Chapter 3

COELOMOCYTE PROFILE
3.1 Introduction

Coelomocytes are the major immune cells of the earthworms. Several functions have been attributed to the coelomocytes. These include the ability to recognize and eliminate foreign materials, primarily by phagocytosis and encapsulation (Stein and Cooper, 1983; Field et al., 2004), and to participate in blood clotting, wound healing and in some aspects of nutrition and excretion. Coelomocytes take part in graft rejection and in the phenomenon of antigen recognition. They are involved in cellular immune reactions and nodulation. (Stein and Cooper, 1981; Armstrong 1991; Valembois et al., 1992; 1994; Cooper and Roch, 1992; Cooper, 1996; Adamowicz and Wojtaszek, 2001).

Earthworm coelomocytes are very convenient to work with and easy to be handled in vitro. They may be retrieved from the coelomic cavity without killing the animals. Therefore, the coelomocytes can be used as convenient model for basic and applied research. Hence earthworms are often used as model soil organisms for ecobiological studies.

The coelom in earthworms is a large cavity that extends through the length of the body, and is filled with coelomic fluid. It is surrounded on the outer side by the parietal peritoneum and on the inner side by the visceral peritoneum covering the alimentary canal. Transverse septa divide it into segmental portions. The peritoneum covering these
septa is similar in structure to that covering the inner surface of the muscle layers. In a few species, the peritoneum in the septa is so much thickened that it almost fills the coelom in this region. In *Lampito mauritii* and *Dichogaster annae*, it is in general, thin and membranous. The septa are usually perforated by pores, which allow the coelomic fluid to pass freely between segments, although some septa effectively isolate each body segment and make it virtually self sufficient with its own supply of blood, nervous system, excretory system and even gonads. Stephenson (1930) stated that sphincters are not present in most genera of Megascolecidae. Other authors have suggested that most earthworms have septa with at least one sphinctered opening.

The coelomic fluid is usually milky white, but may sometimes appear yellowish in *L. mauritii* and *D. annae*. The coelomic fluid of *Eisenia foetida* smells of garlic, hence the name of this species. The consistency of the coelomic fluid differs between different species of earthworms and also depends upon the humidity of the air in which the worms live. Thus it is thicker and more gelatinous in worms in dry situations than in those from wetter habitats. In many areas of the body, the visceral peritoneum is specialized to form bright yellow or orange coloured chloragogen tissue. Chloragogen is involved in glycogen and lipid synthesis and storage, amino acid deamination, and the synthesis of ammonia and urea. It has high iron concentration and may be the site of haemoglobin synthesis or breakdown. Coelomocytes are derived from the peritoneum.
or from specialized structures associated with the epithelium (Valembois, 1971).

Many earthworms eject coelomic fluid through the dorsal pores, in response to mechanical or chemical irritation, or when subjected to extremes of heat or cold. Some species, such as *Megascolides australis* can eject fluid to a height of 10 cm and *Didymogaster sylvaticus* (known as the squirter earthworm) to a height of 30 cm. Coelomic fluid is also expelled through the dorsal pores at times of stress and may have several functions such as preventing desiccation, promoting cutaneous respiration or providing protection from predators (Vail, 1972).

The coelomic fluid contains many different kinds of particles in suspension. The inorganic inclusions are mainly crystals of calcium carbonate. But the corpuscular bodies in the coelomic fluid of lumbricid worms include mainly the phagocytic amoebocytes, which can engulf waste materials. The ceolomic fluid of the earthworms exhibits antibacterial, hemolytic and haemagglutinating activities mediated by proteins (Stein et al., 1990; Lassegues and Valembois, 1994). These proteins were called fetidins (Milochau et al., 1997).

