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Lysosomal responses as a sensitive stress index in biomonitoring heavy metal pollution

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ABSTRACT: Lysosomal responses in digestive cells of *Mytilus galloprovincialis*, collected from a relatively clean area (La Spezia, Italy) and a heavy metal polluted one (Scarlino, Italy), were tested over 1 yr. Lysosomal characteristics were also measured in mussels transferred for 6 mo to the polluted site and in mussels from both populations kept for 4 wk in indoor tanks with clean running seawater. Organisms exposed to high environmental levels of heavy metals showed a reduced lysosomal membrane stability and an enhanced production of lipofuscin. Enlarged secondary lysosomes were observed in transplanted mussels. Depuration in clean water caused a slight but significant increase in lysosomal membrane stability of contaminated animals from Scarlino, which, however, remained below the levels observed in the controls. Lipid content in digestive cells of transplanted organisms was lower than in natural mussels from both populations. No evidence of fatty degeneration was observed as a result of heavy metal exposure. These observations confirm the sensitivity of lysosomes to environmental metal pollutants and support the utility of stress indices based on the responses of these organelles in biomonitoring studies.

INTRODUCTION

Marine molluscs are extensively used in biomonitoring studies due to their ability to concentrate both organic and metallic pollutants (Goldberg 1975, 1980, 1986, Phillips 1980). Recently many authors have outlined the importance of environmental impact assessment programs including methods which measure the biological effects of pollutants on the health condition of organisms (Bayne 1980, Widdows et al. 1981, Bayne et al. 1982, Livingstone 1982, Moore 1985). Many studies have been carried out to develop stress indices at different levels of biological organization (Scott & Major 1972, Abel 1976, Hrs-Brenko et al. 1977, Widdows 1978, Bayne et al. 1980, Axiak & George 1987, Axiak et al. 1988, Vega et al. 1989). In fact, these biological responses can be considered as biomarkers of toxicity to central metabolic organs such as the digestive gland of mussels.

Investigations at the subcellular level can reveal alterations at an early stage of response, before integrated cellular damage shifts to the level of organ or whole animal physiological processes. In many instances, the earliest detectable alterations are associated with lysosomes (Moore 1985), a particular class of subcellular organelles mainly involved in the intracellular digestion of food, cellular defence mechanisms, protein and organelles turnover, and regulation of secretory processes. Lysosomal alterations have been reported in several marine invertebrates (i.e. mussels and periwinkles) exposed to xenobiotics such as polycyclic aromatic hydrocarbons (Moore et al. 1980, 1982, 1985, 1986, 1987, Lowe et al. 1981, Moore 1982, 1988).

It is known that lysosomes are also involved in the metabolism of heavy metals (Sternlieb & Goldfischer 1976). Metal sequestration was observed in the lysosomes of mussel tissues by several authors (George et al. 1976, 1978, Moore & Lowe 1977, Schulz-Baldes 1978, Lowe & Moore 1979, Harrison & Berger 1982, Viarengo et al. 1987). As the digestive gland of mussels is a target organ for heavy metal accumulation and its lysosomal system is highly developed (Owen 1972), this organ represents a useful model for investigating the responses of lysosomes to heavy metal pollution along the coastal areas.

However, for correct use of a stress index in the assessment of disturbances due to pollution, it is indispensable to know whether the response investigated exhibits natural fluctuations related, for example, to the seasonal cycle of the animals.

In the present work the lysosomal membrane stability was tested in the digestive gland of 2 populations of

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Mytilus galloprovincialis, from a clean and a heavy metal polluted area respectively, as well as in those maintained in clean running seawater. Lysosomal responses were also assayed in mussels transplanted from the clean to the polluted area.

MATERIALS AND METHODS

Experimental design. Lysosomal responses were tested at intervals over a period of 1 yr in mussels from 2 different areas of the Tyrrhenian Sea: Scarlino, highly polluted by heavy metals of industrial origin, and La Spezia, where mussels were obtained from a local marine farm.

