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Expression of the heat shock protein Hsp70 in chloride target cells of mayfly larvae from motorway retention pond: A biomarker of osmotic shock

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Abstract

The aim of this work was to study, by an in toto immunohistochemical technique, the expression pattern of the heat shock protein, Hsp70, in the widely used bioindicator species *Cloeon dipterum* (Linnaeus 1761) (Ephemeroptera, Baetidae), living in a motorway retention pond. All sampling and measurements have been performed from March 2002 to March 2003. The water physicochemical analyses have revealed a large increase in Na^+ and Cl^- concentrations after the de-icing road surface in winter related to motorway maintenance that correspond to an osmotic shock (from 3.1 to 105.7 mg L^{-1} for Na^+ and from 3.5 to 193.9 mg L^{-1} for Cl^-). An expression of Hsp70 was observed in the chloride cells only during the osmotic shock. In contrast, the gill insertions were Hsp70 immunoreactive in specimens collected all along the year. For comparison, the expression of Hsp70 was investigated in specimens collected in a temporary pond. *C. dipterum* larvae living in this pond, not submitted to such osmotic shock, do not express Hsp70 neither in chloride cells nor in gill insertions. Likewise, the expression of Hsp70 was not detected in these structures during the drying period when the abiotic conditions become progressively stressful (elevation of temperature and anoxia). As chloride cells play a key role in osmoregulation, their functional integrity is crucial for the survival of the mayfly larvae in occasionally salty freshwaters. According to the well known protective role of the Hsp70 stress proteins, it is likely that the induction of Hsp70 may protect the chloride cells from osmotic shock injuries resulting from the increase in salinity. So, the Hsp70 induction in chloride cells is designed as a useful biomarker of osmotic shock. The in toto immunohistochemical detection of Hsp70 allows to characterize both the exposure situation and biological effects in target cells induced by stresses. This method could be used as a complementary qualitative approach in the biomarker actual concept. Finally, this investigation that combines this osmotic shock biomarker and this kind of bioindicator species would be a helpful tool for the monitoring of freshwater ecological systems.

Keywords: Chloride cell; *Cloeon dipterum*; Heat shock protein Hsp70; Osmotic shock; Mayfly larvae; Motorway retention pond

1. Introduction

Since the 1970s, many toxicological studies have been done using mayfly larvae (Wielgolaski, 1975; Van Wijngaarden, 1993; Admiraal et al., 2000; Fialkowski et al., 2003). The mayfly *Cloeon dipterum* (L. 1761)

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(Ephemeroptera, Baetidae) is one of the most common species among the aquatic insect larvae of Western Europe (Sowa, 1975; Bazzanti et al., 2003). It is found in most of all natural and artificial lenitic freshwaters, such as ponds, rock-pools and ditches. The larvae are mostly primary consumers and actively involved in processing and cycling of nutrients in freshwater systems (Merritt et al., 1984). Although these aquatic invertebrates have been routinely used as biological indicators, biochemical markers in ephemeropterans have been largely neglected. It has been proposed that metabolic pathways and reactions that allow aquatic invertebrates to persist under adverse environmental conditions can be considered as biomarkers to characterize either the exposure situation itself or the effects induced by stresses (Triebskorn et al., 2002). Furthermore, regarding population and community structures in apparently healthy ecosystems, the biomarkers could reveal subtle alterations that may result in long-term irreversible community changes (Hyne and Maher, 2003). It is well known that the biomarker response must be carefully interpreted as it can vary, for a given species, according to the studied tissue or organ (Chapple et al., 1997) and to the development stage (Feder and Hofmann, 1999). So, in quantitative biomarker approach, such as 7-ethoxy-resorufin-*O*-deethylase (E.R.O.D.) or acetylcholinesterase activity measurements or quantification of stress proteins in some organs of vertebrates or molluscs, the major difficulties encountered with the use of mayfly larvae as early warning sentinels in ecological risk assessment are the small size of the larvae and their number of development stages. As the biomarker quantitative study requires large amounts of larvae at the same development stage, it is particularly difficult to achieve such biomarker approach with these small organisms for field monitoring.

