

## Using clones and copper to resolve the genetic architecture of metal tolerance in a marine invader

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Bryozoan, contamination, factor analytical modeling, genetic correlation, genetic variance and covariance, modular organism, trade-off.

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### Abstract

The global spread of invasive species may be facilitated by adaptation to the practices that humans use to manage those species. For example, marine invertebrates that adapt to metal-based antifouling biocides on ship hulls may be more likely to be introduced to and establish in metal-polluted environments. We tested this idea by studying clonal variation in tolerance to, and ability to recover from, exposure to copper in a widespread invasive marine bryozoan, *Watersipora subtorquata*. We cloned colonies of this organism to independently test multiple environments in a genotype by environment design, and then created a genetic variance–covariance matrix. Genotypes were exposed to a gradient of copper concentrations and growth measured during exposure and after a recovery period. There was a significant genotype  $\times$  environment interaction in growth during exposure and recovery. We found clonal variation in tolerance and ability to recover from exposure to copper, with growth during exposure apparently trading off against growth after exposure. A weak genetic correlation between growth during and after exposure further indicated that they are separate traits. Overall, the genetic variation within this population indicates that there is considerable potential for adaptation to copper, but this comes at a cost to growth in unpolluted environments.

## Introduction

The impacts of nonindigenous species (NIS) on native communities are often detrimental, ranging from reducing biodiversity to substantial habitat modification by ecosystem engineers (Wilcove et al. 1998; Crooks 2002). As a result, the question of “what makes a species a successful invader?” is both fundamental and of vast practical relevance. It requires an understanding on a number of levels, including the physiological traits that mediate a species’ ability to successfully invade a new environment (i.e., its “invasiveness”; Sakai et al. 2001), the ecological interactions that mediate invasion (White et al. 2006), and the evolutionary dynamics involved in adaptation to the new environment (Facon et al. 2006).

Tolerance to metal pollution is one trait that may facilitate invasion in contaminated environments (Zhang et al. 2008; Dafforn et al. 2009; Liu and Pang 2010) and is likely to be advantageous at multiple stages of the invasion process, in-

cluding entrainment by a vector, transport, establishment, and spread (Piola et al. 2009). Invasive species have long been recognized as useful subjects in which to study evolution (Grinnell 1919; Lee and Gelembiuk 2008), but research into how they adapt to novel stressors, such as metal pollution, remains rare (Galletly et al. 2007). Therefore, it is important to understand both the genetic basis of metal tolerance and whether tolerance trades off against fitness under unpolluted conditions (Pease et al. 2010; McKenzie et al. 2011).

Metal pollution can result from many anthropogenic activities, ranging from industrial and mining waste (Apte and Day 1998; Gale et al. 2003) to agriculture (Keskin 2010). The impacts of metal pollution can be dramatic, the most obvious being a significant reduction in species richness (Pollard and Yuan 2006; Johnston and Roberts 2009). In contrast, tolerance to metal pollution may evolve rapidly in some species (Macnair 1987; Medina et al. 2007), usually as a result of site-specific pollution exposing a population to intense selection for multiple generations (e.g., mountain birch [Eranen 2008],

wolf spider [Hendrickx et al. 2008], and aquatic oligochaete [Klerks and Levinton 1989]).

Recently, tolerance to copper pollution has been associated with a number of invasive species, most predominantly in the marine environment (Dafforn et al. 2009). The marine environment is particularly susceptible to copper pollution, receiving additional input from sewage discharge, urban runoff and antifouling biocides (Scanes 1996; Fabris et al. 1999; Valkirs et al. 2003). A greater prevalence of NIS has been observed in habitats impacted by metal pollution (Piola and Johnston 2008; Dafforn et al. 2009; Crooks et al. 2010); furthermore, comparisons between analogous indigenous and NIS have identified greater tolerance in the latter (Piola and Johnston 2009). This ability to tolerate copper is thought to be a consequence of species introductions via hull fouling, a major vector for invasive marine species (Wasson et al. 2001; Floerl and Inglis 2005), due to the long-term use of copper-based antifoulant biocides on ship hulls (Piola et al. 2009).

Introductions via fouling on copper-painted vessel hulls can select for tolerance at numerous stages of the invasion process (Sakai et al. 2001; Piola et al. 2009). Initially, a species must either adapt or be preadapted to contaminated conditions prior to transportation (Henery et al. 2010), conditions that may potentially be highly toxic (Valkirs et al. 2003), then endure and survive the journey. This is followed by introduction and establishment into a new environment that may be impacted by metal pollution, as many harbors, ports, and marinas are (Birch and Taylor 1999; Matthiessen et al. 1999; Dafforn et al. 2009). As a consequence, exposure to copper may fluctuate dramatically during the life of an organism. Inconsistent exposure regimes may select for genotypes that are capable of responding to these fluctuating conditions (Lee and Gelembiuk 2008), in comparison to tolerance evolving in response to site-specific pollution where conditions are potentially more consistent (Klerks and Levinton 1989).

