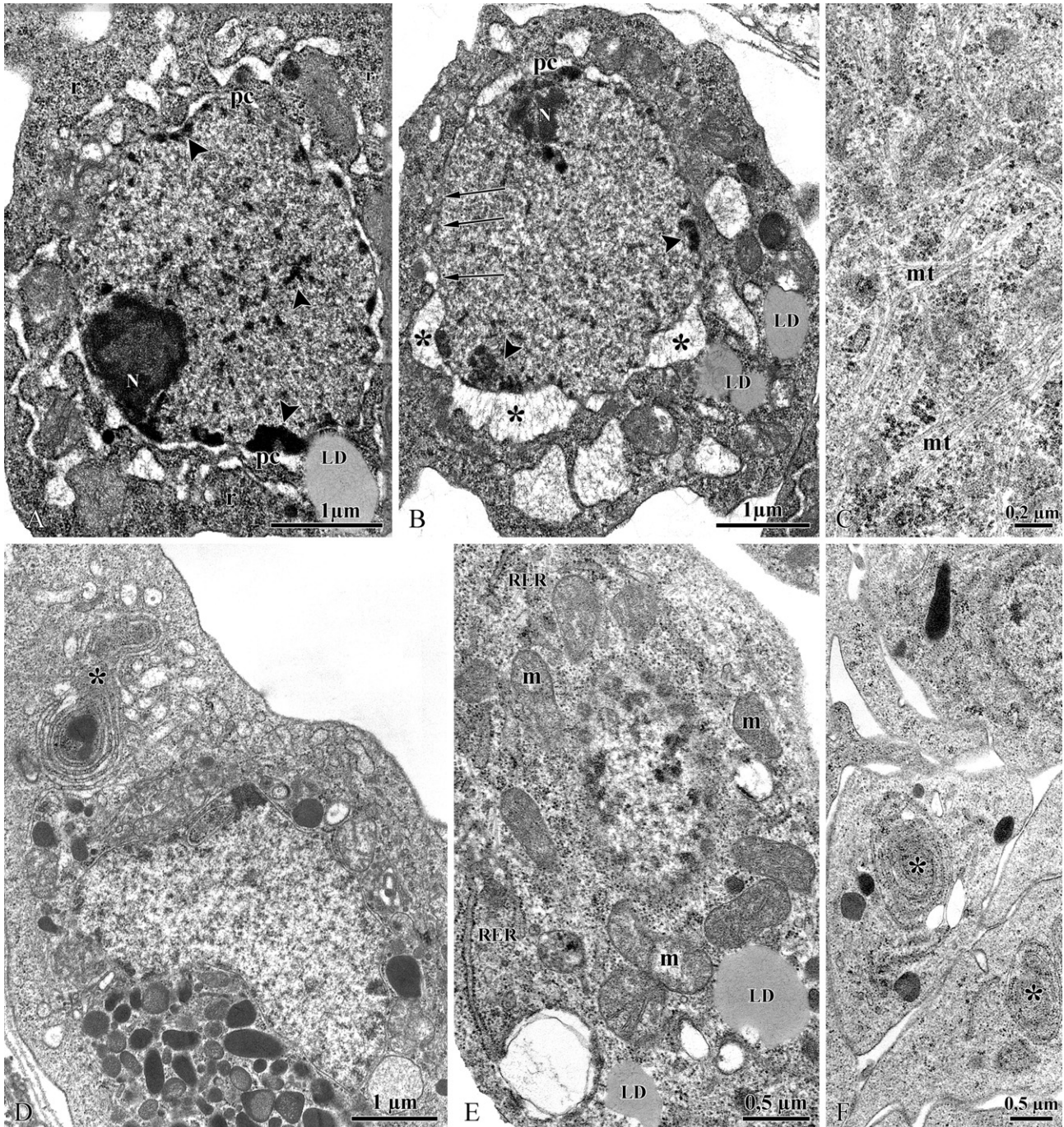
Fig. 2. General subcellular features of *P. dominulus* hemocytes. TEM. (A–B) Nuclear region. Note the nucleoli (N) and the chromatin forming electron-opaque patches (arrowheads). The perinuclear cisternae (pc) form small groups of vesicles (arrows) and finely dispersed material (asterisks). r: ribosomes; LD: lipid droplets. (C) Detail of a hemocyte showing microtubules organized in bundles (mt). (D–F) Cytoplasmic region. Note the well developed rough endoplasmic reticulum arranged in enlarged cisternae (RER) or rolled up in concentric ring-like figures (asterisks). m: mitochondria.

