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Biomarker responses to sewage pollution in freshwater mussels (*Diplodon chilensis*) transplanted to a Patagonian river

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Field and laboratory experiments were combined to evaluate biomarker responses of *Diplodon chilensis* to sewage pollution. Mussels from an unpolluted area in Lacar lake (S0) were caged at a reference site (S1) and at two sites with increasing sewage pollution (S2, S3) in Pocahullo river (all in Argentina). After 1 month, gill (g) glutathione S-transferase (GST) and catalase (CAT) activities, and lipid peroxidation (TBARS) were found to be significantly elevated in S3, gGST being positively correlated with fecal bacteria (FC) concentration. Digestive gland (dg) enzyme activities were depressed and dgTBARS were increased in all transplanted mussels. After 3 mo, most variables returned to control levels in S1 mussels except for dgCAT and dgTBARS. After seven months, GST and CAT activities of S0 and S3 mussels were evaluated in the laboratory, before and after acute exposure (8 h) to high fecal bacteria concentration ([FC] in S3x 2). gGST increased in both groups, while dgGST responded only in S3 mussels. gCAT and dgCAT activities were similarly increased by acute exposure in both groups. Our results suggest that gGST and gCAT are suitable biomarkers for high FC pollution regardless of previous exposure history. In addition, we show that dgCAT is sensitive to the acute increase in FC load, both in naive and long-term exposed individuals, while dgGST becomes responsive after long-term acclimatization.

Keywords: Fecal coliforms, oxidative stress, detoxifying defenses, short and long-term response.

Introduction

Sewage discharges release significant loads of microorganisms, metals, and organic/inorganic contaminants, which can be harmful to the aquatic biota.[1–3] The use of biochemical, cellular, tissue and organism alterations as biomarkers, allows the detection of contaminant elements that may adversely affect wild populations.[4–7]

Mussels are widely used in monitoring programs because they are easy to handle and their filtering habits lead to efficient exposure, intake and bioaccumulation of contaminants from the water column.[4,6,8,9] To perform exposure experiments in the field, transplantation of caged mussels from a reference wild population to contaminated sites is recommended in order to reduce the effect of physiological variation among individuals.[5] Additionally, it is necessary to perform simultaneous studies in a natural population from a clean zone, to avoid confounding effects due to natural biological functions, such as feeding performance and reproductive state.[10,11]

General biomarkers are recommended for the initial stages of monitoring studies.[4] Antioxidant and detoxifying defenses as well as condition parameters can be very sensitive in revealing a prooxidant condition and/or presence of toxicants.[7,9,12–14] The antioxidant enzyme Catalase (CAT) reacts against peroxisomal H_2O_2 to avoid the cellular damage that could be caused by increased production of this reactive oxygen species (ROS). Glutathione S-Transferase (GST) is a detoxifying enzyme that catalyzes the conjugation of reduced glutathione (GSH) with endogenous or exogenous toxic compounds. When the rate of ROS production exceeds the rate of their neutralization by antioxidant systems, there is increased oxidative damage to cellular targets. This imbalance can be assessed by estimating the levels of thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation.[15] The enzyme protein phosphatase 1 (PP1) is actively involved in the modulation of diverse physiological functions such as cell division, meiosis, cell cycle arrest, apoptosis, protein

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and glycogen synthesis, actin reorganization and stress recovery.[16] Considering its importance for cell function regulation, PP1 activity can be regarded as a potential marker for metabolic response.

Sewage-water pollution may affect the activity of multiple enzymes, thus causing significant damage. In caged mussels (*Perna perna*), exposure to domestic effluents causes an increase in digestive gland GST activity, while no effect has been observed observed on CAT activity.[19] On the other hand, high CAT activity and TBARS concentration have been registered in the bivalves *Scrobicularia plana* and *Cerastoderma edule* exposed to untreated sewage discharges and industrial effluents.[20] In addition, somatic growth of mussels exposed to sewage effluents may be increased due to the higher availability of digestible organic matter.[12,14] However, somatic growth could be reduced by tissue loss due to cell damage caused by metals like copper, [9] autophagy [5] and/or consumption of energy reserves due to starvation and /or activation of protection mechanisms.[4,17,18] Characterizing biochemical and somatic responses and their potential change upon exposure to polluted environments is an essential step for determining if a given biomarker is suitable for short- and long-term biomonitoring.