Although the effects of substantial runoff from motorways on the diversity of aquatic invertebrates has been evaluated (Blasius and Merritt, 2002), works concerning biomarker responses are crucially needed. In the present study, an in toto immunohistochemical method has been adapted to detect the presence of a biomarker in target tissues or specialised cells in larvae of *C. dipterum* collected in a motorway semi-permanent stormwater pond. The results were compared with those obtained in *C. dipterum* larvae collected in a temporary pond. Both freshwater ponds have been selected for (1) their similar range of conductivity and (2) their different variation in the physicochemical water parameters. Both the motorway semi-permanent and the temporary ponds are astatic habitats with large variations of abiotic parameters (temperature, ionic concentrations, pH and dissolved O_2 level). Organisms living under such conditions have developed survival strategies that enable them to live in the gradient between homeostasis and death. Among these survival strategies, stress proteins belonging to the heat shock protein (Hsp) family play a key role: their induction is the signal of exposure to conditions that alter intracellular proteins (Parsell and Lindquist, 1993; Freeman et al., 1999; Jean et al., 2004). Because many anthropogenic or natural stressors induce the expression of the 70 kDa Hsp (Hsp70) and because these stress proteins have been evidenced in all organisms studied, Hsp70 proteins have been proposed as biomarker of many environmental stresses (Feder and Hofmann, 1999; Yoshimi et al., 2002; Ait-Aissa et al., 2003). Therefore, the ubiquist Hsp70 protein has been retained in the present investigations.

The aim of the present study was to propose a qualitative approach in the biomarker concept, which could allow to characterize both the exposure situation and biological effects in target cells induced by stresses and that could be applied to small organisms. The choice of *Cloeon* larvae as a model to analyse the effects of an osmotic stress in freshwater systems and the use of immunohistochemical qualitative detection of a biomarker, Hsp70, in the field of environmental monitoring are discussed.

2. Material and methods

2.1. Choice of the aquatic ecosystems

The semi-permanent stormwater retention pond ("Grans" pond, GRA) is characterized by an increase of NaCl levels in winter resulting from the de-icing road surface related to motorway maintenance (Scher and Thiéry, 2005; Scher et al., 2005). "GRA" pond is located along the motorway A54 (Salon de Provence, France). A temporary pond chosen for comparison, that was previously physicochemicaly characterized by Heurteaux and Chauvelon (2004), has been selected for its classical pattern of physicochemical parameter variation as compared to other Mediterranean temporary ponds (Thiéry, 1991). The temporary pond is located in the pool–karst complex of "Valliguières" (VAL, near Nîmes, France).

The electrical conductivity (in triplicate), pH and water temperature were measured in the field with WTW® material in the water column from March 2002 to March 2003 at midday. For water analyses, 500 ml (15 samples for GRA, 8 samples for VAL) were brought back to the laboratory in polypropylene vials. The cations concentrations were determined by atomic absorption using SPECTRA A 640 Varian® with automatic SP-5 sample

