

GENETIC MECHANISMS OF POLLUTION RESISTANCE IN A MARINE INVERTEBRATE

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Abstract. Pollution is a common stress in the marine environment and one of today's most powerful agents of selection, yet we have little understanding of how anthropogenic toxicants influence mechanisms of adaptation in marine populations. Due to their life history strategies, marine invertebrates are unable to avoid stress and must adapt to variable environments. We examined the genetic basis of pollution resistance across multiple environments using the marine invertebrate, *Styela plicata*. Gametes were crossed in a quantitative genetic breeding design to enable partitioning of additive genetic variance across a concentration gradient of a common marine pollutant, copper. Hatching success was scored as a measure of stress resistance in copper concentrations of 0, 75, 150, and 350 µg/L. There was a significant genotype × environment interaction in hatching success across copper concentrations. Further analysis using factor analytic modeling confirmed a significant dimension of across-environment genetic variation where the genetic basis of resistance to stress in the first three environments differed from that in the environment of highest copper concentration. A second genetic dimension further differentiated between the genetic basis of resistance to low and high stress environments. These results suggest that marine organisms use different genetic mechanisms to adapt to different levels of pollution and that the level of genetic variation to adapt to intense pollution stresses may be limited.

Key words: *ascidians; copper stress; genotype × environment interactions; marine invertebrates; pollution resistance; reaction norms; Styela plicata.*

INTRODUCTION

Environmental variation over spatial and temporal scales is a universal feature of natural environments and a significant determinant of population dynamics (Hoffmann and Parsons 1991). Currently, one of the most powerful agents of selection acting on marine organisms is pollution stress (Cairns and Niederlehner 1996, Levinton et al. 2003). Generally, organisms can avoid stress (through movement away from the area), or they are forced to adapt (Hoffmann and Parsons 1991). Many marine invertebrates have larvae that are poor swimmers relative to current speeds, and a sedentary adult stage (Shanks 1995, Young 1995). Consequently, most marine invertebrates have limited ability to avoid pollution and must adapt to environments that can change rapidly over both spatial and temporal scales (Teasdale et al. 2003). Examples of adaptation to pollution can be seen in many marine invertebrates, e.g., bryozoans (Piola and Johnston 2006), arthropods (Donker and Bogert 1991, Groenendijk et al. 1999), and annelids (Grant et al. 1989, Klerks and Levinton 1989). While there is strong evidence for localized and rapid adaptation to stress, increased resistance often incurs fitness tradeoffs (e.g., Hoffmann and Parsons 1991).

Therefore investigating the genetic basis of traits across multiple environments is essential to understand the ecological and evolutionary consequences of anthropogenic toxicants.

Differences in the genetic basis of traits (e.g., for stress resistance) across environments can be inferred by two complementary methods. One approach, the investigation of genotype × environment (G × E) interactions, is common in economically-important plants and animals, where performances of traits associated with yield are of main concern between different geographical locations (e.g., Duque-Vargas et al. 1994, Ramdoyal et al. 2000, Tahir et al. 2006). Significant G × E interactions are also widespread in natural populations (e.g., Rawson and Hilbish 1991, Fanara et al. 2006). The second, related, approach in understanding genetic changes in traits with environments is the measurement of genetic correlations across environments (Falconer 1952). Genetic correlations complement G × E interaction studies as they allow quantification of interactions and facilitate understanding of the complex relationships across multiple environments (Smith et al. 2001, Sgro and Blows 2004). The application of genetic correlations across multiple environments is particularly useful in marine ecosystems as environmental factors are often found as gradients and not as discrete units (Endler 1977). Changes in the genetic basis of traits across environments have been reported in many taxa, suggesting it is likely to occur in marine invertebrates, however, results from the few

Manuscript received 14 December 2006; revised 23 April 2007; accepted 9 May 2007. Corresponding Editor: K. Stokesbury.

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studies examining marine invertebrates have been mixed (Newlon et al. 2003, Todd et al. 2004, Toro et al. 2004). Furthermore, studies examining the genetic basis of pollution resistance in marine organisms remain rare.

Copper pollution is a common stress in coastal marine ecosystems as it is found in numerous sources including urban runoff, sewage, industrial and mining waste, shipping activities and antifouling paint (Paulson et al. 1989; reviewed in Johnston and Keough 2005). Relative to other heavy metals, copper is one of the most toxic to marine invertebrates (Reichelt-Brushett and Harrison 2005), and in many species it causes lethal and sublethal effects, particularly in early life history stages (Reichelt-Brushett and Harrison 2000, 2005, Xie et al. 2005). Copper pollution has the potential to cause significant effects on ecosystems as concentrations can be highly variable over even small areas, exposing organisms to a wide range of stress levels (Teasdale et al. 2003).

