

**HEMOCYTE TYPES AND TOTAL AND DIFFERENTIAL  
COUNTS IN UNPARASITIZED AND PARASITIZED  
*Anastrepha obliqua* (DIPTERA, TEPHRITIDAE) LARVAE**

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Received August 31, 2001 – Accepted November 12, 2001 – Distributed November 30, 2002

(With 25 figures)

**ABSTRACT**

The hemocyte types, in addition to total and differential hemocyte counts were studied in parasitized and unparasitized *Anastrepha obliqua* larvae at the beginning and at the end of the third instar. In both developmental phases, in parasitized and unparasitized larvae, prohemocytes, plasmatocytes, granulocytes, adipohemocytes, spherulocytes and oenocytoids cells were observed. Mitotic figures indicate prohemocytes as stem cells. Prohemocytes, plasmatocytes and granulocytes are the most numerous cells in the hemolymph of *A. obliqua*. Difference in the total number of hemocytes was observed between unparasitized and parasitized larvae at the end of the third instar, but not at the beginning.

*Key words:* hemocytes, *Anastrepha obliqua*, fruit fly, parasitism.

**RESUMO**

**Tipos de hemócitos e contagem total e diferencial em larvas parasitadas e não parasitadas de *Anastrepha obliqua* (Diptera, Tephritidae)**

Os tipos de hemócitos e as contagens total e diferencial foram estudados em larvas parasitadas e não parasitadas de *Anastrepha obliqua* pertencentes ao início e ao final da terceira fase. Em ambas as fases do desenvolvimento, tanto em larvas parasitadas quanto nas não parasitadas, foram observados pró-hemócitos, plasmatócitos, granulócitos, adipo-hemócitos, esferulócitos e oenocitóides. A presença de divisões mitóticas indica os pró-hemócitos como células-tronco. Pró-hemócitos, plasmatócitos e granulócitos são as células mais numerosas na hemolinfa de *A. obliqua*. Foi observada diferença no número total de hemócitos entre larvas parasitadas e não parasitadas apenas no final da terceira fase.

*Palavras-chave:* hemócitos, *Anastrepha obliqua*, mosca-da-fruta, parasitismo.

## INTRODUCTION

*Anastrepha obliqua*, a member of the Tephritidae family (Insecta, Diptera), is a pest of tropical fruits, since its larvae, feeds on the fruit pulp (Bressan, 1987), turning them non viable for consumption, causing considerable economic losses. This fruit fly species is parasitized by some parasitoid wasps (Malavasi *et al.*, 1980; Wharton, 1989; Canal *et al.*, 1996). This could be used in their biological control. The exit of the control method, however, is directly linked to the successful development of endoparasitoids into the larval bodies, which depends on the ability to avoid encapsulation by the host immune system.

The cellular component of the capsules surrounding the parasitoids is formed by hemocytes (Ratcliffe, 1993), whose concentration in the hemolymph changes in parasitized larvae (Nappi & Carton, 1986; Eslin & Prévost, 1998). The population of circulating hemocytes may indicate whether the host defense system was activated or not (Brehélin, 1982), and whether any depression in the number of hemocytes contributes to the protection of the parasite (Rizki & Rizki, 1980, 1992).

As the hemocytes are the cells involved in defense reactions in insects, they are very useful to understand the host-parasite interactions.

Since, the characterization of the hemocytes of *A. obliqua* is rarely encountered in the literature, we describe here the hemocyte types, as well as the total and differential hemocyte counts in the hemolymph of parasitized and unparasitized larvae of this fruit fly.

## MATERIAL AND METHODS

### Insects

*Anastrepha obliqua* larvae were obtained from infested fruits of *Spondias lutea* (cajá-mirim) collected in the gardens of the Universidade de São Paulo, in Ribeirão Preto, SP, Brazil. Only third instar larvae were used because they are larger and more abundant in the fruits collected than the first instars. Due to the long duration of the third instar (about 14 days), these larvae were divided as belonging to the beginning and the end of the third instar. They

were identified according to Teles da Silva (1978).

### Hemocytes characterization

For light microscopy (LM), the larvae were washed in distilled water and placed on ice for immobilization. Hemolymph was obtained by cutting laterally the anterior region of the larvae with a micro scissors. Hemolymph was bled directly on a glass slide and allowed to dry in natural air conditions for 20-30 minutes. During this time the hemocytes adhered to the glass. Cells were then fixed in methanol for 5 min. After natural air-drying of the fixative, hemocytes were stained with Giemsa-Rosenfeld for 3-4 minutes and slides were rapidly washed with bidistilled water. After air drying the slides were dehydrated and mounted in Entellan.

