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Molecular and organism biomarkers of copper pollution in the ascidian *Pseudodistoma crucigaster*

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Abstract

We studied the effects of pollution in the colonial ascidian *Pseudodistoma crucigaster* at organismal and suborganismal levels. Our goal was to find early biomarkers to detect some effect of pollution before changes in community structure or species composition occur. We examined the effect of Cu on the production of heat-shock proteins, defence metabolites, growth rates and presence of resistance forms. We performed a transplant experiment to a Cu polluted harbour and observed negative growth and presence of resistance forms but not depressed production of toxic metabolites or an increase in stress proteins (hsp) in the ascidian. In a laboratory experiment, stress proteins were induced only under half the Cu concentration found in the harbour. We conclude that hsp can be used in this ascidian as an early warning system for sublethal pollution but that the response is inhibited above a threshold of the stressing agent, which may vary among species.
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Keywords: Cu pollution; Hsp; Natural toxicity; Biomarkers; Colonial ascidians; *Pseudodistoma crucigaster*

1. Introduction

The study of the health of aquatic ecosystems is usually performed at the community level, using changes in structure descriptors (e.g. Johnson et al., 1993; Sheppard, 1995) or at the species level, using the presence or absence of bioindicator species (JGESAMP, 1995). Nevertheless, the need for earlier and more efficient warnings of contamination has increased the use of molecular biomarkers, which can potentially signal a disturbance before its effects are visible at the community level (Di Pomerai, 1996; Depledge et al., 1995).

Cells respond to heat shocks and other stresses, such as those resulting from pollution, by the synthesis of an array of proteins that protect them from cellular damage (Lindquist, 1986; Morimoto et al., 1990; Sanders, 1993). Changes at molecular level occur at a threshold far less toxic than those that can be detected by classical monitoring and bioassays so that molecular biomarkers can be considered as early warning systems for sublethal effects of contamination (Cochrane et al., 1991). Among the most commonly used biomarkers of effect are the heat-shock proteins (hsp), which are induced under different stresses (heat, salinity, UV irradiance, organic pollutants, heavy metals).

Relationships between changes produced at the molecular level and those produced at organism level by pollutant-induced stress have been reported in several invertebrates. For instance, an increase in hsp synthesis is associated to an inhibition of scope for growth in *Mytilus* (Sanders et al., 1991). Köhler et al. (1992) found elevated hsp70 levels related to adverse effects on survival, fecundity and offspring production in the Isopod *Oniscus asellus*. In the sponge *Suberites domuncula*, a positive correlation has been found between accumulation of heavy metals such as cadmium and zinc and an increase of hsp synthesis (Müller et al., 1998).

Ascidians are a good diagnostic group whose distribution may reflect some of the prevailing factors affecting benthic assemblages (Papadopoulou and Kanias, 1977; Turon, 1990; Vazquez and Young, 1996; Naranjo

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et al., 1998). As filter feeders, they process large amounts of water and they can accumulate toxicants such as heavy metals or hydrocarbons in their tissues (Monniot et al., 1993, 1994). For this reason they are considered good indicators of water quality. *Pseudodistoma crucigaster* Gaill 1972 is a colonial perennial ascidian with slow growth (Turon and Becerro, 1992). It is common in the western Mediterranean, where it inhabits preferentially the first meters of sublittoral assemblages although it is not found in polluted environments (Turon, 1987). Its morphology varies from encrusting to massive depending on the habitat (Turon, 1990). *P. crucigaster* synthesizes an array of secondary metabolites that feature antimitotic, cytotoxic and antibacterial properties (Martin and Uriz, 1993).

Changes in production of secondary metabolites have been assessed in several benthic species (algae, sponges, bryozoans...) and have been generally related to environmental or physiological factors (Becerro et al., 1995; Uriz et al., 1996; Turon et al., 1996; Martí, 2002), but pollutants can decrease the synthesis of toxic metabolites (Agell et al., 2001; Caralt et al., 2002).

