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Hemocitical responses to environmental stress in invertebrates: a review

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Abstract Although invertebrates are recognized by the great facility to accumulate pollutants present in their environment and many of them are used as sentinel species in biomonitoring studies, little is known about the impact of toxicants on the immune system of these animals. In this regard, hemocytes play a fundamental role: these cells circulate freely through the hemolymph of invertebrates and act on the recognition of foreign material to the organism, mediating and effecting the cellular defense, such as phagocytosis, nodulation, and encapsulation. Different morphological types can be recognized but still there is controversy among the researchers about the exact classification of the hemocytes due to the diversity of techniques for the preservation and observation of these cells. In the present study, a review on the main hemocyte responses to environmental stress in different invertebrate organisms is presented, emphasizing the contamination by heavy metals. It is discussed parameters such as: alteration in the number of cells involved in the defense reaction, phagocytic activity, lysosomal responses, and production of reactive oxygen species.

Keywords Hemocytes · Immunity of invertebrates · Cellular defense · Environmental stress · Heavy metals

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Immune system

It is of fundamental importance for any organism the capacity to recognize the presence of foreign substances through its immunological system. Two defense systems against infectious agents have been selected during evolution: innate immune system or natural immunity and adaptive immune system or acquired immunity. The innate system can be found in all multicellular animals, while the acquired system is phylogenetically young, being only found in vertebrates (Mandato 1998; van de Braak 2002).

Invertebrates have a complex and efficient innate immune system, which can be subdivided in humoral and cellular defense responses. The humoral response is closely related to the synthesis of antimicrobial peptides (Lowenberger 2001), production of reactive oxygen and nitrogen intermediates (Bogdan et al. 2000), besides the activation of enzymatic cascade that regulates coagulation and melanization of hemolymph, release of anti-stress proteins and of certain mole-

cules whose probable function is the opsonization and the iron sequestration (Muta and Iwanaga 1996; Gillespie et al. 1997; Jiravanichpaisal et al. 2006). Now, the cellular defense involves responses mediated by different morphological types of hemocytes, cells that circulate freely in the hemolymph (Correia 2008).

There are indications that the humoral and cellular responses are well coordinated between themselves. Probably, there is an overlap between them, since several humoral factors affect the hemocytes function and these, in turn, are an important source of many humoral molecules (Erold-Erickson et al. 2000; Lavine and Strand 2002; Jiravanichpaisal et al. 2006).

Hemocytes have the ability to discriminate foreign agents to the organism, mediating the recognition of the material; phagocytosis, nodulation, and encapsulation; cytotoxic reactions; coagulation of the hemolymph; and injury repair (Lavine and Strand 2002; Tzou et al. 2002; Falleiros et al. 2003; Correia 2008), it is also observed agglomerations of hemocytes around the stressor agent in a kind of "trap" (Jiravanichpaisal et al. 2006). They are also involved in the storage and distribution of the nutritive material (Russo et al. 2001; Silva et al. 2002) and in the tissue formation (van de Braak 2002).

The terminology of hemocytes is not uniform; the diversity of shapes and functions that these cells can perform in the different species studied, the different methods of preservation and observation and the divergences regarding the criteria used to distinguish the morphologic types make any attempt of comparison very difficult (Gupta 1979; van de Braak 2002). Therefore, the hemocytes classification remains quite controversial nowadays, since, in many times, the researchers end up adopting their own terms to identify cellular types already described by other authors.

In a review made by Johansson et al. (2000), it is described three morphological types of circulating hemocytes in decapods: hyaline cells, semi-granular cells, and granular cells. In the freshwater shrimp the hyaline cells are small, spherical, and may contain few granules and are capable of phagocytosis. The semi-granular cells have variable number of small eosinophil granules and are responsible for the encapsulation, presenting limited phagocytic activity. The granular cells do not act in the phagocytosis and are full of eosinophil granules, which contain activators of prophenoloxidase (Jiravanichpaisal et al. 2006).

Both semi-granular and granular cells may undergo degranulation when in contact with molecules that are not recognized by the immune system, releasing their content to the extracellular media. Both can be also cytotoxic and promote the lysis of foreign cells (Jiravanichpaisal et al. 2006).