In the present study an attempt has been made to identify and characterize the different types of cells in the coelomic fluid and to make an enumeration of these in the two species, *L. mauritii* and *D. annae*. 
3.2 Materials and Methods

The earthworms, *L. mauritii* and the *D. annae* were collected from the field by tilling the soil and by hand picking. These were brought to the laboratory and maintained in separate earthen pots. The pots were filled with alternating layers of cowdung and leaf litter to a depth of 1 to 1½ ft. To prevent the escape of the earthworms, the pots were covered with cotton cloth, which also allowed ventilation. After an initial period of restlessness, both the species of earthworms seemed to get acclimatized to the new surroundings. The adult clitellated earthworms were then drawn from the pot for collecting the coelomic fluid.

3.2.1 Coelomocyte Retrieval

Coelomic fluid was collected from the two species of earthworms separately. The method of Roch (1979) was used for collecting the coelomic fluid. The worms were given electric shock using a 5 V (DC) cell at intervals for a period of 1 minute. The exudate containing coelomocytes were used for total and differential counts and for cytological observations. The creamy-white to yellowish coelomic fluid was collected over cavity slides before drawing the fluid into haemocytometer. The coelomic fluid was diluted as per the procedure adopted by Archer (1977). Samples of ten worms from each of the two species were studied.
3.2.2 Coelomocyte Counts

The fluids with the extruded cells were then used for coelomocyte counts. For the determination of total counts of coelomocytes, the coelomic fluid was diluted with dilution fluid, i.e. earthworm Ringer solution with (Drewes and Pax, 1974) 3% acetic acid (Table 3.1). The coelomocyte counts were made using haemocytometer under the light microscope (Rushton, 1945). Replicate counts were made on coelomic fluid samples from ten worms each belonging to the two species.

For differential count, smears were prepared from the coelomic fluid. The air dried smears were then stained with Giemsa/Wright's stain. The stained slides were then observed under the oil immersion objective of a research microscope (Olympus) for morphological features. Photomicrographs of selected samples were taken. Differential counts of the coelomocytes were made by preparing ten sample slides for each species of earthworms belonging to *L. mauritii* and *D. anae*. 

The results were statistically analyzed with Student's t test for independent data. Probability level of *P*<0.05 is considered statistically significant.
<table>
<thead>
<tr>
<th>Contents</th>
<th>Composition (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>77.0</td>
</tr>
<tr>
<td>K⁺</td>
<td>4.0</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>6.0</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1.0</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>43.0</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>26.0</td>
</tr>
<tr>
<td>Tris</td>
<td>2.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>55.0</td>
</tr>
<tr>
<td>mOSM</td>
<td>167.0</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Table 3.1 Composition of earthworm Ringer solution (Drewes and Pax, 1974)
3.3 Results

In general, three categories of coelomocytes have been characterized in the coelomic fluid of *L. mauritii* and *D. annae*. These include phagocytic amoebocytes, granulocytes and eleocytes. Other inclusions in the coelomic fluid included breakdown products of the corpuscular bodies, protozoa, and nematode parasites and bacteria. Immature stages of coelomocytes of varying size and of different cell lineages could be seen in both the species.

Although morphology of the coelomocytes of the two species differed considerably, the total counts and differential counts of the two species did not show any significant difference.

3.3.1 *Lampito mauritii*

Total Count

Total count of coelomocytes in *L. mauritii* is presented in (Fig. 3.1 and Table 3.3).

Differential Count

The proportion of the different types of cells is given in Figure 3.2 and Table 3.4. The main features of the different types of cells are summarized in Table 3.2.