Styela plicata is a solitary ascidian found in disturbed habitats subject to frequent copper stress. Using a quantitative genetic breeding design, we examined the genetic basis of stress resistance across multiple copper environments through scoring the ability of embryos to hatch into larvae. We adopt new multivariate approaches to the analysis of multi-environment genetic experiments (Smith et al. 2001) to explore the genetic relationships across an environmental gradient.

MATERIALS AND METHODS

Field collection and preparation procedures

Styela plicata is a solitary ascidian commonly found fouling pontoons in boat harbors. Adult individuals were collected from a population located in Manly Boat Harbor, Brisbane, Australia (27°28'1" E, 153°10'29" S). Individuals were kept in the laboratory and stored in seawater collected from the same location up to a maximum of five days with aeration.

Glassware and plastics used in the experiment were cleaned by first soaking in a 5% nitric acid solution for 12 hours, then washing in distilled water and air dried. All seawater used in the experiment was filtered using a vacuum filtration unit and chemical solutions were prepared with filtered seawater. Copper crystals were dissolved in seawater to make a concentrated stock solution of 1000 µg/L. A second stock solution of 100 µg/L was prepared from diluting the initial concentrate. Copper environments were made from dilution of the appropriate stock solutions with seawater. Specimen jars used for different copper environments were soaked in the respective concentration for 12 hours prior to use.

Gamete collection

Gametes were collected using standard "strip spawning" methods (Marshall et al. 2000). While *S. plicata* is a simultaneous hermaphrodite, eggs and sperm were collected from separate individuals. Gonads were obtained by cutting open the tunic, discarding the branchial basket and stomach and placing the internal

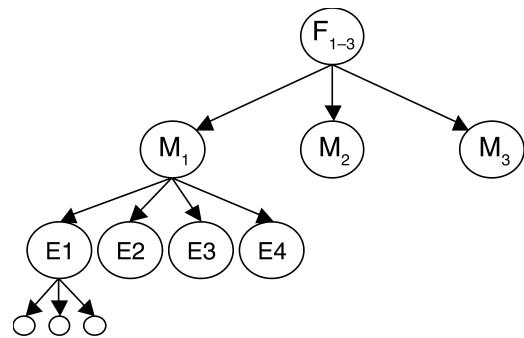


FIG. 1. Experimental design. Eggs from three females (F_{1-3}) were each split into three groups that were then each fertilized by sperm from a different male (M_1 , M_2 , or M_3), as in the North Carolina (II) design (Lynch and Walsh 1998). Zygotes from all male–female combinations were each split into four groups and placed in a gradient of copper environments (E_1 – E_4). Within each copper environment, the fertilized eggs were divided into three replicates. A single experimental block consisted of three females crossed with three males.

tissue in a Petri dish with a few drops of seawater. The gonads were then gently squashed and larger pieces of tissue were removed. The gonad extract was poured through a 100-µm filter into a small beaker where the eggs were retained inside the filter but the sperm and excess seawater passed through into the beaker. The filter containing the eggs was partially submerged in 0.5 mol/L potassium chloride (KCl) for 30 seconds to kill any remaining sperm before rinsing in seawater. Potassium chloride prevents self-fertilization but does not affect egg viability (B. Galletly and D. Marshall, unpublished data). Eggs were collected from all females first, then sperm was collected and kept in high concentrations to minimize sperm aging (Bolton and Havenhand 1996, Marshall and Evans 2005).

Fertilization

Sperm solution was added to the eggs in three batches at five-minute intervals. Gradual addition of sperm allows for the formation of blocks that decrease the incidence of multiple sperm fertilizing the same egg (polyspermy) and, thereby increase overall fertilization success (Styan 1998). The gamete solution was then left for an hour to maximize fertilization before being rinsed in seawater to remove sperm.