Several authors rely only on the absence or presence of cytoplasmatic granules and on their relative size to characterize and classify the morphological types of hemocytes. This kind of classification is very common in species of crustacean, such as in the study conducted by Barraco and Amirante (1992), in which the hemocytes of the species *Squilla mantis* were identified as hyaline cells (few granules), small granule hemocytes and large granule hemocytes.

Nowadays, the most widely accepted classification is based on a review study conducted by Gupta (1985), in which the author groups the hemocytes of insects into seven main types: pro-hemocytes, plasmatocytes, granulocytes, spherulocytes, adipohemocytes, oenocytoids, and coagulocytes.

Pro-hemocytes exhibit high mitotic index and are considered stem cells that will differentiate into specific cell lines (Mandato 1998; Falleiros et al. 2003). They are small, round or ovoid cells with a large nucleus located centrally and cytoplasm restricted to a narrow band around the nucleus; plasmatocytes are polymorphic, variable in size, with cytoplasmatic projections and may be binucleated; granulocytes have granulations that frequently mask the presence of the nucleus; spherulocytes generally are larger than the plasmatocytes and granulocytes, having the cytoplasm full of spherules limited by membranes of varied sizes that can cover the nucleus; adipohemocytes are round, variable in size, with lipid drops in the cytoplasm; however, its identification is controversial since there is a hypothesis that these cells would be variants of plasmatocytes or granulocytes with accumulation of lipid (Bombonato and Gregório 1995; Falleiros 1995; Faraldo 2000; Negreiro et al. 2004); oenocytoids are large cells,

Table 1 Functions of the hemocytes, according Gupta (1985) classification

Hemocyte types (according Gupta 1985)	Function in immunity	References
Pro-hemocytes	Stem cells	Mandato (1998) Lavine and Strand (2002) Falleiros et al. (2003) Negreiro et al. (2004) Jiravanichpaisal et al. (2006)
Plasmatocytes	Phagocytosis Nodulation Encapsulation	Russo et al. (2001) Lavine and Strand (2002) Negreiro et al. (2004) Andrade et al. (2004) Jiravanichpaisal et al. (2006)
Granulocytes	Recognition of foreign material Phagocytosis Nodulation Encapsulation	Pech and Strand (1996) Gillespie et al. (2000) Silva et al. (2002) Lavine and Strand (2002) Negreiro et al. (2004) Ribeiro and Brehélin (2006)
Spherulocytes	Tissue renewal Transport of hormones Production of proteins Nutrition	Ratcliffe et al. (1985) Negreiro et al. (2004) Correia (2008)
Adipohemocytes	Unknown	
Oenocytoids	Production of the phenoloxidase enzyme	Mandato (1998) Lavine and Strand (2002) Jiravanichpaisal et al. (2006)
Coagulocytes	Coagulation process	Negreiro et al. (2004) Xylander (2009)

round with a small nucleus in relation to the cytoplasmatic volume; and the coagulocytes have the cytoplasm with various vacuoles of different sizes and that disintegrate easily in vitro (Carneiro and Daemon 1997, 2001; Correia et al. 2005; Correia 2008).

Negreiro et al. (2004) comment that the function of the granulocytes and plasmatocytes are related, mainly, to the phagocytosis and formation of capsule and nodule, while the oenocytoids are cells that produce the phenoloxidase enzyme. The spherulocytes would be related with tissue renewal, transport of hormones, production of certain proteins of the hemolymph, and nutrition. Coagulocytes would be responsible for the release of substances that trigger the coagulation process. Now, the pro-hemocytes, as they are considered stem cells, would not participate directly of the defense processes (Andrade et al. 2004).

However, according to Pech and Strand (1996), immune functions performed by a morphological type of hemocyte in a particular species may be carried out by a different cellular type in another species.

The Table 1 shows the different functions of hemocytes in immunity response.

Hemocytes "versus" stress

Total and relative number of cells

The success of a defense response depends on the quantity of hemocytes and on the cellular types involved in the process (Russo et al. 2001). In this sense, monitoring the number of hemocytes as a measure of stress and/or physiologic condition has been suggested for some invertebrates (Truscott and White 1990; Coles et al. 1995; Pipe et al. 1999; Lorenzon et al. 2001; Mayrand et al. 2005; Correia 2008).