Tolerance to contaminants has generally been assessed at a population level (Johnston 2011), although research has begun to assess tolerance at the level of the individual and the genotype (McKenzie et al. 2011). Detailed individual-level studies are important because without heritable individual-level variation, selection cannot cause evolutionary adaptation to conditions. Studies of the heritability of tolerance usually involve testing parents and offspring, as full or half siblings, to estimate the narrow-sense heritability of the trait (Galletly et al. 2007; Pease et al. 2010). An easier way of measuring variation among genotypes in response to a variety of conditions, at least in modular organisms, is to use clonal fragments of the same individual in multiple environments in a genotype by environment ( $G \times E$ ) design (Newlon et al. 2003; Monro and Poore 2009). Many invasive marine species are clonal organisms that can reproduce sexually but grow vegetatively. In these organisms, an individual can be cloned into genetically identical fragments (e.g., ascidians [Newlon

et al. 2003]). These clones can then be individually exposed to different environments, thus testing a genotype's response to multiple contaminant concentrations while maintaining independence and precluding any influence of a previous exposure. Because of this property, modular organisms are often useful subjects for the study of genetic variation and architecture—especially when physiology or logistic considerations prevent the use of traditional breeding designs. Using a  $G \times E$  design to test for an interaction, it is possible to infer whether the genetic basis of traits differs across environments by looking at the specific combinations of genetic and environmental factors that influence a trait (Falconer and Mackay 1996). The presence or absence of genetic correlations between environments will also determine whether there is a cost associated with tolerance.

The invasive bryozoan *Watersipora subtorquata* (d'Orbigny 1852) is highly tolerant to copper (Piola and Johnston 2009) and has been found to actively recruit into high copper environments (McKenzie 2010). This bryozoan has a cosmopolitan distribution; its native range is uncertain but thought to be in the Caribbean (Mackie et al. 2006). Capable of growing on copper-based antifoulant paints, it provides a less toxic secondary surface upon which other organisms often settle, facilitating the transfer of other hull-fouling sessile invertebrates beyond their natural distribution (Floerl et al. 2004). It reproduces sexually, releasing lecithotrophic larvae that settle within approximately 24 h of release (Wisely 1958; Marshall and Keough 2004). Upon settlement the larvae metamorphose into a single zooid, or ancestrula, from which a colony forms by vegetative growth (Fig. 1). Each zooid within the colony is a genetic clone of the founding larvae, capable of feeding and reproducing, with a connective system between them (Shepherd and Thomas 1982). Being a modular organism, individual colonies/genotypes can be fragmented into clonal replicates that are capable of recovery and subsequent growth, making it an ideal test species with which to examine environmental tolerances.

Here, we study the among-clone variation in copper tolerance in *W. subtorquata*, a species in which selection is likely to fluctuate between periods of high copper exposure and low copper. We asked whether there is an interaction between genotype and environment (i.e., copper contamination) on colony growth, as growth is an important predictor of fitness.

## Materials and Methods

### Genotype culturing and cloning

To investigate copper tolerance in an invasive species, *W. subtorquata* colonies were reared from the offspring of field-collected adult colonies. Initially, gravid colonies were collected from Burraneer Marina Port Hacking (34°7'S, 151°10'E), an estuary south of Sydney in New South Wales, Australia. Port Hacking is a partially urbanized estuary, impacted by anthropogenic pollution. Due to urbanization



**Figure 1.** Numerous *Watersipora subtorquata* colonies growing in high densities on artificial substrate in Sydney Harbour, NSW, Australia. *W. subtorquata* is a red encrusting bryozoan that can grow into foliose clumps as shown above.

within this section of the estuary, copper accumulation in experimentally deployed oysters have previously found copper levels to be elevated approximately three times beyond natural oceanic levels (copper:  $57.6 \mu\text{g g}^{-1}$  dry weight of oyster tissue; Dafforn *et al.* 2009). Colonies were collected in May 2008 and maintained in individual containers at  $20^{\circ}\text{C}$  for up to three days without light. Spawning was induced by exposing the colonies to light and stopping aeration for approximately 1 h, stimulating the release of larvae. Each container, containing a single-field collected colony, was lined with an acetate sheet (that had been presoaked in seawater for a minimum of 24 h) upon which multiple larvae from that parent colony settled on. Once larvae had successfully settled and metamorphosed, the acetate sheets were sectioned and individual recruits were cultured in separate containers. Multiple siblings were maintained from each parent colony, although only one sibling from each colony was used for this copper tolerance experiment. This sibling was later chosen from each family based on its size and ability to be cloned by fragmentation. Each individual was maintained in a separate aerated container and fed the microalga *Isochrysis galbana* (clone T.Iso) five days a week, with seawater changed on a weekly basis. Culturing was conducted in a constant temperature room set at  $20^{\circ}\text{C}$ , using filtered sterilized seawater (details below). Individuals were maintained as such until they had grown large enough on the acetate sheet for the colony to be cut into 12 pieces, or clonal replicates. This number of clone fragments per genotype was decided by the size of the