In most slides immature stages of the coelomocytes could be noticed. Many cells were also found to be in different phases of cellular aging.
Fig 3.1  Total count of coelomocytes in *L. mauritii* and *D. annae*
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Percent of Total population</th>
<th>Shape</th>
<th>Size (approximate diameter in μm)</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
<th>Pseudopodia Number and shape</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>position</td>
<td>Size in μm</td>
<td></td>
</tr>
<tr>
<td><em>L. mauritii</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoebocyte I</td>
<td>42.6 ± 2.5</td>
<td>Irregular</td>
<td>8.0 – 10.5 μm</td>
<td>central</td>
<td>4 – 5.2 μm</td>
<td>hyaline few lobopodia</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granolocyte</td>
<td>28 ± 2.2</td>
<td>rounded</td>
<td>9.5 – 10.75 μm</td>
<td>eccentric</td>
<td>4.0 – 4.9 μm</td>
<td>hyaline numerous filopodia</td>
</tr>
<tr>
<td>Eleocyte</td>
<td>26 ± 0.5</td>
<td>rounded</td>
<td>17.0 – 20.5 μm</td>
<td>eccentric</td>
<td>2 μm</td>
<td>with numerous chloragosomes rarely few lobopodia</td>
</tr>
<tr>
<td><em>D. annae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoebocyte I</td>
<td>47 ± 3.2</td>
<td>Irregular</td>
<td>9.2 – 12.4 μm</td>
<td>central</td>
<td>4.2 – 5.6 μm</td>
<td>hyaline few lobopodia</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granolocyte I</td>
<td>26 ± 1.5</td>
<td>rounded</td>
<td>9.6 – 11.6 μm</td>
<td>eccentric</td>
<td>4.5 – 6 μm</td>
<td>hyaline numerous filopodia</td>
</tr>
<tr>
<td>Eleocyte</td>
<td>27 ± 2.2</td>
<td>rounded</td>
<td>18.4 – 22.5 μm</td>
<td>eccentric</td>
<td>2 μm</td>
<td>with numerous chloragosomes rarely few lobopodia</td>
</tr>
</tbody>
</table>

Table 3.2  Coelomocytes characteristics of *L. mauritii* and *D. annae*
Fig 3.2  Differential count of coelomocytes in *L. mauritii* and *D. annae*
### Table 3.3 Total count of coelomocytes in *L. mauritii* and *D. annae*

<table>
<thead>
<tr>
<th>Earthworms</th>
<th>Total count of cells (in m$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. mauritii</em></td>
<td>$46 \times 10^3$</td>
</tr>
<tr>
<td><em>D. annae</em></td>
<td>$41 \times 10^3$</td>
</tr>
</tbody>
</table>

### Table 3.4 Differential count of coelomocytes in *L. mauritii* and *D. annae*

<table>
<thead>
<tr>
<th>Coelomocytes</th>
<th><em>L. mauritii</em> (in percentage)</th>
<th><em>D. annae</em> (in percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoebocytes</td>
<td>$42.6 \pm 2.5$</td>
<td>$47 \pm 3.2$</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>$28 \pm 2.2$</td>
<td>$26 \pm 1.5$</td>
</tr>
<tr>
<td>Eleocytes</td>
<td>$26 \pm 0.5$</td>
<td>$27 \pm 2.2$</td>
</tr>
</tbody>
</table>
Amoebocytes

These were amoeboid cells possessing slender cytoplasmic projections, the pseudopodia. The nucleus was moderate sized (4 – 5.2 μm) and polymorphic. The cytoplasm was usually non-granular. The diameter of the body of the cells ranged from 8.0 – 10.5 μm. Many of these phagocytic elements were found to be engorged with various types of foreign bodies, including bacteria and yeasts. They comprised 42.6 ± 2.5 percent of the total cell population. Based on the number and shape of pseudopodia present, the amoebocytes could be distinguished into two sub-types: type I and type II. Type I cells had one or two or a few short and blunt pseudopodia called lobopodia. Type II cells had several, slender, irregularly distributed filopodia (Fig. 3.3).

Vacuolated and non-vacuolated amoebocytes were seen. The vacuolated amoebocytes (Fig. 3.4) were found to outnumber the non-vacuolated cells. More than 50% of all amoebocytes were seen to be involved in phagocytosis (Fig. 3.5).