For Scanning Electron Microscopy (SEM), hemolymph was bled directly on termanox discs after cutting the cuticle. The hemolymph slides were allowed to dry for 20-30 minutes. Then, they were fixed in 70% ethanol for 10 minutes and placed in a chamber to dry for a week. Small pieces of termanox containing hemocytes were mounted on stubs, coated with gold and examined in a JSM 5200 SEM.

### Hematology

Hemolymph was obtained from the *A. obliqua* third instar larvae by cutting their anterior region with a micro scissors. After bleeding, the larvae were dissected to verify the presence of parasitoids (eggs or larvae).

Hemocyte counts were performed on individual larvae. Total hemocyte counts (THCs) were done by applying diluted hemolymph (1  $\mu$ l hemolymph added to 5  $\mu$ l 0.1M phosphate buffer, pH 7.2) to a Neubauer hemocytometer. THCs were expressed as number of cells per  $\mu$ l of hemolymph. Differential hemocyte counts (DHC) were realized on hemolymph slides stained with Giemsa-Rosenfeld. One hundred fifty cells identified from four randomly selected fields were counted per larvae. DHCs were expressed as the mean of each hemocytes type in the total cells counted.

Differences in the THC and DHC results were tested with Student's t-test ( $p \leq 0.05$ ), using Sigma stat. 2.0 software.

## RESULTS

### *Hemocyte types*

Six well-defined hemocyte types were distinguished in the hemolymph of parasitized and unparasitized *A. obliqua* larvae at the beginning and the end of the 3rd instar: prohemocytes, plasmatocytes, granulocytes, adipohemocytes, oenocytoids and spherulocytes.

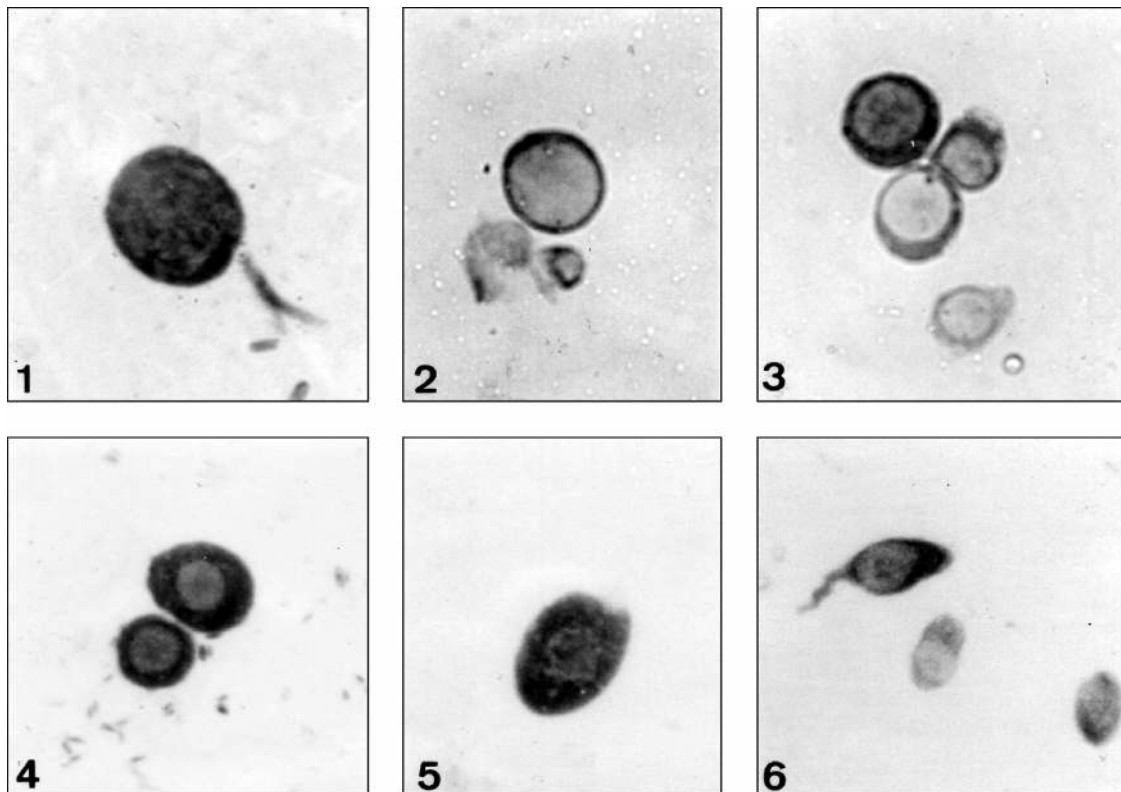
### *Prohemocytes (PR)*

These cells are the smallest hemocytes encountered in the hemolymph. They can be spherical (7.5-13.12  $\mu\text{m}$  in diameter) or oval in shape (5.62  $\times$  7.5  $\mu\text{m}$  to 13.12  $\times$  22.5  $\mu\text{m}$ ). The large and centrally located nucleus (5.6-11.25  $\mu\text{m}$  in diameter) nearly fills the cell so that the