Heavy metals are considered pollutants in potential since they are widely distributed in the environment, be it by natural or anthropogenic

sources and they have been pointed out as a threat to the health and survival of several marine, aquatic, and terrestrial animals (Lorenzon et al. 2001).

According to Robinson and Ryan (1988), hemocytes can transport metals intracellularly (within the cytoplasm or in lysosomal vesicles). Hemocytes of bivalves are known to accumulate high levels of heavy metals, mainly in lysosomes (Matozzo et al. 2001); the most common response observed in these animals exposed to different heavy metals is the increase in the number of hemocytes in circulation (Pipe and Coles 1995).

In a study conducted by Pipe et al. (1999), in which the authors investigated the effects of copper exposure on the immune system of the marine mussel *Mytilus edulis*, cell count by a hemocytometer showed an increase in the number of circulating hemocytes as well as an alteration in the proportion of different morphologic types; while the percentage of eosinophilic cells suffered a significant reduction, the quantity of basophilic cells increased in higher concentrations of the metal. Nevertheless, this increase in the quantity of circulating hemocytes was not significative in the highest concentrations of copper; the authors discuss that this may be due to the high toxicity of the metal, causing cell death, or due to migration of the hemocytes from circulation to tissues injured by the exposition.

In a similar study, using the same methodology, Coles et al. (1995) had already investigated the effects of cadmium exposure on the immune system of *M. edulis*. The results also showed an increase in the total number of circulating hemocytes; on the other hand, the exposition to the metal did not provoke significative changes in the proportion of the different morphological cell types.

Mayrand et al. (2005) conducted a study in which it was analyzed the hemocyte responses of *M. edulis* when it was transferred from an industrialized place to a region without contamination by industry. It was verified by counting the number of cells by a hemocytometer that the mussels exposed for a long period to the conditions of industrialization presented a higher quantity of hemocytes when compared to native mussels of a clean area, and that after the transference from a contaminated place to another non-contaminated, the number of hemocytes was reduced.

David et al. (2008) analyzed morphological alterations in gills of *Mytella falcata* from three sites of a estuary with different contamination levels. Among the alterations it was verified an increase in the number of hemocytes in the hemolymph vessels as well as invasion of the hemocytes in the gill epithelium (Fig. 1). The authors discuss that such tissue responses indicate defense mechanisms and may be related to the

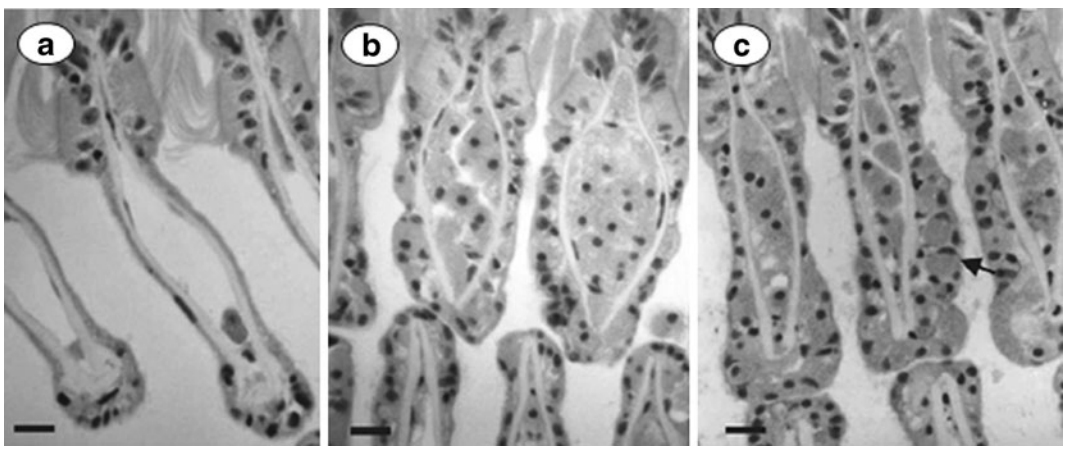

Fig. 1 **a**–**c** Histological sections of *Mytella falcata* gill filaments. **a** Animals from less impacted site; **b**, **c** animals from more impacted site; **b** hemolymph vessel filled with hemocytes; **c** invasion of hemocytes into the epithelium (*arrow*). Scale bars = 10 μm (David et al. 2008)