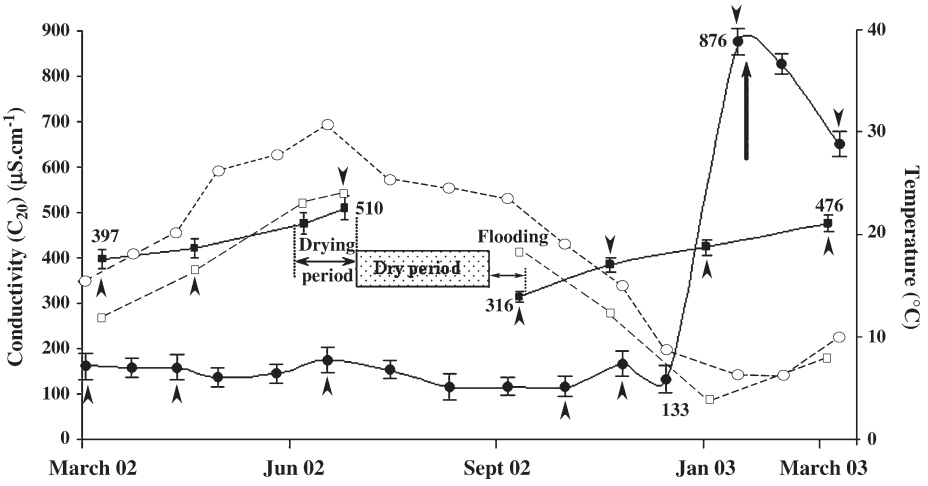

Fig. 1. Evolution of the conductivity (lines, C_{20}, µS cm^{-1}) and of the temperature (dotted lines, °C) in the semi-permanent motorway retention pond (circles, GRA) and in the temporary pond (squares, VAL) from March 2002 to March 2003. In GRA, an important conductivity peak (876.3 ± 5.1 µS cm^{-1}) in January 2003 was the major event (arrow). Conductivity increases by a 6.6 factor in only one month. For animals living in VAL, the stressful event is due to the drying period. The conductivity has increased progressively by a 1.3 factor from March 2002 to July 2002 and has reached 510.3 ± 1.9 µS cm^{-1}. From the end of July to the end of September 2002, the pond was dry. Between the flooding (October 2002) and February 2003, the conductivity mean ± SEM was 400.2 ± 67.2 µS cm^{-1}. ↑: conductivity peak in the motorway pond; arrowhead: sampling of *Cloeon dipterum*.

preparation system. The anions concentrations were determined by chromatography on DIONEX® DX 120 with AS50 sample preparation system. Both analyses were routinely single data with a 2–3% precision that is controlled by the electroneutrality and the ionic balance.

2.2. Animals

The larvae of mayfly, *C. dipterum*, were collected using a hand net (125 µm mesh) between March 2002 and March 2003. The animals collected in GRA and VAL have been fixed in the field in 0.1 M phosphate buffer (PB), pH 7.2 containing 1% paraformaldehyde. *C. dipterum* larvae were isolated under stereomicroscope in the laboratory and maintained in the same fixative solution at 4 °C. They have been sampled according to their size and segregated following to their respective larval stage according to the biometry of their metathoracic femur and interocular gap. Larvae of stage 5 have been selected for this study. This stage was chosen because it is the first stage to exhibit wing-pads and to be species identifiable (Macan, 1970).

2.3. Experiments

Before immunohistochemical study, larvae cuticle has been cleaned by ultrasonication (4 s). The efficiency of this treatment has been verified by scanning electron microscopy (S.E.M.). For S.E.M., animals were critical point-dried, sputter coated with gold (20 nm) and examined with an environmental S.E.M. (Philips® XL 30).

For each collected sample, ten randomly larvae of stage 5 were selected for immunohistochemical detection of Hsp70. They were then rinsed in phosphate buffer (PB) containing 1% Triton X-100 during two days. Seven specimens were used for "in toto preparations". They were carefully dissected under a binocular microscope. In order to allow the ability of the antibody to interact with intracellular proteins, the appendages were removed and the cuticle was medio-dorsally cut. To precise the expression pattern of Hsp70, immunohistochemical experiments were done on frozen sections: three specimens were (i) embedded in Cryomount® (Histolab, Göteborg, Sweden), (ii) frozen in liquid nitrogen and (iii) cut with a cryomicrotome (20 µm). "In toto preparations" and frozen sections were then pre-incubated for 35 min at 20 °C in 0.1 M PB containing 1% Triton X-100, 10% goat serum and 2% bovine serum albumin (PB-T buffer) to saturate non-specific sites. They were incubated overnight at 4 °C in a dilution (1 : 2000) of a primary antibody against Hsp70 raised in rabbit (Interchim, Montluçon, France) in PB-T buffer. The anti-Hsp70 antibody recognizes the 70 kDa stress inducible Hsp70 isoform and does not cross-react with the constitutive Hsc70 isoform. "In toto preparations" and frozen sections were then rinsed three times and were incubated for an hour at 20 °C in the dark, with a secondary antibody (1 : 200), donkey anti-rabbit IgG fluoprobes® 488-conjugated (Interchim) diluted in PB-T