Moreover, little is known about heavy metal effects on ascidians. Cima et al. (1996) observed a decrease of viability in *Stylea plicata* embryos in response to heavy metals. In the same species, some studies have focused on how copper and tributyltin (TBT) affect the inmune reaction of cells (Raftos and Hutchinson, 1997). Recently, an accumulation of copper about 2.5 times higher than in controls has been reported for ascidians inhabiting polluted habitats (Caralt et al., 2002) and the effect of metal pollution on ascidian hemocytes has been evaluated (Radford et al., 2000).

The aim of this study was to assess the relationship between the various responses to stress produced by copper, from molecular to organism levels, using the ascidian *P. crucigaster* as a model species. In particular, we explored potential of the use of hsp proteins to predict sublethal stress, by field and laboratory experiments. Several individuals of *P. crucigaster* were transplanted to a polluted area where copper was the main contaminant (Pinedo, 1998; Cebrian et al., 2003), while others were maintained in their natural habitat. Organism-level variables such as growth rates, changes in morphology, and appearance of resistance forms were monitored, and molecular responses such as the production of bioactive substances and the synthesis of heat-shock proteins were assessed. The accumulation of heavy metals by the ascidian was also analyzed. Additional laboratory experiments were conducted to test the effects of varying concentrations of copper on the production of heat-shock proteins. Our goal was to evaluate the uses of heat-shock proteins to predict damage at higher levels of organization in an organism sensitive to stress by copper pollution.

2. Methods

2.1. Transplant experiment

A field transplant experiment was carried out in the Blanes sublittoral (western Mediterranean) at two different zones (clean and polluted, respectively) close enough to minimise damage and stress during transport. The control site was a vertical North-facing rocky wall from 4 to 7 m in depth. The polluted site selected was in the inner side of the Blanes harbour breakwater, 500 m from the control zone, and consisted of a vertical concrete wall with similar facing and depth. Both walls received similar incident light, as measured by a LiCor sensor (Cebrian et al., 2003). Other physical and chemical variables, such as sedimentation rates, nutrients, particulate organic matter and trace metals in the water were monitored monthly at both sites, and the results were described in detail in Cebrian et al. (2003). Briefly, most variables behaved similarly in both zones, except copper concentration, which was significantly higher at the contaminated site (ca. 97 μg/g in sediment).

In November 1999, we collected 50 individuals from the clean (control) zone. A total of 25 individuals were transplanted to the Blanes harbour, and the remaining 25 were transplanted to the same clean zone as a control for the possible effects of transplantation. Moreover, 25 further individuals were labelled and left untouched to be used as absolute controls. All transplanted specimens were taken with their substrate and placed in separate bowls to be glued in the new site using a two-component epoxy resin (IVEGOR®) within 1 h. Transplants were monitored monthly, and after six months (May 2000) all the individuals still alive were collected. The colonies collected were fragmented in three pieces. One of them was frozen to −80 °C for heat-shock protein analysis, while the other two fragments were freeze-dried for assaying the species natural toxicity (Martin and Uriz, 1993) and analyzing heavy metal accumulation, respectively.

2.2. Growth

We monitored growth of the colonies (absolute control, transplant control and harbour transplant) by taking close-up photographs with an underwater camera at the beginning and at the end of the experiment. The experimental period was chosen to coincide with the seasonal phase of active growth of this species (Turon and Becerro, 1992). Colony outlines were traced from the photographic slides and digitised. The area and perimeter of each colony was then obtained through the SigmaScan software (Jandel). From these values, we calculated a monthly growth rate with the formula:

$$Gr = \frac{(A_t - A_0)}{(A_0 * t)}$$

Where A_t and A_0 were the final and initial area of the colonies, respectively, and t was the number of months of the experiment. We computed also a shape parameter from the equation:

$$\mathrm{Sh} = \frac{S_a}{S_c},$$

where S_a is the area of the ascidian colony, while S_c is the area of a circle of equivalent perimeter. This index ranges between 0 and 1, the latter value corresponding to a perfect circle and lower values indicating irregular outlines. The relatively flat morphology of the colonies allowed us the use of area as an indicator of growth (Turon and Becerro, 1992). On the other hand, the shape parameter is a circularity index, which can be informative of the physiological state of invertebrate modular organisms (Becerro et al., 1994; Cebrian et al., 2003).