Granulocytes

Granulocytes were cells with prominent granules in their cytoplasm (Fig. 3.6). The granules were polymorphic and were seen distributed throughout the cytoplasm. The diameter of these cells ranged from 9.5 – 10.75 μm. They comprised 28 ± 2.2 percent of the total cell population. They had relatively smaller nuclei measuring 4.0 – 4.9 μm and low nucleo-cytoplasmic ratio.
Fig. 3.3  \textit{L. Mauritii} - Amoebocytes

A - Type I,  \hspace{1em} B - Type II
F - Filopodium, L - Lobopodium, N - Nucleus
Fig. 3.4 *L. mauritii*: Vacuolated amoebocyte

N - Nucleus, V - Vacuole, F - Filopodium,
Fig. 3.5 *L. Mauritii* - A type II vacuolated ameobocyte in Phagocytosis of the stain globules

SG - Stain globules, V - Vacuole

Fig. 3.6 *L. mauritii* - granulocytes and amebocytes

G - Granulocyte, A - Ameobocyte
Eleocytes

These are cells with large, distinct vesicles surrounded by a single membrane. The diameter of eleocytes ranged from 17.0 – 20.5 μm. The membrane-bound vesicles called chloragosomes almost completely fill the cytoplasm (Fig 3.7). These were of variable size measuring 0.4 – 1.6 μm. In *L. mauritii*, the number of these vesicles was far fewer than that in *D. annae*. In most eleocytes, an array of chloragosomes was found to be adhered to the outer surface of the nuclear membrane, completely covering and masking the nucleus. The nucleus was oval or spherical and located eccentrically. The eleocytes were the least represented (26 ± 0.5 percent) cells in the coelomic fluid of *L. mauritii*. The number of these cells was also considerably less than that in *D. annae*. The eleocytes had no pseudopodia. These were very delicate cells, which get disintegrated easily upon contact with the substratum. The cytoplasm was almost devoid of any organelles.

3.3.2 Dichogaster annae

Total Count

The total coelomocyte count of *D. annae* was slightly less than that of *L. mauritii* (Fig 3.1). But this difference was not significant.

Differential Count

The proportion of the different types of cells is represented in Figure 3.2. The type I and type II amoebocytes together constituted the
Fig. 3.7 *L. mauritii* - An eleocyte showing the eccentric nucleus and the prominent chloragosomes

N - Nucleus, Chr - chloragosome
largest group of coelomocytes. The immature stages of the amoebocytes and granulocytes were also observed.

Amoebocytes

The amoebocytes of *D. annae* were morphologically distinct from those of *L. mauritii*. Type I cells have one or two short and blunt pseudopodia called lobopodia. Type II amoebocytes have many, slender terminating filopodia (Fig. 3.8). However, the number of filopodia was far less than that in the amoebocytes of *L. mauritii*. The diameter of the cells ranged from 9.2 – 12.4 μm. The nucleus was relatively large measuring 4.2 – 5.6 μm. The amoebocytes comprised 47 ± 3.2 percent of the total population.

Both vacuolated and non-vacuolated amoebocytes were seen. Phagocytosis was observed occasionally.

Granulocytes

The granulocytes had relatively bigger nuclei and low nucleo-cytoplasmic ratio. They had prominent granules in their cytoplasm (Fig 3.8). The diameter of these cells was 9.0 – 11.6 μm. The nucleus was globoid or ovoid, measuring 4.5 – 6 μm. The granulocytes comprised 26 ± 1.5 percent of the total cell population (Fig. 3.9). The granulocytes were the least represented coelomocytes in *D. annae*. 