well discernible at TEM (Fig. 2E). A large amount of rough endoplasmic reticulum is present, arranged in stacks and characterized by enlarged cisternae filled with flocculent material (Fig. 2E), mainly in hemocytes from young wasp larvae. In later stages, RER appears frequently developed in long and narrow cisternae, occasionally rolled up in typical concentric ring-like figures (Fig. 2D and F). Free ribosomes are abundant and often set in clusters (Fig. 2A), especially in hemocytes poor in phagolysosomes or granules. Golgi apparatus, when evident, has the appearance of an active organelle, with both its saccular and vesicular parts well discernible (Fig. 3A). Commonly, lipid droplets may be present (Fig. 4D), especially in older cells.
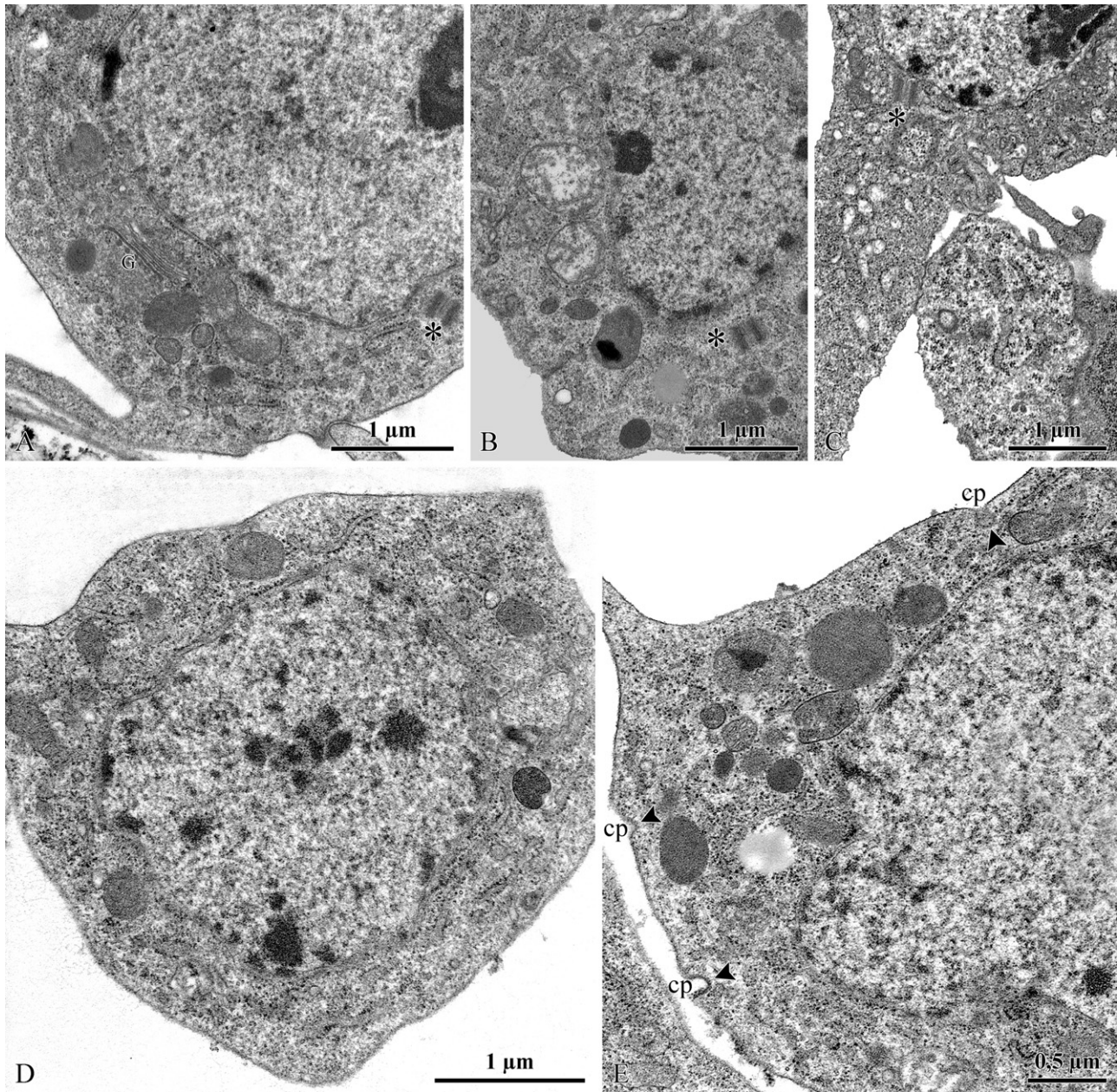
Fig. 3. The prohemocyte. TEM. (A–C) Details of prohemocytes showing a centriole in longitudinal section (asterisks). G: Golgi apparatus. (D) Whole section of a prohemocyte. (E) Coated pits (cp) on the plasma membrane of a prohemocyte: note the presence of the clatrin protein (arrowheads) on the cytoplasmic side of the forming vesicles.