Lysosomal membrane stability (based on hydrolase latency) was also measured in mussels transplanted for 6 mo from La Spezia to Scarlino and in organisms from both the populations allowed to depurate for 4 wk. This part of the work was carried out at the Interuniversitary Centre of Marine Biology (Livorno), where the system included depuration tanks (13 × 1 × 1 m) supplied with running unfiltered seawater at a flow rate of 5 l s^{-1}. Water temperature was approximatively 14 °C and salinity 37 ‰.

Metal analysis. Digestive glands dissected from 20 specimens (5 ± 1 cm in shell length), were subdivided in 5 samples, each constituted by the organs of 4 mussels, and stored at –30 °C until processed for analysis.

Samples were dry weighed and then digested with nitric acid (BDH Aristar 70 %), first at room temperature for 8 h, then at 120 °C for 10.5 h in teflon vessels.

The same procedure was also used for lobster standards (provided by National Research Council, Canada) to check the accuracy of the analytical methods.

Cu, Mn, Fe, Pb, Zn were determined by Atomic Absorption Spectrophotometry (IL model S11 equipped with a deuterium background corrector and an IL model 755 graphite furnace).

Lysosomal responses. Small pieces of digestive glands, rapidly excised from 10 mussels, were placed on cork chucks, immersed in hexane precooled to −70 °C in liquid nitrogen, sealed by double wrapping in parafilm and stored at −80 °C.

Lysosomal membrane stability was tested in 10 µm unfixed duplicate cryostat sections by determining the lysosome acid labilization period which is the time of pretreatment of serial sections at pH 4.5 and 37 °C required to artificially labilise the lysosomal membrane. At that time, a substrate of the assayed enzyme can penetrate through the membrane into the lysosomes and react with the lysosomal enzyme. The enzyme-substrate is then made visible by staining with a diazonium salt. When maximum staining intensity is achieved lysosomal stability (in min) can be calculated.

The acid hydrolase β-N-acetylhexosaminidase was tested according to Moore (1976, 1988) as described below.

Sections, cut in a Leitz 1720 cryostat (−27 °C cabinet temperature and knife packed in dry ice), were pretreated at 37 °C in 0.1 M citrate buffer (pH 4.5) containing 2.5 % NaCl in order to labilize the lysosomal membrane (Bitensky et al. 1973, Moore 1976). Pretreatment times of 0, 2, 5, 10, 15, 20, 25, 30 min were applied to the serial sections. The slides were then incubated for 20 min at 37 °C in 50 ml of 0.1 M citrate buffer (pH 4.5), containing 20 mg naphthol AS-BI-N-acetyl-β-D-glucosaminide (Sigma) previously dissolved in 2.5 ml N-N-dimethylformamide (instead of 2-methoxyethanol as in the original method), 1.5 g NaCl and 3.5 g of Polypep (Sigma), a low viscosity polypeptide acting as a section stabilizer (Bitensky et al. 1973, Moore 1976). Sections rinsed in 3 % NaCl at 37 °C for 2 min were then transferred to 0.1 M phosphate buffer (pH 7.5) containing the diazonium coupler Fast Blue RR or Fast Violet B (1 mg ml^{-1}) for 10 min at room temperature. After a brief rinse in tap water, slides were fixed in 4 % calcium formol (+ 2.5 % NaCl) and mounted for microscopical examination.

The labilization period was determined according to Moore (1988) by microscopical assessment of the pretreatment time required to produce maximal staining intensity in the series of sections.

Cryostat sections were also stained for lipofuscin according to Schmorl (Pearse 1972), and for unsaturated neutral lipids with the Oil Red O reaction (Bancroft 1967).

Statistical analysis. Data were compared using Student's *t*-test for heavy metal concentrations, and Mann-Whitney *U*-test for lysosomal membrane stability (Moore et al. 1979, Moore 1988).