Most Patagonian lakes and rivers are chemically diluted oligotrophic systems,[21] and are recognized as valuable aquatic resources with social, economic, and ecological relevance. Particularly, in North-West Patagonia, population growth threatens to affect water quality and biodiversity in several water bodies through land-use practices and sewage discharges.[8,14,22] For example, the river Pocahullo crosses the city of San Martín de los Andes and flows into Lacar lake (50.28 km^2 of surface and a maximum depth of 277 m). The river and its tributaries receive untreated effluents from diffuse sources (septic tank infiltrations and horse-cattle farming) as well as point source discharges of primary treated sewage (sedimentation ponds) in different parts of its upper and middle courses. Near the river mouth, a sewage tertiary treatment plant (STTP) discharges treated effluents into the river, acting as a major point source of pollution. As a result, increasing chemical and bacteriological deterioration has been detected in Pocahullo river and tributaries[23] and in the lake area immediately downstream of the river mouth.[14]

Diplodon chilensis is a slow-growing, long-lived mussel, which is widely distributed in Andean freshwater bodies of Argentina and Chile.[24–26] Sabatini et al.[14] have detected oxidative stress and oxidative damage in wild individuals collected from the polluted area of Lacar lake (described above). Accordingly, the following hypotheses are proposed: (1) geographical variations in the concentration of pollutants and time of exposure induce differential physiological and biochemical changes in bivalves; (2) antioxidant and detoxifying enzymes in gills and digestive glands of bivalves show differential responses to sewage pollution; and (3) acute enzyme responses to high bacterial loads are modified by acclimatization to polluted sites.

In this work, exposure of caged *D. chilensis* to clean and polluted areas of Pocahullo river were combined with acute exposure in the laboratory to detect physiological responses in gills and digestive gland to water quality and environmental changes, and to determine the suitability of such responses as pollution biomarkers.

Material and methods

Study sites

Adult *D. chilensis* were collected by a diver from a sandy bottom at 3 m of depth in Yuco (S0), an unpolluted area of Lacar lake about 20 km from the city of San Martín de los Andes (40°10′S, 71°31′30″W) (Fig. 1). Field exposure was carried out at three sites in the Pocahullo river basin (Fig. 1). The upstream reference site (S1) was situated in a rural area in Maipú-Calvuco stream, the main affluent of Pocahullo river (40°07′20.3″S, 71°14′57.4″W). This site was slightly affected by cattle farming. S2 was also in Maipú-Calvuco stream, 8 km downstream from S1, where diffuse farming and domiciliary effluents as well as point primary-treated effluents are discharged from sparsely populated suburban areas (40°08′48.74″S, 71°19′35.2″W).

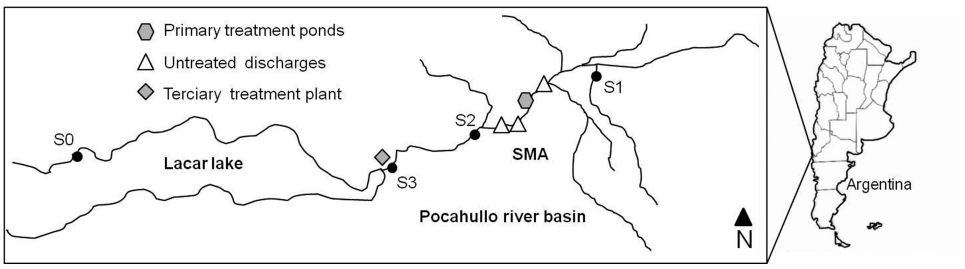

Fig. 1. Sites of mussel extraction and wild population control in Lacar lake (S0) and for short and long-term exposure of caged *Diplodon chilensis* (n = 6 cages per site; each cage containing 15 mussels) in the Pocahullo river basin (S1: Upstream reference site; S2: Untreated and primary-treated sewage discharges; S3: Tertiary-treated sewage discharges).

S3 was located 20 m downstream from the effluent discharge of the main city sewage tertiary treatment plant (STTP) (40°10′S, 71°20′60″W), near the mouth of Pocahullo river.

Field study

A total of 270 individuals (58.76 ± 7.6 mm shell length) were immediately sorted into groups of 15 individuals and placed into 18 cages (iron structure covered with plastic mesh, 40 × 40 × 15 cm). The resulting density was similar to that of natural populations of the S0 area, 187 individuals m^{-2}.[26] Six cages were placed at each site (S1-S3), at a depth of 0.5– 1 m.

The study was carried out from August 2009 to April 2010. After one and three months, six mussels (1 per cage) were collected from each site, transported to the laboratory at low temperature and processed. The remaining mussels were allowed to acclimatize in Pocahullo river for 7 mo. On the basis of the results obtained in the first two samplings, mussels placed in S3 were selected for the laboratory experiment.

Laboratory study

A laboratory experiment was performed in order to evaluate the suitability of enzyme biomarkers for detection of acutely increased sewage-water pollution after acclimatization at a polluted site. We collected 12 mussels from S3 and 12 mussels from S0 and placed them individually in aerated plastic containers with 2 L of water obtained 10 m downstream of the STTP discharge, at 10°C. The bacterial concentration of this medium (1410 MPN 100 mL^{-1}) was about two-fold higher than that at S3 at the time of mussel collection (600 MPN 100 mL^{-1}). GST and CAT activities were measured before (T_0, n = 6) and after (T_8, n = 6) acute (8 h) exposure to concentrated sewage-water.