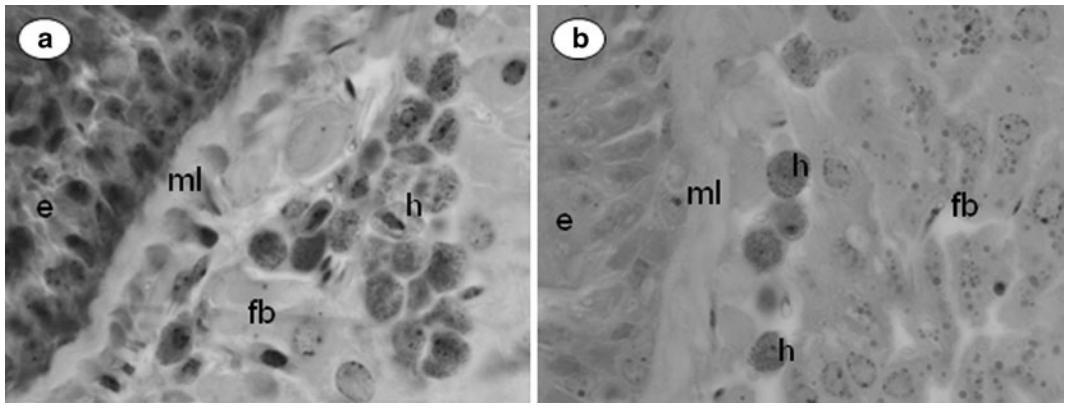

Fig. 2 **a**, **b** Histological sections of the midgut of *R. padbergi* stained with hematoxylin and eosin. Group exposed to sewage sludge. Note the agglomeration of hemocytes in (**a**). *e* epithelium, *fb* fat body, *h* hemocyte, *ml* muscular layer. Magnification, ×1,200

reabsorption process of old or injured epithelial cells.

Radford et al. (2000) studied the effects of metal pollution in the behavior of the hemocytes of the tunicate invertebrate *Styela plicata*. They observed that the animals exposed to the toxicants presented an increase in the circulating hemocytes. Furthermore, the immunohistochemical analyses of the pharynx showed differences in the distribution of the cells in the tissue of the exposed animals in relation to the non-exposed animals, which was not observed in the control group. The authors discuss that such cell accumulation may indicate an inflammatory process involved in the repair of the epithelial lesion.

Similar hemocitical responses were observed through histological analysis in the diplopod *Rhinocricus padbergi* exposed to substrate containing sewage sludge. Some studies have shown an increase in the number of hemocytes between the fat body cells of midgut of exposed animals as well as formation of several agglomerations of these cells (Figs. 2 and 3), indicating a tissue injury with inflammatory process (Perez and Fontanetti 2008; Godoy and Fontanetti 2009; Nogarol and Fontanetti 2010).

Auffret and Oubella (1997) exposed oyster of the species *Crassostrea gigas* to xenobiotics identified in a polluted estuary. They observed changes in the hemocytes aggregation behavior