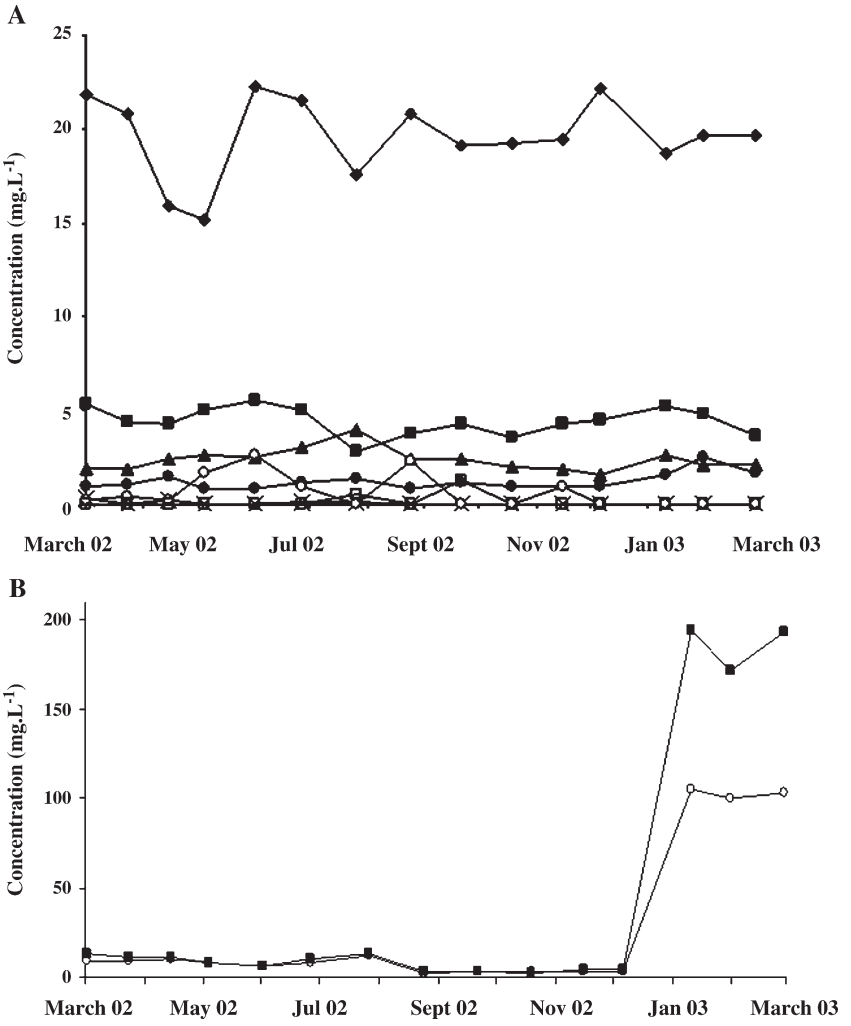

Fig. 2. Evolution of some ion concentrations (in mg L^{-1}) in the semi-permanent motorway retention pond (GRA). (A) Concentrations in Ca^{2+} (♦), Mg^{2+} (●), K^{+} (▲), F^{-} (×), SO_4^{2-} (■), NO_3^{-} (○) and NO_2^{-} (□). Note that the concentrations were almost constant all along the year. (B) Concentrations in Cl^{-} (■) and Na^{+} (○). Note the large increase in the concentrations from December 2002 to January 2003.

buffer. After rinsing in PB, they were mounted in an antifading medium (Gel/Mount®, Biømeda corp., Foster city, CA, USA) and observed under an epifluorescence microscope (Leica® M1560).

The negative control Hsp70 was done by incubating the specimens without the primary antibody. Immunostaining was totally abolished in absence of the primary antibody.

3. Results

3.1. Physicochemical parameters

In GRA, the conductivity (C_{20}) was almost constant from March 2002 to December 2002 (mean±SEM in µS cm^{-1} = 144.3±21.4) (Fig. 1). In one month, from December 2002 to January 2003, the conductivity was largely increased to reach a maximum of 876.3±5.1 µS cm^{-1}. The dissolved oxygen varied from 3.6 mg L^{-1} in July 2002 to 15.8 mg L^{-1} in November 2002. During the year, the temperature varied from 6.2 to 30.5 °C (mean±SEM in °C = 17.6±8.6) (Fig. 1). The concentrations in Ca^{2+}, Mg^{2+}, K^{+}, F^{-}, SO_4^{2-}, NO_3^{-} and NO_2^{-} were almost constant (mean±SEM in mg L^{-1} = 19.6±2.6; 1.2±0.5; 2.4±0.6; 0.05±0.09; 4.4±0.8; 0.62±0.89; 0.12±0.35, respectively) (Fig. 2A). The concentrations in Na^{+} and Cl^{-} are almost constant from March 2002 to December 2002 (mean±SEM in mg L^{-1} = 6.3±3.3; 7.3± 4.2, respectively). But, Na^{+} and Cl^{-} concentrations were considerably increased from December 2002 to January 2003 (Fig. 2B) corresponding to the conductivity peak (Fig. 1), from 3.1 to 105.7 mg L^{-1} for Na^{+} and