Other frequently detected cytoplasmic elements are microtubules (Fig. 2C), dispersed or arranged in bundles and normally located in close proximity to the plasma membrane and then numerous coated pits (Fig. 3E), visible at different moment of the pinocytotic process and characterized by the presence of the clatrin protein in the cytoplasmic side of the forming vesicles. In three cases we noticed a centriole in longitudinal section (Fig. 3A–C): these observations are in line with the mitotic figures identified at light microscopy and confirm hemocytes ability to multiplicate while circulating in the hemolymph. The three hemocytes provided with centrioles present recurrent features also observed in other

cells and remind us of the prohemocyte, the stem cell which gives rise to the other types (Brehélin and Zachary, 1986; Franchini et al., 1996). These hemocytes present rounded shape, little size (diameter <5 μm), high nucleus/cytoplasm ratio, absence of granules and few small phagolysosomes (Fig. 3D).

3.1.2.4. Phagolysosomes. They are membrane-limited inclusions highly irregular in shape and filled with heterogeneous material: lamellated degraded cellular components or other residues (Fig. 4A, C). These vacuoles are tied with the autophagic-digestive activity of the cell and they
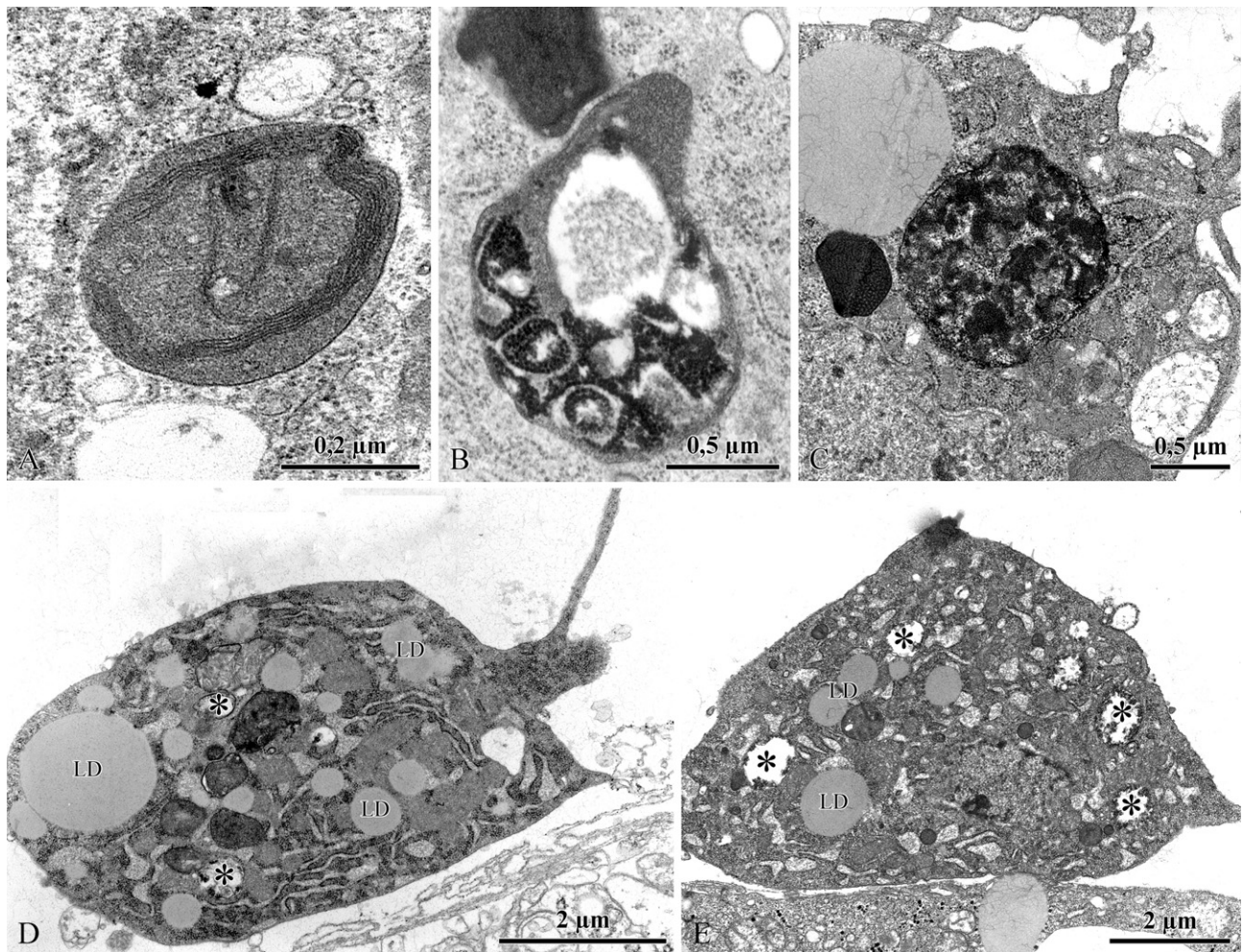
Fig. 4. The plasmatocyte. TEM. (A–C) Different kind of phagolysosomes filled with heterogeneous material: lamellated (A) degraded cellular components (B) or other residues (C). (D and E) Whole agranular cells: note the abundance of phagolysosomes (asterisks) and lipid droplets (LD).

