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DIFFERENTIAL ACCLIMATION OF JUVENILE SEA URCHINS TRANSPLANTED ACROSS A METALLIC TRACE ELEMENT GRADIENT WITHIN THE BAY OF TOULON (FRANCE)

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ABSTRACT Urbanized harbors and embayments scattered along marine shorelines act as important sources of a wide range of chemical contaminants, which include metallic trace elements (MTE). Within the semi-enclosed Mediterranean Sea, the high number of such areas might locally challenge the persistence of coastal marine species. In recent decades, biomonitoring studies, using sentinel species such as mussels, have increased knowledge of coastal contamination and provided a number of contentious potential environmental surveillance biomarkers, which contributed to a better comprehension toward the acclimation abilities of marine organisms, to actual anthropogenically derived environments. The aim of this study was to characterize the influence of the chemically, multi-contaminated, Bay of Toulon (France) on the young fauna, using juvenile sea urchins *Paracentrotus lividus* as a proxy. Hatchery-produced individuals were immersed, for 1 mo, within the bay, at four contrasted sites for MTE concentrations. At the end of the experiment, growth and transcript abundances of key genes, involved in the acclimation process, were assessed. Contrasted phenotypic and molecular responses were observed evidencing a significant environmental influence. Specifically, under the high metallic stress, a dwarfing response was observed, associated with a high level of metallothionein and a low level of hsp response. In addition, the level of DNA methyltransferase transcript abundance was inversely correlated with the level of MTE concentrations, highlighting that the chronic exposure to metallic contaminants would probably increase the risk of genetic mutations, such as those consecutive to transpositions. Overall, this study indicates that further in-depth research into the mechanisms driving plasticity and adaptive changes at the population level is required.

KEY WORDS: *Paracentrotus lividus* sea urchin, acclimation, stress, transplantation, juveniles, metallic trace elements, gene expression, hsp, DNA methyltransferase

INTRODUCTION

In many coastal marine areas, benthic species are exposed to a range of anthropogenically derived pollutants (Halpern et al. 2008), which may critically challenge the dynamics and persistence of populations, assemblages and, in a more global sense, could affect all aspects of ecological processes (Andral et al. 2004, Thébault et al. 2005, Holmstrup et al. 2010, Manzo et al. 2010, Buono et al. 2012).

Among the contaminants of human origin, metallic trace elements (MTE) are of major importance, particularly within anthropized embayments, where they have often been detected at high concentrations (Richir & Gobert 2016). Metallic trace elements are environmentally persistent pollutants, known to produce reactive oxygen species (ROS) (Ercal et al. 2001) and have a deleterious impact on a wide range of critical biological processes, such as protein misfolding, endocrine disruption, or telomeric attrition (Malhotra & Kaufman 2007, Sharma et al. 2008, Iavicoli et al. 2009, Georgescu et al. 2011, Stauffer et al. 2017).

Across the multi-contaminated embayment of Toulon (France), MTE concentration gradients have been detected within dissolved, particulate, and sedimentary fractions (Baumard et al. 1998, Tessier et al. 2011, Pougnet et al. 2014, Wafo et al. 2016, Guigue et al. 2017). The levels of metallic

pollutants were also similar to those found in other harbors around the world, which makes this embayment a good model to assess the differential acclimation abilities of metazoan organisms to contrasted sites within anthropized harbors (Haynes & Johnson 2000, Fatoki et al. 2012).

Detecting the relative influence of MTE, with *in situ* experiments, within a complex mixture of pollutants that is probably under-characterized, remains, however, challenging. Yet, several studies performed within the bay have assessed chemical contamination, either with direct measurements (Tessier et al. 2011) or by using mussels as bio accumulators (Baumard et al. 1998, Andral et al. 2004). Worldwide, in recent decades, biomonitoring studies such as these have increased the knowledge of coastal contamination and provided a number of potential, although contentious, biomarkers for environmental surveillance, which nevertheless have also yielded interesting results related to acclimation at the molecular level.

In this study, the sea urchin *Paracentrotus lividus* was used to monitor the differential modalities of acclimation across contrasted habitats, contaminated with MTE, within the Toulon embayment. This species first develops as planktonic larvae that passively disperse within the water column, until they settle in potentially contrasted habitats for the water quality. The larvae then metamorphose into adult-featured juveniles, which will contribute to the maintenance of the coastal ecosystem, as grazers (Lozano et al. 1995, Tomas et al. 2004, Boudouresque & Verlaque 2007). Both pelagic larvae and benthic urchins must cope with their surrounding environments, which potentially affect both demography and genetic composition of the

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population, by the selection of the more adapted individuals (Pigliucci 1996, Hofmann & Todgham 2010); however, this species is known for its high plasticity that enables it to colonize highly contrasted, even polluted, habitats (Ebert 1996, Warnau et al. 1995, Soualili et al. 2008). Adult-shaped sea urchins, like other echinoderm species, possess a water vascular system that allows movement and grasping of food particles and the filling of the coelomic cavity that contains coelomocytes (Barnes 1982). Contaminants are absorbed directly from the available food and through water filtering and can be bio-accumulated (Warnau et al. 1995, Radenac et al. 2001, Danis et al. 2005), which makes this organism a good model to use as a potential sentinel of water quality, and to assess the relation between environment and the physiology of acclimation (Soualili et al. 2008, Salvo et al. 2014). Although most similar studies were performed on adult individuals, which are likely to be resistant to a range of stressful conditions, studies focusing on early juvenile stages remain scarce.

A wide range of chemical and physical stressors have been laboratory-tested on *Paracentrotus lividus* species. In most cases, these studies were conducted on developing embryo, with fluctuations of gene expression being assessed and considered as a proxy of phenotypic acclimation. Of these genes, most were related to the chemical defense (Goldstone et al. 2006, Marrone et al. 2012, Russo et al. 2013, Varrella et al. 2014).

Here, the impact of chronic exposure to contaminated environments on early juveniles *Paracentrotus lividus* was assessed through measures of growth rate and transcript abundances of key genes involved in stress, detoxification, cell cycle control, homeostasis, and methylation.

MATERIALS AND METHODS

Ethical Statement

Sea urchins *Paracentrotus lividus* (Lamarck) were collected during the legal harvesting period according to French legislation. All animal procedures were performed in compliance with the guidelines of the European Union directive 609/86. The field study did not involve endangered or protected species.

Production of Juveniles

Adult *Paracentrotus lividus* were collected at the Embiez Island by scuba diving, transported in an insulated box to the hatchery within an hour of harvesting, and stabilized in rearing tanks with circulating, filtered seawater (FSW) until required for experimentation. Adults were induced to spawn by gentle mechanical agitation. Following shedding, eggs were collected in FSW, sieved through a 100- μ m mesh filter, washed twice in FSW, and kept in FSW until use. Concentrated dry sperm was collected and kept undiluted at 4°C until use. The eggs and sperm of five females and five males were used. Fertilization was performed in 1L FSW by mixing all batches of eggs with 100 μ L of mixed sperm. Fertilization rates were assessed after 1 h. Larvae were reared at three larvae/mL in 80-L cylindrical-conical tanks with 60 L FSW. The FSW consisted of natural seawater that flowed successively through sand filters (mechanical and biological), cartridge filters with 50- μ m and then 5- μ m meshes, and a UV filter. Filtered seawater was renewed continuously. Temperature and salinity were maintained at $18 \pm 1^\circ\text{C}$ and 38 ppm, respectively. When they reached the pluteus

four-arm stage, larvae were fed twice a day *ad libitum* with a mixture of *Pavlova lutheri*, *Chaetoceros calcitrans*, and *Isochrysis galbana*. Competent larvae were obtained by 18 days. When about 70% of the larval pool reached this stage, larvae were transferred into raceways containing *Ulva lactuca* on which they completed metamorphosis to the juvenile stage in a week. Juveniles were kept in raceways for up to a month. Before transplantation, *Ulva* with small sea urchin juveniles were transferred into 2-mm mesh pouches placed in perforated cages to allow free water circulation and diffusion within the pouches.