Genotype-by-environment interaction

Styela plicata is a free-spawner so eggs can be split into groups and fertilized across multiple males in a North Carolina (II) breeding design (Fig. 1). The North Carolina (II) design is a factorial design where sperm from each male accesses the eggs of each female, enabling variance to be partitioned into additive genetic, maternal, interaction, and error components (Lynch and Walsh 1998). Eight blocks were performed over a period of four months (the duration of the *S. plicata* reproductive season), where each block consisted of

the eggs of three females each combined with the sperm of three males, yielding nine crosses. Fertilization success was kept approximately equal (within 5%), across all combinations within a block. Within each male–female combination, zygotes were exposed to a gradient of nominal copper concentrations of 0, 75, 150, and 350 $\mu\text{g Cu/L}$. The embryos were left to develop in these copper environments in a constant temperature cabinet at 23°C for 10.5 hours before being fixed with 12% formalin (by volume). Individuals were scored as successful (hatched without deformities) or unsuccessful (unhatched or hatched with deformities assumed to significantly affect their survival). Unhatched larvae were considered unsuccessful as pilot studies indicated embryos still developing after 10.5 hours had arrested growth and generally would not hatch. For every male–female–copper environment combination, there were three individual replicates (specimen jars) and ~ 80 embryos were assessed per replicate yielding a total $\sim 75\,000$ embryos that were evaluated over the whole study.

Statistical analysis

Individuals in each specimen jar were counted and classed as successful or unsuccessful. Unfertilized eggs were not included in any analysis. Hatching success was standardized (using mean = 0, standard deviation = 1). Analysis of covariance (ANCOVA) with homogeneity of slope test using Type III sums of squares was used to test for a $G \times E$ interaction.

Falconer (1952) first proposed that the same trait expressed in different environments could be treated as a suite of different traits. Using this theory, the genetic relationships among environments in stress resistance can be investigated by determining the additive genetic variance covariance (\mathbf{G}) matrix across environments and visualizing the eigenvectors of the matrix as genetic dimensions. The SAS MIXED procedure was used to implement restricted maximum likelihood (REML) to estimate variance components (SAS Institute 1998). Environment (copper concentration) was included in the model as a fixed factor. Block was also included as a fixed factor to account for variation between blocks. The effect of male (within block), female (within block) and male \times female interactions (within block) were set as random factors. Diagonalization of \mathbf{G} simplifies the interpretation by determining eigenvectors that describe the major axes of genetic variation across the environments measured. Eigenvectors are independent combinations of the environments and describe a percentage of the variance from the highest percentage, g_{\max} , through to the lowest percentage, g_n (Arnold 1992). Eigenvalues represent the amount of genetic variance explained by that eigenvector.

One approach to analyze \mathbf{G} was to determine the significance of individual genetic correlations between environments using -2 log-likelihood scores. This method was not applicable in the current study because

interpretations of bivariate correlations are not as informative as a multivariate approach in the presence of more than two environments. Alternatively, an approach developed for the analysis of plant multi-environment trial data was applied in this situation (Smith et al. 2001). Smith et al. (2001) detailed the application of multiplicative mixed models for analyzing environments as traits, allowing quantification of the genetic relationship among multiple environments and testing the significance through factor analytic modeling. Factor analytic modeling can be used to determine how many factors (or genetic dimensions) are required to describe the genetic variation among environments (Smith et al. 2001, Hine and Blows 2006). Factor analytic modeling reports the fit of the model (-2 log-likelihood score) for the number of factors specified, using a factor analytic covariance structure rather than unstructured covariance at the sire level. In order to determine the number of significant genetic dimensions, the fit of the model was determined for three through to zero genetic dimensions (the fourth dimension would not converge). Each genetic dimension was then tested for significance by a χ^2 test. The significance of the n th dimension is tested by comparing the model with n dimensions to a model with $n - 1$ dimensions. For example, to test the significance of the third dimension, the model with three dimensions was compared to the model with two dimensions. The χ^2 value is the difference in -2 log-likelihood scores between the two models and the degrees of freedom are the difference in the number of covariance parameters associated with the two models. If the test produced a significant χ^2 , then removing the n th dimension produced a significantly inferior model. This approach was performed for each dimension except for going from 1 to 0 dimensions. In this case, a parms statement, which allows specification of the values for covariance parameters, was used to read in values from the single factor model and hold the four male parameters to zero. The -2 log-likelihood scores were then used in a χ^2 with four degrees of freedom.

RESULTS

The proportion of larvae that hatched successfully decreased with increases in the concentration of copper (Fig. 2). The mean proportion of larvae that successfully hatched within each copper concentration over all blocks was 0.66, 0.62, 0.37, and 0.04 within 0, 75, 150, and 350 $\mu\text{g Cu/L}$, respectively.