Water quality

Physicochemical and bacteriological analyses were carried out at each sampling event on water samples from S0, S1, S2 and S3. During long-term exposure (5–7 months), water quality was monitored only in S0 and S3.

Water temperature (°C) was recorded at each site. Water samples (n = 3) to measure fecal coliform bacteria concentration (FC) were collected in sterile containers and maintained at 4°C until analysis by MPN 100 mL^{-1} method.[27] For chlorophyll *a* concentration (Chl *a*), water samples (n = 3) were filtered through glass fiber filters (Whatman GFF [Buenos Aires, Argentina], 0.45 μm pore). Then, acetone extraction was carried out at 4°C for 24 h, the extracts were centrifuged at 4000 × *g* for 15 min, and absorbance was read at 644 and 647 nm. Chl *a* (μg L^{-1}) was calculated according to Lichtenthaler.[28] To measure particulate organic matter (POM), water samples (n = 3) were filtered through Whatman GFF, 0.45 μm pore, previously dried at 60°C for 24 h, weighed and then ashed at 450°C for 2 h. POM content (mg L^{-1}) was calculated according to Juhel et al.[29] Monthly averages of flow and precipitation rates at Pocahullo river were provided by the Aquatic Biology Station of San Martín de los Andes.

For physicochemical analysis, water samples (n = 2) were collected in polyethylene bottles (pre-washed with 5% HCl solution) and kept at −20°C. Total nitrogen (TN μg L^{-1}) was measured by a cadmium reduction method, after acid persulfate digestion at 120°C for 55 min (HACH [Loveland, CO, USA] method 8192). Sulphate (SO_4^{-2} mg L^{-1}) was measured by a turbidimetric method [27] and turbidity (FAU) by Attenuated Radiation Method (Hach Method 10047). Absorbance measurements were carried out with a Hach DR /4000 spectrophotometer.

Tissue sample preparation

Each individual was placed on ice, weighed (g) and its total shell length (mm) was measured. Mussels were then opened by adductor muscle incision, and gills and digestive gland were removed. Both organs were weighed and homogenized (Omni 1000 motorized homogenizer at 20,000 rpm) in cold 100 mM sodium phosphate buffer (PB), pH 7.0, 1:5 w/v, containing protease inhibitor (0.2 mM phenylmethylsulfonil fluoride, PMSF, Sigma, St. Louis, MO, USA). The homogenates were centrifuged for 15 min at 11000 × *g* at 4°C and supernatants were used for biochemical analysis. The digestive gland mass /shell length ratio (DGM /SL) was calculated as an indicator of metabolic condition.

Biochemical analyses

GST activity was measured according to Habig et al.[30] Gill (20 μL) or digestive gland (40 μL) supernatants were mixed in PB, pH 6.5, with 10 μL of reduced glutathione (Sigma, 100 mM GSH in PB) and 10 μL of 1 chloro-2,4 dinitrobenzene (Sigma, 100 mM CDNB, in ethanol). The change in absorbance at 340 nm was followed for 5 min. One GST Unit was defined as the amount of enzyme needed to catalyze the formation of 1 μmol of GS-DNB /min at 25°C. Results were expressed as U GST per mg of protein. CAT activity was measured according to Aebi.[31] Changes in absorbance at 240 nm were read for 30 s, using 40 μL of gill or digestive gland supernatant in H_2O_2 solution (10 mM in potassium phosphate buffer 50 mM, pH 7). One CAT Unit was defined as the amount of enzyme needed to catalyze the hydrolysis of 1 μmol of H_2O_2 min^{-1} at 25°C. Results were expressed as U CAT per mg of protein. PP1 activity was measured according to Carmichael and An.[32] Absorbance was read at 412 nm for 40 min. One PP1 Unit was defined as the amount of

enzyme needed to catalyze the dephosphorylation of 1 μmol of para-nitrophenyl phosphate (disodium salt, Merck) per min at 25°C. Results were expressed as U PP1 per mg of protein.

Damage to lipids was estimated according to Fraga et al.,[15] by the thiobarbituric acid reactive substances method (TBARS). Supernatants from gill or digestive gland were mixed with thiobarbituric acid (TBA, Sigma) solution and incubated at 95–100°C for 15 min. The mixture was cooled and centrifuged, and the supernatant absorbance was read at 535 nm. TBARS concentration was estimated using an extinction coefficient of 156 $mM^{-1}cm^{-1}$. Results were expressed as μmol TBARS per mg of protein. Total protein content was measured according to Bradford,[33] using a bovine seroalbumin standard curve.

Statistical analysis

Data were presented as mean ± standard error. Normal distribution and homogeneity of variance were checked by Bartlett's test and Levene's tests, respectively. When statistical assumptions were not met, values were transformed by $Log_{10}(x)$ or $Log_{10}(x+1)$, when appropriate. Results were tested by two-way ANOVA followed by Newman–Keuls *post hoc* comparisons. Significant differences were assumed when $P < 0.05$. Spearman's rank coefficient was applied to analyze correlations between biological and water quality variables (significance $P \leq 0.05$).