Transplantation and In Situ Parameters

In June 2015, one pouch was immersed for a month at a depth of 2 m, at four sites within the Bay of Toulon: X41, considered as a control site with regard to MTE concentration; X15, located in the vicinity of aquaculture farms; Missiessy (MIS) and Canal Suffren (CS), which are both located inside the Navy area zone (Fig. 1). These sites were chosen according to a concentration gradient of MTE (Tessier et al. 2011). At these sites, a set of seawater parameters were recorded weekly (Suppl. data 1). In further analysis, parameters that provided evidence or signs of differentiation among sites based on seawater probe measurements were retained: temperature (T), salinity (S), chlorophyll (Chloro), O₂%, total O₂, dissolved organic carbon (DOC), total nitrogen (TN), and measurements of MTE concentrations (nM) in both dissolved and total fractions: Zinc (Zn), lead (Pb), cadmium (Cd), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), and arsenic (As).

At the end of the experiment, juveniles were collected and kept in an icehouse for 1 h. Then, sea urchins were euthanized by immersion in RNAlater (Qiagen), counted, and the diameter of their test was measured. Finally, individuals were divided in two roughly equal parts for further DNA and RNA purifications.

Genotyping, Genetic Diversity, and Parentage Analysis

Genomic DNA of juveniles and progenitors were purified using the Quick-DNA 96 Plus Kit (Zymo Research), according to the manufacturer's instructions. Concentration was determined by measuring the absorbance at 260 nm using a

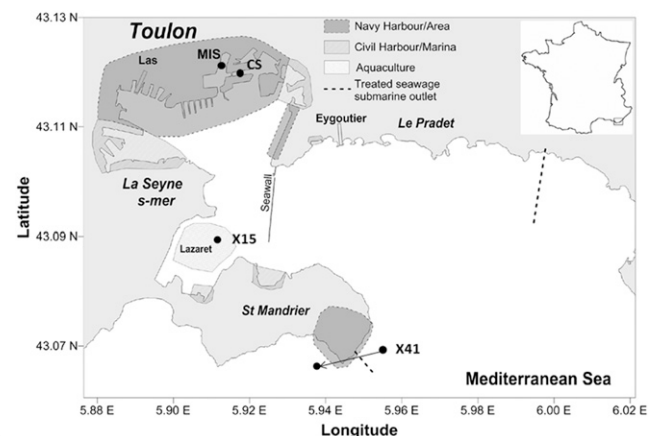


Figure 1. Toulon embayment (left) and immersed cages (right). Black dots show immersion sites. At the end of the first week of experimentation, the cage at X41 was displaced.

NanoDrop-1000 spectrophotometer (Thermo Scientific) and then adjusted to 20 ng/ μ L. DNA purity and integrity were assessed by measuring the 260/280 absorbance ratio using a NanoDrop-1000 spectrophotometer (Thermo Scientific) and by visualizing the DNA on 1% agarose gel electrophoresis.

Individuals were genotyped at seven microsatellite loci as previously described (Couvray & Coupé 2018). Amplified fragments were resolved using an ABI 3730XL Genetic Analyzer (Applied Biosystems, Carlsbad, CA) by Genoscreen (Lille, France). Samples were run together with the LIZ600 DNA ladder. Peaks were sized with the STRand Analysis software (<http://www.vgl.ucdavis.edu>).

Juveniles were then assigned to their progenitors using Cervus 3.0.7 (Kalinowski et al. 2007), and potential genetic structure among sites and size (F_{ST} estimates) were assessed with Arlequin software (Excoffier & Lischer 2010).

Gene Expression by Real-Time qPCR

Individuals whose test size ranged from 3.5 to 5 mm were chosen to assess the highest potential variability in gene expression within the studied population of juveniles, given that the maximum genetic diversity was obtained within this size range.

Total RNA was extracted from whole individuals with 1 mL of TRIzol reagent (Invitrogen) according to the manufacturer's instructions until the collection of the aqueous supernatant phase, which had been further processed with the RNeasy Mini Kit (Qiagen) following the supplier's indications with an on-column DNA digestion. The RNA integrity was assessed on 1% agarose (w/v) gel. All RNA samples displayed sharp ribosomal RNA bands with a 28S/18S ratio of 2:1. RNA concentrations were determined by a NanoDrop-1000 spectrophotometer (Thermo Scientific) and adjusted to a final concentration of 100 ng/ μ L.

Reverse transcription reactions were carried out using the Omniscript RT Kit (Qiagen) with added RNAsin (1 μ L, Promega) in a 20- μ L final volume. Negative RT controls were performed for each sample to ensure that RNA samples were not contaminated with DNA.

The qPCR reaction mixtures contained 5 μ L of 2 \times FastStart Essential DNA Green Master (Roche), 10 pmoles of each primer, and 1 μ L of cDNA in a final volume of 10 μ L. Amplifications were carried out with a LightCycler LC480 (Roche) apparatus with the following conditions: a first denaturation step at 95°C for 10 min, and then 45 cycles of 95°C for 15 sec and 60°C for 1 min. Dissociation curves were automatically performed and showed a single amplification product with no primer dimers. No-template qPCR-negative controls were performed for each of the primer pairs. Quantitative PCRs were performed in duplicate.

Standard curves for each transcript were obtained using serial dilutions of a reference cDNA. Calculated PCR efficiencies were similar for both housekeeping and target genes, which were higher than 90%.

Transcript relative abundances were obtained from standard curves, then normalized with the *polyubiquitin* and *gapdh* housekeeping genes, following Roche's instructions. Transcript abundances, for each individual, are presented relative to the mean expression measured at site X41, considered as a control site (Fig. 1).

As recommended by Kozera and Rapacz (2013), the relevance of both reference genes was checked by assessing the dispersion of within-site mean Ct, observed across the differently contaminated sites, which resulted in dispersions of Ct lower than 1, for both genes.

The primer pairs used in this study are presented in Appendix. The *polyubiquitin* gene was chosen to normalize gene expression. For all other gene transcripts, primer pairs were determined from the EST library of *Paracentrotus lividus* (<http://goblet.molgen.mpg.de/cgi-bin/webapps/paracentrotus2008.cgi>) using Primer3Plus software. The specificity of each primer was verified with BLASTN. Dissociation curves showed a single amplification product and no primer dimers.

Statistical Analysis

Potential correlations between environmental factors were first looked for with a principal component analysis (PCA). Then, one factor of a pair was removed from the dataset when the Pearson correlation value was found significant and higher than 0.9 to prevent any correlation bias due to the limited number of measurements. The total fraction of MTE; dissolved fractions of Cd, Fe, and Co; and all the aforementioned seawater probe parameters were finally retained, resulting in a refined dataset containing 18 factors (column) and 20 sampling dates (rows). Principal component analyses were then performed with this new dataset and the clustering analysis using factorial discriminant analysis (FDA) (XLSTAT).

Differences in mean sizes of the sea urchins' test diameters among sites were assessed by a student *t*-test. Differences were considered significant when *P* value < 0.05.

For each gene, differences in transcript abundances were tested using a Kruskal–Wallis test followed by a Dunn's post hoc multiple comparison test, to determine significant differences (XLSTAT). Corrected *P* values for multiple comparison were automatically calculated. Mean differences among groups (either "size" or "sites") were considered significant when the *P* value was less or equal to the corrected one (XLSTAT).

