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SHORT COMMUNICATION

Monitoring of the immune efficiency of *Mytilus galloprovincialis* in Adriatic sea mussel farms in 2006: regular changes of cytotoxicity during the year

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Abstract

By monitoring the course of hemolymph cytolytic activity in *Mytilus galloprovincialis* during 2006, we have observed important fluctuations in the percentage of cytotoxic animals over the year. The changes seem to be correlated with seasonal variations in the temperature, but observations in mussels kept in aquaria indicated that this parameter is not the main cause of the fluctuations. Data presented here suggest that normal levels of cytotoxicity can be predicted in a population for a specific period of the year, therefore confirming the value of this parameter in determining the immune efficiency of mussels at a given time.

Key words: *Mytilus galloprovincialis*; cytotoxicity; immune efficiency

Introduction

The presence of hemolytic molecules in the hemolymph of molluscs has been reported on several occasions (Wittke and Renwrantz, 1984; Merker and Levine, 1986; Hubert *et al.*, 1997), but the natural target of these molecules has still not been clarified. It is conceivable that hemolyitic factors can be included in the humoral component of invertebrate innate immunity (Hubert *et al.*, 1997), but there is very little information about the relationship between hemolytic activity and immune efficiency in the mussel (Malagoli and Ottaviani, 2005). Recently, the hemolytic activity of the bivalve *Mytilus galloprovincialis* has been seen to be influenced by stressful and pathological conditions imposed either in laboratory aquaria (Malagoli and Ottaviani, 2005) or encountered in mussel farms (Franchini *et al.*, 2005). In order to take hemolytic activity as a valid parameter for evaluating whether the immune efficiency of mussels is compromised in particular circumstances, it is important to know how this activity changes during the year.

This report provides data on fluctuations in hemolymph cytotoxic activity in mussels from the Adriatic Sea in Italy during 2006. Moreover, comparison of the present results with data collected in 2005 (Malagoli *et al.*, 2005) and with seasonal variations in water temperature suggest that hemolymph cytotoxicity is a parameter subjected to seasonally-regulated fluctuations.

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Materials and Methods

Animals

Specimens of the bivalve mollusc *Mytilus galloprovincialis* were obtained monthly from local fishermen in the Cesenatico area (FC, Italy). After their collection, 40 animals were used to obtain the hemolymph immediately, while the remaining specimens were maintained in the laboratory aquaria in artificial seawater (temperature 16 ± 1 °C, pH 8.0 ± 0.2 and salinity 35 ± 1 psu). After 14 days, a further 40 mussels were sacrificed and the hemolymph withdrawn.

Hemolymph preparation and cytotoxicity assay

The detailed procedure for the hemolysis assay is described elsewhere (Malagoli and Ottaviani, 2005). In short, the hemolymph was collected by gently aspirating with a sterile syringe inserted between the mussel valves and filtered into sterile tubes using 0.2 μm sterile filters. Hemolytic activity was evaluated by checking the cytolysis of human A positive erythrocytes obtained after washing the whole blood at least three times in 9 vol. of sterile NaCl 0.9 %. Subsequently, the erythrocytes were

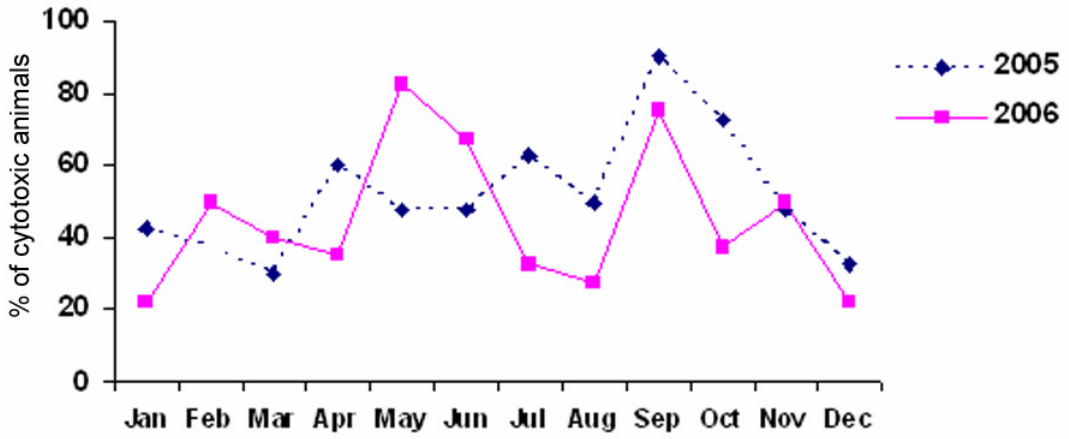

Fig. 1 Course of mussel cytotoxicity during 2005 and 2006 in the of Cesenatico area. Please note that the value for February 2005 is missing (Malagoli *et al*., 2005).

re-suspended in sterile TBS (50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, pH 8.5) at a final concentration of $2x10^9$ cells/ml. Five hundred μl of filtered hemolymph were added to 500 μl of erythrocyte suspension and then incubated for 1 h at 25 °C (Hubert *et al*., 1997; Malagoli and Ottaviani, 2005). After incubation, samples were centrifuged at 3000xg for 5 min at 4 °C, and the optical density (OD) of the supernatants was evaluated by measuring absorbance at 541 nm with a Helios β spectrophotometer (Spectronic Unicam, Cambridge, UK). Samples with an OD above the fixed OD threshold level of 0,5 where considered cytotoxic (Malagoli and Ottaviani, 2005). The experiments were repeated twice in duplicate for each animal. All chemical reagents came form SIGMA-Aldrich (St Louis, MO, USA).