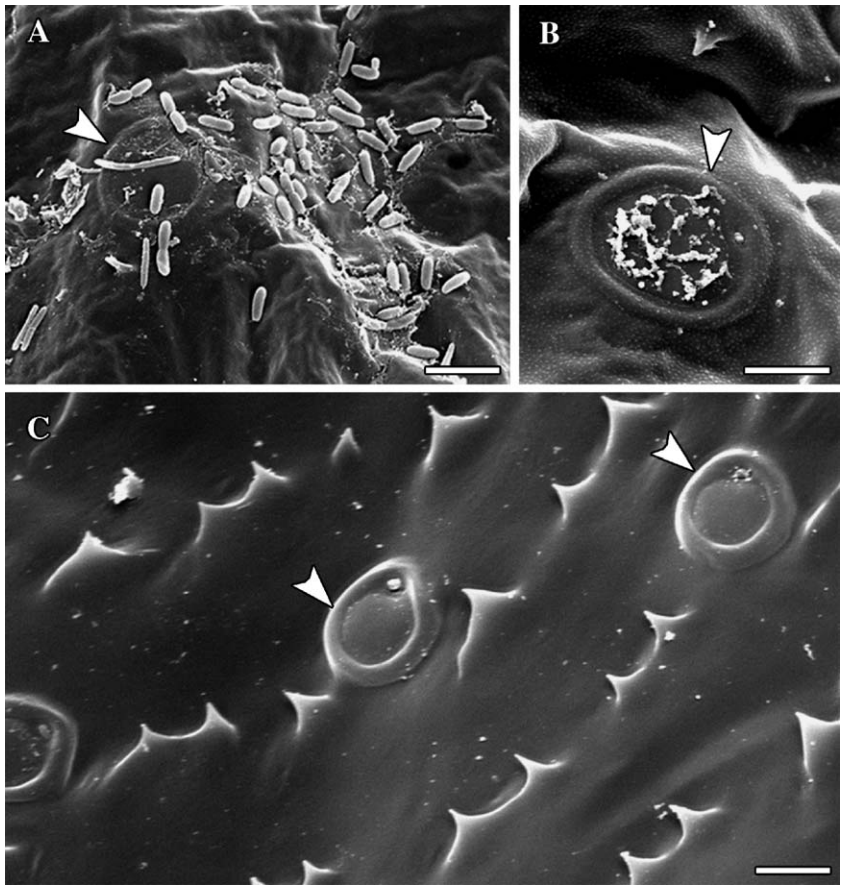

Fig. 3. Scanning electron micrographs of *Cloeon dipterum* larvae chloride cells (arrowheads). (A–B) Detail of the cuticle before cleaning by ultrasonication (4 s) showing bacteria (A) and coated materials (B). (C) Two chloride cells after cleaning by ultrasonication (4 s). Scale bars = 2 μm.

from 3.5 to 193.9 mg L^{-1} for Cl^{-}). All along the year, pH varies from 7.4 to 9.3.

In VAL, the conductivity was slightly and progressively increased from March 2002 (397.1 ± 4.7 μS cm^{-1}) to the end of July 2002 (510.3 ± 1.9 μS cm^{-1}) just before complete water evaporation (Fig. 1). From the flooding, at October 2002, to March 2003, the conductivity has progressively increased from 316.0 ± 2.0 to 476.1 ± 2.0 μS cm^{-1} (Fig. 1). The Na^{+} and Cl^{-} concentrations were stable and have reached a maximum of 4.34 and 9.37 mg L^{-1}, respectively, before the dry period. During the period of the study, temperatures were ranged from 4.1 to 24.3 °C (Fig. 1) and pH was almost constant between 7.6 and 8.2. The dissolved oxygen varied from 4.3 to 13.6 mg L^{-1}.

3.2. S. E. M. observations

Fig. 3 shows detail of the cuticle before (A, B) and after (C) cleaning. Before ultrasonication, the cuticle showed many bacteria (Fig. 3A) and coated materials (Fig. 3B) at the vicinity of chloride cells, which were no more present after the treatment (Fig. 3C). It is obvious that the cleaning method confers good results and must be applied before immunoassay.