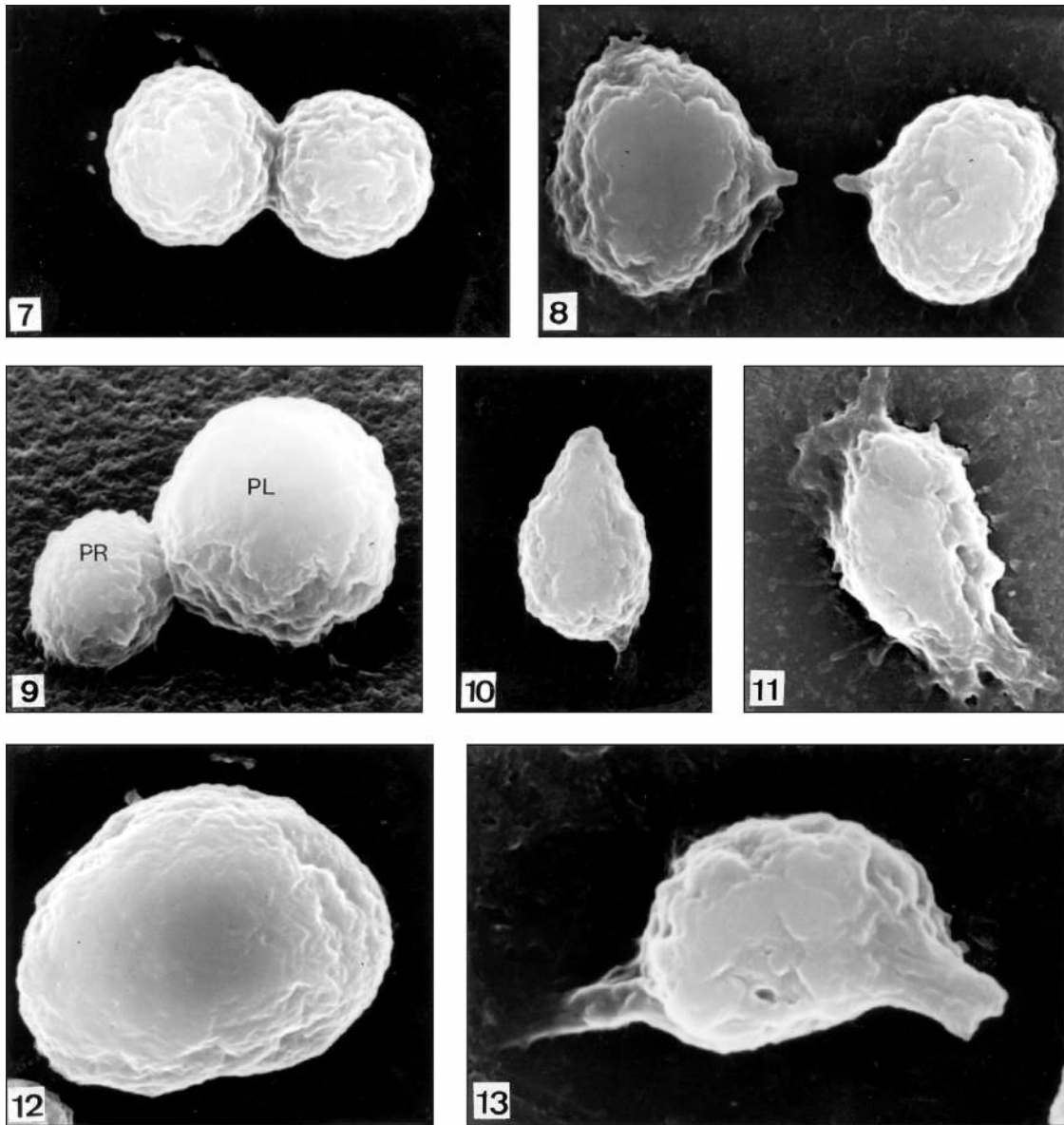
cytoplasm occupies a narrow area around the nucleus, which shows a spherical shape (Figs. 1-3). The surface of the cell, under the microscope, seems to be slightly irregular. They were the unique hemocyte type presenting features suggestive of mitotic figures (Figs. 7-8).

