

Short larval exposure to low level of copper has long-lasting latent effects on juvenile performance in the sea urchin *Evechinus chloroticus*

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ABSTRACT: Exposure to environmental stressors such as pollutants early in life may have latent effects visible only in later life stages. Sea urchin *Evechinus chloroticus* larvae were exposed briefly (2 d) to 4 treatments of low levels of copper (2 to 10 $\mu\text{g l}^{-1}$). Juveniles were re-exposed to a further short pulse of copper (10 $\mu\text{g l}^{-1}$) to investigate potential resistance to pollution. No major direct effects of larval exposure to copper were observed on larval growth, survival or settlement success. However, complex latent effects started to appear from 8 d post-settlement onwards in individuals exposed as larvae to the highest concentration of copper. Juveniles had strongly impaired growth from 8 to 25 d post-settlement, with average radial length and spine to body ratio decreasing by 24 % for both variables. In addition, juveniles previously exposed to the highest tested concentration of copper as larvae were more vulnerable to a subsequent pulse, with up to 4 times higher mortality in groups previously exposed to copper during the larval stage. On the other hand, survivors had a 35 % higher growth rate than naive juveniles (i.e. no previous exposure). These results demonstrate that exposure to a concentration of copper similar to that found in many polluted bays and harbours (10 $\mu\text{g l}^{-1}$) for as little as 48 h in early life impacts juvenile performance and leaves them more vulnerable to a subsequent exposure. This highlights the importance of considering latent effects when evaluating the impacts of pollution.

KEY WORDS: Latent effects · Copper toxicity · Sea urchin · Early life stages · *Evechinus chloroticus*

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INTRODUCTION

In organisms with complex life cycles such as most marine invertebrates, early life stages play a major role in adult population dynamics where both larval quantity and quality drive adult abundance and performance (Gaines et al. 1985, Johnson 2008). In the last 20 yr it has become increasingly evident that larval quality can be highly variable due to environmental stresses encountered in early life (Pechenik et al. 1998, Shima & Swearer 2009). In addition to directly impacting larval mortality or growth, some effects may cross the metamorphic boundary and continue affecting performance in later life (i.e. carry-over effects), while other effects originating in

the larval stage may become visible only in later life stages (i.e. latent effects; Pechenik 2006).

Sub-lethal exposure of embryonic or larval stages to a variety of stressors has been found to cause substantial carry-over or latent effects on juvenile performance. These stressors include poor nutrition (Phillips 2002, 2004, Chiu et al. 2007), low salinity (Diederich et al. 2011, Montory et al. 2016), low oxygen (Segura et al. 2014) and ocean acidification (Dupont et al. 2013). It is clear that understanding the impact of environmental disturbances on early life stages and their cascading effects on later life stages is crucial for informing predictions of intensifying human impacts on marine systems. Yet despite this, very few studies to date have examined pollution as

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a potential driver for carry-over or latent effects in marine invertebrates (Pechenik 2006, Kimberly & Salice 2014), even though it is one of the most important anthropogenic disturbances in coastal ecosystems.

Among the myriad common pollutants entering coastal waters, copper is one of the most toxic to aquatic life (Bryan 1971, Flemming & Trevors 1989, US EPA 2007, Watson et al. 2008). While extensive ecotoxicity studies using embryos and larvae have been carried out on copper and other metals (e.g. Fernández & Beiras 2001, Doyle et al. 2003, Bielmyer et al. 2005, Rouchon & Phillips 2016), the effects of metal pollution in the context of ecologically relevant exposure regimes are still poorly understood (Mayer-Pinto et al. 2010). Indeed, most toxicity studies are very short term (days) and use unrealistic concentrations and/or prolonged exposure. In the field, marine larvae are exposed to relatively low levels of copper in most countries (see Table S1.1 in Supplement 1 at www.int-res.com/articles/suppl/m579p067_supp.pdf) and are unlikely to be chronically exposed to toxicants, except perhaps in enclosed bays or harbours with low water renewal. Thus, one of the most common sources of pollution affecting planktonic larvae is river plumes and other storm or wastewater outputs that channel terrestrial run-off to the sea during rain events. Barring extreme episodes, typical exposure to pollutants from run-off is probably on the order of a few days at the most (Johnston & Keough 2002, Phillips & Shima 2006, Rouchon 2015), and there is evidence from a variety of taxa that even short exposure of early life stages to a stressor can have strong effects on later life stages (Pechenik et al. 2001, Ng & Keough 2003, Diederich et al. 2011, Montory et al. 2016).

One of the specific ways in which early sub-lethal exposure to a pollutant such as copper may have a latent effect is how it mediates responses to a subsequent exposure to that same contaminant later in life. On one hand, survivors may be physiologically damaged and thus more vulnerable to further stress, resulting in poor growth, survival or reproductive success (Ng & Keough 2003, Kimberly & Salice 2014, Fischer & Phillips 2014). On the other hand, early exposure may result in acclimation via physiological change that leads to higher tolerance to later re-exposure (Klerks & Weis 1987). Although there is evidence that parental exposure to stressors can cause transgenerational resistance in offspring (Marshall et al. 2008, Kimberly & Salice 2015, Ross et al. 2016), the degree to which acquired resistance occurs across the metamorphic boundary is not clear for marine invertebrates. In fish, acclimation to pollutants generally involves physiological processes that are quickly

lost after exposure to the contaminant has stopped (Dixon & Sprague 1981, Brinkman & Woodling 2014).