initial colonies. Once large enough (approximately 1.5 cm in diameter), each colony/genotype was sliced into 12 pieces approximately  $8 \text{ mm}^2$  using MicroPoint FeatherLite<sup>(TM)</sup> scissors (to reduce tissue damage), ensuring that each piece had a distinct and equally sized growing edge. Hereafter, each cloned colony is referred to as genotype. Each clone fragment was then attached to the bottom of a petri dish (35 mm diameter) using a minuscule smear of superglue on the bottom of the acetate. Once cemented, the petri dish was rinsed once before being filled with seawater containing *I. galbana* at a concentration of  $10^5$  cells  $\text{mL}^{-1}$ , which was replaced every 24 h from this point on. Colonies were allowed to recover for seven days before commencing the copper tolerance assay.

### Copper treatments

Copper solutions were prepared using analytical grade copper II sulphate anhydrous. The experimental concentrations were prepared on a daily basis from a stock solution of  $1 \text{ g L}^{-1}$  Cu in Milli-Q water. From this, an initial solution of  $1000 \mu\text{g L}^{-1}$  Cu in Milli-Q water was prepared daily from which the experimental treatments 25, 50, 75, 100, and  $125 \mu\text{g L}^{-1}$  Cu in seawater were then diluted. These treatments were chosen to represent the range of concentrations that copper-based antifouling paints can leach over time (Valkirs *et al.* 2003). For a food source *I. galbana* was incorporated as a component of the copper and control solutions at  $10^5$  cells  $\text{mL}^{-1}$ , with new cultured microalgae used on a daily

basis. All equipment was acid washed in 5% nitric acid for a minimum of 24 h then thrice rinsed in Milli-Q water prior to use. All seawater used throughout this experiment was filtered to 5  $\mu\text{m}$  (Pentair Mechanical Filter Module, Life-gard Aquatics, Cerritos, CA, USA) and sterilized using a UV light sterilizer (SMART UV Sterilizers 50 W, Emperor Aquatics, Pottstown, PA, USA). Salinity, pH, and dissolved oxygen were monitored for all solutions using a YSI 556 MPS<sup>®</sup> (Yellow Springs, OH) before being used experimentally to ensure no differences between treatments. Water samples were collected from each treatment every three days throughout the exposure period to determine the total Cu. From these, three replicates of three of the treatments: control, 50, and 100  $\mu\text{g L}^{-1}$  ( $n = 3$ ) were randomly chosen for analysis. These samples were analyzed at the Australian Government National Measurement Institute in Sydney, Australia, using ICP-AES (detection limit of 5  $\mu\text{g L}^{-1}$ ). These analyses found copper concentrations to be close to the nominal values (control  $< 5 \mu\text{g L}^{-1}$ , 50 =  $54.3 \pm 0.9 \mu\text{g L}^{-1}$  and 100 =  $106.7 \pm 3.3 \mu\text{g L}^{-1}$ ). Hence, further analysis of the random samples was not deemed necessary.

### Copper tolerance assay

After recovery from the cloning process, two clone fragments from each genotype were randomly assigned to each of the six experimental treatments (environments), control seawater, 25, 50, 75, 100, and 125  $\mu\text{g Cu L}^{-1}$  ( $n = 2$  replicates per concentration within a genotype). Clone fragments were exposed to the appropriate solution for a nine days period, with the treatment solution replaced daily with fresh solution. After the exposure period, the clone fragments recovered for a period of nine days, with all treatments given the control solution containing microalgae ( $10^5 \text{ cells mL}^{-1}$ ) on a daily basis. A total of 11 genotypes were cloned and tested for copper tolerance that, due to differing growth rates between genotypes, were tested in three time blocks (January, February, and May 2009). Based on the methods and techniques developed by Piola and Johnston (2009), this regime (with a nine days exposure and nine days recovery period) and copper concentrations aimed to mimic the fluctuating conditions that a colony may be exposed to such as pulse and press pollution events. It was also a sufficient time period for growth to be exhibited, that is, for new zooids to bud then become fully formed zooids (L. A. McKenzie, personal observation).

Throughout the experiment clone fragments were photographed to assess zooid survival and growth as an indicator of copper tolerance, using a dissecting microscope (Leica Model: M205C) and digital camera (Leica Model: DFC290). After the initial cloning recovery period, the clone fragments were photographed on day 1 when the copper exposures began, day 10 when the recovery period began, and day 18 at

the end of the recovery period. Images were digitally analyzed, measuring the area of live zooids to the micrometer using Leica Application Suite (LAS Version 3.6). From these photos, two periods of growth were calculated; (1) growth during exposure, and (2) growth during recovery, as the change in area of live zooids relative to the initial area of live zooids at the beginning of each period. A growth rate of 1 denotes no change in area,  $> 1$  is an increase, and  $< 1$  a decrease. Due to the method of cloning, live zooids were easily delineated with fragments growing flat against the base of the petri dish. Only live zooids were measured, which were tinged orange and had obvious internal organs. Zooids damaged on the edge of the fragment while cutting were not included in the measurements even if still orange after the initial recovery period, as they were incapable of growth and had reduced survival.