2.3. Toxicity analysis

We used MICROTOX® (Microbics, Carsbad, CA, USA), a standardised method described in (Kaiser and Ribó, 1988), to quantify the species natural toxicity, which is produced by an array of toxic secondary metabolites synthesized by the ascidian. This method measures light production by the bioluminescent deep-sea bacterium *Vibrio fischeri* = *Photobacterium phosphoreum*, and detects decreases of phosphorescence produced when the bacteria are placed in contact with a toxic substance. MICROTOX® measurements correlate well with other ecologically relevant tests and perform best in terms of precision and repeatability (Becerro et al., 1995). Fragments of colonies from all experimental conditions were freeze-dried, weighed, ground in a mortar and extracted three times (1 h each) with methanol. The three extracts were pooled and the solvent evaporated under reduced pressure (crude extract). Crude extracts include the amount of compounds produced by the ascidian, among which there are those potentially toxic. These crude extracts were weighed and resuspended through sonication in artificial seawater for toxicity analyses. They were assayed at an initial concentration of 2000 μg/ml relative to ascidian dry weight. Four decreasing concentrations (with a dilution factor of two) were tested after incubation for 5 min at 15 °C. The concentration of crude extract that produces 50% in light decrease (which is assumed to represent the death of 50% of phosphorescent bacteria) was calculated (EC_{50}). This EC_{50} was used in this study as an indicator of the sample toxicity.

2.4. Heavy metals analysis

In order to quantify accumulation of heavy metals in *P. crucigaster* tissue, fragments of freeze-dried colonies from the transplant experiment were ground in a glass mortar. We monitored the concentration of Cu, Pb, Cd and Hg. We also analysed V as this element is known to be concentrated by ascidians. Teflon tubes to be used in the analyses were previously cleaned with pure oxygenated water (H_2O_2) and nitric acid (HNO_3, 65%), rinsed with deionised water and then dried. Approximately 0.1 g of each colony (DW) was weighed, placed in one of the Teflon vials with 3 ml of 65% HNO_3, and 1 ml of H_2O_2, and put into a stove at 95 °C during 20 h for digestion. After that, samples were transferred to a set of weighed fingerbowls. 6 ml of milli-Q water was added to the digestion in Teflon vials and emptied into the corresponding fingerbowls, which were weighed again. Solutions in the fingerbowls were diluted (1:20) with HNO_3 1% and 10 ppb of Rh was added as an internal standard. Solutions were measured against a calibration prepared using 1 blank and 4 increasing concentrations of commercial standards of every element. Appropriate concentrations of standards were selected according to the element. Blanks were used to check for possible contamination.

Standards, samples and blanks were analysed in an inductively coupled plasma mass spectrometer (Perkins–Elmer Elan 6000 model). Total heavy metal accumulation in tissue was expressed as μg of metal per gram of dry weight of tissue (ppm).

2.5. Laboratory experiment

As copper was the main metal present in the harbour, a second experiment was carried out in the laboratory in order to find out whether copper at several concentrations produces a stress response in *P. crucigaster*. Thirty individuals of this species selected at random were taken from the control site and transported to the laboratory in coolers. The specimens were placed in 800 ml individual bowls at a constant temperature of 18 °C. Controls contained filtered, aerated seawater. Treatments consisting of 15 and 30 μg/l of copper solutions were changed daily to maintain nominal copper concentrations. These concentrations were selected to match the one potentially present in the harbour (30 μg/l) and one-half of it. The experiment lasted for five days and samples were then stored at −80 °C.

2.6. Hsp analysis

Stored individuals of *P. crucigaster*, both from the field transplant and the laboratory experiments, were homogenised on ice using as buffer a calcium- and

magnesium-free solution (pH 7.3) containing 20 mM HEPES, 500 mM NaCl, 12.5 mM KCl supplemented with 1 mM dithiothreitol (DTT), phenylmethylsulfonide (PMFS) 1 mM Tripsin Inhibitor and 1% Igepal Ca-630 (Sigma). Homogenates were then centrifuged at 15,000*g* for 1 h in a refrigerated centrifuge. Supernatants were immediately frozen to −80 °C. Total protein content was determined following Lowry et al. (1951) method using BSA as a standard. Samples were boiled for 5 min after the addition of SDS-PAGE sample buffer.