Sea urchins (Phylum Echinodermata) are common and ecologically important members of subtidal reef communities. They are also highly sensitive to pollutants, such that they are often used in ecotoxicity studies, and as indicator species for marine systems around the world (US EPA 2002, ASTM 2012). Many urchin species have planktonic larvae for up to several weeks, and during this relatively long phase larvae may be impacted by coastal runoff when dispersing through river plumes or polluted harbors (Phillips & Shima 2006). In southern California, anthropogenic contaminants in run-off have had major effects on population connectivity in another echinoderm, the bat star *Patiria miniata*, via toxic effects on larvae that cause mortality high enough to act as a dispersal barrier (Puritz & Toonen 2011). In echinoids, most studies on pollutants have focused on early pluteus formation, occurring between fertilisation and the 2-armed pluteus stage, which takes one to few days post-fertilisation depending on the species (King & Riddle 2001, Fernández & Beiras 2001, Doyle et al. 2003, Bielmyer et al. 2005, Kobayashi & Okamura 2005, Ghorani et al. 2013) and little is known about the potential for carry-over effects of larval exposure to pollution on post-metamorphic life stages.

The aim of this study was to test for carry-over and latent effects of ecologically relevant pulses of copper pollution (i.e. short-term, low concentrations such as in run-off after a rain event) using early life stages of the sea urchin *Evechinus chloroticus* as the focal species. *E. chloroticus* is a common, endemic species of sea urchin in New Zealand, found primarily on hard substrates in shallow subtidal habitats, distributed around the New Zealand mainland and Snares and Chatham Islands (Dix 1969, Shears & Babcock 2007). Similar to urchins in other systems, *E. chloroticus* is a dominant grazer that can shape community structure of subtidal rocky reefs by mediating the abundance of macroalgae (Andrew 1988, Schiel 1990, Villouta et al. 2001, Shears & Babcock 2003).

By global standards (e.g. Table S1.1 in Supplement 1), New Zealand waters are not highly contaminated, and few studies have evaluated the impacts of water-borne contaminants on New Zealand species. Nevertheless, pollution has still been identified as a major threat to coastal invertebrates such as *E. chloroticus* (Morrison et al. 2009, Freeman et al. 2010). Rouchon & Phillips (2016), in one of the few studies examining effects of contaminants on New Zealand marine species, found that *E. chloroticus* larvae are more vulnerable to metals, especially copper,

compared to other urchin species. Here, we used short pulses of copper concentrations derived from field measures in low- to moderately polluted areas of New Zealand and elsewhere in the world to examine potential carry-over and latent effects of larval exposure on surviving juveniles for up to 40 d post-settlement. We then tested whether juveniles that had been exposed to copper as larvae developed resistance to it by re-exposing them at 25 d post-settlement to another short pulse of copper, and comparing the responses with those of naive juveniles that had never been exposed to copper.

MATERIALS AND METHODS

Metal concentrations

Larvae were exposed to 1 of 4 concentrations of copper ('ANZECC', 'field', 'field × 2' and 'high'), either early or late in their development, plus a control treatment of no exposure (Table S1.2 in Supplement 1). Copper exposure lasted for 48 h in all treatments. The 'early' group was exposed to elevated copper at 4 d post-fertilisation and the 'late' group at 11 d post-fertilisation. There were 3 replicate jars for each combination of copper concentration and timing of exposure, as well as the control.

The ANZECC concentration was based on the current recommended water quality value for 95 % species protection in New Zealand (ANZECC 2000). The 'field' concentration was based on the maximum dissolved copper concentration measured from 29 seawater samples collected following major rain events across 4 sites in the Wellington region between September 2011 and January 2012 (Rouchon 2015). The 'high' concentration was chosen as a realistic level occurring in moderately polluted water such as near large cities (Table S1.1 in Supplement 1). Metal stock solutions were made from reagent grade copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and aliquots were added to the jars in amounts that would result in the desired final concentration for each treatment. Actual dissolved copper concentrations for each treatment were determined by recreating experimental conditions without urchin larvae in January 2013 using the same protocol, culture medium, glassware and stock solution as during the actual experiment. Filtered seawater (FSW) was analysed for background copper 4 times between 2012 and 2014. Water samples were analysed by Eurofins Environmental Laboratory Services, Lower Hutt, New Zealand. Dissolved copper data are given in Table S1.2 in Supplement 1.