RESULTS

Concentrations of Cu, Mn, Fe and Pb in the digestive gland of mussels were higher at Scarlino than at La Spezia, whereas Zn did not show significant differences (Table 1).

Lysosomal membrane stability differed considerably ($p < 0.05$) in mussels from the 2 study sites (Fig. 1). The labilization period of the lysosomal membrane ranged with a few exceptions from 20 to 25 min in mussels from La Spezia (Fig. 1A), while much lower values (2 to 6 min) were seen in mussels from Scarlino (Fig. 1B).

Mussels transplanted from La Spezia to Scarlino for 1 wk showed a reduction ($p < 0.05$) of the lysosomal membrane stability to levels comparable to those observed in native mussels at Scarlino (Fig. 1C).

No changes were observed in mussels from La

Table 1. *Mytilus galloprovincialis*. Heavy metal concentrations in μg g^{-1} dry weight (mean ± SD, n = 5) in the digestive gland of mussels from La Spezia and Scarlino. Differences between mean values: • = $p > 0.05$; •• = $p < 0.05$

Location	Cu	Mn	Fe	Zn	Pb
La Spezia	15.6 ± 0.6	19.1 ± 5.2	605 ± 88	118 ± 17	6.1 ± 1.3
Scarlino	22.2 ± 1.1 ••	198 ±68 ••	3150 ± 468 ••	153 ± 26 •	152 ± 32 ••

Spezia during the 4 wk of depuration (Fig. 2A), whereas a slight but significant increase of lysosomal membrane stability ($p < 0.05$) was observed in mussels

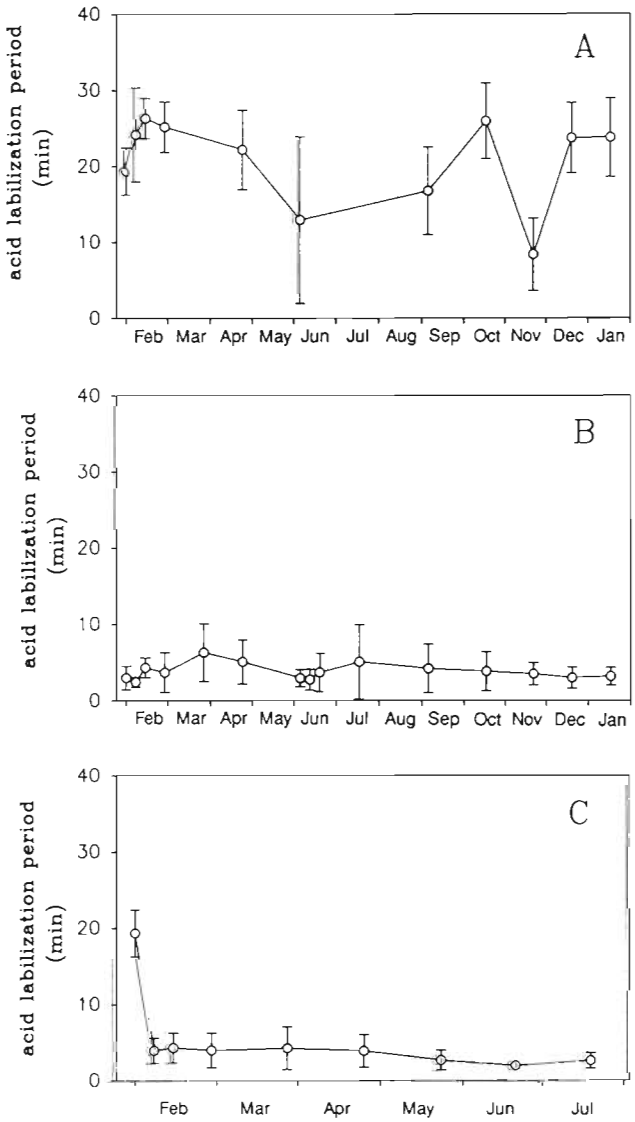

Fig. 1. *Mytilus galloprovincialis*. Responses of lysosomal membrane stability in digestive gland of (A) mussels from La Spezia, (B) mussels from Scarlino, and (C) mussels from La Spezia transplanted to Scarlino. (Mean ± SD, n = 10)