3.3. Immunohistochemical observations

In GRA, *C. dipterum* larvae collected in January and in March 2003, when the Na^{+} and Cl^{-} concentrations were increased, always displayed Hsp70 immunopositive chloride cells (Fig. 4). Hsp70 have been detected in the chloride cells of the abdomen (Fig. 4A, B) and in the chloride cells of the gills (Fig. 4C). In all other sampling points, the *C. dipterum* chloride cells were not Hsp70 immunoreactive. Immunolabelled areas have been found at the level of the insertion of the gills outward and during the conductivity peak, i.e., all along the year (Fig. 5A, B).

In VAL, animals collected at the end of the drying process of the pond (summer), when the abiotic parameters are drastic (elevated temperature and low level of

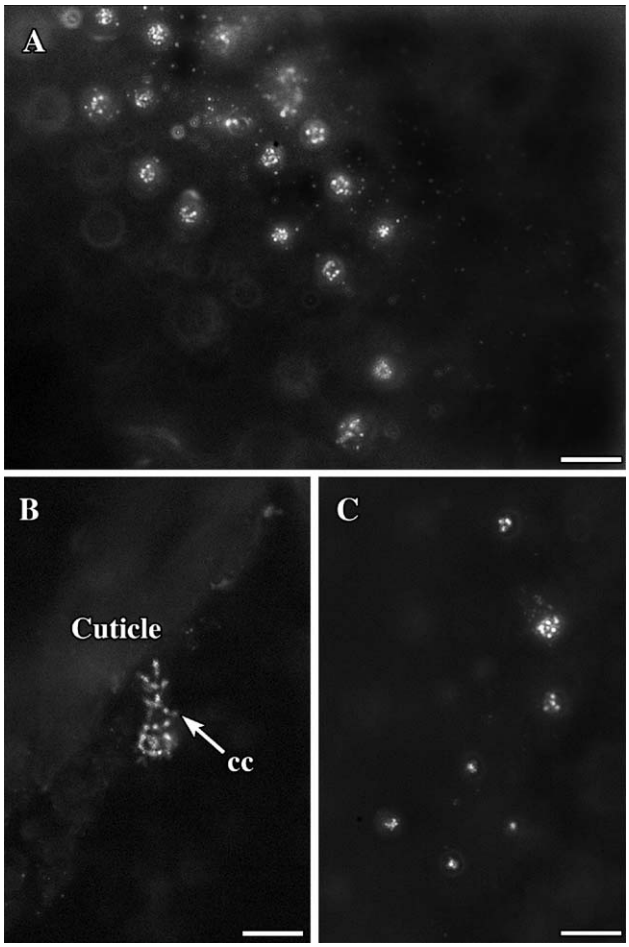

Fig. 4. Hsp70 immunoreactive chloride cells in *Cloeon dipterum* larvae collected in winter during the osmotic shock in the semi-permanent motorway retention pond (GRA). (A) "In toto preparation" showing many immunoreactive chloride cells on the abdomen. (B) Transversal frozen section through an abdominal immunoreactive chloride cell (cc). (C) Immunoreactive chloride cells on a gill. Scale bars = 10 μm.

oxygen), do not express Hsp70 proteins either in the chloride cells or at the level of the gill insertions (Fig. 5C).

4. Discussion

In *C. dipterum* larvae from motorway retention pond (GRA), some Hsp70 immunoreactive structures were found either outward and during the osmotic shock. However, although the immunolabelling was present at the level of the gill insertions, whatever the sampling moment, the chloride cells were Hsp70 immunoreactive only during winter. The winter period corresponds to the highest conductivity in the pond, which increases in less than a month by a 6.6 factor. This conductivity peak is explained by the large increase in both Na^+ and Cl^- concentrations. This unusual event in a freshwater pond is related to human activity as it results from the de-icing road surface during motorway maintenance. The increase in salinity is obviously a major stress for animals living in freshwater habitats. The osmotic shock can disrupt biochemical processes, can modify cellular homeostasis and can cause stunted growth or death. To withstand fluctuations in the abiotic environmental conditions, animals have developed capacities of adaptation, like cellular responses. One of the most common mechanisms for reacting to deleterious conditions is that of cellular stress response which involves the rapid synthesis of a set of heat shock proteins (Hsps) (Lindquist, 1986; Feder and Hofmann, 1999; Schill et al., 2004). Hsps often include constitutive as well as stress-inducible isoforms. Their role in unstressed cells consists mostly in regulating protein homeostasis (e.g., degradation of abnormal proteins), in directing the folding and assembly of others proteins, and in transport of proteins within the cell (Lindquist, 1986; Parsell and Lindquist,