Results

Water quality

Table 1 summarizes water quality data obtained from Lacar lake (S0) and Pocahullo river (S1-3), in the short-term samplings (1–3 months). S1 and S0 showed similar characteristics, except for turbidity ($P < 0.05$) and temperature ($P < 0.001$), which were higher in the river than in the lake. During this period, FC was higher at S2 and S3

Table 1. Water quality of the Lake control (S0) and Pocahullo river basin sites (S1: Upstream reference site; S2: Untreated and primary-treated sewage discharges; S3: Tertiary-treated sewage discharges).

	Sites	*September*	*December*
FC (MPN 100 mL^{-1})	S0	0 a	32 ± 8.00 a
	S1	15.5 ± 4.5 a	11 ± 0.00 a
	S2	600 ± 100 b	500 ± 0.00 b
	S3	1500 ± 100 c	1500 ± 100 c
Chl a (μg L^{-1})	S0	2.46 ± 0.00	0.92 ± 0.49 a
	S1	1.81 ± 0.00	0.53 ± 0.17 a
	S2	0.34 ± 0.00	1.79 ± 0.37 a
	S3	1.43 ± 0 .00	3.27 ± 0.14 b
POM (mg L^{-1})	S0	7.32 ± 0.00	16.5 ± 2.50
	S1	16.62 ± 2.33	21.99 ± 6..34
	S2	15.14 ± 0.86	14.37 ± 1.37
	S3	16 ± 1.00	18 ± 0.00
TN (μg L^{-1})	S0	177 ± 0.00	135 ± 0.02 a
	S1	177 ± 0.06	118 ± 0.00 a
	S2	206 ± 0.08	177 ± 0.6 a
	S3	354 ± 0.12	678 ± 0.09 b
SO_4^{-2} (mg L^{-1})	S0	2.72 ± 0.00 a	1.75 ± 0.11 a
	S1	2.40 ± 0.20 a	1.44 ± 0.14 a
	S2	2.01 ± 0.56 a	1.88 ± 0.09 a
	S3	3.39 ± 0.43 b	3.31 ± 0.99 b
Turbidity (FAU)	S0	1.5 ± 0.5 a	1.5 ± 0.5 a
	S1	6.5 ± 1.5 b	5.5 ± 0.5 b
	S2	5.0 ± 1.0 b	6.5 ± 0.5 b
	S3	5.0 ± 1.0 b	8.0 ± 0.0 b
T (°C)	S0	8.85 ± 0.05 a	13.4 ± 0.10 a
	S1	6.4 ± 0.00 b	8.1 ± 0.10 b
	S2	5.95 ± 0.50 b	8 ± 0.00 b
	S3	6.4 ± 0.00 b	8.5 ± 0.50 b
**Flow rate (m^3 sec^{-1})	Pocahullo river basin	8.79	4.51
**Precipitations (mm)		66.10	33.20

*Fecal coliform bacteria (FC); chlorophyll *a* (Chl *a*); particulate organic matter (POM); total nitrogen (TN); sulphates (SO_4^{-2}). Different bold characters denote significant differences ($P < 0.05$) among sites for a given time. Results are expressed as mean ± SE.
**Unpublished data provided by the Aquatic Biology Station, San Martín de los Andes.