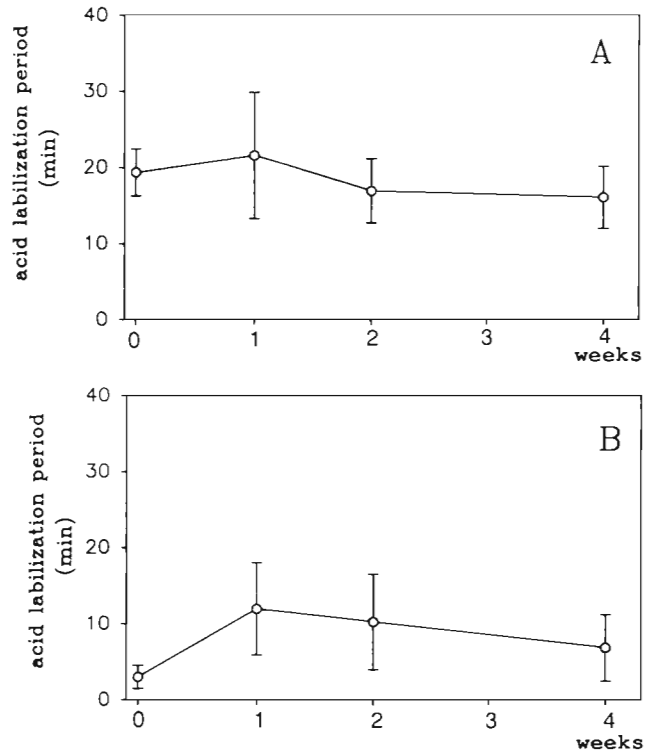

Fig. 2. *Mytilus galloprovincialis*. Effects of depuration in clean running seawater on lysosomal membrane stability in mussels from (A) La Spezia and (B) Scarlino. (Mean ± SD, n = 10)

from Scarlino (Fig. 2B). However, this increase remained below the level observed in controls.

Lysosomal perturbations in mussels exposed to environmentally high levels of heavy metals was also inferred from evidence of an enhanced amount of lipofuscin in tertiary lysosomes (Fig. 3). In addition, mussels transplanted to Scarlino also developed enlarged secondary lysosomes (Fig. 4).

Seasonal differences in the content of unsaturated neutral lipids were observed in the digestive gland of natural mussels; maximum of staining occurred in summer and minimum in winter and autumn, with similar patterns in the 2 populations. However, mussels transplanted during the winter from La Spezia to Scarlino failed to increase their lipid content, and the maximum summer staining was not observed (Fig. 5).