Larval assays

Broodstock *E. chloroticus* (8 to 20 cm test diameter) were collected from an unpolluted site: Kau Bay, Wellington (41.2882° S, 174.8326° E) in November 2012. We reared larvae at Victoria University Coastal Ecology Laboratory (VUCCEL) for 28 d in a system of continuously stirred glass jars (described in Strathmann 1987). Each jar contained 2 l FSW that was pre-filtered (15 μm) and then filtered through a 0.2 μm paper cartridge. Initial densities of embryos were relatively low (1 embryo ml^{-1}). Jars were placed in a flowing seawater bath to maintain a near constant temperature of $16 \pm 1^\circ\text{C}$; pH ranged between 7.97 and 8.02 throughout the experiment in all jars. Dissolved oxygen in this setup was typically above 90 % with a minimum of 85 % as measured in pilot studies. Larvae were the product of sperm and egg stocks combined from several individuals (5 males and 2 females). The spawning procedure followed ASTM (2012) and is described in Rouchon & Phillips (2016). FSW in jars was renewed 3 times wk^{-1} by inverse siphoning. Copper was added to the relevant treatments by adding a small volume of a stock solution following a water change. After 48 h exposure, larvae in all treatments, including control, were gently sieved and transferred to a fresh jar to remove any traces of copper. From 4 d post-fertilisation, following each water change, larvae were fed with *Dunaliella tertiolecta* at a concentration of 8000 cells ml^{-1} of culture. *Dunaliella* stocks were rinsed thoroughly by 3 successive centrifugations ($1201 \times g$ for 5 min) and resuspensions in clean FSW before being fed to the larvae, to remove traces of algal culture medium that was rich in essential metals. All jars were sampled at 4 and 11 d post-fertilisation and again near the end of the larval stage at 25 d (early timing group) or 27 d (late timing group) post-fertilisation. Jars were randomly numbered and all measurements were done blind with regard to the treatment classification.

Settlement

Settlement containers consisted of 2 l plastic containers with mesh sides (80 μm) to allow for water circulation. Larvae in replicate jars were pooled to remove variation among jars and then divided into 3 replicate containers for each copper level and timing of exposure, and 12 control replicates. Containers were placed into large plastic trays (10 randomly assigned containers per tray) filled with FSW and oxygenated with 2 airlines tray $^{-1}$. Trays were placed in a

flowing seawater bath to maintain a near constant temperature of $16 \pm 1^\circ\text{C}$. Each settlement container was inoculated with benthic diatoms (mixed species) 8 d prior to settlement to provide a settlement cue and food for young juveniles (Xing et al. 2007). Benthic diatom cover remained abundant throughout the experiment with visible but scarce grazing tracks. Complete water renewals were done 3 times wk^{-1} . Temperature was measured daily while pH and dissolved oxygen were measured weekly in trays. All parameters were constant across trays. All treatments except those from the high copper level were placed in settlement containers after 28 d of larval development. Larvae in high copper treatments, both early and late exposure groups, appeared to be delayed (see Fig. S2.1 in Supplement 2 at www.int-res.com/articles/suppl/m579p067_supp.pdf) and were therefore given 5 additional days (33 d post-fertilisation) to achieve similar development as the other treatments (i.e. >75% of normal larvae being at the 8-armed stage) before being transferred to the settlement containers. Time post-settlement was counted from the first day that larvae were placed in settlement containers. Larvae were given 8 d undisturbed to settle after which any remaining larvae were removed from the containers.

Juvenile re-exposure

Half of the control replicates (6) were exposed to copper for the first time at the juvenile stage (i.e. naive group), and the remaining 6 were kept in clean FSW (i.e. control group). At 25 d post-settlement, juveniles from all larval treatments except for controls were exposed to a high level ($10 \mu\text{g l}^{-1}$) of copper for 2 d. Juveniles were then placed into clean FSW until 40 d post-settlement when mortality, growth and normal development were assessed. Experimental setup and maintenance routine were the same as described for juveniles.

Response variables and data analyses

Larval performance

Direct effects of copper treatments on larvae were evaluated by (1) larval survival, (2) normal development, (3) delayed development, (4) larval growth and (5) larval morphometrics. More details concerning data analysis of all larval variables are given in Supplement 2 because they were not the main focus of this study.

Juvenile performance after larval exposure

We observed juveniles under a dissecting microscope ($40\times$ magnification) to determine (1) settlement success, (2) mortality at 25 d post-settlement and (3) growth from 8 to 25 d post-settlement. Juvenile growth was measured by 2 variables: body growth (radial growth) and spine growth. Juvenile morphometrics were measured from photographs (20 juveniles container^{-1}) at $25\times$ magnification using the software ImageJ.

The effect of larval experiences on juvenile performance was evaluated by fitting an initial model including all of the following factors and covariates: copper concentration and timing of copper exposure (main factors), larval survival, proportion of normal larvae, larval size, proportion of larvae with a rudiment and rudiment size (covariates). All larval traits were measured at the end of the larval stage and averaged per treatment, as larvae from replicate jars were pooled before settlement. In addition, 2 other covariates were used: initial larval density in settlement containers (initial density) for settlement success and size at 8 d post-settlement analyses; and juvenile density at 8 d post-settlement (juvenile density) for juvenile survival and growth analyses.