### Statistical analysis

To determine whether copper tolerance differs between genotypes and to test for a  $G \times E$  interaction, we analyzed growth rate during exposure and recovery separately using a nested three-factor analysis of variance (random: block and genotype (block), fixed: environment). The within-cells error term is constituted by the variation between the clone replicates within each environment (Hughes 1992). Data were checked for normality and homogeneity of variances using frequency histograms and residual plots. Tests for homogeneity of variances, such as Levene's test, were inappropriate because there were only two replicates within an environment for each genotype. Data were analyzed untransformed. These and the following analysis of variance (ANOVA) analyses were run in the statistical program PASW Statistics version 18.

To explore whether tolerance to copper involves multiple traits or costs, we analyzed growth across the multiple environmental treatments using factor analytical modeling, with the two growth periods; during exposure and recovery, analyzed separately. The genetic variance-covariance matrix ( $G$ ) across copper treatments was estimated using Restricted Maximum Likelihood (REML, in the proc MIXED routine in SAS), followed by factor analytic modeling in order to estimate the eigenvectors of  $G$  and test their significance. Hine and Blows (2006) outline the rationale for this approach and Galletly *et al.* (2007) apply it in a similar way to that used here. Block and environment were included in the model as fixed variance effects, while genotype (block) was estimated as a random effect. Our estimates of genetic variances and covariances are estimates among clones, and thus contain all sources of genetic (co)variance, rather than simply the additive component.

To ascertain whether the genetic basis of growth is common within an environmental treatment across the two growth periods (exposure and recovery), we calculated genetic



correlations ( $r_g$ ) for each environment with the ANOVA method used by Astles *et al.* (2006). Variance components were extracted from two-way ANOVAs performed for each environment, with genotype and growth period as the two factors. To determine the 95% confidence limits of our estimated  $r_g$ , we resampled the data with replacement using PopTools 3.0 (Hood 2008), and used the distribution of pseudo- $r_g$  values as bootstrap confidence intervals. This method was first developed by Monro and Poore (2009), and we direct the reader to that paper for more information. Heritability estimates for each environment within each period are presented Table S1 and were calculated according to Becker (1984).

### Measurement efficacy

To determine that any effects found were not caused by measurement error, measurement efficacy was quantified by repeatedly measuring random fragments. This was done for 30 fragments, with three measurements taken per fragment, to compare variation within and between fragments using a one-way ANOVA. We checked data for normality and homogeneity of variances using frequency histograms, residual plots, and Levene's test. Data were found to have a bimodal frequency distribution and unevenly spread residuals, which were corrected by a natural log transformation, although variances were still found to be heterogeneous after transformation. Therefore, we analyzed the transformed data and accepted significant results when  $P < 0.01$ . Fragments were found to differ significantly in size, with minimal within fragment variance, and measurement repeatability was  $99.97 \pm 0.007\%$  indicating that measurement efficacy was high (Table S2). Initial size did not have a significant effect on growth ( $P = 0.158$ ) (Table S3).

### Results

All clone fragments survived the cloning process and exhibited signs of growth and repair during the period post fragmentation. There was no mortality during and after exposure to copper, with all clone fragments surviving even in the highest concentration environments. Most genotypes exhibited new zooid formation, with negative growth (i.e.,  $<1$ ) usually being attributed to death of older fully formed zooids that were behind the growing edge. When growth was impacted and zooid formation either reduced or halted, budding zooids began turning black, although there were no obvious deformities or mutations observed.

Growth during the exposure period varied between genotypes, with a significant  $G \times E$  interaction between genotype and copper environment (Table 1A). Growth was more than 1 for the control and  $25 \mu\text{g L}^{-1}$  Cu treatment, although there were two exceptions, with a genotype in each of these environments that had a growth rate of less than 1 (Fig. 2A). Multiple genotypes that maintained size or exhibited growth