Samples of equal amounts of protein (15 μg for the field experiment and 25 μg for the laboratory experiment) were loaded on a 10% running gel with a 4% stacking gel. For Western blot procedures, gels were electroblotted onto nitrocellulose membranes (Bio-Rad) using 20 mM tris, 15 mM glycine, and 20% methanol (v/v) as transference buffer. The membranes were blocked with TBS containing 0.5% gelatin, 0.2% Tween-20 and 0.1% sodium azide for 30 min. After that they were probed with a 1:2500 dilution of monoclonal antibody directed against bovine brain hsp70 (Sigma). Blots were rinsed twice in TBS and then blocked with 1% BSA in TBS containing 0.5% gelatin and 0.2% Tween-20 for 30 min. The membranes were then incubated for 1 h in a 1:5000 dilution of alkaline phosphatase-conjugated goat antimouse IgG (Sigma). Excess of secondary antibody was removed by two washes in 0.2% Tween-20 in TBS. Finally, to visualise the bound sites of the antibody, the substrates p-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used. Western blots were scanned with Bio-Rad Fluor-S™ MultiImager equipment, and the band density was quantified using the software Quantity One (Bio-Rad). Thus, semiquantitative data on protein amounts are provided since it was not possible to use pure proteins as an internal standard.

2.7. Data analysis

For the analysis of growth, shape index, crude extract yield, toxicity and heavy metal concentrations we used standard ANOVAs after checking for normality and homoscedasticity of the data. Non-parametric equivalents (Kruskal–Wallis test) were applied when necessary (detailed in Results).

For the analysis of hsp data in the field transplant, one replicate each of absolute control, transplant control and treatment was assayed in the same Western blot for comparison. Similarly, in the laboratory experiment we included in the same Western blot one control, one treatment at a concentration of 15 μg/l of copper and one treatment at 30 μg/l of copper. Data from both field and laboratory experiments were analysed by a Randomised Block Design taking blots as the blocking factor.

3. Results

3.1. Growth, shape changes, resistance states and mortality

Notable differences in growth rates were observed between treatments (Fig. 1). Active growth was recorded for the specimens left untouched and for the transplant controls (monthly growth rates, 0.239 ± 0.041 and 0.224 ± 0.044, respectively, mean ± SE). On the other hand, colonies transplanted to the harbour showed negative growth (-0.091 ± 0.010). A Kruskal–Wallis test showed that the differences between groups were highly significant ($p < 0.0001$) and post hoc comparisons (Dunn's method) revealed that only the group transplanted to the harbour was significantly different from the other two.

The general shape of the colonies was quite circular, with shape index values above 0.7 (Fig. 2), as typical of the species (Turon and Becerro, 1992). The shape of the colonies remained fairly constant irrespective of the treatment during the study period, and no significant differences in shape index were detected at the end of the study (ANOVA, $p = 0.183$).

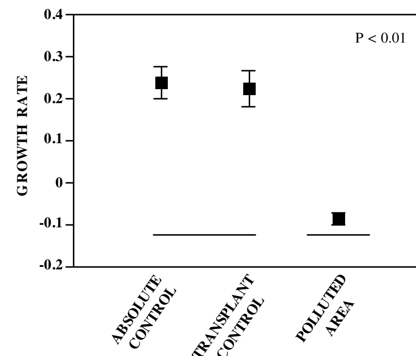

Fig. 1. Monthly growth rates of *Pseudodistoma crucigaster* (field experiment). Vertical bars represent standard errors. Growth rates which proved not significantly different in a Tukey test are joined by horizontal lines.

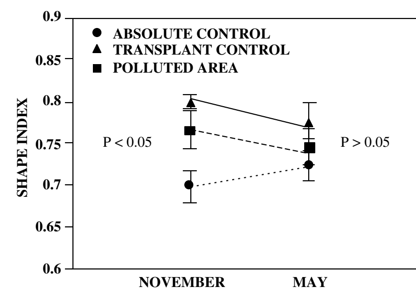

Fig. 2. Mean circularity index of *Pseudodistoma crucigaster* at the beginning (November) and the end of the experiment (May).