The best model was selected from the initial model (full model) described above, using a stepwise regression based on Akaike's information criterion (AIC) for each juvenile performance variable. Best models were then analysed using ANCOVAs with the selected factors and covariates, which are presented in the 'Results' section. Interactions were included only when selected in the best model. Settlement success and juvenile survival were arcsine square-root transformed to meet model assumptions. Partial R^2 values for each component of the model were estimated by hierarchical partitioning (Chevan & Sutherland 1991, Mac Nally 1996) using the 'hier.part' package in R (Walsh & Mac Nally 2013). Containers 10 and 13 (both treatment controls) were not measured at 8 d post-settlement and were removed from the analysis.

Juvenile performance after re-exposure

Juvenile performance was evaluated 2 wk after re-exposure to copper (40 d post-settlement) by (1) mortality, (2) growth (both radial and spine) from 25 to 40 d post-settlement and (3) normal development at 40 d post-settlement. All juveniles in each con-

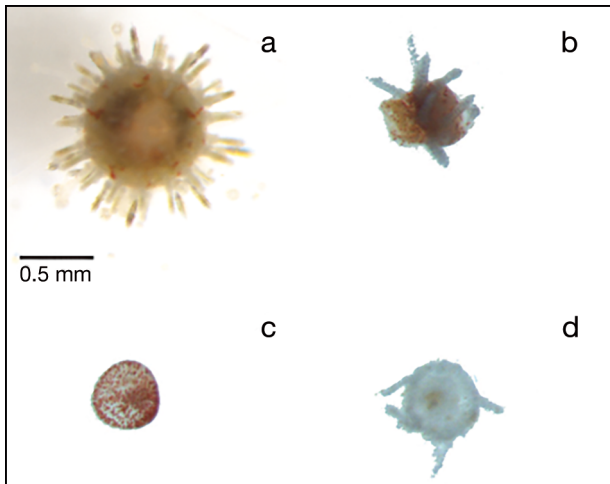


Fig. 1. Developmental categories of *Euechinus chloroticus* juveniles. All juveniles pictured are 25 d post-settlement. (a) Normal: spherical body with pigmentation and well developed spines and tubefeet; (b) irregular: strongly irregular body, typically with a large protrusion on top of the test (pictured); (c) spineless: juvenile completely lacking spines; (d) ghost: juveniles lacking pigmentation in the body but with moving tubular feet or spines

tainer were rated for normal development and were deemed as 'abnormal' when body shape was strongly irregular, or lacked spines or pigmentation (Fig. 1) when observed under a dissecting microscope (40 \times magnification).

For all variables, 2 independent hypotheses were tested separately: comparison between naive juveniles (i.e. juveniles exposed for the first time at 25 d post-settlement) and controls (i.e. juveniles never exposed to copper); and the effect of larval experience on juveniles exposed to copper at 25 d post-settlement for the second time (ANZECC, field, field \times 2 and high groups) compared with controls. Best models for the later analyses were selected as described for juvenile performance and then analysed using ANCOVAs, with copper level and timing of exposure as the main factors and larval traits as the covariates. Mortality was arcsine square-root transformed to meet ANCOVA assumptions. In addition, final radial length (at 40 d post-settlement) was analysed using a mixed effect linear model, with container as the random effect and individual juvenile as the residual. Model fit, partitioning of variance and contrasts were calculated as described for juvenile performance. All statistical analyses were performed using the software R v.3.1 with package 'multcomp' (Hothorn et al. 2008) for pairwise comparisons and package 'nlme' (Pinheiro et al. 2015) for mixed effects models.

RESULTS

Larval survival, growth and development

Overall, there were no major effects of copper exposure during larval development on any of the variables measured before metamorphosis (i.e. larval survival, normal development, delayed development, growth and morphometrics). The only exception was a minor delay in development of larvae in the high copper concentration. Larvae in this treatment recovered to values similar to controls within 3 d. See details in Supplement 2.

Juvenile performance after larval exposure

Settlement success

Settlement ranged from 3.5 to 68%. It was significantly driven by the timing of copper exposure and rudiment size at the end of the larval stage (timing: $F_{1,31} = 47.60$, $p < 0.001$; rudiment size: $F_{1,31} = 39.79$, $p < 0.001$). Overall, the model explained 61% of the observed variation (multiple R^2), of which 58% was explained by timing of exposure and 42% by rudiment size. Juveniles exposed as young larvae had higher settlement success than juveniles exposed later during the larval stage (early exposure: $28 \pm 15\%$; late exposure: $18 \pm 15\%$). In both timings of exposure groups, settlement success increased with rudiment size at the end of the larval stage (Fig. 2).