Genotype-by-environment interaction

The presence of a $G \times E$ interaction was observed by graphing reaction norms of genotypes, that is, the performance of each genotype in each environment of interest (Lynch and Walsh 1998). In this case, the reaction norms of genotypes are mean standardized success of paternal half-siblings from all blocks, plotted across all environments (Fig. 2). Paternal half-siblings

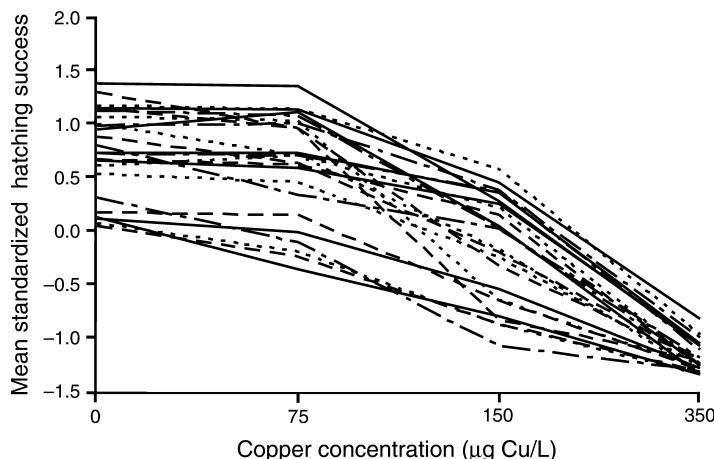


FIG. 2. Reaction norms for each genotype across copper concentrations. Lines represent the value for each genotype or mean of paternal half-siblings ($n = 24$ genotypes).

were used to partition the phenotypic variance in stress resistance into genetic and non-genetic sources of variation, and in particular the paternal (male) contributions are free of maternal effects. There was a significant $G \times E$ interaction between male and copper environment (male \times concentration, $F_{23, 802} = 2.639$, $P < 0.001$), using ANCOVA.

Genetic correlation across environments

A multiplicative mixed-model approach was used to estimate the **G** matrix across environments. A visual inspection of **G** (Table 1) showed that as copper concentrations increased, the additive genetic variance in hatching success was generally low, with the genetic variance in the highest stress environment being an order of magnitude lower than the other three environments. Environments are numbered in order of increasing copper concentration from E1 (0 $\mu\text{g Cu/L}$) to E4 (350 $\mu\text{g Cu/L}$). The two “benign” environments (E1 and E2) were had a strong genetic correlation (~ 0.90) and across the two “harsh” environments (E3 and E4) also had a high genetic correlation (~ 0.73 ; Table 1).

The **G** matrix was diagonalized and the first eigenvector (g_{max}) explained a total of 80.5% of the additive genetic variance. Each subsequent eigenvector (g_2 to g_4), contributed 16%, 2.5%, and 1% of the variance, respectively (Table 2). The first eigenvector, g_{max} , distinguished low-medium stress from high stress as the highest copper environment, E4, had a low loading in comparison to the first three environments (Table 2). The second eigenvector further distinguished between low and high stress, where E1 and E2 have negative loadings and the E3 and E4 have positive loadings.

Factor analytic modeling tested the statistical significance of the genetic dimensions described by **G**. Out of four dimensions only the first was significant (Table 3; $P = 0.021$) and, therefore, only g_{max} can be considered to display significant genetic variance.

The first two dimensions were displayed in a biplot to visualize the genetic relationships between multiple environments (Fig. 3). In the biplot, the squared length

of a vector is the variance explained by the two dimensions, while the cosine of the angle between vectors is the genetic correlation between them in this two-dimensional space (Smith et al. 2001). Therefore, vectors orientated in the same direction have a high correlation and subsequently, as the angle between vectors increases, the genetic correlation decreases. Accordingly, environments 1 and 2 which are adjacent on the copper concentration gradient have vectors of similar direction and magnitude (Fig. 3), reflecting the high pair-wise genetic correlation between them (Table 1). Environments 3 and 4 also have vectors in a similar direction as one another, and the genetic correlation between these environments is also high (Table 1). Therefore, the genetic basis of stress resistance is different between levels of high and low stress. Note that the genetic correlations indicated by the angles between any pair of vectors in the biplot will not coincide exactly with the bivariate genetic correlations in Table 1. This is because Table 1 contains the entire four-dimensional space, while Fig. 3 is a two-dimensional representation of 96.5% of the estimated genetic variance in Table 1.

DISCUSSION

We investigated the genetic basis of pollution resistance among multiple environments in a marine invertebrate using a quantitative genetic approach. Genotype-by-environment interactions have previously

TABLE 1. Genetic variance covariance (**G**) matrix of hatching success in the four environments.