A clustering analysis based on transcript abundances was performed using an FDA considering the whole set of genes (XLSTAT).

RESULTS

Environmental Data

The FDA highlights the differentiation among sites, mainly driven by the metallic contamination (Fig. 2 left), and the relatively low variability of the water quality within sites, during the immersion. Whereas CS and MIS are obviously separated, the ellipses of X41 and X15 partially superimpose, indicating a lesser divergence of those two sites (Fig. 2 right). [F2]

When analyzing the MTE specifically, marked differences among sites were observed for zinc, lead, and copper, which indeed presented the highest and/or most contrasted concentrations among sites (Table 1 and Appendix). [T1]

After a month of immersion, the cages and *Ulva* were covered by large amounts of resuspended sediment at the MIS and CS sites, unlike at the X41 and X15 sites (Coupé, personal observations).

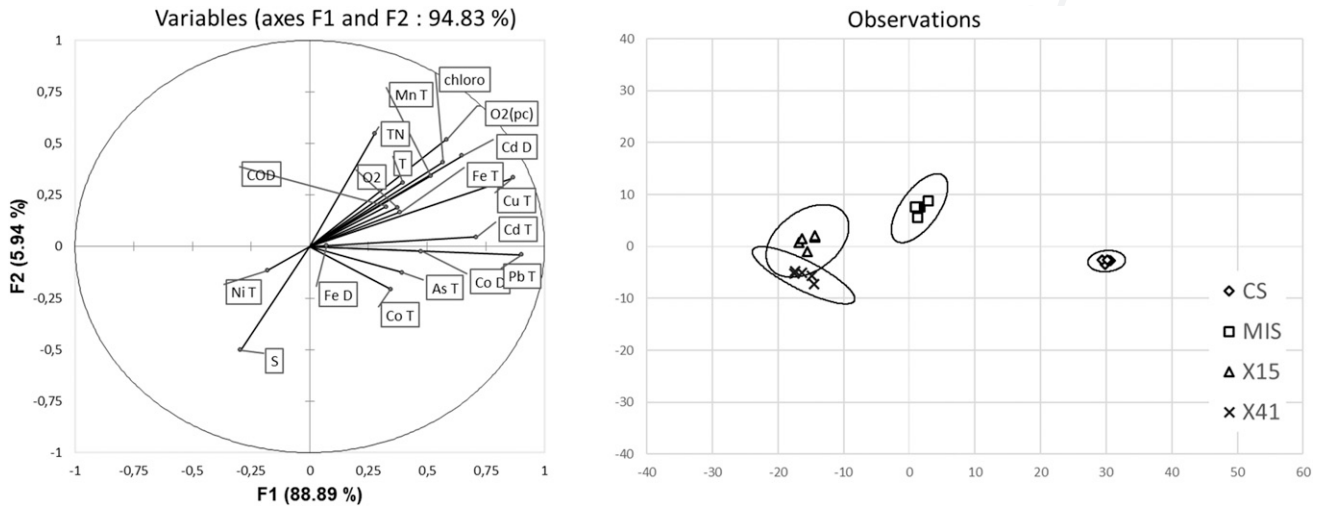


Figure 2. Factorial discriminant analysis of the four sites regarding water parameters. Measures of seawater physicochemical parameters and MTE concentrations were performed once a week during the immersion experiment (5 measures). The FDA has been performed on a refined dataset that considered the most contributing variables.

Environmental Influence on the Diameter Size and the Number of Recaptured Sea Urchins

Two hundred and sixty-six juveniles were recaptured. Overall, the mean diameter size was 4 mm with a lower and upper 95% confidence interval of 3.8 and 4.2 mm, respectively. A high variability in the diameter of sea urchins was observed within each site, with smallest and largest observed individuals sized at 1.2 and 10.4 mm, respectively (Fig. 3). The mean size of juveniles harvested at each site was found significantly higher than the mean size of juveniles before immersion, suggesting that individuals could grow evenly under contrasted environmental conditions.

There was no difference in mean size between the hatchery and the control immersion site (X41); however, the results highlighted that the bay environment had an effect on abundance and/or test size at the X15, CIS, and MIS sites compared with X41. Specifically, the more polluted sites resulted in more recaptured juveniles (228 of 267 were found at MIS and CS), but test sizes appeared negatively correlated to concentrations of dissolved lead, zinc, and copper within the bay environment (Pearson; $P = -0.999$; $P < 0.027$), suggesting that these are stressing environments. Taken together, these results highlight

that the two sites with the highest levels of MTE concentrations, had nonetheless beneficial conditions for growth and survival.

Assessment of Genotypic Diversity

All juveniles from X41 and X15, 49 from MIS, 29 from CS, and 25 from the hatchery were genotyped, representing a total of 142 individuals. They all had been assigned to the parents used at the hatchery. Among the five females and five males used to produce juveniles, three females and four males had contributed, in similar proportions, for 93.5% and 97.2% of the whole progeny, respectively. Eleven of the 25 possible genotypes represented 89.4% of the progeny. The maximum genotypic diversity was found in the set of juveniles whose test sizes ranged between 3.5 and 5 mm (data not shown).

The size dispersion of juveniles produced by each of the three main contributing females displayed a Gaussian distribution. The two other less contributing females produced significantly smaller juveniles, suggesting a potential maternal effect (ANOVA; $P = 0.0051$). No maternal effect was observed with the biggest individuals. On the contrary, there was no significant difference in the size of juveniles produced by each of the five males, which shows that there is no paternal effect.

TABLE 1.

Mean metal concentrations (nM) observed during the experiment, within dissolved and total fractions, at each site.

Sites	Cu		Pb		Zn	
	Dissolved	Total	Dissolved	Total	Dissolved	Total
X41	4.92 (1.25)	5.57 (1.43)	0.26 (0.15)	0.29 (0.11)	16.64 (7.81)	17.05 (4.60)
X15	25.36 (6.63)	29.38 (7.73)	1.10 (0.19)	1.69 (0.247)	49.14 (10.12)	51.70 (10.32)
MIS	71.78 (19.39)	84.02 (23.54)	1.41 (0.18)	2.45 (0.59)	129.97 (28.34)	135.44 (27.05)
CS	93.33 (21.80)	113.92 (29.79)	4.64 (0.872)	7.18 (2.23)	289.65 (45.92)	377.24 (57.59)

The table focuses on Zn, Pb, and Cu, which resulted in the highest among site concentration variability (minimum max/min ratio >17.5 vs. less than four for the other metals). Standard errors are presented in brackets.

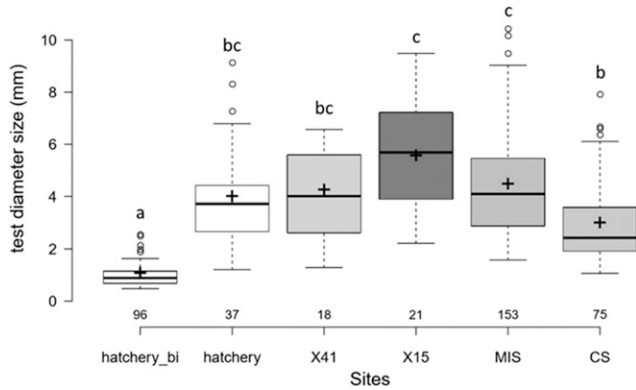


Figure 3. Distribution of test diameter within sites before and after immersion. Limits of the boxplot represent the first, median, and third quartiles, with Tukey whisker extents (dashed lines). Means are represented by a plus sign. “Hatchery_bi” refers to juveniles produced at the hatchery and measured before immersion (“bi”). The number mentioned above the horizontal axis indicates the total number of recaptured juvenile sea urchins. Letters represent Dunn’s post hoc results.