appear very numerous and of large size (up to 1.6 μm diameter) in hemocytes from *P. dominulus* specimen at the end of the larval development. Phagolysosomes are well diffused and they are prevalent in those agranular cells i.e., lacking in granules (Fig. 4D and E) that are different from the prohemocytes for the size (diameter >5 μm) and the nucleus/cytoplasm ratio (much lower): we refer to these cells as "plasmatocytes" for the analogy with what was observed in other insects.

3.1.2.5. Granules. These membrane-limited inclusions, rather regular in shape, are quite variable in size (min 0.1 μm max 0.9 μm) and electron density. Their function is still not clear but they seem involved in the defense reactions processes of the organism against intruders: frequently they were observed while releasing their content in the medium through exocytosis (Fig. 5G). When abundant, granules identify a precise kind of hemocyte: the granular cell (Fig. 5A and H). A primary distinction is between structured and unstructured or amorphous granules: these categories were already employed in the past to characterize

other insect groups (Scharrer, 1972; Butt and Shields, 1996; Ribeiro and Brehélin, 2006).

Structured granules present electron-opaque filaments which are arranged in bundles stacked at different angles within the granule (Fig. 5C and E). The filaments resemble microtubular structures in cross-sections (0.0131 ± 0.0039 μm diameter) and their opacity is extremely variable. These inclusions are prevalently circular, ovoid or kidney-shaped and some images help to uncover the steps of their formation, as observed by Scharrer (1972) too. First tubular elements appear loosely scattered or restricted nearby the bounding membrane (Fig. 5D); then they progressively fill the entire inclusion body by forming densely packed array of bundles. In some cases the filaments do not invade the whole granule and the central zone become occupied by a granular or filamentous matrix of variable electron density.