	E1	E2	E3	E4
E1	0.0301	0.04	0.0156	0.0055
E2	<i>0.9071</i>	0.0647	0.0327	0.0083
E3	<i>0.4585</i>	<i>0.6542</i>	0.0386	0.0094
E4	<i>0.4789</i>	<i>0.4927</i>	<i>0.7252</i>	0.0043

Notes: Additive genetic variances are on the diagonal and in bold, while covariances are above the diagonal and correlations are in italics below. Environments are in order of increasing copper concentration from E1 (0 $\mu\text{g Cu/L}$) to E4 (350 $\mu\text{g Cu/L}$).

TABLE 2. Diagonalization of the four environments, showing eigenvalues, loadings of each environment (or trait), and the percentage of the total variance explained by each eigenvector.

Eigenvector	Eigenvalue	Percentage of total variance	E1	E2	E3	E4
g_{max}	0.11086	80.5	0.46668	0.74854	0.45496	0.12210
g_2	0.02209	16.0	-0.44074	-0.26447	0.83780	0.18411
g_3	0.00359	2.5	0.62184	-0.50688	0.03617	0.59588
g_4	0.00112	1.0	-0.44864	0.33589	-0.29964	0.77209

Note: Environments are from E1 (0 µg Cu/L) to E4 (350 µg Cu/L) in order of increasing copper concentration.

been observed in marine invertebrates in response to varying location (Newlon et al. 2003), and along a depth cline (Todd et al. 2004). However, not all studies on marine invertebrates show significant $G \times E$ interactions. Toro et al. (2004) exposed *Mytilus chilensis* to different geographical locations and found no $G \times E$ interaction in shell height and growth rate. This is the first study to find a $G \times E$ interaction with regards to pollution resistance in a marine invertebrate.

Genetic correlations are complementary to $G \times E$ analysis as they allow quantification of the relative genetic differences across environments and estimate how much of the genetic contribution to stress resistance is shared between environments. Determination of the G matrix across environments showed high genetic correlations between environments that were similar in copper concentration and low genetic correlation between environments that were at extreme ends of the gradient. Sgro and Blows (2004) also found this general pattern, using a similar analytical approach, in *Drosophila serrata* exposed to a laboratory temperature gradient. The decrease in genetic correlation between very different environments suggested a dichotomy in genetic mechanisms of stress resistance between low vs. high stress environments. Guidelines for copper concentrations in marine waters generally fall between the copper levels of E1 and E2 used in this experiment (e.g., Australian and New Zealand Conservation Council 2000). Therefore, environments consistent with these guidelines would not select for different genetic mechanisms of stress resistance in this population. However, in reality, harbors and marinas probably exceed these

guidelines often (reviewed in Johnston and Keough 2005) and are likely to result in the differential selection of resistant genotypes. Whether pollution resistant genotypes have lower performance overall is unclear but it is clear that different genetic mechanisms will be engaged in such environments (Shirley and Sibly 1999).

Levels of additive genetic variance decreased across the copper gradient. The highest copper environment had very low levels of additive genetic variance across environments in comparison with the other environments. Therefore, there is little genetic variation available for adaptation to extreme copper stress. However, marine invertebrates have been shown to rapidly evolve resistance to extreme levels of cadmium stress (Klerks and Levinton 1989, Levinton et al. 2003), indicating there is genetic variation in some traits to some types of extreme stress. Whilst studies have generally found additive genetic variance is decreased in a high stress environment, exceptions are common and the underlying mechanisms remain unclear (Hoffmann and Merila 1999).

Changes in the genetic basis of a trait across environments can be examined in detail through measuring changes in gene expression across environments. Studies investigating gene expression across environments have shown that different conditions can

TABLE 3. Factor analysis of dimensions showing -2 log-likelihood scores, covariance parameters, and significance for removing a dimension.

No. factors (dimensions)	-2 log-likelihood	Covariance parameters	χ^2	df	P
3	344.9	39	1.9	2	0.386
2	346.8	37	3.7	3	0.296
1	350.5	34	11.6	4	0.021
0	362.1	34			

Notes: The -2 log-likelihood scores and covariance parameters are associated with the model describing the number of dimensions in the same row. The χ^2 value, degrees of freedom, and P value are the results of a χ^2 comparing the model with n dimensions to the model with n - 1 dimensions.