The genetic diversity of the progeny population was highly homogeneous among sites and size class (no significant F_{ST} values).

Analysis of Transcript Abundances after Immersion

Relative Abundances and Expression Profiles Clustering

The most abundant transcripts within each individual encoded *growth hormone (gh)* and *metallothionein (mt)* consistent with the juvenile stage of sea urchin development and the level of MTE observed within each immersion site. Similarly, although in a lesser extent, *sod [Cu/Zn]*, *dnmt1*, and *p38mapk* were also highly expressed genes, demonstrating what intracellular ROS juveniles had to cope with at each site and the potential environmental influence on genome methylation (Fig. 4).

F4

The FDA could separate the individuals in agreement with the differentiation of immersion sites (Fig. 5 right), considering

F5

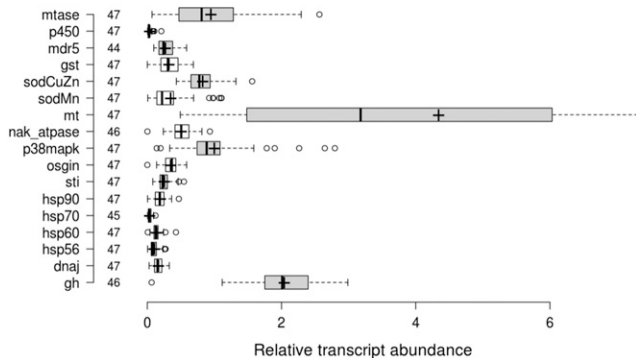


Figure 4. Distribution of relative transcript abundances. Transcripts abundances are given relative to the mean abundances of *gapdh* and *polyubiquitin* transcripts. Limits of the boxplot represent the first, median, and third quartiles, with Tukey whisker extents (dashed lines). Means are represented by a plus sign. The number of individuals is mentioned at the right of the vertical axis.

the whole set of genes. The first axis of the FDA accounts for 52.7% of the observed variability and is mainly driven by the hsp response (*hsp70*, *hsp90*, and *sti1*). The second axis accounts for 34.5% of the variability and is mainly driven by the *metallothionein* and *dnmt1* genes (Fig. 5 left). The results hence reveal that sea urchin exposed to the highest metallic stress, globally trigger a specific metallothionein stress response, and a significantly reduced heat shock response. Noteworthy, these sea urchins also repress the expression of *dnmt1* gene. By contrast, lower metallic stresses resulted in a slighter upregulation of the metallothionein and a potentially higher hsp response. Sea urchins immersed in the control site (*i.e.*, X41) and X15 globally display similar transcription profiles. Overall, overlapping ellipses reveal, nevertheless, that some individuals could produce similar transcriptomic responses, although experiencing contrasted environmental stress (Fig. 5 right).

Among Sites Variability

Significant among site differences in mean transcript abundances were found for 9 of the 17 gene transcripts assessed, which were involved in the heat shock response, detoxification, osmoregulation, and epigenetic modifications (Appendix). For some of those genes, high variance in relative gene expression was observed among sea urchins within the sites, without any correlation with a maternal or paternal effect.

Genes involved in a heat shock response (hsp70, hsp90, hsp60, hsp56, dnaJ, and sti1). Significant differences in the mean abundance of all transcripts among sites were detected (Appendix). Specifically, gene expression was globally upregulated at sites located within the bay compared with the control site. Moreover, the variability of gene expression probably increased with the level of MTE contamination.

Similar patterns of gene expression were observed for *hsp70*, *hsp90*, and *sti1* among sites. The highest mean expressions were found at MIS, which was less contaminated than CS with MTE. On the contrary, the mean level of expression of *dnaJ* was higher at CS than MIS, although the mean level of expression was skewed by some high values.

Compared with the control site, *hsp60* and *hsp56* were found to be similarly upregulated in individuals that were immersed within the bay. The variability of expression was similar among sites.

Overall, the genes were slightly upregulated, with the maximum increase in the mean expression observed for *hsp70* at MIS, with an approximately 4.2-fold increase in mean expression compared with the control site (Fig. 6).

F6

Genes involved in detoxification (metallothionein, p450, sod [Cu/Zn], sod [Mn], gst, and mrp5). Significant changes in mean expression among sites were observed for the *metallothionein (mt)* gene only, and a positive correlation between the transcript abundance and the MTE gradient could be evidenced (Spearman, $r = 0.74$; $P < 0.0001$) (Fig. 6 and Appendix). There was a clear influence of the sites within the bay. Indeed, X15 and MIS were found to be similar with about a 5-fold increase in mean expression compared with the control and similar variability of expression within sites, whereas *mt* transcript abundances found in juveniles immersed at CS were, in average, 12-fold higher than the mean expression at the control site. The similar level of *mt* gene expression found between X15 and MIS

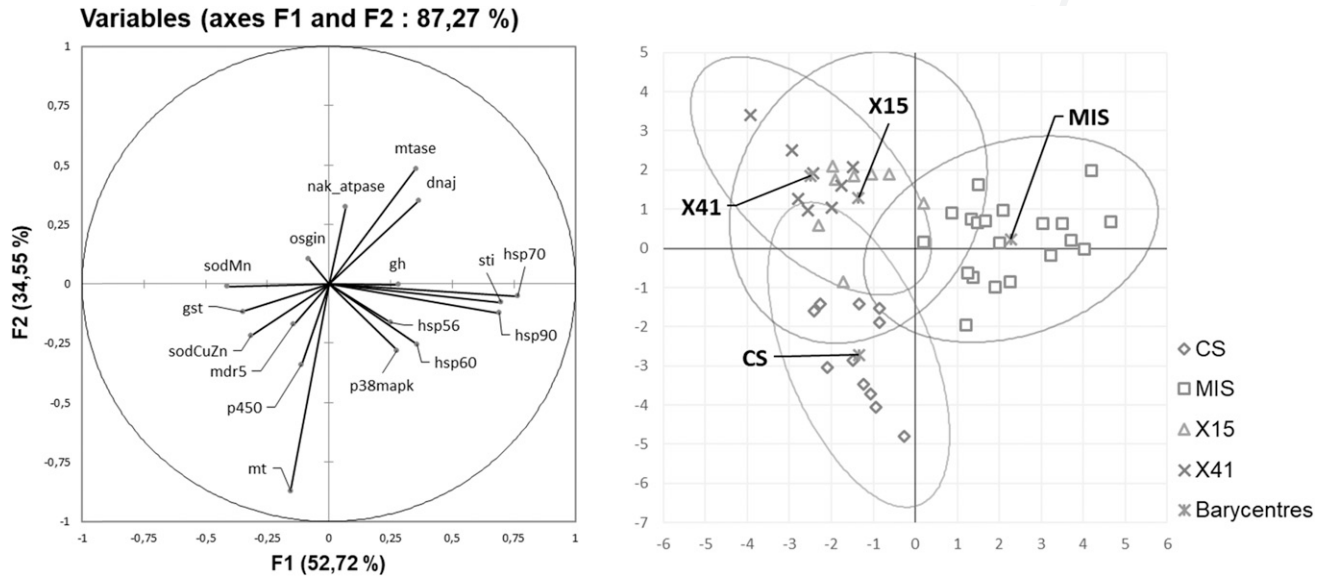


Figure 5. Factorial discriminant analysis of the four sites regarding relative transcript abundances. The analysis has been performed based on the transcript abundances of the whole set of genes. Each symbol represents a juvenile sea urchin.

appears consistent with a similar level of total metallic load at both sites, suggesting an unspecific response to heavy metals. In addition, the variability was low at the control site, compared with those within the bay.