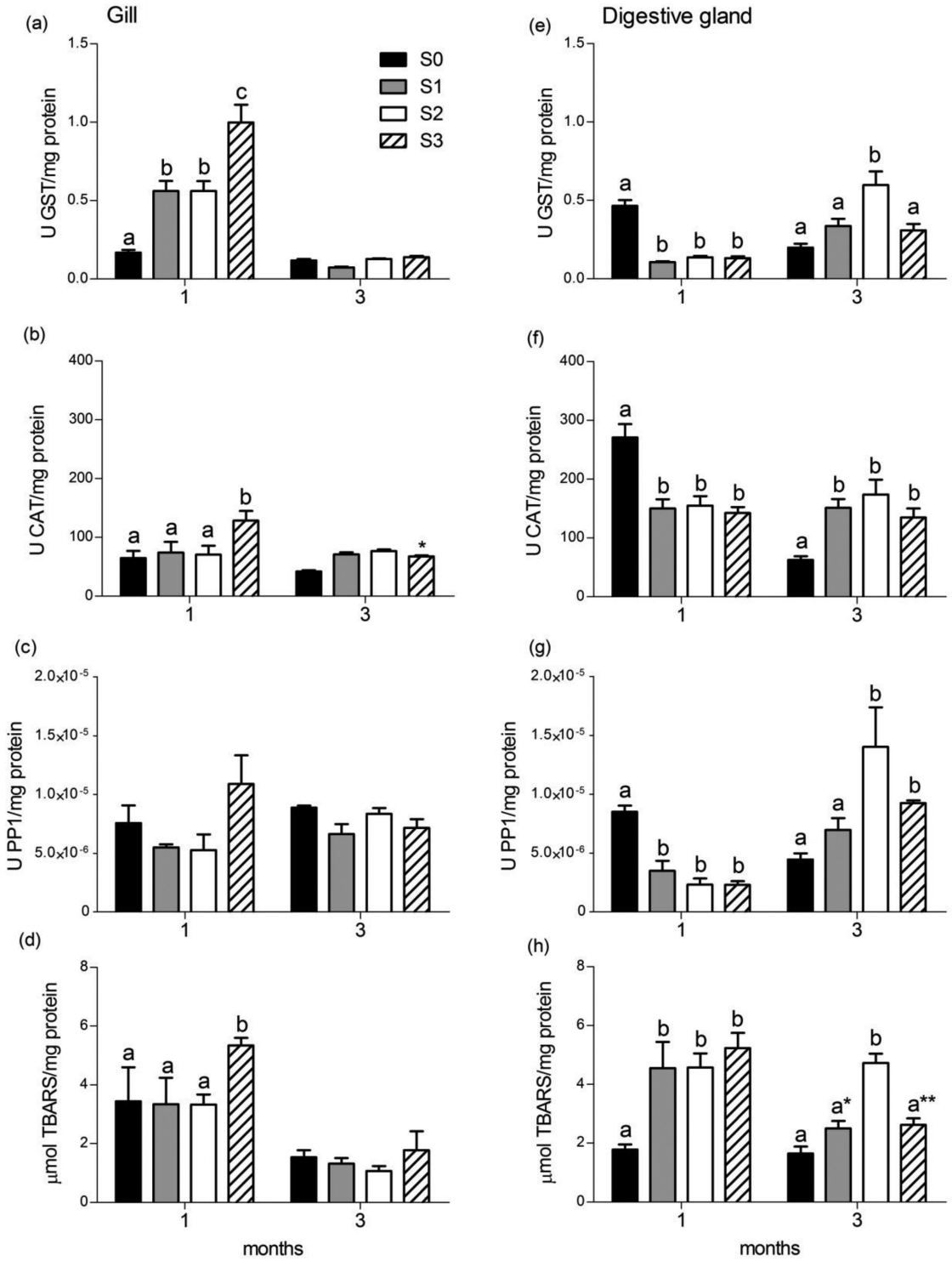

Fig. 2. Short-term effects (1–3 months) on gill (a–d) and digestive gland (e–h) enzyme activities and lipid peroxidation in *Diplodon chilensis* (S0: Lake control; S1: Upstream reference site; S2: Untreated and primary-treated sewage discharges; S3: Tertiary-treated sewage discharges). Different characters denote significant differences ($P < 0.05$) among sites, for a given time; *denotes significant differences ($P < 0.05$) between months for S3 in (b) and for S1 in (h); **denote ($P < 0.01$) between months for S3. Results are expressed as mean ± SE.

than at S0 and S1 ($P < 0.05$), with higher FC at S3 than at S2 ($P < 0.01$). Chl *a* and TN were significantly higher at S3 than at the other sites in the first month ($P < 0.05$), while SO_4^{-2} loads were elevated at this site in both samplings, as compared with S0-S2 ($P < 0.05$). POM did not show any significant variation among sites.

During the long-term exposure period, FC at S3 was always significantly higher than at S0 ($P < 0.001$) and showed pronounced temporal variations from the fifth month of exposure (24000 vs. 22 ± 2 MPN 100 mL^{-1}) to the seventh (600 vs. 0 MPN 100 mL^{-1}). This peak in FC at S3 in the fifth month was coincident with peaks in TN (2743 ± 0.15 μg mL^{-1}, $P < 0.001$) and SO_4^{-2} (6.38 ± 0.04 mg mL^{-1}, $P < 0.05$) at the same site. These TN and SO_4^{-2} concentrations were also significantly higher than those measures at S0 (236 ± 0.06 μg mL^{-1}; 1.63 ± 0.00 mg mL^{-1}, respectively). No significant differences for TN, SO_4^{-2} and POM between S3 and S0 were observed in the rest of the period (data not shown).