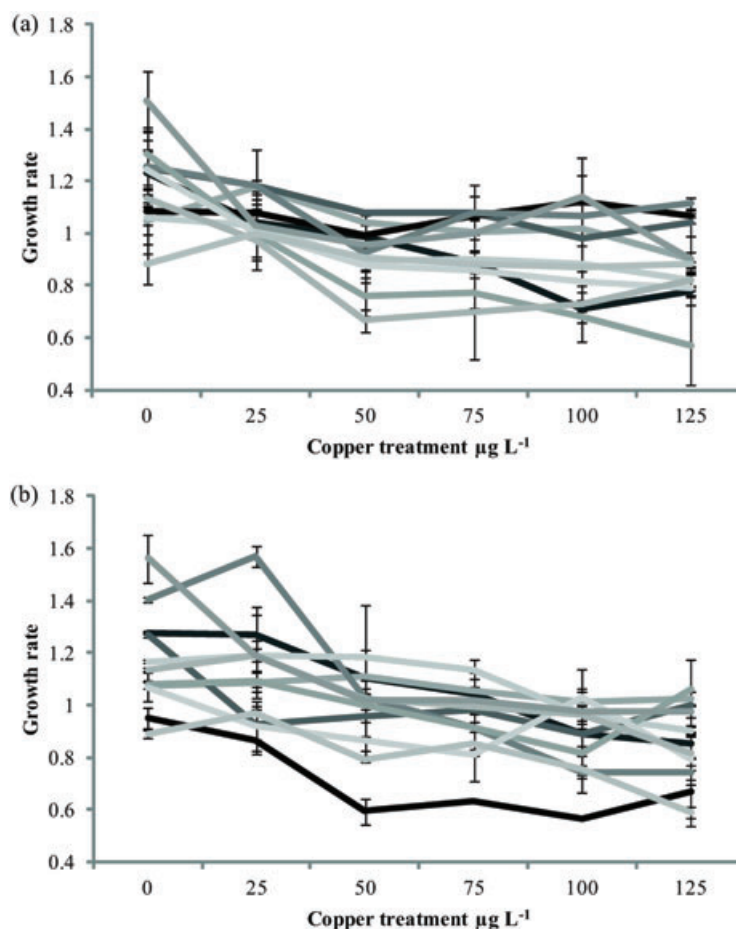
**Table 1.** ANOVA results for growth during (A) exposure and (B) recovery, showing the significant  $G \times E$  interaction.  $P$ -values in bold represent significant differences at  $\alpha = 0.05$ .  $P = 0.000$  denotes values  $<0.001$ .

Source	df	MS	F	P
(A) Growth during exposure				
Copper	5	0.31	6.28	<b>0.007</b>
Block	2	0.43	8.18	<b>0.011</b>
Genotype (block)	8	0.02	1.11	0.381
Block $\times$ Copper	10	0.05	2.42	<b>0.024</b>
Genotype (block) $\times$ Copper	40	0.02	1.71	<b>0.027</b>
Error	66	0.01		
(B) Growth during recovery				
Copper	5	0.34	8.21	<b>0.003</b>
Block	2	0.01	0.03	0.967
Genotype (block)	8	0.28	9.65	<b>0.000</b>
Block $\times$ Copper	10	0.04	1.44	0.200
Genotype (block) $\times$ Copper	40	0.03	2.93	<b>0.000</b>
Error	66	0.01		

in the copper concentrations above  $25 \mu\text{g L}^{-1}$  Cu generally did so in more than one of these environments. The  $G \times E$  interaction is apparent when graphing the reaction norms, or growth of each genotype in each environment (Fig. 2A), where some genotypes showed enhanced growth in lowest copper treatment of  $25 \mu\text{g L}^{-1}$  compared to in the seawater control. There was also a significant interaction between block and copper treatment (Table 1A).

A significant  $G \times E$  interaction between genotype and copper treatment was also found in growth during the recovery period (Table 1B; Fig. 2B), with growth in general still lower in the high-copper environments despite the exposure having ceased. There was no effect of block during the recovery period (Table 1B).

The genetic variance–covariance (G) matrix showed that during the exposure period growth in all environments with copper ( $\geq 25 \mu\text{g L}^{-1}$ ) negatively co-varied with growth in the control environment (Table 2B). Comparatively, these covariances were positive for growth during the recovery period (Table 2B). The diagonalization of the G matrix for growth during the exposure period showed one significant dimension of genetic variance, the eigenvector  $g_{\max}$  ( $\chi^2 = 16.7$ ,  $\text{df} = 6$ ,  $P = 0.011$ ), which explained 61.7% of the genetic variance (Table 3A). This first eigenvector showed a clear difference between the control environment, which had a negative loading, compared to the copper environments (Table 3A), consistent with the pattern of positive and negative covariances. For growth during the recovery period,  $g_{\max}$  was again the only significant dimension within the G matrix ( $\chi^2 = 77.9$ ,  $\text{df} = 6$ ,  $P < 0.001$ ), explaining 80.2% of the genetic variation (Table 3B). Compared to the previous G matrix, all environments were positively loaded on  $g_{\max}$ . The presence of only one significant dimension in each G matrix suggests



**Figure 2.** Reaction norms for each genotype across the six environments, (A) during the exposure period and (B) during the recovery period. Each line represents the mean response per genotype  $\pm$  SE. A growth rate of 1 denotes no change in area,  $>1$  is an increase, and  $<1$  a decrease. There were 11 genotypes used.

**Table 2.** Genetic variance covariance (G) matrix of growth (A) during exposure and (B) during recovery. Genetic variances are on the diagonal and in bold, while covariances are above the diagonal. Environments are in  $\mu\text{g L}^{-1}$  Cu.