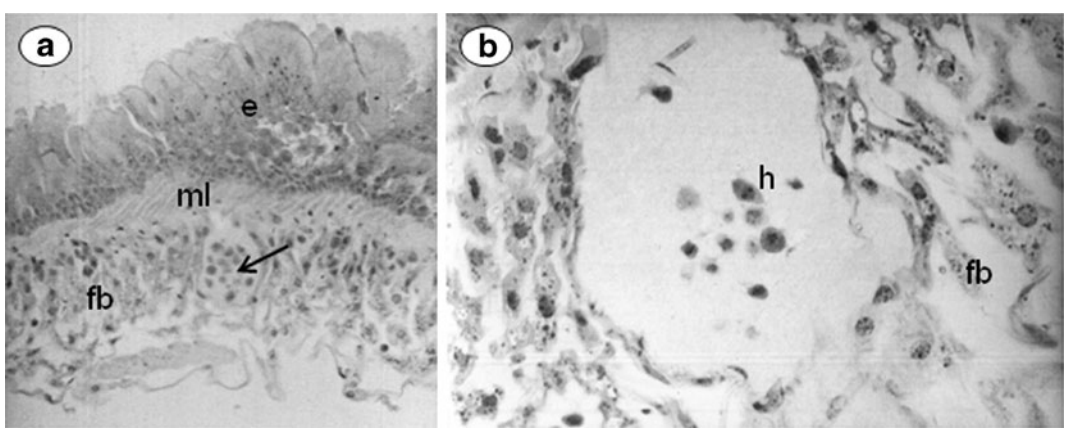

Fig. 3 **a**, **b** Histological sections of *R. padbergi* midgut exposed to sewage sludge. *e* epithelium, *fb* fat body, *h* hemocyte, *ml* muscular layer, *arrow* in (**a**) agglomeration of hemocytes. Magnification, **a** ×100; **b** ×1,000 (Godoy and Fontanetti 2009)

according to the chemical used and advocated that the aggregation activity of these cells may be altered by the stress induced by environmental contamination.

Increase or decrease in the total number of blood cells are considered a common responses to stressors present in the environment.

There are reports that the increase in the number of these cells in response to a determined type of stress can be reversible (Oubella et al. 1993). This suggests that such increase happens due to stimulation of the hemocytes migration from the tissues (Cajaraville et al. 1996), and not by cellular proliferation, which may or may not result in alterations in the proportion of the different morphological types, depending on the distribution of the types of hemocytes in the organism tissues (Coles et al. 1995; Pipe et al. 1999).

There are still divergences among the researchers in relation to the existence or not of the proliferation of circulating hemocytes. Probably, the variation in the number of hemocytes is regulated by release of cells from the hematopoietic tissue complemented by cellular migration between the several tissues of the organism. Hematopoiesis is the process by which the hemocytes mature and enter in the circulation; they are constantly produced to replace those that died or that were metabolically damaged. However, the rate that this process occurs can be altered by the influence of several environmental factors (Johansson et al. 2000; Jiravanichpaisal et al. 2006).

In a study carried out by Sorvari et al. (2007), in which it was analyzed the effect of heavy metals on the immune response of ant colonies of the species *Formica aquilonia*, it was verified that moderate levels of heavy metals led to an increase in the encapsulation performed by the hemocytes, while high doses suppressed the immune system.

Pipe et al. (1999) alert that although the increase in the number of hemocytes is a common response to environmental stress it cannot be seen as a rule. According to Pipe and Coles (1995), the decrease in the total number of hemocytes under stress conditions can be consequence of cellular lysis, reduced recruitment or movement of the cells from the circulation to the tissues.

According to Lorenzon et al. (2001), exposure to pollutants may cause an expressive diminishing in the amount of circulating hemocytes in the hemolymph. The authors exposed the shrimp *Palaemon elegans* to different concentrations of heavy metals diluted in saltwater and concluded that the number of circulating hemocytes counted in a hemocytometer significantly decreased in the animals exposed to the different heavy metals tested. They argue that the change in the quantity of hemocytes will also depend on the exposure time to the pollutant, the pollutant nature, its concentration, and of the species chosen for the study.

Victor (1993) investigated the behavior of the hemocytes in the crab *Paratelphusa hydrodromous* exposed to sub-lethal doses of cadmium. The total count of cells in the hemocytometer showed a drastic reduction in the number of circulating hemocytes, while the differential count demonstrated a variation in the percentage of morphological types after the exposition; the stress reduced the quantity of hyalinocytes and eosinophilic granulocytes and increased the number of pro-hemocytes and intermediary granulocytes, according to the classification adopted by the author. In the referred study, the gills of the exposed crabs were also analyzed histologically, being verified the hemocytes aggregation around the injured tissue and invasion of granulocytes in the gill epithelium.

Similarly, specimens of the crab *Cancer irroratus*, collected in a site of sewage discharge, also presented hemocytes aggregation in the gills, indicating a probable answer to the contamination by heavy metals (Greig et al. 1982).