As for the general state of the colonies, as assessed visually, the individuals transplanted to the harbour were clearly in a worse state than both the controls and the transplant controls. From 25% to 83% of the harbour specimens, depending on the month, were in resistance form, with siphons closed. In contrast, only one specimen of the control colonies (and none of the transplant controls) was observed in resistance form once during the experimental period.

Only 12 colonies (out of 25) of each the harbour transplant and transplant controls were present at the end of the study, while 23 colonies (out of 25) of the 2absolute controls were still in place. The losses in the transplanted specimens were most likely attributable to detaching of colonies from the resin, rather than to natural mortality, as they occurred on the transplanted specimens and not on the absolute controls. Moreover, losses were concentrated in the first 2 months of the experiment, suggesting that colonies that were not well attached were washed away.

3.2. Heavy metals

Only Cu, Pb and V were found in detectable amounts in the samples. Concentrations in the blanks were below detection limits in all metals analysed (<0.2 ppm). For Cu and Pb, there was a marked increase in the concentrations found in the specimens transplanted into the harbour (Fig. 3). Cu values reached 47.1 ± 5.7 ppm (mean ± SE) in the harbour, one order of magnitude higher than the concentration found in the specimens kept outside the harbour. Pb concentration in the tissues of the transplanted colonies reached 15.1 ± 3.0 ppm, while the concentration was well below 5 ppm outside the harbour. For both elements, a Kruskal–Wallis test revealed significant differences among treatments ($p < 0.001$ in both cases), and the post hoc tests (Dunn's test) showed that the colonies inside the harbour had significantly higher values than the absolute and transplant controls, the latter two not being significantly different.

As for V accumulation, again, the highest values were found in the specimens kept inside the harbour (26.3 ± 3.7 ppm), but differences were not so high with respect to the absolute or transplant controls (16.3 ± 4.2 and 12.1 ± 1.3 ppm, respectively). A Kruskal–Wallis test detected a significant effect ($p = 0.011$), which was due to the differences between the harbour transplant and the transplant control (Dunn's test).

3.3. Toxicity

The percentage of crude extract obtained showed an increasing pattern, the specimens transplanted had a higher yield than the absolute controls, and the transplants to the harbour had, in turn, higher values than the transplant controls (Fig. 4A). The values were

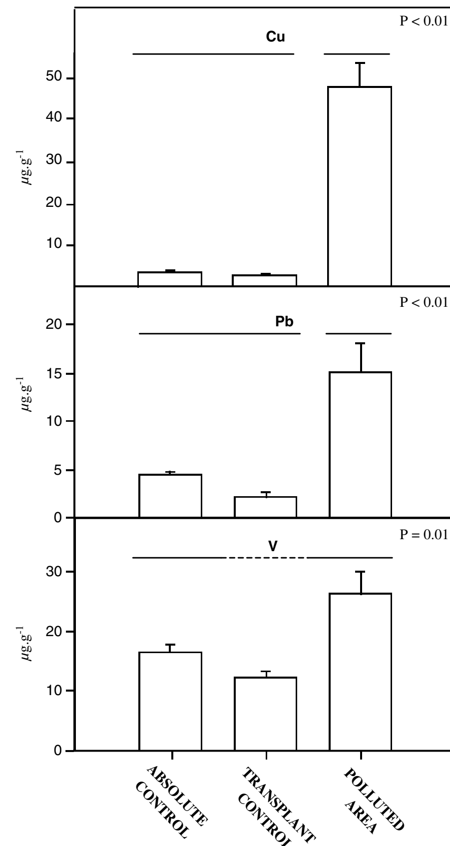

Fig. 3. Comparison of heavy metal accumulation in *Pseudodistoma crucigaster* in the field experiment. Bars represent standard errors. Horizontal lines join treatments that proved not significantly different in a Tukey test.