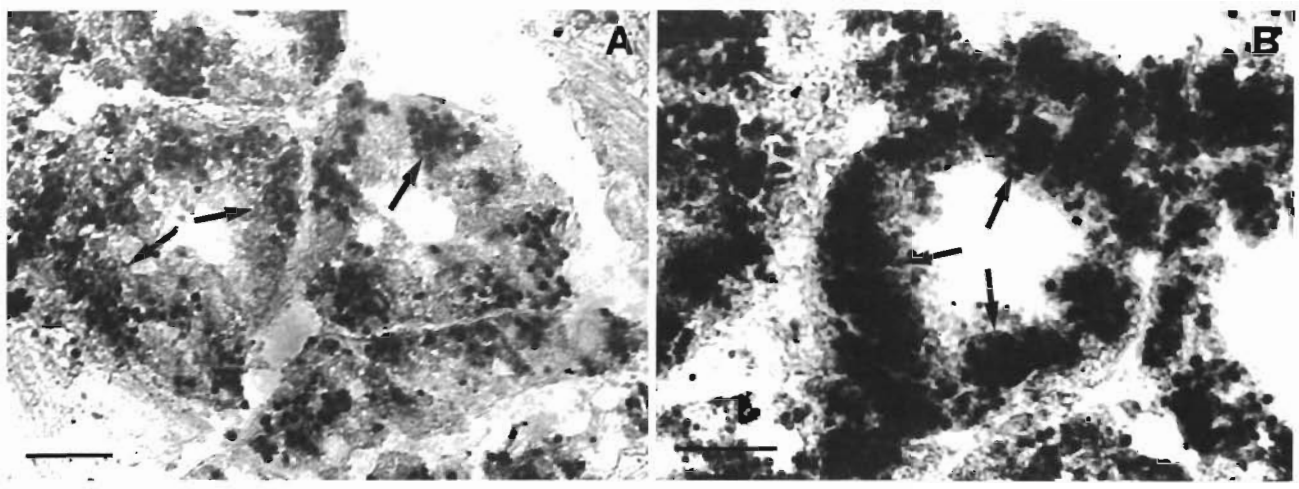

Fig. 3. *Mytilus galloprovincialis*. Digestive tubules showing lipofuscin accumulation (arrows) in tertiary lysosomes of mussels from (A) La Spezia and (B) Scarlino. Scale bar: 20 μm

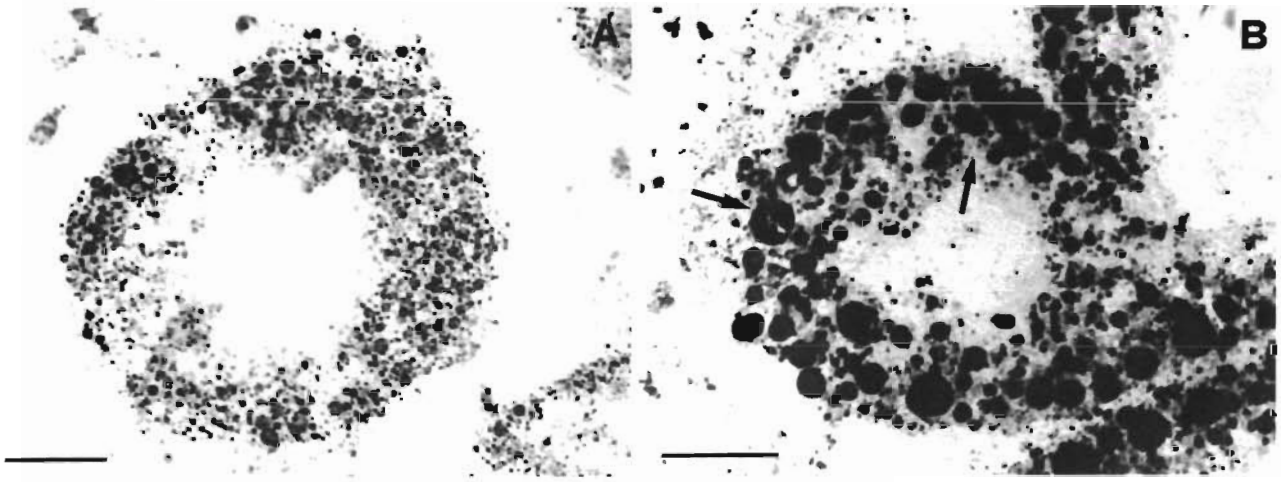

Fig. 4. *Mytilus galloprovincialis*. Digestive tubules reacted for the lysosomal marker enzyme β-N-acetylhexosaminidase. (A) Normal lysosomes and (B) enlarged lysosomes (arrows) in mussels from La Spezia transplanted to Scarlino. Scale bar: 20 μm

DISCUSSION

From this study it appears that concentrations of 5 metals (Cu, Mn, Fe, Zn and Pb) in mussels from La Spezia are similar to values reported for relatively unpolluted areas of the Mediterranean (Lulic & Strohal 1974, Sheppard & Bellamy 1974, Fowler & Oregioni 1976, Majori et al. 1978, Renzoni 1980, Leonzio et al. 1981). On the contrary, mussels from Scarlino were heavily contaminated, especially by lead, iron and manganese. The values presented in this work refer only to February, but similar differences in heavy metal concentrations were observed over the course of 1 yr (author's unpubl. data), and are assumed to be related to the presence in Scarlino of factories which use pyrites and titanium minerals to produce sulphuric acid and titanium dioxide.