On the other hand, a study carried out by Truscott and White (1990), in which the researchers examined the effects of heavy metals (copper, cadmium, and mercury) on the immune system of the crab *Carcinus maenas*, concluded that the hemocytes of the analyzed species are tolerant to the stress by heavy metals, not being observed significative changes in the number of these cells in the exposed animals.

According to Olabarrieta et al. (2001), hemocytes can accumulate toxicants present in the environment, which confer a certain resistance to stress by heavy metals. Ahearn et al. (2004)

verified that ions of zinc are sequestered and captured by circulating hemocytes as a detoxification process in the lobster *Homarus americanus.* In oysters, the hemocytes accumulate copper and zinc in vesicles limited by the membrane (Pirie et al. 1984). However, when exposed to very high concentrations of heavy metals, the hemocytes may suffer deleterious effects, such as the loss of the cytoskeleton integrity.

In general, all the organisms have mechanism of cellular defense that can be involved in the detoxification process of heavy metals, which leads to a certain tolerance to the stress caused by such elements (Hall 2002). Hemocytes of mollusks are known to synthesize metallothioneins after exposure to cadmium (Roesijadi et al. 1997). Metallothioneins are intracellular proteins of low molecular weight, responsible for maintaining the homeostasis of essential metals and are found in animals, higher plants, eukaryotic microorganisms, and in several prokaryotes (Klaassen et al. 1999; Vasak 2005). The fact that they exert oxidant activities suggests that one of their functions is to protect the cell against the action of free radicals (Andrews 2000). Metallothioneins are active in the defense against harmful effects caused by heavy metals, by binding to them by residues of cysteine (Park et al. 2001; Coutinho and Barbosa 2007). Metallothioneins have a key role in the detoxification of heavy metals when their intracellular concentration exceeds that necessary for the metabolic functions (Ahearn et al. 2004).

The increase or decrease in the total and/or differential number of circulating hemocytes in a certain organism may reflect several factors, such as physiological stress, exposure to pollutants, changes in the temperature and salinity, life stage, nutrition level as well as the occurrence of illnesses (Oliver and Fisher 1995; Lorenzon et al. 2001; van de Braak 2002).

Oliver and Fisher (1995) observed an increase in the number of hemocytes counted in a hemocytometer after they experimentally infected two species of bivalves. The authors also verified that the amount of hemocytes varied according to the temperature and salinity in *Crassostrea virginica*. According to van de Braak (2002), the increase in the temperature of the water may elevate the number of circulating hemocytes in the hemolymph due to a higher rate and force of heart pumping, although this is not a rule, there are a conjunction of factors.

Phagocytic activity

Heavy metals, as the other environmental toxicants, are capable of altering the phagocytic activity of the hemocytes. Depending on the exposure time, nature, and concentration of the heavy metal, the phagocytosis carried out by the hemocytes can be stimulated, inhibited or not suffer any kind of modification (Coles et al. 1995).

In a study conducted by Olabarrieta et al. (2001), using a suspension of neutral red-stained zymosan, it was verified that hemocytes of the mussel *Mytilus galloprovincialis* exposed in vitro to cadmium presented a significant increase in the phagocytic ability, showing that the metal can enhance the capacity of cellular defense. Similarly, Coles et al. (1995) and Pipe et al. (1999) observed that hemocytes of bivalves exposed in vivo to certain concentrations of heavy metals may have their phagocytic activity stimulated.

However, Matozzo et al. (2001), studying the effects of copper and cadmium on the hemocytes of the mollusk *Tapes philippinarum*, verified a significative decrease in the phagocytic activity of the hemocytes of the animals exposed to copper, while no inhibition was observed in those cells exposed to cadmium. This shows that the hemocitical response under stress conditions by heavy metals will depend on the nature and concentration of the metal. The phagocytosis index was expressed as the percentage of cells containing ingested yeast particles (*Saccharomyces cerevisiae*).

Auffret et al. (2002) also observed a severe decrease in the phagocytosis of hemocytes of oyster of the species *Ostrea edulis* exposed to cadmium and copper in relation to a control group.

Reduction in the phagocytic activity may be due to changes in the cell cytoskeleton provoked by the heavy metal; the hemocyte under stress lose the ability to adhere to the substrate and interact with foreign particles (Matozzo et al. 2001).