Juvenile survival

Survival after 25 d post-settlement was high, ranging from 78 to 100%. The best model for survival explained 43% of the variation and included timing of copper exposure (but not level of copper), juvenile density and rudiment size (Table 1a). Survival was slightly higher in juveniles exposed to copper early during the larval stage (early group: $96 \pm 6\%$; late group: $92 \pm 8\%$). Timing of exposure contributed to 43% of the multiple R^2 . Juvenile survival declined with density and increased with rudiment size. Both factors accounted for 22 and 23% of the variation, respectively.

Juvenile growth

At 8 d post-settlement, juveniles from the high treatment (late group only) were significantly larger

than those in the control (812 ± 103 vs. 714 ± 70 μm in late control; Dunnett's contrasts: $z = 5.92$, $p < 0.001$). However, from 8 to 25 d post-settlement, juveniles exposed to the high copper level during larval development (both early and late groups) suffered from a negative growth rate ($-24 \pm 3\%$) compared with a

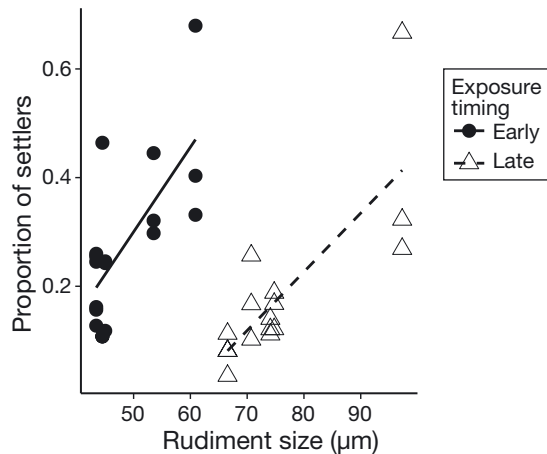


Fig. 2. Effect of timing of copper exposure during larval stage and rudiment size before settlement on *Evecchinus chloroticus* settlement success (i.e. proportion of juveniles observed at 8 d post-settlement). Juveniles in the early treatment group were exposed to various levels of copper as larvae at 4 d post-fertilisation. Juveniles in the late treatment group were exposed later during larval development (11 d post-fertilisation)

Table 1. Effect of larval experience on *Evecchinus chloroticus* juvenile (a) survival and (b,c) growth at 25 d post-settlement. All variables were analysed using ANCOVAs. Juvenile survival was arcsine square-root transformed to meet ANCOVA assumptions. Significant effects ($p < 0.05$) are highlighted in **bold**

Source	df	SS	F	p
(a) Juvenile survival				
Timing	1	0.33	14.07	0.001
Juvenile density	1	0.13	5.46	0.03
Rudiment size	1	0.25	10.82	0.003
Larval size	1	0.04	1.78	0.19
Larval development	1	0.04	1.77	0.19
Residuals	28	0.66		
(b) Radial growth				
Timing	1	0.01	3.71	0.07
Copper level	4	0.28	44.25	<0.001
Rudiment size	1	0.01	5.16	0.03
Larval size	1	0.01	4.71	0.04
Residuals	26	0.04		
(c) Spine growth				
Copper level	4	1.39	7.47	<0.001
Proportion of rudiment	1	0.27	6.00	0.02
Larval development	1	0.15	3.27	0.08
Residuals	27	1.26		

positive growth rate of $+6 \pm 5\%$ in controls (coefficients contrasts: $t = -9.82$, $p < 0.001$). Thus, the average juvenile size per container from the high copper treatment were 24% smaller at 25 d than at 8 d post-settlement (778 ± 95 μm at 8 d vs. 594 ± 93 μm at 25 d; Fig. 3a). In contrast, in control groups the average juvenile size was 6% larger at 25 d than it was at 8 d post-settlement. Consequently, by 25 d post-settlement, juveniles from the high copper treatment (both early and late groups) had become significantly smaller than controls (high level: 594 ± 93 μm , controls: 768 ± 95 μm ; coefficient contrasts: $t = -9.88$, $df = 30$, $p < 0.001$; Fig. 4a). The best model showed a significant effect of level of copper exposure, rudiment size and larval size (Table 1b) that explained