	Control	25	50	75	100	125
(A) During exposure						
Control	<b>0.0169</b>	−0.0001	−0.0067	−0.0045	−0.0063	−0.0111
25		<b>0.0015</b>	0.0091	0.0033	−0.0004	0.0015
50			<b>0.0223</b>	0.0051	−0.0019	0.0038
75				<b>0.0001</b>	0.0039	0.0032
100					<b>0.0009</b>	0.0046
125						<b>0.0042</b>
(B) During recovery						
Control	<b>0.0311</b>	0.0281	0.0384	0.0231	0.0171	0.0151
25		<b>0.0471</b>	0.0513	0.0322	0.0197	0.0137
50			<b>0.0634</b>	0.0490	0.0410	0.0366
75				<b>0.0423</b>	0.0383	0.0317
100					<b>0.0364</b>	0.0298
125						<b>0.0305</b>

that 100% of the genetic variation that we detected is associated with each  $g_{\text{max}}$  and that there are fewer dimensions than originally measured traits. Instead, growth in environments with copper appears to trade-off with growth in non-copper environments (Figs. 2 and 3).

Growth rates were relatively consistent within genotypes in the Control treatments “during” and “after” exposure period (Fig. 3A), indicating that some genotypes grow consistently faster than others in the absence of copper (the genetic correlation between the two periods of growth was 1.08) (Table 4). Yet when exposed to copper, the fastest growing genotypes were not the genotypes that grew fastest after copper exposure ended (Fig. 3B–F). Genetic correlations between growth during and after exposure were also much lower  $r_g$  values (estimated  $r_g$  ranged from 0.25 to 0.25; Table 4), and were significantly different from 1 for at least two of the environments; 50 and 125  $\mu\text{g L}^{-1}$  Cu.

## Discussion

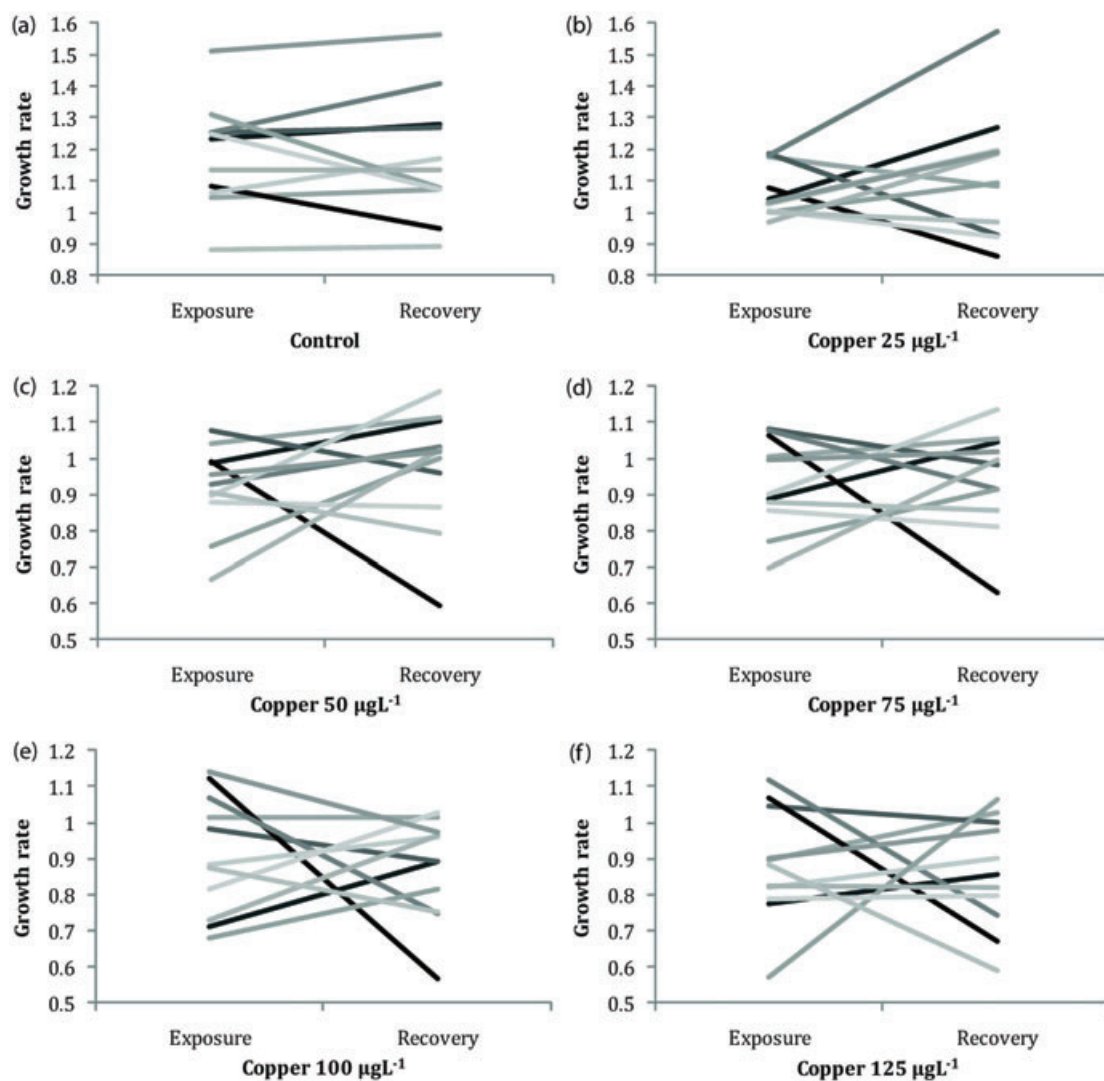
Tolerance to metal pollution may be an important contributor to invasion success for a number of marine species (Piola

