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 hemolytic 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.

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...
re-suspended in sterile TBS (50 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl₂, pH 8.5) at a final concentration of $2 \times 10^9$ 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 were considered cytotoxic (Malagoli and Ottaviani, 2005). The experiments were repeated twice in duplicate for each animal. All chemical reagents came from 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 hemolytic 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 cytotoxicity 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

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**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).

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