### *Plasmatocytes (PL)*

The plasmatocytes are highly polymorphic cells; they show rounded, oval, spindle-shaped or sometimes irregular form, viewed under LM (Figs. 4-6) and SEM (Figs. 9-11). Plasmatocytes are also variable in size. When spherical in shape, they present 13-26  $\mu\text{m}$  in diameter. When oval, they are 26-34  $\mu\text{m}$  long and 15-30  $\mu\text{m}$  wide. The nuclei can be spherical (9-15  $\mu\text{m}$  in diameter) or oval (7-9.5  $\mu\text{m}$  long and 9.5-1.5  $\mu\text{m}$  wide).



**Figs. 1-6** — Hemocytes of *A. obliqua* larvae, after Giemsa-Rosenfeld staining: prohemocytes (Figs. 1-3: X640) and plasmatocytes (Figs. 4-6: X330).



**Figs. 7-13** — Hemocytes of *A. obliqua* by SEM: prohemocytes in mitotic cytokinesis (Figs. 7-18: X5000), plasmotocytes (Figs. 9-11, 9: X7500 and 10-11: X5000), granulocyte (Fig. 12: X5000) and adipohemocyte (Fig. 13: X7500).

The majority of the PLs are mononucleated, but some binucleated cells were occasionally observed. The irregular shape of the cells is due to cytoplasmic extensions. After the Giemsa staining, PLs show a variable number of granu-

les, which can be negative or positively stained. Normally the negative granules are smaller than the positive. PLs and PRs presenting same size and shape can be distinguished one from another by the higher nuclear/cytoplasmic ratio in the latter.

**Granulocytes (GR)**

After Giemsa staining, the GRs are recognized by their numerous small and highly basophilic granules, which are present in the clear bluish cytoplasm. These hemocytes are variable in shape and size. They can be large or small hemocytes and have a spherical (15-22.25  $\mu\text{m}$  in cellular diameter) or oval shape (13-18  $\mu\text{m}$  long and 15-34  $\mu\text{m}$  wide). Spherical and oval GRs present rounded nuclei with 5.6-11.3  $\mu\text{m}$  in diameter. The nucleus also basophilic generally occupies a central position (Figs. 12, 14-16). The phagocytic activity of the GRs was observed in relation to the yeast present in the hemolymph of the flies (data not shown). Cells with intermediate features between PLs and GRs were also observed.

**Oenocytoids (OE)**

These cells can be spherical (22-35.5  $\mu\text{m}$  in diameter) or oval (18.7-26  $\mu\text{m}$  long and 26.5-35.6  $\mu\text{m}$  wide) in shape, and present nuclei with the same general shape of the cell. Spherical nuclei are 7.5-11.3  $\mu\text{m}$ .

After Giemsa staining the OEs exhibit a moderate acidophilia, and reveal a homogeneous cytoplasm containing fine and weak acidophilic granulation (Fig. 17). Cells with intermediate characteristics between GRs and OEs were frequent in the slides observed.

**Adipohemocytes (AD)**

These cells are polymorphic (Figs. 18-21). They can be large or small in size, and have an oval or irregular shape. After Giemsa staining, the cytoplasm shows a high basophilia and a variable number of large refringent lipid-like inclusions, which sometimes obscure the nuclei contour, and appear prominent on cell surface (Figs. 13, 20-21).

**Spherulocytes (SP)**

The spherulocytes are variable in shape; they can present a regular or irregular shape. The cytoplasm is characterized by the presence of highly basophilic or acidophilic spherules and small spherical vacuoles (Figs. 22-23). Cells presenting intermediate characteristics between plasmatocytes

and spherulocytes were encountered in the hemolymph (not showed).