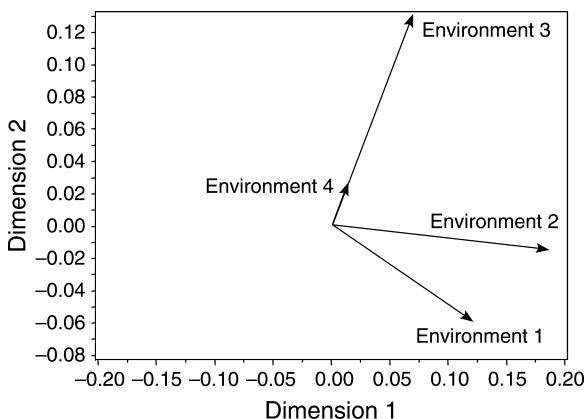


FIG. 3. Biplot of the first two dimensions of genetic variance among the four environments. Each vector relates to a copper concentration, where environment 1 is the lowest copper environment and environment 4 is the highest copper environment.

influence the level of expression of a wide range of genes. Generally, stress genes show significant changes in regulation between stressful vs. non-stressful environments across species (Brown et al. 2006, Knight et al. 2006, Sheader et al. 2006). While there are exceptions (Tanguy et al. 2003, Sorensen et al. 2005), the lack of response in these non-significant studies may be due insufficient stress levels able to stimulate changes in expression. Gene expression studies of particular relevance are those investigating the response of metallothioneins to metal stress. Metallothioneins (MTs) are proteins involved in homeostasis, sequestration and detoxification of metals and are conserved from insects to higher vertebrates (Balamurugan et al. 2004, Selvaraj et al. 2005). While the physiological mechanisms of stress resistance have not been determined in ascidians, it is possible copper concentrations may induce expression of MTs in *S. plicata*, conferring increased resistance to a specific copper environment.

Gene expression studies have often focused on the response of genes to the presence or absence of a stress. Comparisons of multiple environments are few, but the data from these studies supports the genetic dichotomy between low and high stress environments found by the current study. Hansen et al. (2006) compared MT gene expression between fish from three different rivers of increasing copper contamination. The river of highest contamination had significantly higher gene expression of MTs, in comparison with the rivers of medium and low copper contamination. In a manipulative laboratory study, Lukkari et al. (2004) exposed earthworms to a control and three copper polluted soils of increasing concentrations. Using protein concentration as a measure of gene expression, they found that as the copper level in the soil increased, MT concentrations also significantly increased. The MT levels climbed gradually between the first three environments and then had a large spike in the highest copper environment in short term exposure. The results of both Hansen et al. (2006) and Lukkari et al. (2004) showed gene expression levels changed above certain copper levels. However, this trend may be restricted by other specific factors including stress duration as long term exposure to copper did not show the same pattern (Lukkari et al. 2004). Possibly, resistance to copper in *S. plicata* is a threshold trait, where MT gene expression is induced once the copper stress has reached a particular level.

Stress resistance commonly incurs both ecological and direct fitness costs in plants and animals (reviewed in Hoffmann and Parsons 1991, Posthuma and Vanstraelen 1993, McKenzie 2001, Heil 2002). An increased ability to cope with stress may lead to a decrease in an organism's fecundity, survival, competitive ability and resistance to pathogens in the absence of stress. For example, pollution resistance tradeoffs have been shown in *Drosophila melanogaster* resistant to cadmium (Shirley and Sibly 1999). Such costs can cause resistance to be rapidly lost from the population when the stressor is

removed (Levinton et al. 2003). It may be that copper resistance in *S. plicata* also incurs a cost, particularly for resistance to extreme levels of copper. If these trade-offs occur, they may be different between the low (E1 and E2) and high (E3 and E4) stress environments as they have a different genetic basis. Further, resistance to low levels of pollution (E2) may not involve any costs at all, as it has the same genetic basis as an unpolluted environment. However, higher copper concentrations (E3 and E4) may select for alleles that confer a lower performance overall.

Conclusions

Increasing copper concentrations caused a decrease in hatching success, a measure of survival. However, hatching success appeared to have a different genetic basis in high copper concentrations compared with low copper concentrations. Little is known regarding the genetic basis of resistance to, or the genetic consequences of, pollution. This study suggests that marine organisms may adapt to varying intensities of pollution through different genetic mechanisms.

ACKNOWLEDGMENTS

Thanks to K. McGuigan, S. F. Chenoweth, and P. E. Cook for reviewing drafts. Thanks also to R. Allen, L. Barr, and S. Hart for helping to collect *Styela*.

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