Within the second type of granules we include both some elements analogous to what Moran (1971) called "opaque bodies" and other similar inclusion with different degrees of electron density. As a rule, these granules are without any discernable substructure and appear filled with granular-
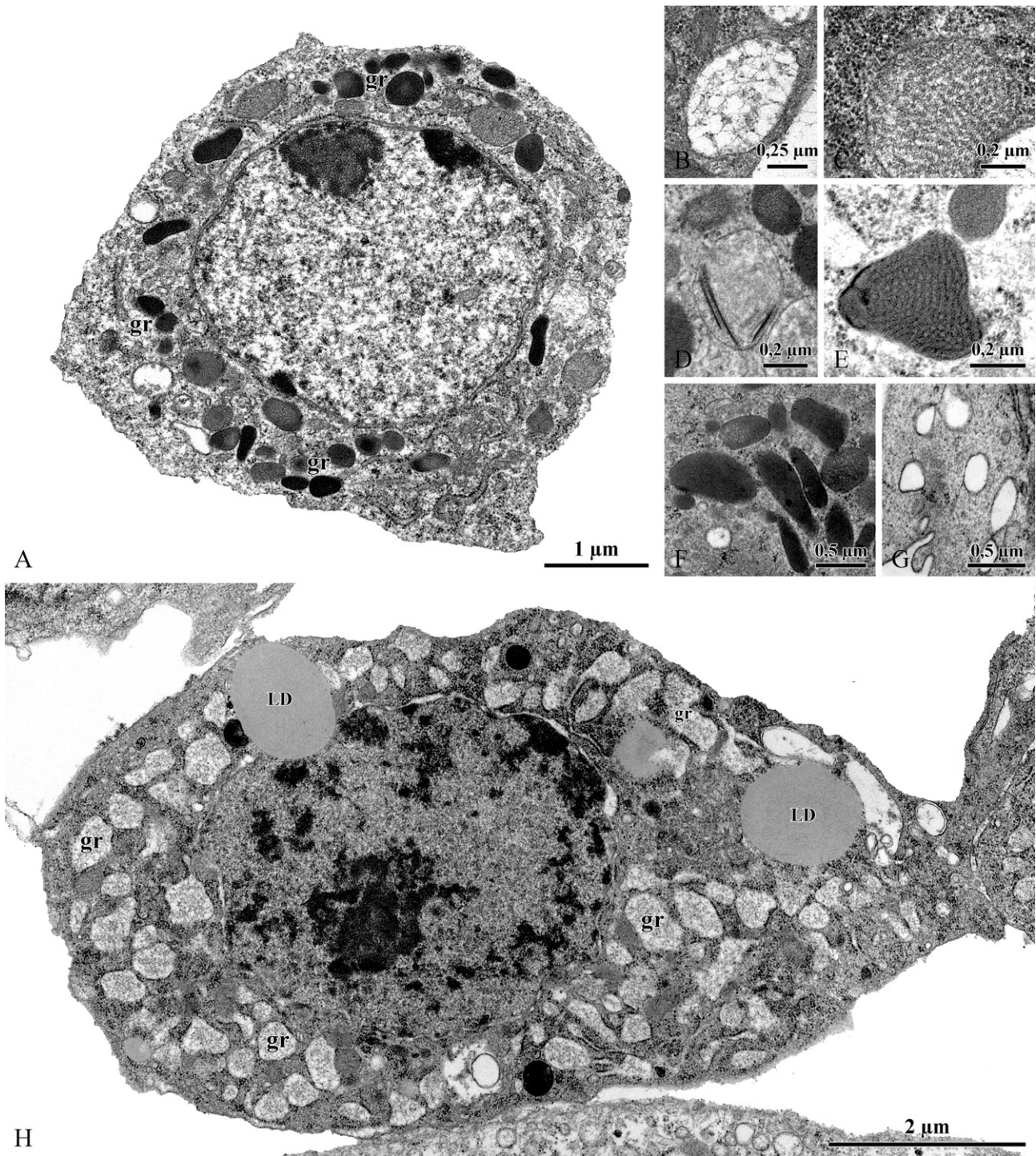
Fig. 5. The granulocyte. TEM. (A and H) Whole cells in different stages of growth: a young granular cell (A), with thin cytoplasm and few small granules (gr) and a mature granulocyte whose large cytoplasm shows many granular inclusions (H). LD: lipid droplets. (B and F) The two types of "opaque bodies": the electron-transparent granules (B) filled with granular-flocculent material and the electron-opaque bodies (H) filled with a dense, homogeneous matrix. (C–E) The structured granules: note the early steps in the formation of the inclusion with first tubular elements appearing nearby the bounding membrane (D). (G) Granular inclusions releasing their content in the medium through exocytosis.

flocculent material (electron-transparent granules; Fig. 5B) or with a dense, homogeneous matrix (electron-opaque bodies; Fig. 5F). Generally these inclusions are larger than the first type and present approximately a circular or ellipsoid shape.