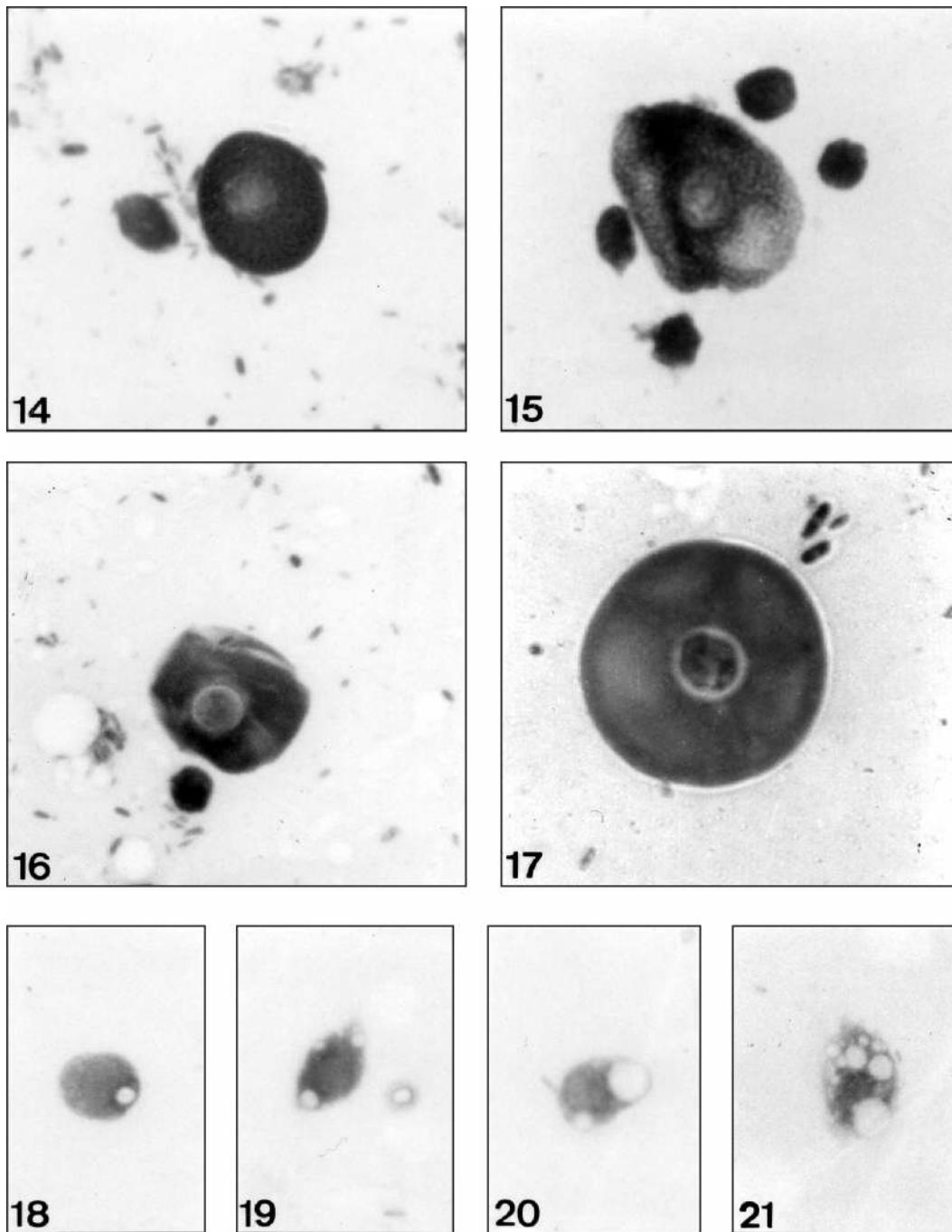
The Figs. 24 and 25 show hemocytes encountered in the hemolymph of *A. obliqua* whose type was not identified.

**Total (THC) and Differential (DHC) Hemocyte Counts**

The mean of the total hemocyte counts of parasitized larvae was not significantly different from unparasitized larvae at the beginning of the 3rd instar, but was significantly higher at the end of the instar (Table 1).

By means of the DHC it was verified that at the beginning of the 3rd instar 97% and 94.9% of the total circulating hemocytes were comprised of prohemocytes and plasmatocytes in both parasitized and unparasitized larvae, respectively (Table 2). The number of prohemocytes was not statistically different in parasitized ( $36.11 \pm 6.82$ ) and unparasitized ( $38.0 \pm 12.55$ ) larvae ( $t = 0.33$ ; g.f. = 13;  $p = 0.05$ ). Similar results were also obtained for plasmatocytes and granulocytes (Table 2). The granulocytes are among the most frequent hemocyte type in the hemolymph, but their relative proportion remained lower than 4% (Table 2). The remaining hemocyte types are present in the circulation, but their proportion was never greater than 1.0% (Table 2).

