

REVIEW

Aspects of eco-immunology in molluscs**V Matozzo***Department of Biology, University of Padua, Padua, Italy**Accepted April 5, 2016***Abstract**

There is increasing interest in the international scientific community in eco-immunology, a relatively new discipline combining the knowledge from immunology, biology, ecology, physiology and biochemistry. The integrative approach of different scientific disciplines provides a useful perspective, mostly from an evolutionary point of view, into the understanding of the basic mechanisms of the immune responses, as well as the complex host-parasite interactions. However, while knowledge concerning factors affecting the immune response is increasing, more efforts should be made to determine the physiological mechanisms that regulate these responses. This need is particularly pressing in investigations in invertebrates because they are model organisms widely used to study the basic mechanisms of innate immunity and to assess environmental health (the immunomarker approach in biomonitoring studies). In this context, this review focuses on some of the eco-immunology aspects of molluscs.

Key Words: eco-immunology; hemocytes; immune responses; molluscs

Introduction

What is the cost of the immune response? This is probably one of the most important questions that stimulates debate among experts in the field. In invertebrates in particular, very few data about the metabolic cost of the immune response are available despite the fact that these organisms have a comparatively simpler immune system compared with vertebrates. According to Schmid-Hempel (2003), these costs may be “*evolutionary costs*” (a variation of a component of the immune system may affect growth and reproduction of an organism and *vice versa*) and “*use costs*” (the cost to maintain the immune function and to deploy a response). These fundamentals suggest that the immune defence can be costly and cannot always be maintained at maximum levels (Ardia *et al.*, 2012). Consequently, determining the cost of the immune response assumes a considerable significance when considering the influence of both extrinsic and intrinsic factors on the immunosurveillance status of organisms. In this regard, it is well known that a relationship exists between stress and the immune responses (Ottaviani and Franceschi, 1996; Lacoste

et al., 2001a, b, c), as well as between the neuroendocrine and immune systems, in both vertebrates and invertebrates (Ottaviani *et al.*, 2008; Demas *et al.*, 2011).

These perspectives have promoted (at least in part) the development of eco-immunology, or ecological immunology (Sheldon and Verhulst, 1996; Rolff and Siva-Jothy, 2003; Ottaviani *et al.*, 2008; Demas and Nelson, 2012). Demas *et al.* (2011) highlighted that in the last few years, the two research areas that have attracted remarkable attention among experts of eco-immunology of both vertebrates and invertebrates are (i) the energetics of immunity and (ii) the relationship between stress and immunity. In terms of the cost, eco-immunology assumes the immune defence must be minimised (Ottaviani *et al.*, 2008). To achieve this goal, organisms can activate pathways, which involve immune and neuroendocrine components (Ottaviani *et al.*, 2008).

Regarding the relationship between stress and immunity, this research area is expanding within the field of eco-immunology (Biondi, 2001; Ottaviani and Malagoli, 2009). However, the debate on this topic is heated because a clear relationship between stress and immunity is not often obvious. In particular, several studies on invertebrates have demonstrated that stressors sometimes increase, while other times decrease, the immune response, depending on several factors, such as the animal

Corresponding author:

Valerio Matozzo

Department of Biology

University of Padua

Via Ugo Bassi, 58/B, 35131 Padua, Italy

E-mail: valerio.matozzo@unipd.it

species tested, the type and duration of stress, and the immune parameters measured.

Despite such controversial data, invertebrates are considered an attractive model to study eco-immunology because of the relatively simple mechanisms that support their innate immune system and their propensity to undergo chemical, biological and physical manipulations, which allows researchers to study the effects of both biotic and abiotic factors on the immune response (Ellis *et al.*, 2011; Cuvillier-Hot *et al.*, 2014). Although some invertebrate species use body structures or barriers (e.g., shells and external mucous substances) to protect against the surrounding environment, including pathogens, they rely on their innate immune reaction to protect against internal non-self materials. Invertebrates lack antibodies, and the innate immune response is not specific to a particular pathogen. However, some authors stated the limit between “innate” and “adaptive” immunity may be fuzzy and artificial (Vinkler and Albrecht, 2011). These authors suggest invertebrates with a long lifespan should have some kind of acquired immunity. Indeed, some invertebrates, including molluscs, can live several decades or more than a hundred years (Bodnar, 2009). For example, the *Arctica islandica* species is a 374-year-old bivalve species (Schöne *et al.*, 2005; Wanamaker *et al.*, 2008). This is an interesting topic that will be a subject for discussion among eco-immunologists in the coming years.