Results and Discussion

The monthly evaluation of the hemolytic activity revealed significant fluctuations in the percentage of cytotoxic animals during the year (Fig. 1). Even if the mean percentage of cytotoxic bivalves is 45 %, two peaks were registered during the year: one at the end of the spring and the second at the end of the summer. Interestingly, the trends in hemolyitic activity in the second half of 2005 and 2006 almost overlapped (Fig. 1). Since no peculiar situations were reported for the area in which the mussels were reared, the comparable results indicate that cytotoxicity in mussel populations is normally subject to fluctuations during the year. Temperature can be considered the most common variable in the mussel farm area assessed in this study. We therefore compared the time course of water temperature with that of cytoxicity in the mussel population (Fig. 2). The two variables seem to be correlated, since the peak in hemolytic activity in the hemolymph corresponded to the two periods in which the temperature either started to rise or to fall (Fig. 2). However, the comparison of cytotoxicity between animals sacrificed immediately after collection and those kept for two weeks in aquaria at a constant temperature indicates that temperature cannot be the main parameter in determining cytotoxicity (Fig. 3). For each month, the percentage

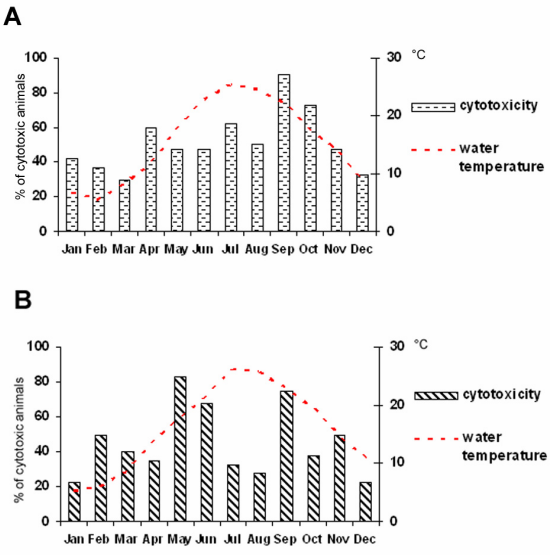

Fig. 2 Comparison between hemolymph cytotoxic activity and temperature variations during 2005 (A) and 2006 (B).

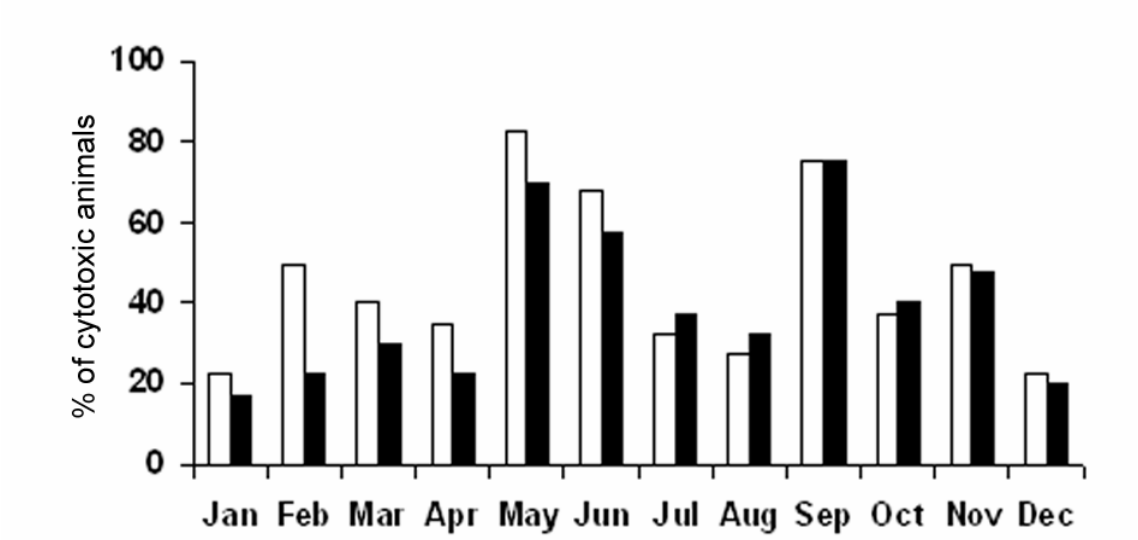

Fig. 3 Comparison between cytotoxicity immediately after collection (white bars) or after a period of conservation in the aquarium at 17 °C (black bars).

of cytotoxic mussels was almost identical in the two groups of animals. The essentially unmodified cytotoxic activity following conservation in the aquarium is in agreement with observations in 2005 (Malagoli *et al.*, 2005), demonstrating that even when mussels are maintained at a constant temperature different to that of the seawater, the level of cytotoxicity among population does not change significantly. In previous articles, we have reported that sudden changes in aquarium temperature can modify the number of animals displaying significant cytotoxic activity (Malagoli and Ottaviani, 2005), but we have also found that when sudden modifications in environmental parameters do not intervene, cytotoxic activity is a relatively stable immune function (Malagoli *et al.*, 2005). Further studies are required to establish whether the component influencing the time course of cytotoxicity is mainly environmental or rather connected to the mussel life cycle.

The mussel's cytotoxic response to seasonal changes would represent a classical example of ecoimmunology. Evolutionary ecologists assume that immunological defences must be minimized in terms of metabolic cost, because there must be a trade-off between maintaining a normal immune response and facing the significant changes in life conditions. From an evolutionary point of view, this balance plays a key role in species survival (Lochmiller and Deerenberg, 2000).

Concluding, mussel cytotoxicity is an activity that changes over the year with a regular time course, meaning that normal levels can be predicted for a given period. Our observations support the idea of using hemolymph cytotoxicity as a useful parameter in evaluating the immune efficiency of mussels at a specific point in time (Malagoli and Ottaviani, 2005).

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