**Table 3.** Diagonalization of the six environments (A) during exposure and (B) during recovery, showing the eigenvectors, loadings of each environment, and the percentage of total variance by each eigenvector. Eigenvectors in bold indicate a statistically significant dimension. Environments are in  $\mu\text{g L}^{-1}$  Cu.

Eigenvector	Eigenvalue	Percent of total variance	Control	25	50	75	100	125
(A) $g_{\max}$	0.0348	61.7	-0.5728	0.2177	0.6523	0.2400	0.1435	0.3476
(B) $g_{\max}$	0.2060	80.2	0.3091	0.3983	0.5641	0.4379	0.3682	0.3168

*et al.* 2009), yet little is known about how invaders have adapted to copper as a novel stressor (Galletly *et al.* 2007). This study aimed to investigate metal tolerance in a marine NIS, *W. subtorquata*, and whether there is genetic variation in

this trait. We found clonal variation in tolerance to a gradient of copper concentrations and the ability to recover from exposure to copper. Growth during and after exposure appears to not to be exactly the same trait, while a trade-off between



**Figure 3.** Reaction norms for each genotype across the exposure and recovery period, within the six environments, (A) control, (B)  $25 \mu\text{g L}^{-1}$  Cu, (C)  $50 \mu\text{g L}^{-1}$  Cu, (D)  $75 \mu\text{g L}^{-1}$  Cu, (E)  $100 \mu\text{g L}^{-1}$  Cu, and (F)  $125 \mu\text{g L}^{-1}$  Cu. Each line represents the mean response per genotype. A growth rate of 1 denotes no change in area,  $>1$  is an increase, and  $<1$  a decrease. There were 11 genotypes used. Please note different y-axes scales.

**Table 4.** Genetic correlations (after Astles et al. [2006]) between the two growth periods: during exposure and during recovery, within each environment. MS is the mean square for genotype (G), genotype  $\times$  environment (G  $\times$  E), and the error (Err), while V is variance. CI is the critical interval;  $\alpha = 0.05$ ,  $n = 2$ ,  $E = 2$ . Environments are in  $\mu\text{g L}^{-1}$  Cu.

	MS <sub>G</sub>	MS <sub>G<math>\times</math>E</sub>	MS <sub>Err</sub>	V <sub>G</sub>	V <sub>G<math>\times</math>E</sub>	$r_g$	Lower CI	Upper CI
Control	0.118	0.014	0.01	0.026	0.002	1.08	0.456	2.718
25	0.053	0.041	0.011	0.003	0.015	−0.25	−4.677	7.340
50	0.045	0.088	0.011	−0.011	0.039	0.22	−0.033	0.379
75	0.029	0.042	0.008	−0.003	0.017	0.16	−2.981	2.995
100	0.034	0.060	0.015	−0.007	0.023	0.22	−1.292	1.699
125	0.029	0.066	0.01	−0.009	0.028	0.25	−0.647	0.356

growth in control and copper environments during exposure suggests a cost to tolerance.

The differences among clones in their ability to grow when exposed to copper and when not exposed to copper suggests that different *W. subtorquata* genotypes are favored during copper pollution events than those favored when pollution is absent. Furthermore, the presence of genetic variation in tolerance to ecologically relevant copper concentrations parallels tolerance and mortality patterns in juvenile *W. subtorquata* (McKenzie 2010; McKenzie et al. 2011). Overall the genetic variation present within this species indicates that further evolutionary change is possible under strong directional selection.

Pollution events can vary dramatically in frequency, intensity, and duration, potentially creating fluctuating selection regimes (Beck 1996). Even within an impacted site disturbance events can cause further spikes in exposure (Knott et al. 2009), due to multiple sources of copper pollution (Weis et al. 1998; Srinivasan and Swain 2007). Hence, selection for copper tolerance will vary depending on the temporal and spatial exposure regimes. It is possible that fluctuating selection might contribute to maintaining the high levels of genetic variation in copper tolerance that we report in this study. Additionally, fluctuating conditions may also select for the ability to survive and recover from a pulse pollution event, compared to a constant exposure regime often experienced in chronically polluted environments (Klerks and Levinton 1989). Consequently a potential mechanism for withstanding pulse pollution events, as a form of adaptive plasticity (Ghalambor et al. 2007), could be to partition growth in response to the duration and intensity of the stress. This may be reflected in the lack of genetic correlation between each copper environment during exposure and recovery, which implies that growth during contamination and recovery afterwards are largely separate traits (Falconer and Mackay 1996). These traits may have then become optimized under different selection regimes, dependant on the duration and intensity of exposure events.