Gene involved in DNA epigenetic modification (dnmt1). The mean abundance of the *dnmt1* transcript was significantly different between sites (Fig. 6). Considering the three polluted sites only, it appears that the mean transcript abundance decreases with

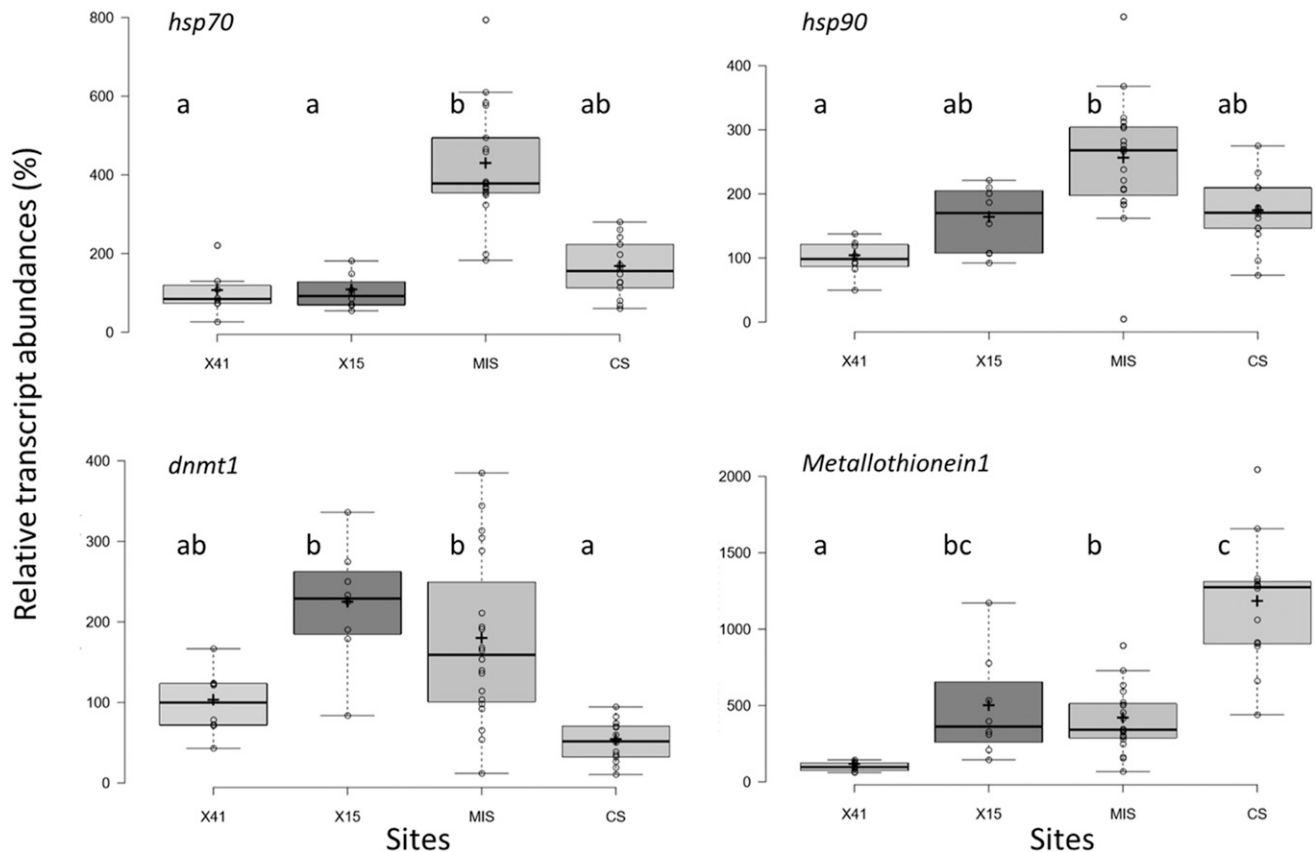


Figure 6. Relative abundances of the major contributing transcripts in population clustering. Transcript abundances are given relative to the mean abundances of *gapdh* and *polyubiquitin* transcripts. Each circle represents a sea urchin juvenile. Limits of the boxplot represent the first, median, and third quartiles, with Tukey whisker extents (dashed lines). Means are represented by a plus sign. Letters represent Dunn's post hoc results.

the level of MTE concentration, until the mean abundance at CS was found to be similar to the control site. This suggests that there is a contrasted regulation of *dnmt1* gene expression triggered by environmental conditions.

Growth, cell cycle regulation, and osmoregulation (p38mapk, growth hormone, oxidative stress-induced growth inhibitor, and Na⁺/K⁺ ATPase pump). These genes were not significantly differentially expressed among sites (Appendix); however, there was a tendency for the upregulation of *p38mapk* in juveniles immersed within the bay (P values < 0.024 compared with the control for a corrected $P = 0.0083$).

DISCUSSION

This study gives a first insight into the influence of chronic exposure of a benthic metazoan species to the contrasted habitats encountered within the Toulon embayment. The methodology relied on the caging of early juvenile sea urchins, and the monitoring of phenotypic parameters, at both organism and molecular levels. The measurement of MTE and seawater parameters, performed weekly, also gave a picture of the features of each habitat that the juveniles were exposed to.

Growth, Abundance, and Selection among Sites

The four experimental sites were under anthropogenic influence. Site X41 was nonetheless considered as a reference site because the MTE concentration here was no different to that of the open sea. The rearing conditions at the hatchery were obviously unrealistic. The three other sites were in a strong MTE contamination gradient across the bay of Toulon. At the end of the experiment, a thick layer of sediment was observed, covering the cages and algae at sites MIS and CS, but not at sites X41 and X15.

At the control site (X41), juvenile test sizes were the same as those reared at the hatchery. Immersion within the bay had beneficial influences compared with the control site, either with regard to test size (X15) or the number of juveniles recaptured (MIS and CS). As large juveniles of similar size at both X15 and MIS could be observed independently of the amount of available sediment, food availability was probably not a limiting parameter. The mean test size among sites within the bay decreased with the MTE contamination, suggesting that the MIS and CS sites were stressful environments. A similar observation has previously been reported in fish species (Canli & Atli 2003). This dwarfing trend is likely to be an evolutionary adaptive response to environmental stress (Morten & Twitchett 2009, Gardner et al. 2011). For instance, Garilli et al. (2015) demonstrated, in two gastropod species, that individuals were smaller when adapted to acidified seawater. They also pointed out that stressed individuals consumed more energy than non-stressed ones, but even so, at the whole-animal level, the energy demands of smaller stressed individuals was much lower than that of their heavier congeners living under normal conditions. In addition to this observation, the number of recaptured juveniles was much higher at MIS and CS, suggesting that the presence of large amounts of sediment probably had a role in the survival of small juveniles (Broekhuizen et al. 2001); however, this hypothesis cannot be ascertained with certainty because an equal amount of *Ulva* was added to each immersed pouch, which means that the number of juveniles immersed at

each site had not been determined accurately. Nevertheless, although a high variance in density among pouches cannot be excluded, the number of sea urchins immersed within each pouch likely exceeded the maximum number of recaptured individuals.