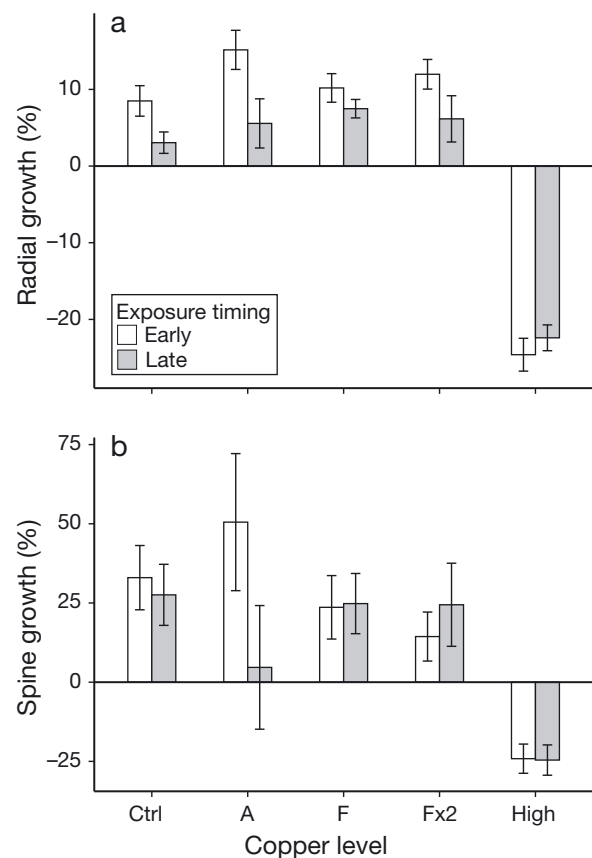


Fig. 3. Effect of level and timing of copper exposure during larval development on *Evecchinus chloroticus* (a) radial growth and (b) spine growth at 25 d post-settlement. Growth is relative to juveniles size at 8 d post-settlement. Copper levels during larval stage: control (Ctrl): no added copper; ANZECC (A): $2 \mu\text{g l}^{-1}$; field (F): $3 \mu\text{g l}^{-1}$; field $\times 2$ (Fx2): $6 \mu\text{g l}^{-1}$; and high: $10 \mu\text{g l}^{-1}$. Juveniles in the early treatment group were exposed to copper as larvae at 4 d post-fertilisation. Juveniles in the late treatment group were exposed later during larval development (11 d post-fertilisation). Error bars: SE of the mean ($n = 3$). Note the difference in scale on the y-axes; na: not applicable

93% of the variation in growth, with copper level accounting for most of the explained variation (71%). Juvenile radial growth was positively related to larval size and rudiment size, with both factors accounting for 11 and 9% of the explained variance, respectively.

Juvenile spine growth followed a similar pattern to that of radial growth, with a multiple R^2 of 56%. Level of copper exposure was the most important factor, accounting for 83% of the explained variance (Table 1c). Juveniles exposed to high level of copper during the larval stage had a negative spine growth, with the average spine length being $24 \pm 7\%$ shorter at 25 d than it was at 8 d post-settlement (8 d: $380 \pm 94 \mu\text{m}$, 25 d: $285 \pm 52 \mu\text{m}$; coefficients contrasts: $t = -4.92$, $p < 0.001$). By contrast, controls had positive

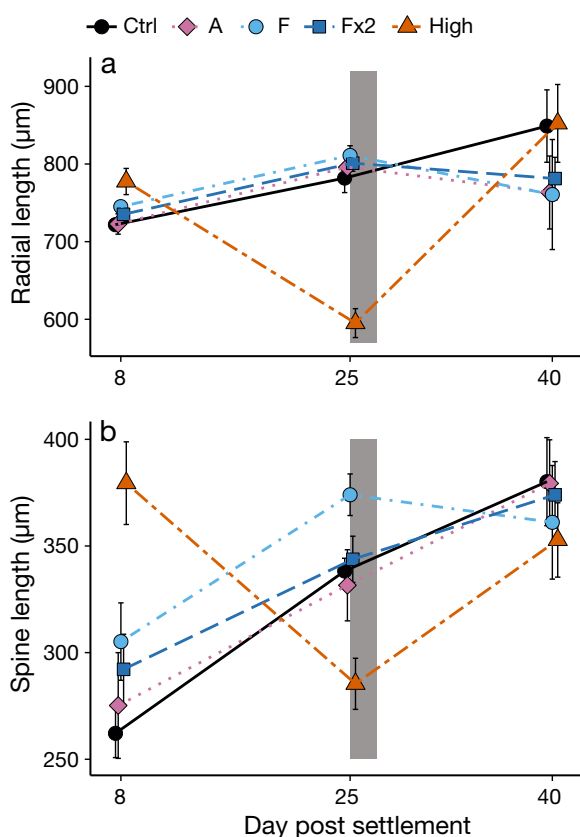


Fig. 4. *Evechinus chloroticus* (a) radial length and (b) spine length from 8 to 40 d post-settlement, averaged by containers, showing the latent effects of copper exposure during the larval stage on juvenile size. Copper levels during larval stage: control (Ctrl): no added copper; ANZECC (A): $2 \mu\text{g l}^{-1}$; field (F): $3 \mu\text{g l}^{-1}$; field $\times 2$ (Fx2): $6 \mu\text{g l}^{-1}$; and high: $10 \mu\text{g l}^{-1}$. Shaded area: exposure to a 2 d pulse of $10 \mu\text{g l}^{-1}$ copper at 25 d post-settlement for all juveniles (except controls). Juveniles in the early and late treatment groups (i.e. exposed as larvae at 4 [early] and 11 [late] d post-fertilisation) were pooled because timing of exposure was not significant (Tables 1b,c & 2b,c). Error bars: SE of the mean (n = 3)

spine growth with an average spine length $31 \pm 22\%$ longer at 25 d than it was at 8 d post-settlement (Fig. 3b). At 25 d, juveniles from high level also had shorter spines than controls (high level: $285 \pm 52 \mu\text{m}$, controls: $334 \pm 66 \mu\text{m}$; coefficient contrast: $t = -2.85$, $df = 30$, $p = 0.01$; Fig. 4b). The proportion of larvae with a rudiment and normal larval development at the end of larval development were included as covariates and accounted for less than 9% of the total R^2 .