In appearance there are no specific patterns in the distribution of the two types of granules: their frequency changes from cell to cell and many hemocytes show both types while others can present only one type. Moreover there is a great variability in morphology and degree of electron density and
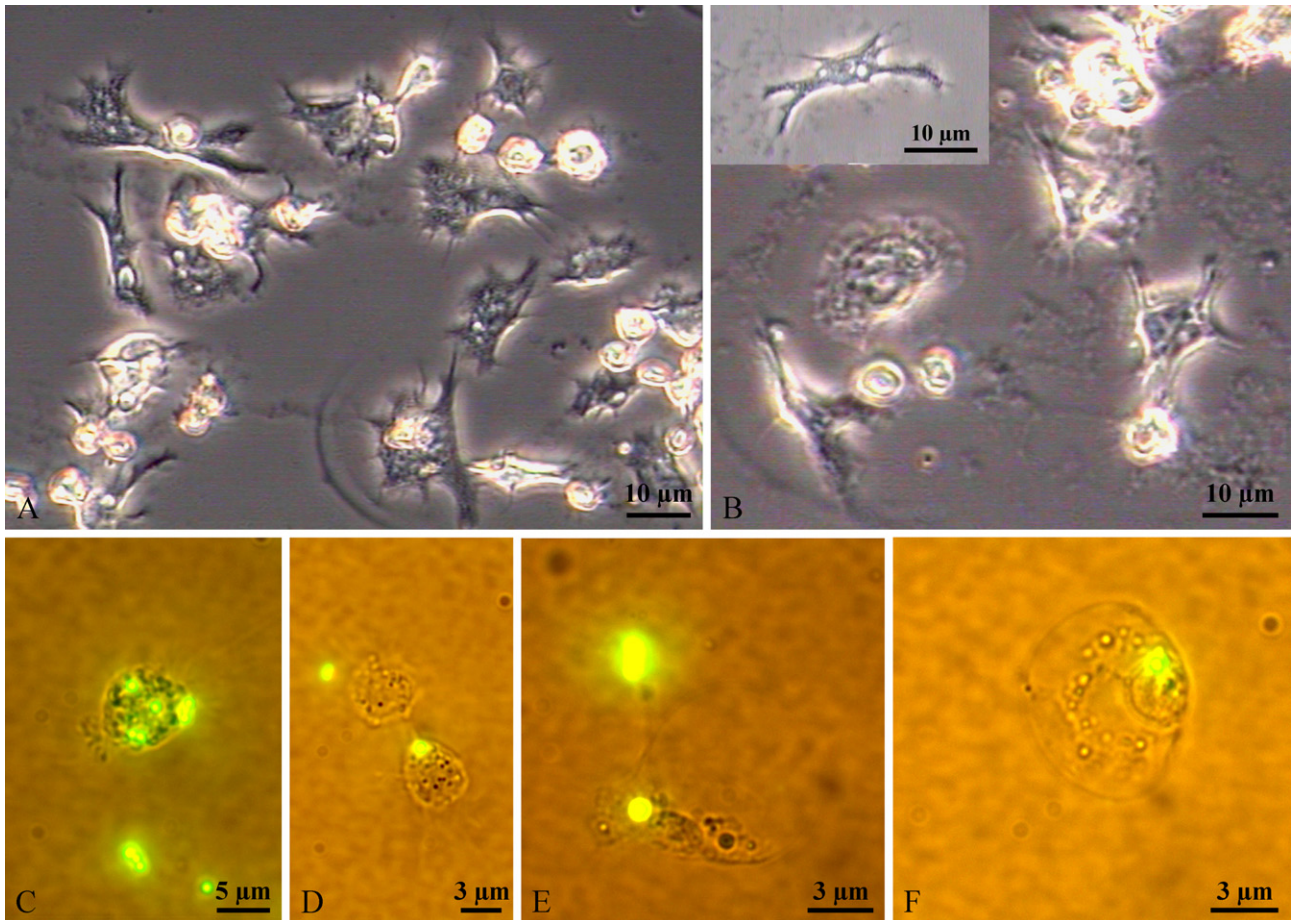
Fig. 6. Functional tests. (A and B) Adhesion on glass slides. Here are evident the two types of hemocytes yet described: the type "a" or granular cell, small, spherical and very refractive at phase contrast and the type "b" or plasmatocyte, which is larger, less refractive and shows a bidirectional fibroblastic extension (B, inset). (C–F) Phagocytosis of fluorescent beads.

we detected many intermediate forms between structured and amorphous granules: occasionally, unstructured granules revealed "masked" microtubular elements, as Scharrer (1972) had already observed. All these considerations support a strong relationship between the two types of inclusions and let us suppose that one type can convert into the other rapidly.

3.2. Functional tests

3.2.1. Adhesion on glass slides

In monolayers, most of *P. dominulus* hemocytes are able to adhere to a glass slide after 20 min incubation (Fig. 6A and B). We have observed two modalities of spreading according to as many cellular morphologies, recurrent in equivalent proportions. Some small hemocytes, very refractive in phase contrast, keep spherical and do not spread extensively: they adhere to the substrate with thin pseudopodia and lamellipodia which sometimes encircle the cell. Other hemocytes, on the contrary, larger and less refractive than the previous ones, spread rapidly and become thin cells characterized by a bidirectional fibroblastic extension (Fig. 6B, inset): they develop numerous pseudopodia and chiefly long and wide lamellipo-

dia. Their cytoplasm varies from round to enlarged, elongated and spindle-shaped.