Hence, intraspecific competition for food may have taken place at the X41 and X15 sites, but the explanation for this observation is as yet undetermined. One explanation could be that bigger individuals secrete or indirectly induce the release of a growth inhibitor, which could interrupt the development of small juveniles, thus increasing the risk of their predation. In such a case, sedimentary particles at MIS and CS could have quenched this putative signaling hormone, thus allowing small individuals to grow. Although this hypothesis cannot be confirmed, as discussed previously, a link between hormonal signaling and growth has previously been established in sea urchin species (Heyland & Hodin 2004), suggesting that environmental cues are likely to influence population densities and growth rates.

Gene Expression Profiles

To assess the potential plasticity within a recruited population, the cohort of juveniles whose test size ranged between 3.5 and 5 mm were considered, as harboring the maximum genetic diversity. Moreover, larger or smaller individuals might have biased the gene expression analysis because of their differentially developed organs (Kozera & Rapacz 2013). Although this cohort does not represent the full plasticity that could be expected within a genuine population of young recruits, the gene expression results have nonetheless highlighted potentially high variance in gene expression within and among sites. In similar future experiments, it would be interesting to investigate a much larger number of progenitors.

The analysis of gene expression was performed on well-known genes potentially involved in acclimation processes (Marrone et al. 2012). The transcript profiles observed within individuals at the end of the experiment would most likely reflect acclimation processes to recent changes within each site (Hofmann & Todgham 2010), although any long-term effects of some environmental factors on gene expression could not be excluded (Jaenisch & Bird 2003).

The FDA revealed that sea urchin juveniles could develop differential acclimation processes at the molecular level, independently of the genotype or size. This is consistent with the well-known plasticity of this species (Ebert 1996). Nevertheless, the ellipses of the FDA overlapped, reflecting that the molecular response was not as homogenous within each site, except for the control site. This might be related to pair site similarities, evidenced by PCA based on either MTE or probe parameters alone (data not shown). Indeed, when one considers MTE, MIS was more like X41/X15 than CS. On the contrary, focusing on probe parameters specifically, MIS and CS were quite similar. Thus, one explanation could be that there is a differential susceptibility to the range of environmental drivers encountered within the sites (Killen et al. 2013). Another possibility could be that juveniles acclimate to non-homogenous microenvironments within their cage. This could contribute to the apparent observed plasticity at a very small spatial scale (Lavy et al. 2016). Within the cages, the inside and outside of the *Ulva*-containing pouches could be different non-homogenous microenvironments, the inner one being better protected or buffered by the

algae compared with the outer. Therefore, it would be interesting to test this hypothesis and eventually further decipher the relative contribution of the surrounding micro-local effect on individual plasticity in the molecular acclimation response.

Immersed juveniles produced high amounts of growth-related transcripts (*gh* and *p38mapk*), as would be expected considering their developmental stage. Moreover, the *dnmt1* gene was more highly expressed than the other genes (except within individuals immersed at CS), which was also consistent with genome methylation occurring at each genome replication (Jones & Liang 2009). Among the detoxification genes, juveniles highly expressed *mt* and *sod* [*Cu/Zn*] genes, reflecting the stressful environment within the Toulon embayment, and consistent with the high concentration of MTE and the production of potential ROS that they could trigger (Ercal et al. 2001). Comparatively, there was a much lower abundance of *hsp* transcripts, indicating that MTE likely constituted the main environmental stress juveniles had to cope with, irrespective of the site in the Toulon embayment.

When considering among site variations specifically, the levels of expression of the metallothionein gene were quite different. Such a result was expected regarding the critical cytoprotective function of metallothionein against nonessential heavy metals, such as cadmium and lead (Ragusa et al. 2013, 2017, Kim & Kang 2017) and MTE concentrations. The increase in *mt* transcripts have also been reported in similar environmental studies and in similar proportions in the gray mullet fish (Oliveira et al. 2010), juvenile sea bass (De Domenico et al. 2011), rockfish (Kim & Kang 2017), and mussels (Franzellitti et al. 2010). There was no difference among the sites for all of the other detoxification genes surveyed, suggesting that there are no or very limited differences in oxidative stress (*sod* and *gst*) among sites, resulting from exposure to high concentrations of MTE, as previously reported (Regoli & Principato 1995). Thus, the levels of *metallothionein* gene expression appear to be sufficient to limit the deleterious effect of ROS, potentially triggered by MTE. Furthermore, genes encoding *p450* and *mrp5* did not show any difference among sites, suggesting that the levels of these expressed proteins were sufficient to face organic pollutants, whatever their potential concentration differences within the bay; however, although not significant, a tendency with the increase in *mrp5* gene expression within the MTE pollution gradient of the bay was detected. This result is consistent with reported measurements of several classes of organic pollutants, found in sediment surface layers (Gómez-Gutiérrez et al. 2007).

Patterns of heat shock protein gene expression were also different among sites, although less pronounced than with *mt*, but consistent with similar studies performed, for instance, on the mussel *Mytilus galloprovincialis* (Franzellitti et al. 2010) and oyster *Crassostrea gigas* (Choi et al. 2008). Unexpectedly, *hsp70* was only upregulated at MIS compared with the control site; however, at least similar levels of expression were also expected at CS, which was more contaminated with MTE. Furthermore, both CS and MIS were expected to be subjected to a wider range and higher intensity of pollutants than X41 and X15. Similar patterns were found for *hsp90* and *sti1*, which both interact with *hsp70* (Song & Masison 2005), except that their mean transcript abundance seemed better correlated with the level of contamination (*i.e.*, within the area encompassing X41 to MIS). The difference in *hsp70* transcript abundances between CS and

much less MTE-contaminated sites is challenging to explain, as *hsp70* induction is expected to reflect the organism's sensitivity to contaminants. A number of different classes of inorganic and organic contaminants do indeed trigger the expression of *hsp70*, either at nontoxic or cytotoxic levels, through different toxicity mechanisms (Ait-Aissa et al. 2000, Rajeshkumar et al. 2013); however, the highest tolerance to MTE had also been associated with decreased levels of *hsp70*, for instance, in *Daphnia* (Haap & Köhler 2009) and *Danio rerio* larvae (Scheil et al. 2010). The obtained results tend to agree with those of previous studies demonstrating that in organisms chronically exposed to high levels of metal contaminants, *hsp70* was less involved in the cytoprotective process than *mt* (Urani et al. 2007). Furthermore, from an evolutionary perspective, Zanger et al. (2000) argued that the low *hsp70* level observed in chronically metal-stressed soil arthropods was a result of natural selection. Although this evolutionary trait has never been specifically assessed in *Paracentrotus lividus*, selection acting on regulatory regions could not be excluded, at least within the Mediterranean basin, where anthropogenically contaminated sites are regularly encountered along the coast, specifically for instance in the region of Toulon, from Genova to Fos-sur-Mer. The smaller variability of *hsp70* expression observed in CS compared with MIS appears consistent with this idea. With these assumptions taken together, the high level of *hsp70* found at MIS could in fact result from other stress factors. Among the abiotic parameters tested as potential stress factors, the salinity at MIS was significantly lower than that at the other sites. An increase in *hsp70* transcripts to low salinity has indeed been reported in the sea cucumber *Apostichopus japonicas* (Meng et al. 2011), although Madeira et al. (2014) observed that hypo salinity hindered the temperature induced expression of the *hsp70* gene.

Overall, *hsp* and *mt* gene expressions clearly demonstrated the expected environmental stress gradient within the Bay of Toulon, which potentially are not uniquely due to metallic load (Baumard et al. 1998, Andral et al. 2004, Guigue et al. 2017).