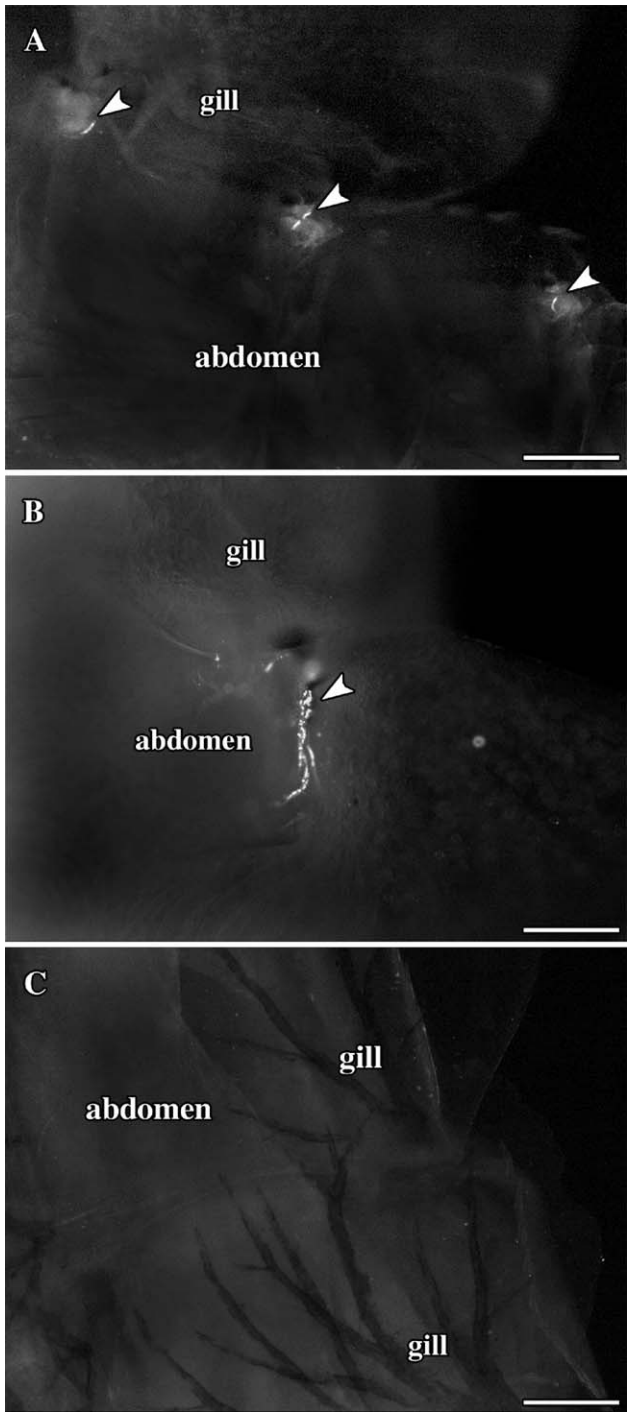

Fig. 5. Hsp70 immunoreactivity in "in toto preparations" of *Cloeon dipterum* larvae. (A, B) Hsp70 immunoreactive cells at the level of the gill insertion (arrowheads) in specimens collected in the semi-permanent motorway retention pond (GRA) outward the osmotic shock (spring) (A) and in specimens collected in GRA during osmotic shock (winter) (B). (C) No Hsp70 immunoreactivity is detected in animals living in the temporary pond (VAL) and collected during the drying period. Scale bars (A,C)=50 μm, (B)=100 μm.

1993; Sanders, 1993). Induction of stress proteins as Hsps is referred to an emergency response following exposure to many stressors (Lindquist, 1986; Parsell and Lindquist, 1993). Osmotic stress has been found to induce expression of Hsps in several organisms (Smith et al., 1999; Spees et al., 2002; Todgham et al., 2005). From