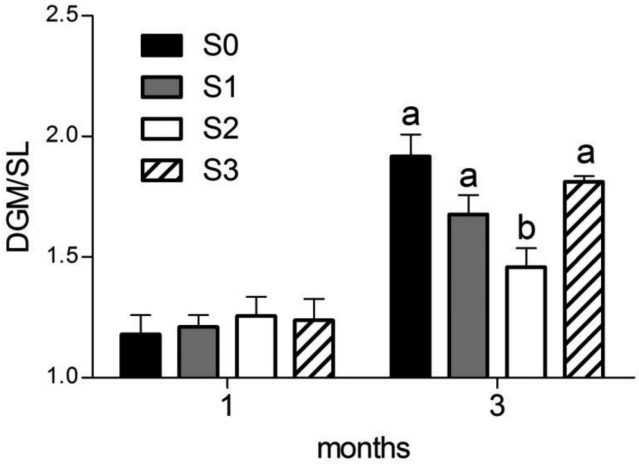

Fig. 3. Short-term effects (1–3 months) of sewage-water on DGM/SL of *Diplodon chilensis* (S0: Lake control; S1: Upstream reference site; S2: Untreated and primary-treated sewage discharges; S3: Tertiary-treated sewage discharges). Different characters denote significant differences ($P < 0.05$) among sites, for a given time. Results are expressed as mean ± SE.

Field study

Significant geographical and temporal variations in gill GST activity (gGST) were observed in caged bivalves (interaction, $P < 0.001$) (Fig. 2a). After 1 mo, gGST had increased in mussels transferred to the three sites of the river, as compared with those in the lake control (S0) ($P < 0.01$). In addition, gGST was significantly higher in S3 than in S1 or S2 ($P < 0.05$), yet no difference was observed between S1 and S2. At this point in time, gGST and FC were significantly correlated ($r^2 = 0.92$, $P < 0.05$). After 3 mo, gGST recovered control levels in all transplanted mussels.

For gill CAT activity (gCAT) two-way ANOVA yielded significant differences for site, time, and interaction effects (interaction, $P < 0.05$). Comparison among sites indicated that gCAT was significantly higher in S3 mussels than in those of S0-S2 ($P < 0.05$). No significant difference among sites was detected in the third month (Fig. 2b). There were no significant differences in gill PP1 (gPP1) activities (Fig. 2c). Gill TBARS level (gTBARS) increased in the first month only in mussels transplanted to S3 ($P < 0.05$). This variable did not differ among sites in the third month (Fig. 2d).

All the enzyme activities measured in digestive gland (dg) showed significant differences among sites and between times (interaction, $P < 0.001$). In the first month, all the transplanted mussels suffered a general depression of dgGST ($P < 0.05$), dgCAT ($P < 0.05$) and dgPP1 ($P < 0.01$) with respect to S0 (Fig. 2e-g). dgGST was negatively correlated with turbidity ($P < 0.05$; $r^2 = -0.85$). In the third month, dgGST reached S0 levels in S1 and S3 mussels but significantly increased in S2 ($P < 0.001$), while dgCAT was higher in all transplanted mussels than in those of S0 ($P < 0.05$).

dgPP1 recovered S0 values in S1 and significantly increased in the two polluted sites ($P < 0.01$). dgTBARS increased in all transplanted mussels in the first month ($P < 0.001$), whereas in the third month this variable was significantly higher at S2, as compared with S0 ($P < 0.005$), and to S1 and S3 ($P < 0.05$). In S1 and S3 mussels, dgTBARS decreased significantly in the third month ($P < 0.05$ and $P < 0.01$, respectively), and did not differ from those of S0 mussels (Fig. 2h). DGM/SL decreased in S2 mussels in the third month ($P < 0.01$) (Fig. 3).

Laboratory study

At T_0, gGST was similar in S0 and S3 mussels. Exposure to a high bacterial load ([S3] × 2) for 8 h (T_8) produced a ~3-fold increase in enzyme activity in both groups ($P < 0.001$ between times) (Fig. 4a). In contrast, dgGST showed a differential response between S0 and S3 mussels after acute exposure ($P < 0.0001$). At T_8, the activity of this enzyme increased 13-fold in S3 mussels ($P < 0.001$) but remained unchanged in those of S0 (Fig. 4b). Both, in gill and digestive gland, CAT activity was significantly higher in S3 than in S0 mussels ($P < 0.0001$), and significantly increased ($P < 0.0001$) at T_8 (Figs. 4c,d).

Discussion

Field study

D. chilensis showed different biochemical responses in gills and digestive gland upon transplantation from lake to river conditions. This tissue-specific response is probably due to differences in the degree of exposure and in pollutants