Dnmt1 gene expression gave contrasting results, with the X15 and MIS sites being upregulated compared with the control, with high within-site variance. An increase, such as this, in transcript abundance related to the higher level of MTE contamination appears to be consistent with zinc-induced regulation of gene expression (Zhang et al. 2012). Zinc activates the metal response element-binding transcription factor-1 in the upstream *dnmt1* gene, triggering its overexpression. When exposed to other metals such as cadmium or copper, the intracellular redistribution of zinc is induced, which binds metal response element-binding transcription factor-1 and consequently activates *dnmt1* expression (Kimura et al. 2009). Although the upstream structure of the *dnmt1* promoter in the genome of *Paracentrotus lividus* was not analyzed in this study, the observed relative abundances globally fits with this regulation mechanism under the influence of either one or more heavy metals because mean transcript abundances at X15 and MIS were higher than that at the control site; however, within the bay, mean *dnmt1* transcript abundance decreased with increasing metal concentrations, and the mean transcript abundance observed at CS was even lower than that of the control. This unexpected pattern might result from a combination of site-specific stresses that eventually repress *dnmt1* expression.

In vertebrates, DNA methyltransferases are essential enzymes that establish and maintain the methylation of some

cytosine residues (*i.e.*, mainly those of CpG dinucleotides) within the genome, throughout the cell replication process (Goll & Bestor 2005). Although *dnmt1* is primarily involved in the maintenance of methylation patterns (Sharif et al. 2007), *de novo* activities have also been observed in the maintenance of genome stability and in the development of tumor cells (Chen et al. 1998). The overexpression of *dnmt1* is often associated with *dnmt1 de novo* activity, which generally results in the hypermethylation of promoters of cancer suppressor genes, hence suppressing their expression and favoring tumorigenesis (Rhee et al. 2002, Peng et al. 2006). The reduction in CpG methylation, due to the inactivation of the *dnmt1* enzyme, also resulted in growth defects, cell death, activation of transposable elements, genome instability, and hence, the development of cancer (Walsh et al. 1998, Xu et al. 1999, Jackson-Grusby et al. 2001, Eden et al. 2003, Gaudet et al. 2003).

Environmental stressors are known to activate transposable elements and influence the number of repeated sequences, thus increasing genome instability (Kale et al. 2005, Schmidt & Anderson 2006), although it has been reported that such mechanisms may be associated with better fitness (Casacuberta & González 2013).

The upregulation of *dnmt1*, observed at X15 and CS, was possibly due to an acclimation response to potential risks from deleterious genome modifications. Thus, the decrease in *dnmt1* across the contamination gradient might increase the risk of genome modifications, thus increasing susceptibility to diseases. In future studies, it would be interesting to assess the influence of pollutants on both the reactivation of transposable elements and genome stability. As discussed previously, the decrease in *dnmt1* expression also correlates with the decrease in the test size observed across the bay.

Potential differences in genome or region-specific methylation among sites, which could have been correlated with differences in *dnmt1* expression, were not assessed. In further research, it would

be interesting to focus on the expression of *methyltransferase* among the gene family and specific methylation of some genome regions, to obtain a better insight into the role of epigenetics in the acclimation and adaptive response (Martinez-Zamudio & Ha 2011, Vandegheuchte & Janssen 2011).

CONCLUSIONS

This study provides a first insight into the impact, on early juvenile *Paracentrotus lividus* sea urchins, of chronic exposure to real environmental sites, within the Toulon embayment. The species demonstrated the high acclimation abilities of such young individuals, although associated with a potentially important trade-off, *i.e.*, to say, their growth potential when under high levels of environmental stress. It is still questionable whether younger aged (*i.e.*, immediate postlarvae) and larval individuals would acclimate as well.

Among the set of genes that have been investigated, only a fraction displayed differential expression, of which the DNA methyltransferase 1 suggesting a critical role of epigenetic modification in the stress response. Hence, future studies should be performed to test the hypothesis that transposable elements are possibly expressed, with the risk of genome integration under stressful conditions. Finally, as this study investigated gene expression profiles, it reasserts that regulatory genomic regions and transacting factors are critical toward local adaptation. The study of these actors at the population level would probably contribute to give a more precise overview of the evolutionary potential of the species.

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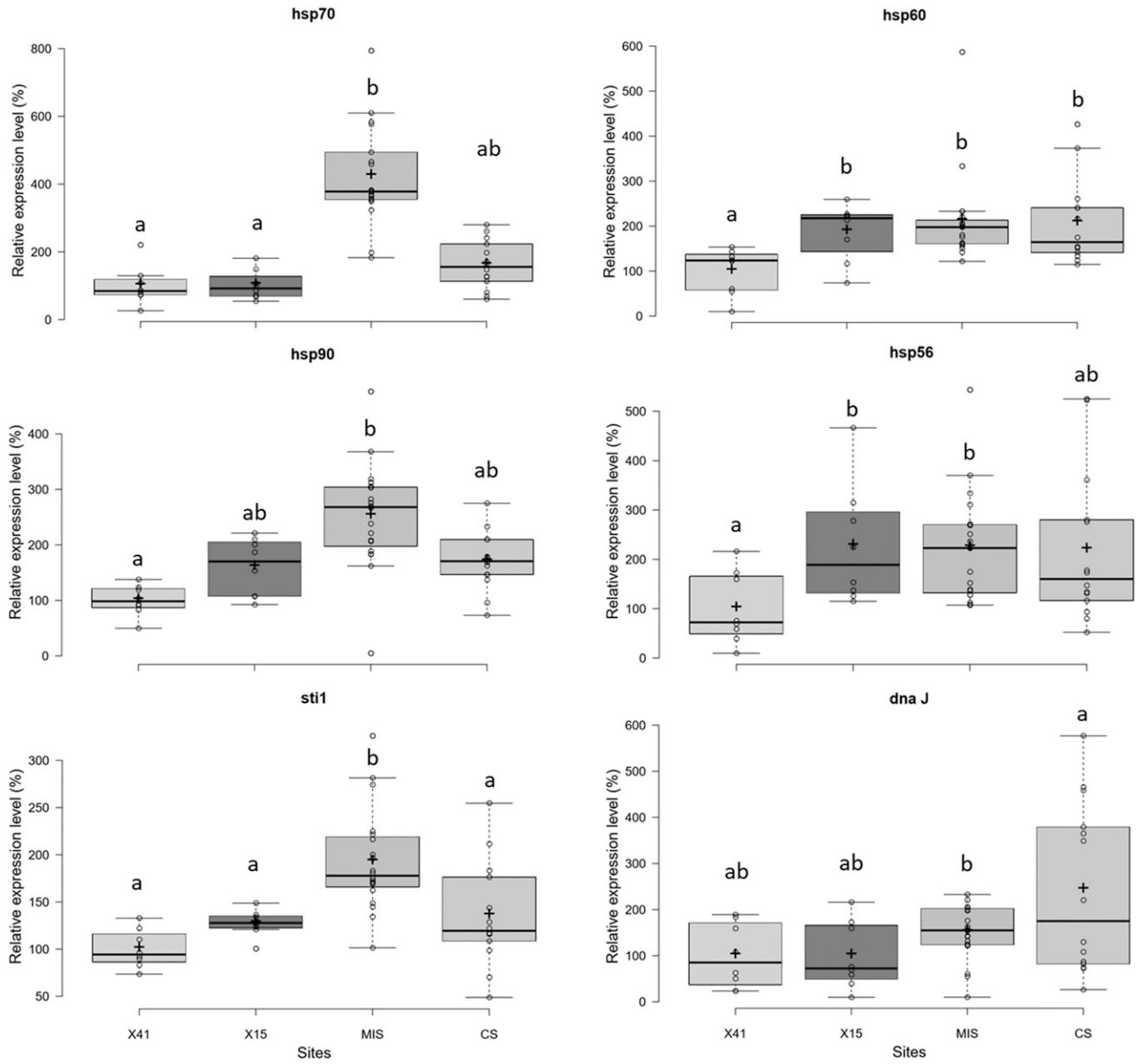
List of Primers