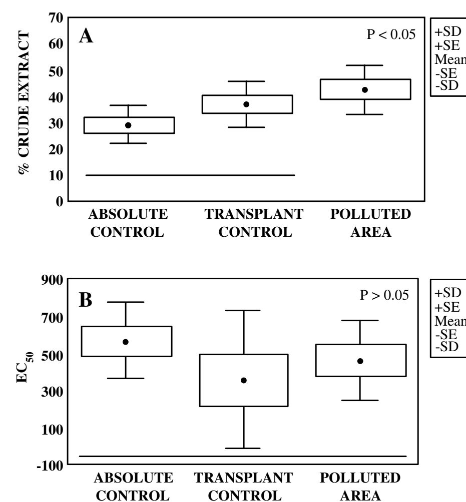

Fig. 4. (A) Percent of crude extract of*Pseudodistoma crucigaster* in the field experiment. (B) Mean toxicity (in EC_{50} values) of *Pseudodistoma crucigaster* in the same field experiment. Boxes and vertical bars are standard errors and standard deviations, respectively. Values that proved not significantly different in a Tukey test are joined by horizontal lines.

remarkably high, from 29.0 ± 2.4% in the absolute controls to 42.5 ± 3.6 in the harbour. The ANOVA showed significant differences among treatments ($p = 0.014$) and post hoc tests (Tukey HSD) revealed that this pattern was due to the differences between absolute controls and transplants to the harbour ($p = 0.012$).

The toxicity of the extracts, as measured by the EC_{50}, showed quite a different pattern (Fig. 4B). There was a high variance (especially so in the treatment controls) and overall there were no significant differences among treatments (ANOVA, $p = 0.385$).

3.4. Hsp

The monoclonal antibody raised against bovine homologous hsp70, immunoreacted in *P. crucigaster* revealing a single band with a molecular mass of 78 kDa, which confirms the high degree of conservation of this family of proteins.

In the field experiment, a slight but non significant (randomised block design, $F = 0.44$, $p > 0.05$) increase in hsp expression was found in transplanted individuals (both in situ and to the polluted site) with respect to those untouched (absolute control) possibly due to transplant stress (Fig. 5).

In the laboratory experiment, a concentration of 15 μg/l of copper induced significantly more hsp expression than controls and than a concentration of 30 μg/l of copper (Randomised block design, $F = 9.23$, $p < 0.01$) (Fig. 6).

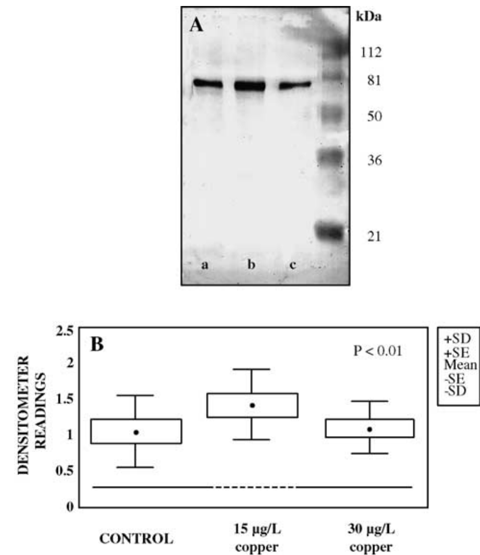

Fig. 6. (A) Western blot of heat-shock protein (HSP78) in *Pseudodistoma crucigaster* from the laboratory experiment: (a) control; (b) individuals incubated in the presence of 15 μg/l of copper, (c) individuals incubated in 30 μg/l of copper. Bands in the right lane represent molecular-weight markers that were run in parallel. (B) Densitometer readings of the heat-shock protein (HSP78) in *Pseudistoma crucigaster* from the laboratory experiment. Boxes and vertical bars are standard errors and standard deviations, respectively. Protein amounts which proved not significantly different in a Tukey test were joined by horizontal lines.