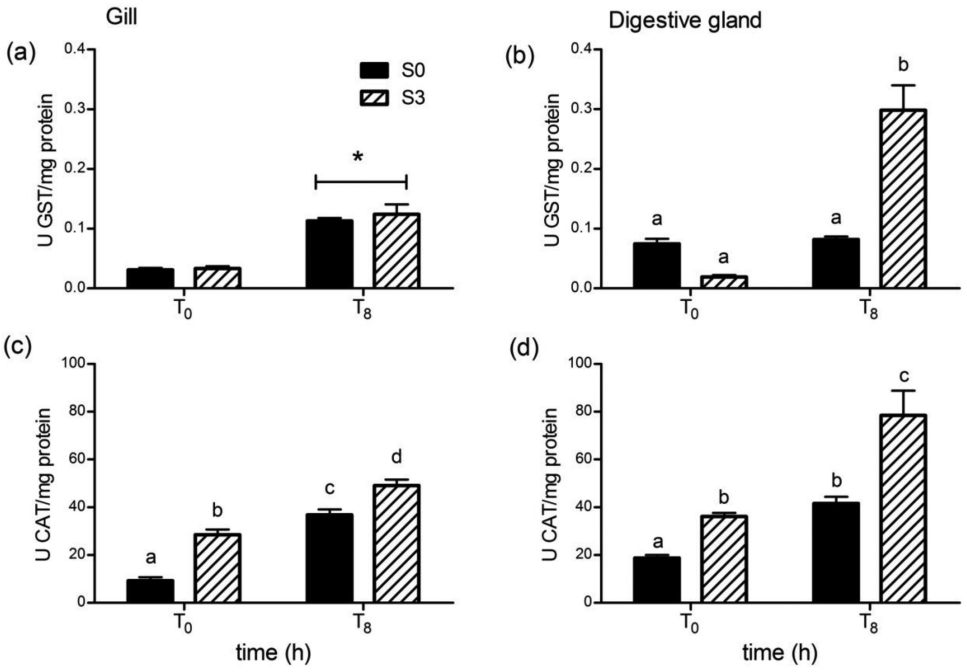

Fig. 4. Acute effects (8 h) of exposure to concentrated sewage-water ([S3 FC] × 2) on gill (a, c) and digestive gland (b, d) enzyme activities of *Diplodon chilensis* control (S0) or previously exposed to sewage pollution in S3, for 7 mo. Different characters denote $P < 0.05$ (c, d) and $P < 0.001$ (b) among sites, for a given time; *denotes $P < 0.05$ between T_0 and T_8. Results are expressed as mean ± SE.

bioavailability.[12,34] Gills are considered suitable organs for studying pollutant effects on mussels due to their high surface area and their direct contact with the surrounding medium.[4,35] However, the results described herein suggest that this suitability depends on time of exposure and on the selected biomarker. For example, most of the gill biomarkers analyzed in *D. chilensis* reflected differences in water quality after one month of exposure to river conditions, but this sensitivity was lost after 3 months.

In this regard, a general trend to increased gill enzymes' activity was noticed after one month of exposure. gGST was elevated in all the mussels transferred to the river (S1-3), as compared with the lake wild population control (S0). Particularly, increased gGST in S1 mussels, which were not exposed to significant pollution, suggests stress due to transplantation. Although no further response of this enzyme was evident in mussels exposed to moderate sewage pollution at S2 (FC = 600 MPN 100 mL^{-1}), gGST was strongly activated in mussels exposed to a higher pollution level at S3 (FC = 1500 ± 100 MPN 100 mL^{-1}). This suggests that the sensitivity limit for gGST under these conditions is between 600 and 1500 MPN 100 mL^{-1} FC.

Farcy et al.[36] have reported similar sensitivity for digestive gland GST in the freshwater mussel *Elliptio complanata* transplanted to sewage-polluted sites. These authors reported increased GST activity in a site with 1950 CFU fecal coliform 100 mL^{-1} but not in a less polluted site (495 CFU 100 mL^{-1}). On the other hand, during the first month, *D. chilensis*g CAT increased only in S3 compared with S0 mussels, and transplantation stress was not evident in S1 mussels. In spite of the observed antioxidant defense system activation, lipid peroxidation (TBARS) was not fully avoided in gills of S3 mussels at this time. Therefore, gGST, gCAT and gTBARS of *D. chilensis* caged for 1 month can be considered suitable biomarkers for high bacterial concentrations.

After 3 months, gGST and gCAT were not affected either by transplantation stress or by bacterial loads. gTBARS were compensated in all sites, although FC concentration in water remained similar to that in the first month. Faria et al.[37] have proposed that in *Corbicula fluminea,* the response to domiciliary and industrial pollution depends on different antioxidants at different times of exposure, maintaining a constant antioxidant potential.

The digestive gland is responsible for intracellular digestion, nutrient distribution, detoxification and elimination of xenobiotics, and accumulation of energetic reserves.[14,38,39] In a laboratory study, Sabatini et al.[14] did not detect significant differences in dgGST between *D. chilensis* fed with *E. coli* or with green algae, until after five weeks. Viarengo et al.[5] have suggested that manipulation and exposure of caged mussels to a different environment, rather than pollutants, cause a stress situation that can alter physiological parameters. Camus et al.[40] have found a depletion of total antioxidant scavenging capacity (TOSC) in *Mytilus galloprovincialis* related to transplantation stress, which is completely

compensated after 1 mo. In this work, the activities of all the enzymes measured in digestive glands of transplanted mussels decreased with respect to S0 after one month of exposure. This effect was similar in S1 and in the polluted sites (S2-3), which most likely reflects transplantation stress and not a response to increased bacterial load, in coincidence with Sabatini et al.[14]

In addition to transplantation stress, the initial reduction in enzyme activity in digestive gland of *D. chilensis* could be a response to higher turbidity in the river than in the lake, since turbidity has been associated with population decrease of this species in Chilean lakes.[25] Ellis et al.[41] have demonstrated that increasing levels of inorganic suspended sediments reduce the filter feeding activity of *Atrina zelandica* after 3 days. In our study, dgGST was negatively correlated with turbidity during the first month of exposure. On the other hand, the decrease in DGM/SL that would be expected to occur during a low-food intake/starvation period [38,41] was not evident in *D. chilensis*.