Gene transcripts	Left primer	Right primer	Size (bp)	Efficacy	Ref
<i>hsp90</i>	CAGAGACCTTTGCCTTCCAG	AATCTTGTCCAGGGCATCAG	114	1.94	<i>This study</i>
<i>hsp70</i>	CAGAACCACGCCAGCTATG	GCTTGGATGCTACTATCGTTG	150	1.976	Marrone et al. (2012)
<i>hsp60</i>	GAATATCCAGTGTACTCCGAC	GCATCAGCTAAGAGGTCAAC	160	1.862	Marrone et al. (2012)
<i>hsp56</i>	GGAGCTATGCTAAGGACATC	CTACAGCCTTAGCGACAGTG	183	1.887	Marrone et al. (2012)
<i>dnaJ (hsp40)</i>	AAGTCAGCGAGGCGTACAGT	GAGCTCAAAGAACGTCTGG	140	1.89	<i>This study</i>
<i>dnmt1</i>	GATCTCGTCAGACGATAGAAG	CTCTTGCTGTGTTAGCATTG	175	1.98	Marrone et al. (2012)
<i>p38mapk</i>	GTGATCAGCTTGCTTGACTG	GTAGATGAGGAACTGGACGTG	144	1.852	Marrone et al. (2012)
<i>sti 1</i>	AGGGCTACCTCAGGAAAGGA	ACGTGCCTCCTTGTTATTGG	110	1.89	<i>This study</i>
<i>Na/K ATPase (alpha subunit)</i>	AGGCTTGCGTCTTCTTGATT	TTCTTCACCAGGCAGTTCTT	123	1.932	<i>This study</i>
<i>Polyubiquitin</i>	CCGGTTTCGATTAGGATCAA	GCCTGGCTACAACACCAAAT	158	1.934	Martin et al. (2011)
<i>gapdh</i>	GATTCCATGGGGTGATAACG	TGATGACCTTCTTGGCTCCT	110	1.909	<i>This study</i>
<i>sod [Cu-Zn]</i>	CATTGGACGATCACTTGTGG	TTGAGATGCCAATGACTCCA	122	1.963	<i>This study</i>
<i>sod [Mn]</i>	AGGGCAAGCACTCACTCTGT	TTCGCTGTTGTCGAGATGAC	130	2.077	<i>This study</i>
<i>Metallothionein</i>	GAACGTGCTCAAATGCTTCA	TGGCAGAAGCGTTGACATAC	144	1.952	<i>This study</i>
<i>Oxidative stress-induced growth inhibitor 1</i>	GAGAAATCTCGGCTGGACTG	GCCCATTATCAAAGCTCAT	187	1.891	<i>This study</i>
<i>Glutathione-S-transferase</i>	TTGAATGACTTCCCAAATTGC	ACATCAGGGCCTATGCAGAC	193	1.897	<i>This study</i>
<i>Growth hormone</i>	CAGGTGCAGGTCTGAGACA	CAACCCCATGAAATTTGGAC	114	1.977	<i>This study</i>
<i>mp 5</i>	AGGGATCAGGACTGTTGTGG	GTGCCATACCAGAAGGCAAT	177	1.887	<i>This study</i>

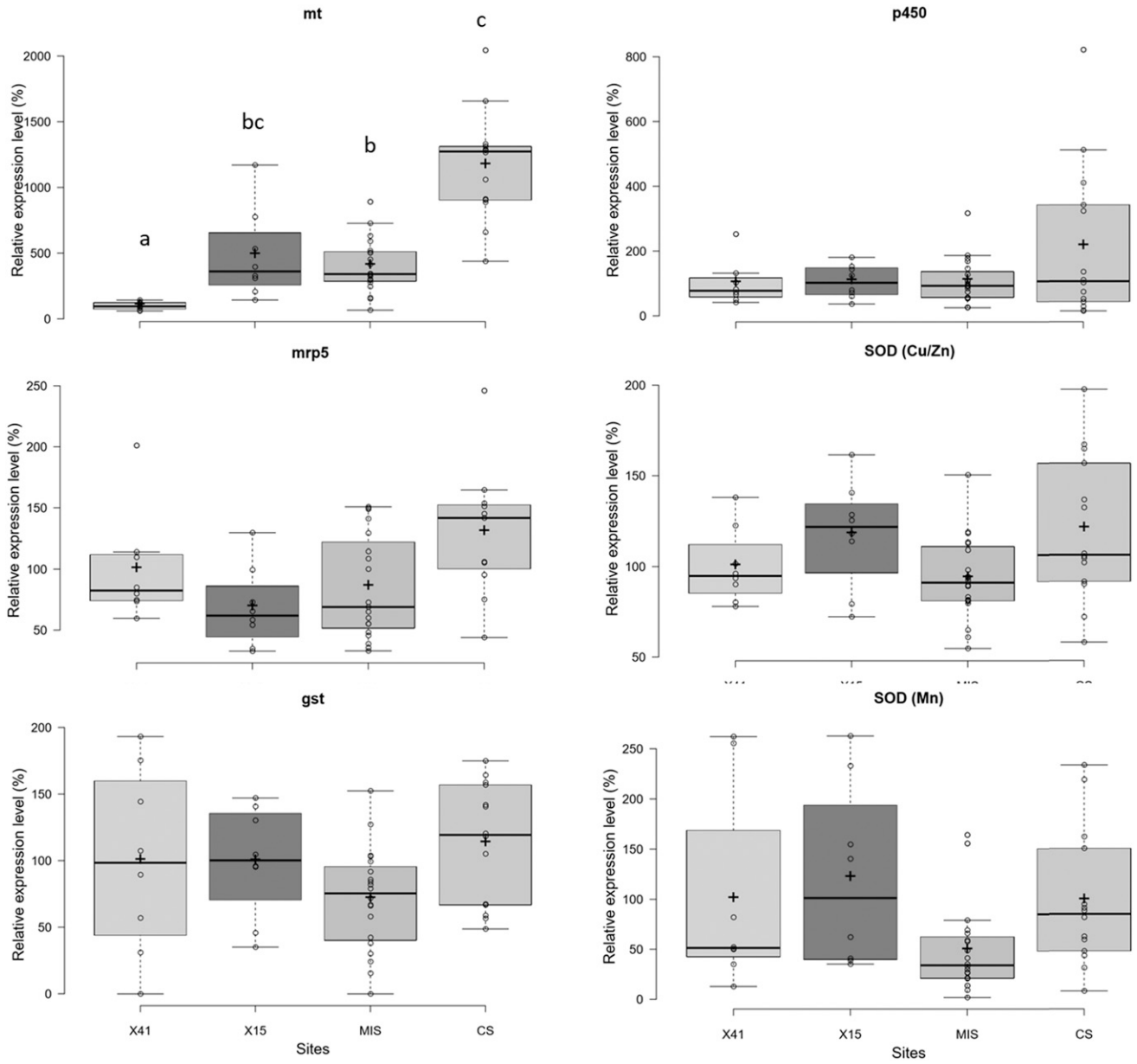
Transcript Abundances

Limits of the boxplot represent the first, median, and third quartiles, with Tukey whisker extents (dashed lines). Means are represented by a plus sign. Open circles represent the relative abundance within individuals. All data are presented relative to the mean abundance observed within the control site (*i.e.*, X41). Letters represent Dunn's post hoc results.

Heat Shock Protein Genes



Detoxification Genes



Methylation, Growth-Related, and Homeostasis Genes