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Fig. 3.8  *D. annae* - Amoebocytes I and II
Am I - Amoebocyte I
Am II - Amoebocyte II

Fig. 3.9 *D. annae* - Granulocytes
Eleocytes

These cells had numerous membrane-bound vesicles called chloragosomes in the cytoplasm (Fig. 3.10 and 3.11). In *D. annae*. The number of these vesicles was far more than that in *L. mauritii*. However, the size of the individual vesicles was comparatively smaller ranging from 0.3 \( \mu \text{m} \) to 1.2 \( \mu \text{m} \). These were large cells ranging in diameter between 18.4 to 22.5 \( \mu \text{m} \). The nucleus was small in size ranging from 2.1 \( \mu \text{m} \) to 2.6 \( \mu \text{m} \). The proportion of these cells in the coelomic fluid was 27 ± 2.2 percent.
Fig. 3.10  *D. annae* - Eleocyte
 Chr - Chloragosome, N - Nucleus

Fig. 3.11  *D. Annae* - Eleocyte - cell membrane burst
 Chr - Chloragosome
3.4 Discussion

Short-term electric shock treatment was found to be relatively safe and convenient method for the retrieval of coelomic fluid. The other methods of coelomic fluid retrieval such as direct withdrawal from the coelomic cavity and by the application of 5% ethyl alcohol were found to have damaging effect on the worms. All the experimental animals survived the electric shock treatment and could maintain good health in culture. However, there may be a transient depletion of cells responsible for defence against the microbial flora of the soil after repeated electric shock treatment. Presumably other cells, such as blood haemocytes (Friedman, 1979) and humoral factors may also help in protecting the animals against soil borne microorganisms.

The coelomocyte counts, both total and differential counts, of *L. mauritii* and *D. annae* did not differ significantly. Kale and Krishnamoorthy (1979) however, reported species-specific variation in the total counts and differential counts of coelomocytes in the five species of earthworms.

The present study has revealed that the morphology of coelomocytes of the two species of earthworms differs considerably. However, in general three different classes of cells - amoebocytes, granulocytes and eleocytes were noticed.
Although the classification of coelomocytes of oligochaetes is still unclear and a common system cannot be applied to all families, the present categorization in *L. mauritii* (Megasolecidae) and *D. annae* (Octochaetidae) agree with the consensus system adopted by Linthicum et al. (1977), Cooper (1996), Admowicz and Wojtaszek, et al. (2001) and Admowicz (2005).

Kale and Krishnamoorthy (1979) had reported that the structure of coelomocytes of five different species of earthworms was almost the same. However, the present observations showed that the two species of earthworms, *L. mauritii* and *D. annae*, had morphologically distinct coelomocytes. The differences in morphology were more pronounced in amoebocytes and eleocytes. The type I amoebocytes of *L. mauritii* had more number of filopodia than that in the amoebocytes of *D. annae*.

The vacuolated amoebocytes are aged cells. In most of the earthworms studied, more than 50% of the amoebocytes were vacuolated. The increased number of vacuolated cells in the earthworms may be correlated with the aging process, since all the earthworms studied were fully mature or aging ones. According to Lavia and Hill (1972) the vacuolation and nuclear pycnosis are signs of cell death. The amoebocytes of *L. mauritii* were more phagocytotic than that of *D. annae*. Although the proportion of the cellular elements was slightly different in the two species.
of earthworms the difference was not significant. The immature stages of coelomocytes found in both the species may represent the different developmental lineages or different developmental stages of the same cell lineage.

Amoebocytes are generally regarded as immune cells involved in phagocytosis and encapsulation. Granulocytes also have limited phagocytic activity. Eleocytes are cells with nutritive and excretory functions. These cells are capable of migration through the body and distribute the nutrients or release these substances into the coelomic fluid, thereby regulating the amount in circulation by homeostatic means. However, the exact functional roles played by the different coelomocytes in earthworms remain yet to be fully understood.

Studies on the morphology and population of different types of coelomocytes of earthworms assume significance since these cells can be used as model systems in analyzing the mechanisms of invertebrate immunity. The characterization of the normal coelomocytes is essential in assessing the way in which these worms respond to the changes in environmental factors. This may also contribute to the utilization of earthworms in monitoring the environmental quality as bioengineers.