Juvenile performance after re-exposure

Naive juveniles

Juvenile mortality 2 wk after re-exposure to copper was less than 8% in controls and did not differ between juveniles exposed for the first time to copper (naive group) and juveniles never exposed to copper (controls) (ANOVA: $F_{1,7} < 0.001$, $p = 0.99$; Fig. 5). Radial growth was lower in naive juveniles than controls (ANOVA: $F_{1,7} = 9.26$, $p = 0.02$). Naive juveniles had a near null growth of $-8 \pm 14\%$ compared with a positive growth of $15 \pm 7\%$ in controls (Fig. 6a). Spine growth in control and naive groups was not significantly different (ANOVA: $F_{1,7} = 1.08$, $p = 0.33$; Fig. 6b). Similarly, there was no significant difference in normal development between naive and control groups (Wilcoxon test: $W = 20$, $p = 0.22$).

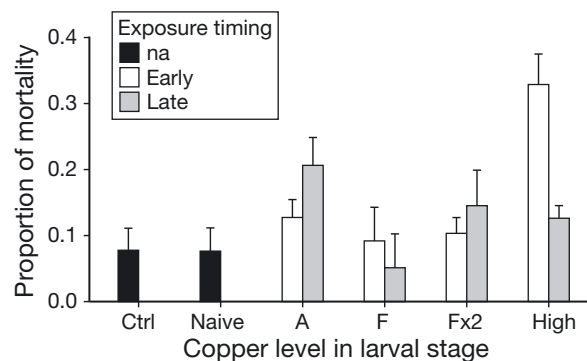


Fig. 5. Effect of copper exposure during larval development on juvenile *Evechinus chloroticus* mortality after subsequent exposure to $10 \mu\text{g l}^{-1}$ Cu at 25 d post-settlement. Copper levels during larval stage: control (Ctrl): no added copper; naive: no added copper; ANZECC (A): $2 \mu\text{g l}^{-1}$; field (F): $3 \mu\text{g l}^{-1}$; field $\times 2$ (Fx2): $6 \mu\text{g l}^{-1}$; and high: $10 \mu\text{g l}^{-1}$. Juveniles in the control group were never exposed to copper. Juveniles in the naive group were exposed to copper for the first time as juveniles. Juveniles in the early treatment group were exposed to copper as larvae at 4 d post-fertilisation. Juveniles in the late treatment group were exposed later during larval development (11 d post-fertilisation). Error bars: SD (n = 3); na: not applicable

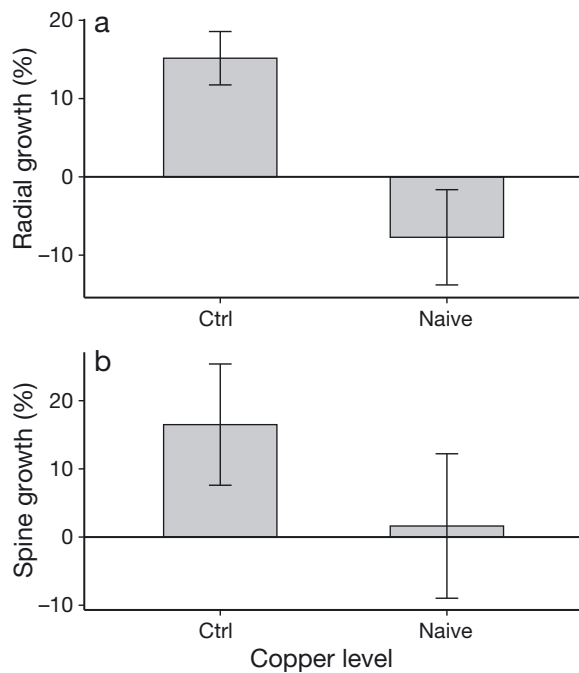


Fig. 6. *Evechinus chloroticus* (a) radial growth and (b) spine growth from 25 to 40 d post-settlement, averaged by containers, showing the direct effects of copper exposure on juveniles. Naive juveniles were raised in control conditions as larvae but exposed to a 2 d pulse of $10 \mu\text{g l}^{-1}$ copper at 25 d post-settlement. Error bars: SE of the mean ($n = 3$)

Juveniles previously exposed to copper as larvae

For juveniles that had been previously exposed to copper during the larval stage, mortality was influenced mainly by level of copper exposure (76% of the explained variance, multiple $R^2 = 45\%$; Table 2a). Mortality was significantly higher in juveniles that had been exposed to ANZECC and high levels of copper, but not for those exposed to field and field \times 2 levels (ANZECC level: $t = 2.16$, $p = 0.04$, high level: $t = 2.18$, $p = 0.04$; Fig. 5). Mortality was more than 4 times higher for juveniles that had been exposed to the high level of copper early during larval development ($33 \pm 8\%$ in early high vs. $8 \pm 9\%$ in naive). Mortality decreased with rudiment and larval size, accounting for 10 and 14% of the explained variability, respectively.