Sauvé et al. (2002) analyzed the phagocytic activity of hemocytes in several species of bivalves exposed to different metals (silver, cadmium,

mercury, and zinc). The results showed a species-specific hemocitical response; the sensitivity to metals varied according to the species, although the organisms were similar and belonging to the same taxonomic group. In some species, low concentrations of mercury, zinc, and cadmium stimulated the phagocytosis; however, in high doses, all the tested metals induced an inhibition. They used flow cytometry to quantify the phagocytosis of fluorescent microspheres by hemocytes.

Indeed, some studies have demonstrated that the hemocytes activity may be stimulated in low concentrations of contaminants and inhibited in high concentrations (Fisher et al. 1990; Pipe et al. 1999).

Lysosomal responses and production of reactive oxygen species

Besides alterations in the quantity of cells mobilized for cellular defense and phagocytic activity, the environmental stress may affect other parameters related to the hemocytes behavior. Giamberini and Pihan (1997) assessed the lysosomal responses of the hemocytes of the mussel *Dreissena polymorpha* experimentally exposed to high concentrations of zinc and lead. The results showed an increase in the number and/or size of the lysosomes in the hemocytes of the exposed animals, although it has not been a response dependent on the dose or the exposure time.

Snyman et al. (2000) investigated the use of lysosomal responses of hemocytes of the common garden snail *Helix aspersa* as bioindicator of stress resulted from the exposition to a fungicide copper based. The authors analyzed the retention time of neutral red, since the efflux of the lysosomal content to the cytosol can be measured by the retention time of such substance. According to this method, the dye is sequestered for the lysosomal compartment when living cells are pre-incubated with neutral red. The assessment of the dye leakage to the cytosol by microscope can characterize damages in the lysosome membranes and the time that the dye takes to reach the cytosol is related to the damage degree of the membranes. The evaluation of lysosome membrane permeability by retention of neutral red is a practical and very used method, since lysosomes are the target of many pollutants (Lowe and Pipe 1994; Viarengo et al. 2007; Freire et al. 2008).

They verified that the retention time of neutral red decreased progressively with the increase in the fungicide concentration, concluding that both the concentration and the exposure time to the toxicant may influence the lysosomal responses of the hemocytes.

Lysosomes are known for their involvement in the accumulation and detoxification of organic and/or metallic xenobiotics (Giamberini and Pihan 1997). According to Pipe (1992) and Winston et al. (1996), the lysosomal compartment is an important place of generation of reactive oxygen species (ROS) inside the hemocyte. ROS production constitutes one of the functions of the hemocytes in the organism defense and may be stimulated by the presence of toxicants in the organism, mainly in moderate concentrations; high concentrations of chemicals may lead to suppression in the production. The hemocytes protect themselves against the oxidative stress by enzymatic antioxidants (superoxide dismutase, catalase, and glutathione peroxidase); the activity of such enzymes may also be affected by contaminants, causing oxidative damages to the cell (Pipe and Coles 1995).

Fisher et al. (2000) analyzed the defense of oyster of the species *C. virginica*, collected in different sites with varied contamination levels. They observed that oyster from sites with higher concentrations of xenobiotics, especially specific metals, exhibited higher generation of superoxide by hemocytes, besides the high density and mobility of these cells. The authors discuss that the increase in the parameters of internal defense in contamination conditions may reflect the detoxification process carried out by hemocytes.

In another study carried out with the species *C. virginica*, in which the animals were exposed to cadmium, Roesijadi et al. (1997) verified that high concentrations of the heavy metal suppressed the production of ROS by hemocytes, suggesting that cadmium is immunotoxic to this species.

Moreover, where it is verified a reduction in the phagocytic activity of the hemocytes due to stress, the metabolic process triggered by it may also be affected; one of this process is the production of

ROS, which may suffer suppression (Auffret et al. 2002).

Final considerations

The study of the components of the immune system may provide a measure of the health state of an organism and of the stress level resulted from environmental contamination (Pipe et al. 1999). According to Wong et al. (1992), the effects of exposition to contaminants may be assessed by cellular and functional parameters of the immune system of sentinel species. In this sense, little has been studied about the impact of different toxicants on the immune system of invertebrates, although they are known for easily accumulate pollutants.

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