Despite the general similarities in the average number and percentage of hemocytes in the two analyzed phases of the third larval instar, some distinct alterations were noted. At the end of the instar, the proportion of prohemocytes and plasmatocytes in parasitized and unparasitized larvae diminishes to 87.8% and 80.5% respectively. In addition, granulocyte proportion increased from 11% in parasitized larvae to 18.1% in unparasitized (Table 2). The proportion of the other cell types remained unaltered, not comprising more than 1%. As showed in Table 2, in this phase the number of prohemocytes was significantly greater in parasitized larvae ( $67.8 \pm 17.14$ ) than in unparasitized ( $49.5 \pm 12.12$ ), but no statistically significant difference was encountered between these larvae in relation to the mean number of plasmatocytes and granulocytes.



**Figs. 14-21** — Hemocytes of *A. obliqua* larvae, after Giemsa-Rosenfeld staining: granulocytes (Figs. 14-16, 14: X270 and 15-16: X410), oenocytes (Fig. 17: X410), and adipohemocytes (Figs. 18-21: X280).

TABLE 1

Total hemocytes counts in the hemolymph of parasitized and unparasitized *A. obliqua* larvae, at the beginning and at the end of the 3rd instar. Results (mean  $\pm$  SD) are given in hemocytes counter/ $\mu$ l.

| Larvae        | Phases of the third instar |                   |
|---------------|----------------------------|-------------------|
|               | Beginning                  | End               |
| Unparasitized | 147.0 $\pm$ 10.6           | 210.0 $\pm$ 144.5 |
| Parasitized   | 345.0 $\pm$ 51.1*          | 505.4 $\pm$ 48.0* |

Means in the same column followed by asterisks differ significantly (t-test: -2.71; d.f.: 12;  $p = 0.019$ ).

TABLE 2

Differential hemocytes counts (means  $\pm$  s.d.) and percentage in the hemolymph of unparasitized and parasitized *A. obliqua* larvae, at the beginning and at the end of the 3rd instar.

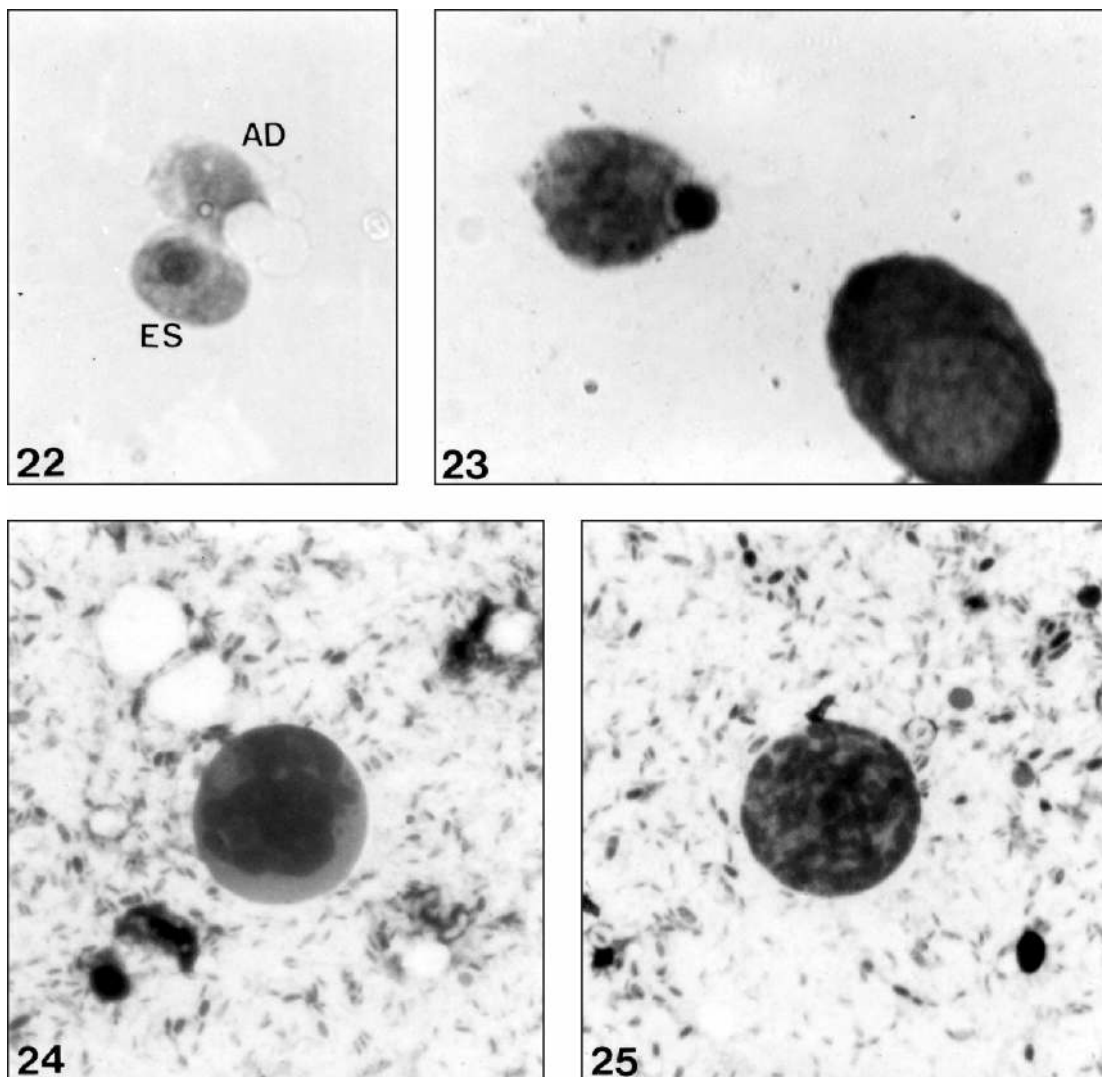
| Phase of the third instar | Hemocytes types | Unparasitized    |      | Parasitized      |      |
|---------------------------|-----------------|------------------|------|------------------|------|
|                           |                 | Mean $\pm$ sd    | %    | Mean $\pm$ sd%   | %    |
| Beginning                 | PR              | 38.0 $\pm$ 12.5  | 37.5 | 36.11 $\pm$ 6.8  | 35.2 |
|                           | PL              | 58.5 $\pm$ 6.8   | 57.6 | 63.33 $\pm$ 4.0  | 61.8 |
|                           | GR              | 3.83 $\pm$ 3.8   | 4.0  | 2.84 $\pm$ 2.0   | 62.4 |
|                           | OE              | –                | 0.3  | –                | 0.1  |
|                           | SP              | –                | 0.3  | –                | 0.1  |
|                           | AD              | –                | 0.3  | –                | 0.4  |
| End                       | PR              | 49.5 $\pm$ 12.1* | 38.0 | 67.8 $\pm$ 17.1* | 50.4 |
|                           | PL              | 55.5 $\pm$ 6.87  | 42.5 | 51.2 $\pm$ 15.5  | 37.3 |
|                           | GR              | 23.7 $\pm$ 13.8  | 18.1 | 15.1 $\pm$ 13.4  | 11.0 |
|                           | OE              | –                | 1.1  | –                | 0.7  |
|                           | SP              | –                | 0.1  | –                | 0.1  |
|                           | AD              | –                | 0.2  | –                | 0.5  |