Because of the size, the shape and the spreading modality, we can correlate the first variety of these adhering hemocytes with "type a" identified at light microscopy and with the granular cell of TEM observations; the other hemocytes, on the contrary, seem to be analogous to optical "type b" and TEM plasmatocytes.

3.2.2. Phagocytosis of fluorescent beads

Most of *P. dominulus* hemocytes were able to phagocytize fluorescent microspheres after 30 min incubation in the medium (Fig. 6C–F). We observed both hemocytes with a singular bead within the cytoplasm (Fig. 6D–F) and other with two or more (up to seven; Fig. 6C).

4. Discussion

The first problem we dealt with while characterizing the hemocytes of *P. dominulus* larvae was the difficulty to homologize our results with those of studies focused on other species which mostly belonged to other insect orders; for-

tunately we uncovered a certain similarity with hemocytes from Lepidoptera which is a well studied group of insects (Price and Ratcliffe, 1973; Brehélin et al., 1978; Brehélin and Zachary, 1983; Butt and Shields, 1996; Ribeiro et al., 1996; Ribeiro and Brehélin, 2006). We have followed a different approach with respect to the procedure adopted in last works on similar matter (for example, Kaaya and Ratcliffe, 1982; Hillyer and Christensen, 2002; Giulianini et al., 2003; Brayner et al., 2005). These Authors normally identify 4–7 different cellular types (variables depending whether they decide to include also prohemocytes and adipocytes or not) and they accurately describe them one by one chasing the following order: general morphological observations, ultrastructural analysis, functional assays. What's more, these studies usually support the "multiple-cell theory" about hematopoiesis and hemocyte differentiation, which suggest the existence of separate immutable cell lines, each differentiating from a single germinal stem, that give rise to the hemocyte types (Akai and Sato, 1973; Gupta, 1985).

In the case of *P. dominulus* larvae, we recognize no more than three types of hemocytes (we do not consider adipocytes) but, due to the substantial uniformity in external morphology and to the strong repetitiveness of most subcellular structures, we feel much closer to a "single-cell" theory (Scharrer, 1972). This is a more unitarian interpretation of the cellular elements of hemolymph which suppose that the various hemocyte types are merely stages, with separate functions, of a single cell line derived from a unique germinal stem i.e., the prohemocyte. This is in line with the great functional versatility of these complex and highly specialized cells which is required in order to achieve to so a disparate multitude of tasks (Rowley and Ratcliffe, 1981; Götz and Boman, 1985): a wide physiological flexibility is necessary to undergo ready transformation in response to environmental stimuli. The single-cell theory relies on the presence of transitional stages of some hemocyte types and on the existence of only prohemocytes and plasmatocytes in tissue culture and hematopoietic organs (Lavine and Strand, 2002). A more recent review on insect immunorecognition (Ottaviani, 2005) supports a limited number of hemocytes involved in defense reactions against invading organisms suggesting that, apart from the prohemocytes, probably two immunocytes (plasmatocytes and granular cells) appear to play a role in immune functions.

In our opinion there is only one feature that marks distinctively a precise hemocyte variety: the granules, which are a prerogative of granular cells. The other two types are agranular cells and they differ each other for the size (prohemocytes are evidently smaller) and for the nucleus/cytoplasm ratio which is much lower in plasmatocytes. In the case of *P. dominulus* no other feature seems to justify the identification of further hemocyte types and this is supported by first cytological investigations on honey bees (Chauvin, 1968), which are other hymenopteran insects presumably close to the wasps as regards to the cellular components of the hemolymph. For many reasons we consider the small, agranular cells as prohemocytes: the size first of all, the thin cytoplasm which is

a proof for a cell that is beginning its growth, the absence of phagolysosomes or granules, whose increase in number and size is a consequence of the activity of the cell, the centrioles (three cases observed). Despite the difficulty to individuate them, which is in line with the low mitotic index generally recorded in other insects (Rowley and Ratcliffe, 1981), these are extremely important organelles since they indicate that the cell has mitotic capacity (this confirms the prohemocyte, able of dividing as stem cell, see Brehélin and Zachary, 1986; Franchini et al., 1996) and because they are a clear sign of hemocytes ability to separate in circle i.e., when already circulating in the hemolymph. One should not take it for granted because still not long time ago it was not clear whether maintenance of hemocyte numbers depended mainly on hematopoiesis or on in circle division. Now we know that both processes probably operate, depending on the species examined and the stage in the life cycle (Rowley and Ratcliffe, 1981; Nappi and Carton, 1986; Lavine and Strand, 2002) even though it has been demonstrated that hemocytes can grow and multiply *in vitro* for an indefinite period, without the involvement of hematopoietic organs (Gupta, 1985).