The eco-immunology of molluscs

Among invertebrates, molluscs are one of the most studied groups in terms of their immune functions. Like other invertebrates, molluscs rely on an innate, non-lymphoid immune system, the main protagonist of which is the hemocyte. The hemocytes circulate freely in the hemolymph, where they play an important role in wound and tissue repair, shell production and repair, and the immune response (immunocytes) (Cheng, 1981; Hine, 1999). In molluscs, the immune reaction towards non-self particles involves several mechanisms, including recognition, phagocytosis, encapsulation, intracellular digestion, and the production of cytotoxic substances and antimicrobial peptides.

This review does not intend to summarise the large amount of data concerning the involvement of mollusc hemocytes in the immune response. Rather, attention is given to some of the eco-immunology aspects of molluscs. As stated above, eco-immunology focuses on the effects of both biotic and abiotic factors on the immune system among taxa. Because the immune defence is energetically costly, it is assumed that organisms under stressful conditions decrease resource allocation to immune system defence (Moret and Schmid-Hempel, 2000). In molluscs, several studies have demonstrated variations in the immune response after exposure of the organisms/cells to environmental contaminants (Cajaraville *et al.*, 1996; Oliver and Fisher, 1999; Donaghy *et al.*, 2009; Matozzo, 2014). However, there is increasing evidence that the immune response of molluscs can be modulated by both biotic and abiotic factors other

than environmental contaminants. For example, variations in the number of circulating hemocytes were recorded in two economically important clam species (*Ruditapes philippinarum* and *R. decussatus*) after challenge with the pathogenic bacteria *Vibrio P1* (experiments with both the bivalve species) and after starvation (experiment with *R. philippinarum* only) (Oubella *et al.*, 1993). The number of circulating hemocytes increased significantly in both species after short-term (0 to 72 h) and long-term (0 to 7 days) challenge experiments, whereas one week of fasting caused a marked reduction (by 65 %) in the number of hemocytes in *R. philippinarum*. To explain these variations, the authors suggested that a reversible migration of hemocytes from the tissues to the hemolymph, or *vice versa*, occurred in clams (Oubella *et al.*, 1993). These cellular mechanisms can be important in the cases of infections, as the capability for hemocyte redistribution in the organism may be interpreted as an increase in the immunosurveillance by providing immunocytes at sites of pathogenic aggression (Oubella *et al.*, 1993). Experimental infection of molluscs with parasites offers a useful tool for the study of specificity and host immune resistance to parasitic infection. In a recent study, Gorbushin and Borisova (2014) implanted echinostomatid rediae, *Himasthla elongata*, to the specific iteroparous long-living host, coenogastropod *Littorina littorea* (common periwinkle). Neither young nor mature rediae survived in the recipient periwinkles in the course of 30 days post-implantation, suggesting a strong immune response of the host. The strong immune response (production of toxic humoral immune factors, encapsulation of the implants and increased hemocyte counts) was already evident during the first week after implantation. Conversely, rediae from the same microhemipopulation showed perfect survival rates in primary *in vitro* axenic cultures. Based on the results obtained, the authors stated that low investment in *L. littorea* annual reproduction would result in increased investment in self maintenance and immune mechanisms, causing the general resistance to the trematode infestation. This resistance should be relatively higher in long-lived iteroparous gastropods compare to semelparous short-lived molluscs, such as pulmonates (Gorbushin and Borisova, 2014).