In this respect, Nicholson and Lam [4] have suggested that in starved bivalves, the mass loss due to reserves consumption can be compensated by increased water uptake. Alternatively, the reduction of enzyme activity recorded in this period could reflect metabolic depression, with lower use of energetic reserves. The observed decrease in antioxidant/detoxifying defenses could be responsible for the increase in lipid peroxidation detected in transplanted mussels, even at lowered metabolic rates.

Transplantation effects on *D. chilensis* were almost completely compensated in digestive gland after 3 mo, since in S1 mussels, most of the variables studied returned to S0 levels by this time, except for dgCAT, which remained elevated at S1-3. On the other hand, in mussels transplanted to S2 and S3, the initial depression of enzyme activities was followed in the third month by a surge of dgGST and dgPP1 with respect to S0 and S1. Similar response patterns have been described by Regoli and Principato [42] and by Franzellitti et al.,[43] who argued that exposure to pollution for short periods may induce a transient decrease in antioxidant defenses, which can be followed by the induction of the antioxidant system.

Interestingly, we have detected peaks in enzyme activities and TBARS in digestive gland of S2 mussels as well as a reduction in DGM/SL in the third month. These changes were not correlated with FC concentration. Additionally, these changes are not likely to be a response to bacterial pollution since previous studies on *D. chilensis* from the same lake showed higher DGM/SL in mussels collected from a sewage-polluted site than in those from non-polluted sites.[14,26] Alternatively, the effect of pollutants not directly related to sewage-water, such as metals or microcystin, could have reduced DGM/SL in this species through tissue damage and/or xenobiotics elimination by merocrine/holocrine mechanisms.[9,13] In addition, the presence of pharmaceutical drugs in sewage-water could have reduced the condition index in exposed mussels.[44]

Laboratory study

The degree of environmental stress to which organisms are exposed is not static, but instead it shifts with changes in the environment, pollution, metabolism, diet, among others. Pre-exposure to low intensity oxidative stress may enhance tolerance to a higher intensity stress.[45] In the present study, *D. chilensis* was exposed to variable degrees of sewage pollution in an impacted site (S3) for 7 mo and then subjected to 8-h exposure to highly polluted water.

Mussels freshly collected from S0 and S3 showed enzyme- and tissue-specific responses. In gills, acute exposure to concentrated sewage-water stimulated both GST and CAT, independently of the previous treatment. This sensitivity matched the responses of gGST and gCAT to a similar bacterial concentration in the first month of the field exposure in S3 (Fig. 2). In addition, the basal gCAT activity was higher in long-term acclimatized mussels, probably due to increased enzyme expression.[34,46,47]

As for dgGST, acclimatization to polluted water probably involved the expression of different enzyme isoforms. This is suggested by the fact that S3 mussels show a low basal dgGST activity, which dramatically rose in response to increased FC concentration, while S0 mussels showed no response to the same stimulus. The inducible GST isoform would remain inactive at mild FC concentration, such as that measured in S3 at the time of sampling for this experiment (600 MPN 100 mL^{-1}), and would respond to acute increases in the bacterial load.

On the other hand, the response of dgCAT was similar to that of gCAT. Basal levels of gCAT and dgCAT were higher in S3 mussels than in those from S0. This enzyme showed a significant increase upon acutely increased FC concentration, both in S0 and S3 mussels, which was consistent with the increased gCAT detected in the short-term field exposure at S3. This suggests acclimatization through increased expression of a unique isoform. However, molecular biology studies should be performed to elucidate the existence of different GST and CAT isoforms in this species and their responses to pollutants.

Conclusions

The physiological response of *D. chilensis* to sewage pollution is tissue-specific and depends on exposure time and on the degree of pollution. In gills, GST, CAT, and TBARS markers clearly respond to short-term field exposure to high bacterial load. However, in digestive gland these markers can be masked by transplantation effects, at least when mussels are transferred from lake to river conditions.

Particularly, we conclude that gill GST and CAT are suitable biomarkers of high FC pollution with or without previous exposure. In the digestive gland, CAT is sensitive to both long-term exposure and acute increase in FC load,

both in naive and long-term exposed individuals, while GST becomes responsive after long-term acclimatization.

Freshwater mussels appear to be suitable candidates for sewage-water biomonitoring. However, biochemical biomarkers should be selected according to the proposed time of exposure, and transplantation effects should be considered.

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