Means in the same line followed by asterisks are significantly different (t-test: -2.90; g.f.: 18;  $p \leq 0.05$ ). AD: adipohemocytes, Gr: granulocytes, OE: oenocytoids, PL: plasmacytes, PR: prohemocytes, SP: spherulocytes.

## DISCUSSION

*A. obliqua* 3rd instar larvae present six hemocytes types in the hemolymph, all easily distinguishable in smears, after Giemsa staining, namely: prohemocytes, plasmacytes, granulocytes, adipohemocytes, spherulocytes and oenocytoids. Under SEM, however, we were able to recognize only the first three types.

Gupta (1985) describes seven basic types of hemocytes, common among the different Orders of insects: the six cellular types identified in the present study plus the coagulocytes. According to Arnold (1974), coagulocytes are very fragile cells that can be observed "in vitro", only. So being, it is not impossible that *A. obliqua* has this hemocyte type and that the utilized methodology has not been able to discriminate it.



**Figs. 22-25** — Hemocytes of *A. obliqua* larvae, after Giemsa-Rosenfeld staining: spherulocytes (Figs. 22-23: X210) and not identified hemocytes (Figs. 24-25: X340).

In spite of the difficulty to compare the hemocytes among different species, the morphology described here for *A. obliqua* and the data already published make this comparison possible.

Prohemocytes are unmistakable because of their small size and large nucleus-cytoplasm ratio. Consequently, prohemocytes of *A. obliqua* certainly correspond to the prohemocytes described by several authors for another insects (Gupta, 1979; Mall & Gupta, 1980; Barraco & Cestari, 1984; Gianotti & Caetano, 1985; Chiang *et al.*, 1988; Fenoglio *et al.*,

1993; Russo *et al.*, 1994; among others). Plasmacytes are the most variable cell in shape observed in the hemolymph smears of *A. obliqua*. They can be rounded, fusiform or completely irregular in shape due to cytoplasm expansions. Plasmacytes emitting filamentous expansions have been described in many dipterans (Whitten, 1964; Barraco & Cestari, 1984). In *A. obliqua*, only plasmacytes present cytoplasm expansions. In the literature, the elongated cells or fusiform cells are identified as vermiform cells (Devauchelle, 1971; Gianotti & Caetano, 1985).