Regarding starvation, Oubella *et al.* (1993) observed a return to initial hemocyte densities once feeding had resumed in *R. philippinarum*. Since hemocytes play an important role in nutrient transport, an accumulation of hemocytes in storage tissues could allow the utilisation of these metabolic reserves by starved molluscs (Oubella *et al.*, 1993). Differential food quality was shown to influence markedly phagocytic activity, reactive oxygen species (ROS) production, and abundance of free-floating hemocytes in *Crassostrea gigas* and *R. philippinarum* (Delaporte *et al.*, 2003, 2006). In the Antarctic bivalve, *Laternula elliptica*, reductions in the number of hemocytes were observed in animals subjected to starvation, compared to constant feeding (Husmann *et al.*, 2011). Conversely, in the same bivalve species, analyses of immune response genes revealed that most transcripts were

more affected by injury (both valves of the animals were cracked and the siphon was cut at two places) rather than starvation (Husmann *et al.*, 2014). Overall, the genes were upregulated in the hemocytes of young, fed individuals after acute injury, while only minor variations in expression were found in young animals under starvation conditions and in older individuals. The authors suggested that the stress response of *L. elliptica* depended on the nature of the environmental cue and on age (Husmann *et al.*, 2014).

Two exhaustive reviews on the effects of changing environmental parameters on the mollusc immune response are provided by Matozzo and Marin (2011) and Anisimova (2013). At the cellular level, for example, phagocytic activity is affected by variations in temperature (Hégaret *et al.*, 2003; Cheng *et al.*, 2004a; Monari *et al.*, 2007), salinity (Cheng *et al.*, 2004b; Matozzo *et al.*, 2007), and air exposure/anoxia (Pampanin *et al.*, 2002; Matozzo *et al.*, 2005). Recently, attention has also been given to the evaluation of the effects of reduced seawater pH (as predicted in a global climate change scenario) on the immune parameters of bivalves (Bibby *et al.*, 2008; Matozzo *et al.*, 2012).

Considering that environmental parameters vary seasonally and the reproductive cycle of animals is regulated by these parameters, seasonal and gender-related differences in the function of the immune system have been investigated in molluscs. For example, a seasonal variation pattern in the parameters of hemocyte function was recorded in clams (*R. philippinarum*) collected from different sites of the Lagoon of Venice (Matozzo *et al.*, 2003). Indeed, variations in the functional response of hemocytes appear closely dependent on seasonal variations in both environmental parameters and the physiological status of clams (Matozzo *et al.*, 2003). In the same clam species, different hemocyte parameters, such as the total hemocyte count (THC), the hemocyte size frequency distribution, the endocytotic activity, and the activities of lysozyme, acid phosphatase, superoxide dismutase (SOD) and catalase (CAT), were measured during the pre-spawning period to assess whether the two sexes reach the spawning period with a different immunosurveillance status (Matozzo and Marin, 2010). That study demonstrated that gender-related differences in immune parameters can occur in clams during the pre-spawning period and indicated that the hemocytes from females were more active compared with those from males (Matozzo and Marin, 2010). A relationship between THC and the temperature-dependent reproductive cycle has been found in *Mytilus galloprovincialis* from the Lagoon of Venice; the hemocyte number was lower in the summer during the spawning period compared with the spring and winter (Pipe *et al.*, 1995). In *Mya arenaria*, the percentage of hemocytes with ingested fluorescent particles (indicative of phagocytic activity) and cell viability varied significantly among the sampling sites (the St. Lawrence Estuary and Saguenay Fjord, Québec, Canada), whereas no influence of gender was observed (Gagné *et al.*, 2008). However, in the same study, a slight gender-related difference in the number of hemocytes was recorded, with females

having somewhat fewer hemocytes compared with males. Spawning can be a stressful condition for the immune response of molluscs. In *Mytilus edulis*, a relationship between lowered phagocytic activity and spawning has been observed (Fraser *et al.*, 2013). In some cases, spawning can reduce the phagocytic activity of mussel hemocytes by 60% when compared with hemocytes from mussels collected after the spawning phase (Fraser *et al.*, 2014). In the same mussel species, the phagocytic activity of the hemocytes from females was significantly reduced after exposing cells to 10^{-5} M of mercuric chloride. Conversely, a significant reduction in the phagocytic activity of the hemocytes from males was recorded at 10^{-4} M of mercuric chloride, a 10-fold higher concentration, suggesting that the hemocytes from females were more sensitive to metal exposure compared with those from males (Brousseau-Fournier *et al.*, 2013).