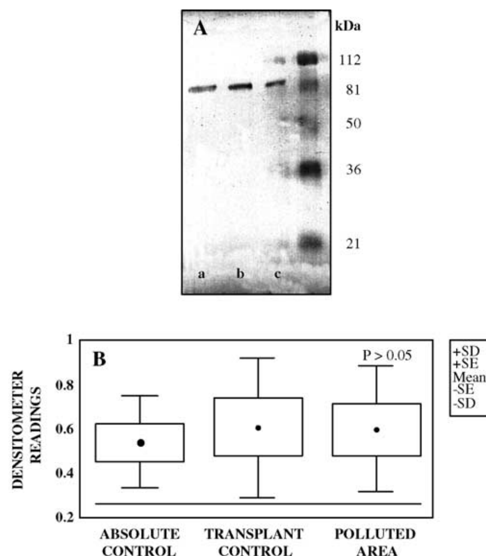

Fig. 5. (A). Western blot of heat-shock protein (HSP78) expression in *Pseudodistoma crucigaster* from the field experiment: (a) absolute control; (b) transplant control; (c) individuals transplanted to the polluted harbour for six months. Bands on the right lane are molecular-weight markers, which were run in parallel to the ascidian samples. (B). Densitometer readings of the heat-shock protein (HSP78) in *Pseudistoma crucigaster* from the field experiment. Boxes and vertical bars are standard errors ant standard deviations, respectively. Protein amounts which proved not significantly different in a Tukey test were joined by horizontal lines.

4. Discussion

Ascidians are benthic filter feeders that abound in harbour environments (Monniot et al., 1985; Lambert and Lambert, 1988). They can accumulate toxins in their tissues (Monniot et al., 1993, 1994) and thus can be used as indicators of water quality (Papadopoulou and Kanias, 1977). Rather than focusing on species adapted to these environments, we used as a test organism a species typical of cleaner waters in the zone in order to ascertain its response to an environment contaminated by copper such as Blanes harbour (Cebrian et al., 2003). The negative growth and the abundance of resistance forms confirm the strong effect of this environment on the colonial ascidian *P. crucigaster*.

Monthly growth rates of between 0.2 and 0.3, as found in the colonies inhabiting outside the harbour, fall well within the values observed during the active period of growth of natural populations of this species in the area (Turon and Becerro, 1992). Conversely, the conditions inside the harbour clearly impeded the normal growth of the colonies, which even featured some shrinkage.

Natural populations of this species during summer feature resistance phases in which the siphonal apertures are sealed by a thick, glassy cuticle (very similar to the phenomenon described by Turon, 1992, in another ascidian). Many colonies inside the harbour were observed in a resistance form (although the experiment was carried out in winter–spring) while resistance states were almost absent from the colonies outside the harbour. This suggests that *P. crucigaster* is unable to filter normally and that responds to the adverse conditions of the harbour by closing the siphonal apertures.

Percent yield values of crude extract were remarkably high in this ascidian as compared with values for sponges (Becerro et al., 2003) or for another ascidian (Caralt et al., 2002). Moreover, crude extract yield increased in both transplant treatments. However, this increase did not correspond to a higher production of toxic compounds, as no significant differences in toxicity were observed among treatments. This indicates that a large fraction of this extract corresponds to other unknown compounds whose production was induced by the transplantation stress.

In the sponge *Crambe crambe* the production of toxic substances was depressed by copper pollution (Agell et al., 2001), and toxicity levels were also lower in populations of the ascidian *Clavelina lepadiformis* living inside harbours than in those of the open littoral (Caralt et al., 2002). In our case we did not find a comparable trend, as both hsp expression and toxicity measures were not significantly different across treatments. The similar levels of toxicity in individuals of the control and polluted sites suggest that chemical defences in this ascidian might be of the "constitutive" type, defined as those with low production cost that are maintained at a more or less constant level (Steinberg, 1994), irrespective of variable environmental conditions. In specimens of the sponge *Crambe crambe* transplanted to the same polluted site, there was an increase in hsp production concomitant with a reduction in toxicity (Agell et al., 2001). This fact suggested the existence of a trade-off between allocation of resources to both functions. This could not be substantiated in our case, as no significant changes, in either hsp or toxic metabolite production, was observed in the transplants to the harbour.

Even if the normal functioning of the colonies was altered inside the harbour, they nevertheless accumulated heavy metals at significantly higher levels than the colonies in their natural environment. Cu is the main metal accumulated in the harbour individuals, followed by Pb. This species accumulates these metals at lower concentrations (in specimens kept inside as well as outside the harbour) than other species that live naturally at both habitats, such as the sponge *Crambe crambe* (Cebrian et al., 2003) and the ascidian *Clavelina lepadiformis* (De Caralt et al., 2002) The two latter are supposed to be more resistant to pollution levels and may withstand higher concentrations of metals in their tissues.