The analysis of lysosomal membrane stability in mussels from Scarlino (both natural and transplanted) clearly indicated a severe dysfunction of the lysosomal system. This phenomenon is presumably related to the excessive levels of heavy metals accumulated in the organelles.

Similar alterations were described in marine invertebrates exposed to xenobiotics such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls and heavy metals (Moore & Stebbing 1976, Moore et al. 1978, Moore 1979, 1988, Harrison & Berger 1982, Moore & Clarke 1982, Livingstone et al. 1986, Moore & Viarengo 1987, Viarengo et al. 1987).

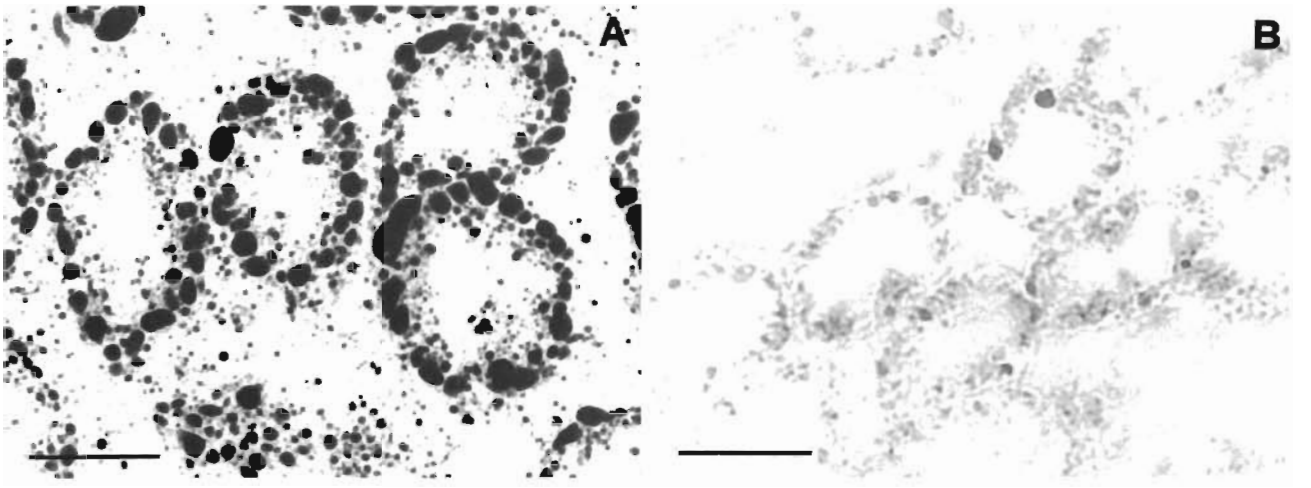

Fig. 5. *Mytilus galloprovincialis*. Digestive tubules showing lipid content in summer. (A) Natural mussels from Scarlino showing numerous large lipid droplets, which were found also in the mussels of La Spezia. (B) Mussels from La Spezia transplanted to Scarlino showing an important reduction of lipid droplets. Scale bar: 50 μm

The severe dysfunction of the lysosomal system in mussels from Scarlino, as indicated by the reduction of membrane stability, probably masked any effects resulting from seasonal differences in environmental conditions.