There is a certain agreement about the origin and function of the typical granular cells inclusions, although accurate knowledges about them are still scanty nowadays. Granules seem to be synthesized by the Golgi apparatus and the final stage of their maturation is apparently the structureless, electron-dense body containing acid mucopolysaccharide-like substances (Rowley and Ratcliffe, 1981; Gupta, 1985; Ribeiro and Brehélin, 2006). With reference to their function, several observations suggest these inclusions are the storage form of the clotting protein, which reacts with the plasma coagulogen (a lipophorin) when released into the hemolymph to form the clot; moreover, it has been supposed that these inclusions contains phenol oxidase, an enzyme implicated in the process of melanization and in the tanning of the cuticle (Moran, 1971; Scharrer, 1972; Butt and Shields, 1996).

The substantial uniformity in the principal subcellular structures of *P. dominulus* hemocytes makes it difficult to classify them as distinct cellular types: these forms show a common history and probably it might be more correct to see them as functional states of a unique cellular line, which begin with the prohemocyte and leads to the granular cell passing through the plasmatocyte. As we know from literature, a main proof to sustain the "single-cell" theory are the "intermediate forms" i.e., hemocytes whose morphology do not satisfy any of these categories completely, because of an overlapping of features. In our observations, we have evidence of many representatives of this cell group. Hemocytes with very few little granules are no more plasmatocytes (= agranular cells) but not yet typical granular cells: they are transient hemocytes, evolving from one form to the other and we suppose they will progressively enrich their cytoplasm with granular inclusions, dependently to the activity of both the cell and the organism. On the other side, the controversial size and the presence of few small phagolysosomes make it difficult to assert whether some particular cells are prohemocytes or plasmatocytes. In

this case functional tests are helpful since they uncover the strong ability of plasmatocytes of adhering on glass slides and phagocytizing fluorescent beads, which are typical functions of a macrophage (Ottaviani, 2005). These observations are strongly supported by the analysis of Gupta (1985) on the evolution of hemocyte types: in taxa were only prohemocytes, plasmatocytes and granular cells are present, first two are evanescent stages and are not discernible as types while the differentiation after granular cells (i.e., spherule cells, oenocytoids, coagulocytes, etc.) is generally accompanied by distinct prohemocytes and plasmatocytes. In addition, in the more highly evolved taxa any of the types may be suppressed.

Hematopoiesis and hemocytes differentiation are still open questions since if we suppose that hematopoietic organs produce just prohemocytes and only prohemocytes can divide in circle, we should accept as a consequence that the hemocyte load of the organism is maintained exclusively by these two processes. We must also consider that hemocytes turn over is quite rapid referring to the autoradiographic studies on the lepidopteron *Galleria mellonella* (Rowley and Ratcliffe, 1981): these Authors counted 3 days for differentiation (from prohemocyte to granular cell) and other 3 days to complete life cycle under *in vitro* conditions, which can quite differ from the situation *in vivo*. The only consideration that we may do after our observation is that during *P. dominulus* life span there must be a change in the mechanism of hematopoiesis at a certain point, probably at the end of larval development, since we found a much lower hemocyte load in the adults than in 3rd/4th instar larvae: this trend is in line with the analysis about the relationship between hemocyte load and age of workers in honey bees (Chauvin, 1968). From an ecological perspective, we might explain this disparity thinking on the immune role of hemocytes and highlighting the importance of fitness maintenance in the whole organism. Adult wasps are provided with a toughened cuticle which is itself an efficient barrier against parasites while larvae are an easier target for invading organisms and must rely on a more competent immune system to survive: this is the reason why they must invest more energetic resources to maintain a greater hemocyte load. Moreover, from the 'ecological immunology' point of view (Siva-Jothy et al., 2005) contrary to larvae adults need to find a balance between the application of resources for survival and reproduction (Meylaers et al., 2007).

Acknowledgments

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