Larvae and juveniles can be more susceptible than adults to contaminants (Xie et al. 2005), and the reduced tolerance

and genetic variation in adult colonies to higher concentrations reflects early life-history mortality. All clones survived exposure to  $50 \mu\text{g L}^{-1}$ , a concentration that is ecologically relevant to early life-history stages, because it resembles the exposure an individual would receive when recruiting to a contaminated surface such as an antifoulant-coated boat hull (Valkirs et al. 2003). *W. subtorquata* larvae actively recruit to copper-polluted surfaces such as these, but then suffer high mortality and consequently experience strong selection (McKenzie 2010). Laboratory studies have also shown that tolerance to similar concentrations is a heritable trait in larval *W. subtorquata* (McKenzie et al. 2011) compared to higher concentrations where all genotypes are unlikely to survive beyond metamorphosis (Wisely 1958; Piola and Johnston 2006b; McKenzie et al. 2011).

The exact mechanisms used to tolerate copper are unknown in *W. subtorquata*, although the synthesis of metal-binding proteins (metallothioneins) has been suggested or identified for many invertebrate species (Amiard et al. 2006). Increased production of metallothioneins is likely to be metabolically costly (Roesijadi 1992). Associated costs can become more apparent when comparing between highly tolerant and intolerant populations, which have been chronically exposed for multiple generations (Shirley and Sibly 1999; Piola and Johnston 2006a). A population of *W. subtorquata* that has experienced strong selection due to copper pollution is likely to exhibit costs associated with tolerance, which may also manifest in traits other than growth. For example, field experiments manipulating copper exposure regimes found that colonies that recruited to, but were then removed from, a copper-polluted environment had lower growth and fecundity than individuals that remained exposed (McKenzie 2010). In this study, growth in copper-free and copper environments during exposure was negatively correlated, suggesting a trade-off between growth in the presence and absence of copper. Clearly, *W. subtorquata* has evolved to tolerate exposure to copper, but this trade-off implies that tolerance is a costly trait to maintain.

The copper concentrations tested in this study represent the range of concentrations that copper-based paints leach



over time (Valkirs *et al.* 2003). It is likely that tolerance to copper in *W. subtorquata* evolved through direct exposure to antifouling paints. Unfortunately, this is unlikely to be solitary incident of tolerance to copper evolving in a NIS; many marine NIS experience similar conditions during entrainment onto a vector, transportation, and introduction (Floerl and Inglis 2005; Clarke *et al.* 2011). Additionally, a number of marine NIS are increasingly becoming associated with metal-polluted environments (Piola and Johnston 2008; Dafforn *et al.* 2009). We suggest that the use of copper-based antifouling paints as a managerial control is not preventing the spread of marine NIS. Instead, we have shown that there is the genetic capacity for NIS to tolerate, and evolve further tolerance to, copper at multiple life-history stages (McKenzie 2010; McKenzie *et al.* 2011). Changing global managerial practices is a difficult task, but an emphasis needs to be placed on finding and implementing methods that do not use metal biocides, to minimize the spread of copper-tolerant NIS. Reducing metal contamination within the marine environment, particularly in ports and harbors that receive high volumes of shipping/boating traffic, could also reduce the retention and spread of copper-tolerant NIS. Unfortunately, these are challenging goals, but they should still be considered in an attempt to minimize the impact and spread of marine NIS.

The NIS *W. subtorquata* appears to have evolved multiple mechanisms to tolerate a range of pollution events. Alternative patterns of growth in response to copper exposure, where growth is partitioned either during exposure or recovery, indicate they have adapted to withstand pulse and press exposure events. This ability to tolerate periodic exposure to high concentrations of copper would explain its persistence in copper-polluted environments, such as harbors, ports, and marinas. Additionally, the variation in tolerance to copper indicates evolutionary potential within *W. subtorquata* to respond to further directional selection. Regardless of whether this species was preadapted to metal-polluted environments prior to being globally transported, it is apparent that tolerance to copper is an advantageous trait that is under selection in anthropogenically impacted habitats. With metal pollution an increasing disturbance on a global scale (Birch and Taylor 1999), we can expect to see a greater prevalence of copper-tolerant populations of invasive species.

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## Supporting Information

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**Table S1.** Heritability estimates ( $H^2$ ) for growth during (A) exposure and (B) recovery within each environment.

**Table S2.** Measurement efficacy.

**Table S3.** Effect of initial fragment size on growth during exposure to copper.

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