mammalian cell studies, it has been suggested that Hsps may function to stabilize proteins before the adaptative synthesis of osmolyte transport proteins involved in protection against osmolytes (Cohen et al., 1991; Sheikh-Hamad et al., 1994). It is obvious from the cited work and the present investigations that osmotic shock induces Hsp70 expression. The expression of Hsp70 in chloride cells, during the peak of salinity in the motorway retention pond (GRA) is particularly interesting. In mayfly larvae, these cells are found in the integument of nearly all body part (Komnick and Abel, 1971). They have been shown to absorb NaCl from hypotonic external concentrations, playing a key role in hyper-regulation (Komnick et al., 1972; Filshie and Campbell, 1984). Furthermore, Wichard et al. (1973) have suggested that the adaptative behaviour of chloride cells is correlated with the osmoregulatory situation and enables animals to live in habitats with different osmolarities. These authors have shown that the chloride cells are more sensitive to increase than to decrease salt concentrations. Under experimental and natural conditions, the gradual increase in freshwater NaCl concentrations leads to a significant reduction in the number of chloride cells in mayfly larvae (Wichard et al., 1973). These authors have reported degeneration of nearly all the chloride cells after experimental exposure to 150 mM NaCl for 24 h. In GRA, although the peak of conductivity is an important stressful event for *C. dipterum* larvae, the NaCl concentrations are about 30 times lower than those used by Wichard et al. (1973). So, the present study demonstrates that the Hsp70 response is very sensitive because it occurs at lower NaCl concentrations than those leading to chloride cells ultrastructural degradations which impair the osmoregulation processes. Therefore, we suggest that Hsp70 induction in chloride cells may have protected these cells against the osmotic shock and so, from osmotic stress injuries. Indeed, protection against environmental stressors afforded by induction of Hsps is thought to accrue from: (1) protection of either nascent polypeptides or mature proteins from denaturation; (2) renaturation of denatured proteins; (3) prevention of formation of protein aggregates, by targeting irreversibly damaged proteins for degradation (Parsell and Lindquist, 1993). The suggested protective role of Hsp70 in chloride cells is essential for the survival of the mayfly larvae during the osmotic shock, as these cells play a key role in osmoregulation.

In contrast to GRA, in the temporary pond (VAL), during the 5 months preceding the dry period, the conductivity increases slightly and progressively only by a 1.3 factor. Moreover, the maximal Na^+ and Cl^- concentrations, at each osmotic stress period, are 20 times lower in VAL than in GRA. In VAL, the animals collected under these stress conditions do not display Hsp70 immunoreactivity in the chloride cells or at the gill insertions. Even if the drying period leads to an osmotic stress, this stress differs mostly from the osmotic shock observed in GRA by its weaker amplitude and its progressiveness. This could explain the absence of Hsp70 induction in chloride cells. The data obtained in the present study support the protective role of Hsp70 proteins in these cells only from drastic osmotic shock. Therefore, in *C. dipterum*, the Hsp70 induction in chloride cells may be considered as a biomarker of an important osmotic shock. In contrast, Hsp70 induction at the gill insertions cannot be considered as such biomarker because it occurs outward and during the osmotic shock and could result from other abiotic factors.

The validation of the present results for others common species of mayfly larvae owing chloride cells will allow applying this immunohistochemical method to numerous lenitic freshwater systems. Such a qualitative method could be complementary of biochemical semi-quantitative or quantitative ones. Advantages and disadvantages of semi-quantitative and quantitative methods have been listed by Cimino et al. (2002). For example, western blotting, the most widely used technique, is time consuming and often complex. The often long and complex procedures that are common to semi-quantitative and quantitative techniques, except for the ELISA technique, need numerous alive specimens for each sampling (Cimino et al., 2002). Our immunohistochemical technique does not require large amount of animals and provides some relevant information on the target organ(s) or cell(s) exposed to stress. For further investigations, such qualitative immunohistochemical technique should be coupled with a quantification image analysis system like that of Machella et al. (2005).

5. Conclusion

The qualitative aspect of the present investigations will be helpful for the development of the biomarker approach within the Arthropoda which, despite of their wide representation among aquatic environments, are until now neglected in biomarker studies (Eckwert and Köhler, 1997; Snyder, 2000). Indeed, the lack of knowledge concerning the relationships between biochemical marker variations and macroinvertebrate population responses has been also pointed out by Hyne and Maher (2003). The present results are the first step toward the use of these mayfly larvae as model in biomarker studies. The interest of this model is that mayfly larvae are pertinent bioindicators. Thus, in the

future, investigations that combine this model at once in bioindicator and in biomarker studies would be a powerful tool to appreciate the impact of anthropogenic environmental changes.

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