The heterogeneous response in lysosomal membrane stability found in summer in mussels from La Spezia, indicated by the large standard deviation (Fig. 1A), could be due to a thermal stress induced by the increase of temperature in the relatively closed and shallow waters of the marine farm during the summer. In this respect, Moore (1976) observed a significant reduction in the latency of lysosomal β-hexosaminidase after exposure of mussels to temperatures of 25 to 28 °C. In addition, he found a greater decrease in summer than in winter, a finding which suggests a possible seasonal difference in lysosomal activity. The data presented here support the hypothesis that mussels are more susceptible to stress in summer than in winter.

The reduction in lysosomal membrane stability observed in November (Fig. 1A) could be related to the reproductive cycle. Although *Mytilus galloprovincialis* in the Mediterranean has a prolonged reproductive period, principal spawning usually occurs in autumn (Berner 1935, Renzoni 1963, Bourcart & Lubet 1965).

Thompson et al. (1974) demonstrated that in *Mytilus edulis* metabolic reserves move from digestive gland to gonad during the later stages of gametogenesis and after spawning. These events, associated with a degeneration in the structure of digestive tubules and an increase of autophagic processes, result in severe lysosomal disturbance (Bayne et al. 1978).

More difficult to interpret are the results concerning mussels maintained for 4 wk in indoor depuration tanks. No significant effects were observed in mussels from La Spezia, thus indicating good environmental conditions (such as level of available food) in the experimental system. Mussels from Scarlino showed only a slight increase in lysosomal stability. However, it must be pointed out that only a slight decrease in trace metal concentrations was observed after the depuration period (author's unpubl. data); this fact could explain the persistance of the lysosomal stress.

The presence of enlarged lysosomes in mussels transplanted to Scarlino showed another feature of the complex lysosomal response to pollutants; lysosomal enlargment provoked by fusion reflect profound alterations in the rate of fusion events in the lysosomal-vacuolar system of the digestive cells (Hawkins 1980, Moore 1985, 1988). In this respect the scarcity of enlarged lysosomes in the natural mussels of Scarlino deserves further investigation.

Lipofuscin is the end product of lipid peroxidation of membraneous components (Davies 1983) and, if accompanied by lysosomal alterations, can be considered a good indicator of increased autophagic processes (Moore 1988). Moreover, lipofuscin granules may contribute to the detoxification of heavy metals which, trapped both chemically and mechanically (George 1983), are eliminated by excretion of residual bodies (Viarengo 1989, Viarengo et al. 1990). The enhanced levels of lipofuscin found in mussels from the polluted environment are in agreement with the data of Viarengo et al. (1987) who observed lipofuscin accumulation in the digestive cells of mussels exposed to Cu.

Furthermore, Moore (1988) reported an accumulation of unsaturated neutral lipids within lysosomes of digestive cells in mussels exposed to organic pollutants. We did not observe a similar effect in mussels exposed to heavy metals in the Scarlino area. *Mytilus*

galloprovincialis from both Scarlino and La Spezia showed a marked seasonal cycle with a maximum lipid content in summer and minimum in winter. Thompson et al. (1974) also found lipids stored in the digestive gland of *M. edulis* during the summer, and a total gland lipid decrease during late August and September.

The very low levels of digestive gland lipids in transplanted mussels, with no appreciable increase during the summer, could be explained by the fact that reserve materials from this organ are rapidly utilized during periods of stress (Thompson et al. 1974). The scarcity of energy reserves available for gametogenesis would probably reduce the reproductive capability of these organisms. Therefore, it is suggested that the difference in gland lipid content of transplanted animals as compared with natural mussels from Scarlino may be due to a different adaptation to the stressed environment.

The present work confirms the sensitivity of lysosomes to environmental pollutants including heavy metals. Alterations of these organelles could interfere with the intracellular digestion of food, the normal turnover of proteins and organelles, and the regulation of fusion processes associated with the lysosomal-vacuolar system. Lysosomal responses, as early warning system for detection of environmental disturbances, could represent a useful tool for biomonitoring studies. However, for a correct interpretation of the results, seasonal variations, mainly due to the reproductive cycle of mussels, should be considered.

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