Vanadium accumulation behaves differently. Values of vanadium in colonies from inside and outside the harbour were of the same order of magnitude. Ascidians accumulate vanadium in their tissues (Hawkins et al., 1983; Michibata et al., 1986), where it fulfils physiological roles (Rowley, 1983; Martoja et al., 1994). These roles may explain the preferential accumulation of this element in this and other ascidian species living in the same habitat (i.e. *Clavelina lepadiformis*, Caralt et al., 2002) in contrast to the lower levels found in other invertebrates such the sponge *Crambe crambe* (Cebrian et al., 2003).

Stress proteins of the hsp70 family respond strongly to both physiological and environmental stresses in vertebrates (Sanders, 1990) and invertebrates (e.g. Koziol et al., 1996). However, this is the first time that an inducible heat-shock protein in ascidians has been identified in a context other than immune recognition (against self-fertilization) events (Marino et al., 1998). The monoclonal antibody used cross-reacted in *P. crucigaster* with a 78 kD protein of the hsp70 family. This antibody had demonstrated in previous works a high affinity for a 78 kD protein in two *Mytilus* species (Sanders et al., 1994) and the echinoderm *Strongylocentrus purpuratus* (Sanders and Martin, 1994).

The amount of hsp constitutively found in all the individuals of *P. crucigaster* analysed, regardless of whether they were at the clean or contaminated site, might be related to the implication of the hsp70 gene in gamete self-incompatibility to prevent self-fertilization in hermaphrodite species (Marino et al., 1998). *P. crucigaster* did not show a significantly different synthesis of stress proteins between individuals from clean areas and those transplanted to the harbour, which indicates that copper pollution at the concentration found at the harbour did not induce hsp synthesis. However, the lack of response cannot be interpreted as a lack of stress of the individuals but rather to a strong metabolic damage, because the macroscopic changes observed in the colonies (i.e negative growth, resistance forms) clearly indicate strong adverse conditions for the species at the polluted area.

The sensitivity of molecular and cellular-organism features is different, and sometimes when the effects are manifested macroscopically, the molecular mechanisms are already collapsed. The levels of copper pollution in the Blanes harbour induced hsp expression in other animals such as the sponge *Crambe crambe* (Agell et al., 2001). Although the response to stress at the molecular level may be used as an early warning signal of what may occur after at higher levels of organisation (Dyer et al., 1991; Ryan and Hightower, 1994), the use of hsp expression as biomarkers of pollution requires caution

because the threshold at which the proteins can be induced varies as a function of the species sensitivity to a given pollutant (Van Straalen, 1994). The existence of a species-specific threshold above which a given pollutant becomes toxic, affecting the cell physiology and concomitantly reducing the overall rate of protein synthesis has been reported previously in rotifers (Cochrane et al., 1991). In these cases the amount of hsp70 is no longer a good indicator (Wheelock et al., 1998) of pollution levels. The laboratory experiment performed supported that the Cu concentration in the harbour was above the threshold for HSP induction. The level of hsp78 increased in individuals under copper concentrations lower than those found at the harbour while this protein was not induced at copper concentrations similar to those at the harbour. This experiment demonstrated that *P. crucigaster* can induce production of stress proteins in response to copper contamination but only at concentrations ca. 15 μg/l.

To summarise, under the harbour conditions strong effects could be seen at the organismal level, reflected by negative growth and formation of resistance stages. We failed to find responses at the molecular level (hsp, toxic molecules) possibly due to a too high stress level. The laboratory experiments confirmed that Cu levels comparable to those of the harbour seem to overwhelm the capacity of the species to respond via the production of stress protein. At lower Cu levels, however, a clear response was observed. Our results show the need for preliminary sensitivity analyses to determine the range of pollutant concentrations within which this biomarker is useful for a given species. We conclude that hsp expression can be a good biomarker for pollution in this group when stress levels are below a certain threshold, above which its induction seems to be inhibited. They are, therefore, highly suitable for studies at concentrations of pollutants sublethal for the organisms and may prove useful to detect stress situations where other biomarkers at the molecular or organismal level are not informative.

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