Granulocytes of *A. obliqua* appear to correspond to the granular hemocyte, cited by Gillespie *et al.* (1997) and reported by other investigators (Arnold & Hinks, 1976; Barraco & Cestari, 1984; Gianotti & Caetano, 1991). They are easily identified by their large size and cytoplasm characteristically filled with basophilic granules in Giemsa or methylene blue-stained smears. In *A. obliqua* larvae, emission of pseudopods was not observed in the granulocytes. This data agrees with the observation of the granulocytes in some insect species (Arnold, 1974; Chiang *et al.*, 1988), but is in disagreement with the characterization given by Olson & Carlson (1974) and Barraco & Cestari (1984), who described these cells as having cytoplasmic extensions.

Adipohemocytes in *A. obliqua* are hemocytes exhibiting a large lipid like vesicle, sometimes large enough to deform the cell. Their characteristics are similar to those observed in other insect species (see Chiang *et al.*, 1988; Russo *et al.*, 1994). Adipohemocytes have been regarded as granulocytes variants by some authors (Price & Ratcliffe, 1974; Kaaya *et al.*, 1986). Oenocytoids can be as large as the granulocytes or larger. In *A. obliqua*, they can be distinguished from the former by their fine granulation and, as occurs in other insects (Barraco & Cestari, 1984), by their acidophilic cytoplasm observed after Giemsa staining. This type of hemocyte is not encountered in all insect species (Russo *et al.*, 1994). Spherulocytes are easily distinguished from the other hemocytes by the presence of small basophilic or acidophilic spherules distributed in the cytoplasm. However, in the present study, spherulocytes do not show size as large as described by other authors (see Fenoglio *et al.*, 1993; Gianotti & Caetano, 1985). Furthermore, those types do not occur in all insects (Russo *et al.*, 1994).

The theories about post-embryonic origin of hemocytes in insects are based on the cellular types encountered in the hemolymph. From the morphological observations presented in this study, a scheme has been devised outlining the possible relationships among the six types of hemocytes in *A. obliqua*. The prohemocyte is the most abundant hemocyte in the hemolymph and also the only one seen in cytokinesis. These facts have suggested a role of stem cells to them and agree

with the theories of Gupta (1985) and Arnold (1979) related to the origins of hemocytes.

The intermediate forms between prohemocytes and plasmatocytes suggest the growth and differentiation of the later from prohemocytes. In a similar way, the origin of granulocytes, adipohemocytes and spherulocytes from plasmatocytes, in *A. obliqua*, is indicated by the presence of numerous cells presenting intermediate forms, and characteristic staining properties. Plasmatocytes, as pluripotent cells capable of producing granulocytes and spherulocytes, have also been suggested in silkworms because of the presence of intermediate forms of hemocytes observed in the hemolymph (Beaulaton, 1979). This potentiality of the plasmatocytes is in agreement with the single-cell origin theory proposed by Arnold (1979). In the dictyopterans species (Fenoglio *et al.*, 1993), however, intermediate features of granulocytes and spherulocytes led to hypothesize a developmental relationship between both, as postulated by Gupta (1985).

According to our findings, the occurrence of cells with intermediate morphological characteristics and staining after Giemsa treatment indicate the transformation of granulocytes in oenocytoids. This fact is in accordance with the theory of Gupta (1985), which proposes granulocytes with potentiality to differentiate in adipohemocytes, spherulocytes and oenocytoids, but disagrees with the single-cell and multiple-cell origin theories proposed by Arnold (1979).

In relation to circulating hemocyte counts, our results showed a greater number of hemocytes in parasitized than in unparasitized larvae at the end of the 3rd instar but not at the beginning. These findings are in agreement with those obtained by Silva (2000), which detected encapsulation process against parasitoids only at the end of the 3rd instar *A. obliqua* larvae. According to DHC this greater number of hemocytes is on account of increase in the prohemocytes number.

The increase in the hemocytes number in the hemolymph of insects is a normal response to parasitism (Nappi, 1981; Eslin & Prévost, 1998). Therefore, the difference in hemocytes population between unparasitized and parasitized *A. obliqua* larvae indicates that the host defense system was activated (Nappi, 1981; Brehélin, 1982).

The present work contributes to characterization of the hemocytes of *A. obliqua*. However, further analysis are still required to bring additional details about the functions of each hemocyte type, in order to understand how the host is able to respond to endoparasitism in a cellular manner.

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