Other environmental characteristics can strongly affect hemocyte parameters in molluscs. In a recent study, clams (*R. philippinarum*) were collected for one year in two sites of the Lagoon of Venice characterised by different environmental conditions: a seaward site close to a Lagoon inlet where high hydrodynamism and frequent shipping activity occur, and a landward site with low hydrodynamism and riverine inputs (Matozzo *et al.*, 2012). The measured hemocyte parameters highlighted an overall better condition for clams collected from the seaward site; however, no clear differences in contamination levels of sediments were observed between the two sites. This study suggests the unique environmental conditions (differences in salinity, total chlorophyll, sediment grain size and organic matter) of the two sampling sites affected the hemocyte parameters in bivalves. In addition, these results indicate animals experiencing different environmental conditions can respond differently to the experimental exposure to contaminants. To test this hypothesis, clams (*R. philippinarum*) were collected from two sites of the Lagoon of Venice with different environmental conditions: Marghera, which is characterised by a relatively high contamination level and is a location where clam fishing is restricted, and Chioggia, a site inside a licensed area for clam culture with lower contamination levels (Matozzo *et al.*, 2013). A number of hemocyte parameters, such as THC, the diameter and volume of hemocytes, and the lysozyme activity in both the hemocyte lysate and the cell-free hemolymph, were measured soon after clam sampling, after 7 days of acclimation in the laboratory and after 1, 3 and 7 days of exposure to copper. The results revealed a persistent difference in the hemocyte response of clams from the two sampling sites before and after exposure to copper, indicating bivalves with a different ecological history respond differently to experimental exposure to contaminants.

Tide or distance from the shore can also influence mollusc hemocyte parameters. It has been demonstrated that the hemocytes of clams (*M. arenaria*) exposed for a longer period of time to the air (low tide) are more sensitive to the toxic effects of metals (Alix *et al.*, 2013). The spatial distribution of bivalves on the shore (upper, middle and lower)

can also affect their immunocompetence. In *M. arenaria*, animals from upper and middle ranges showed markedly lower phagocytic activity compared with those from the lower range (Beaudry *et al.*, 2013). Similarly, bivalves (*M. arenaria*) that are collected closer to the shore can have a higher number of circulating cells compared with clams from beds further offshore (Gagné *et al.*, 2009).

A relationship between population characteristics and the response of immunomarkers has been demonstrated in clams (*M. arenaria*). In particular, clam density was significantly correlated with the viability of hemocytes, while residual clam density (clam density not related to distance from the estuary) was correlated with the viability of hemocytes and the phagocytic activity (Gagné *et al.*, 2008). In addition, the study demonstrated that when clam density was corrected for salinity differences (estuary), there was a significant correlation with hemocyte activity, phagocytosis and cellular energy expenses. Considering that immunocompetence biomarkers are strongly associated with clam population metrics, the authors suggest that the immunomarkers could serve as predictive biomarkers, not only for clam health but also for population health as well.

In conclusion, to date, eco-immunology studies on molluscs have largely dealt with both the constitutive immune defences and the effects of pollutants on immunomarkers. According to Ellis *et al.* (2011), the understanding of the effects (also cumulative) of environmental stressors and biotic factors on mollusc immunocompetence is an important topic, especially when considering that interspecific differences in response to the same environmental stressor can often occur among mollusc species and among different invertebrate species in general. Consequently, in the field of eco-immunology, there is a need to increase knowledge on the effects of a wider range of stressors, alone or in combination. This is particularly true in a climate change scenario, as variations in various environmental factors could occur concurrently. A deviation from the optimum for some environmental factors (*e.g.*, temperature, salinity, oxygen, and pH) may have deleterious effects on the physiological performance of animals, including immunosurveillance. A reduced immunosurveillance of animals can in turn have negative consequences for the entire population. In this context, in order to increase the capability of eco-immunology investigations to predict both the ecological and the evolutionary impacts of environmental stressors, future studies should be performed employing traditional immunological and emerging techniques, such as those of the “omics” disciplines.

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