Radial growth of juveniles was significantly affected by the level of copper exposure during larval development, with a model fit of 74% (Table 2b). Juveniles exposed to high levels of copper during the larval stage had a 4-fold greater growth rate 2 wk after subsequent copper exposure ($43 \pm 16\%$ growth in high level vs. $9 \pm 13\%$ in naive juveniles; contrasts:

Table 2. Effect of larval experience on *Evechinus chloroticus* juvenile (a) mortality and (b,c) growth 2 wk after re-exposure to copper. All variables were analysed using ANCOVAs. Juvenile mortality was arcsine square-root transformed to meet ANCOVA assumptions. Significant effects ($p < 0.05$) are highlighted in **bold**

Source	df	SS	F	p
(a) Juvenile mortality				
Copper level	4	0.39	4.01	0.006
Rudiment size	1	0.12	5.83	0.02
Larval size	1	0.10	4.87	0.04
Residuals	22	0.45		
(b) Radial growth				
Copper level	4	1.12	14.7	<0.001
Juvenile density	1	0.05	2.41	0.14
Larval size	1	0.05	2.48	0.13
Residuals	22	0.42		
(c) Spine growth				
Copper level	4	0.30	2.12	0.11
Residuals	24	0.84		

$t = 6.24$, $p < 0.001$; Fig. 4a). Radial length at 40 d post-settlement was not significantly different across copper concentrations (2-way ANOVA on mixed effect model; $\chi^2 = 9.53$, $df = 5$, $p = 0.09$), timing of exposure ($\chi^2 = 0.32$, $df = 1$, $p = 0.57$), or their interaction ($\chi^2 = 10.64$, $df = 5$, $p = 0.06$; Fig. 4a). Spine growth, on the other hand, was unaffected by copper exposure or larval history (Fig. 4b, Table 2c).

Normal juvenile development was high in all containers, with most juveniles having a regular body shape (91 to 100%), and was unaffected either by juvenile re-exposure to copper or by larval traits and life history (best model was the null model, Kruskal-Wallis test on copper level: $\chi^2 = 0.37$, $df = 4$, $p = 0.98$).

DISCUSSION

This study showed that short exposure to $10 \mu\text{g l}^{-1}$ of copper during larval development has complex latent impacts on *Evechinus chloroticus* juvenile performance, visible only from 8 d post-settlement. Perhaps even more importantly, surviving juveniles were more vulnerable to a subsequent exposure to the same stressor. Unexpectedly, in the absence of further exposure, larvae exposed later, and juveniles, were more affected than larvae exposed early during larval development. It should be noted that only the highest tested copper concentration produced responses but doses were still relatively low and brief, consistent with levels commonly found in the field.

Latent effects of larval exposure to copper on juveniles

At 8 d post-settlement, juveniles were larger and had longer spines relative to body size when they had been exposed to the highest concentration of copper in late larval development. However, this initial positive effect soon after settlement was cancelled by a dramatic decrease in both body size and spine length between 8 and 25 d post-settlement in both groups exposed to copper early and late during larval development. As a result, by 25 d post-settlement, juveniles that had been in the high copper level groups as larvae were substantially smaller than controls.

Juveniles were not individually followed during this experiment and thus a decrease in average size may be due to selective mortality of larger individuals. However, the reduction in average size was large (24%), with very little mortality (<5%, corresponding to an average of 3 ind. container⁻¹) suggesting that it is more likely that juveniles had actually shrunk. Shrinkage in test diameter of adult sea urchins as a result of food limitation has been reported for some species, including *E. chloroticus*, although not for young juveniles (Dix 1972, Levitan 1988, Constable 1993). In the current study, food abundance in settlement containers was not directly measured; however, a broad estimation of benthic diatom cover was recorded and no difference was noted among treatments. In addition, benthic coverage appeared more than sufficient for grazing pressure (i.e. well-developed brown film with sparse grazing tracks) and almost all juveniles had visibly full guts at 25 d post-settlement. Furthermore, growth was not related to juvenile density, as would be expected if food limitation was a factor. This study provides the first evidence that test shrinkage in urchins might occur as a result of environmental stressors other than food limitation. Furthermore, for these very young juveniles it is a latent effect of larval experience, not their current experience as is the case for shrinkage due to food limitation.

While there was no difference in juvenile survival for those from different larval copper concentrations in our experimental setting, growth impairment in the field is likely to result in higher subsequent mortality due to predation. Indeed, young juveniles of many benthic species are thought to be the most at risk of predation (Hunt & Scheibling 1997). Predators of juvenile urchins typically include crabs, demersal fish and sea stars (Hunt & Scheibling 1997, Clemente et al. 2013). Although little is known about predation

patterns on small juveniles due to inherent difficulties in studying highly cryptic early life stages, Clemente et al. (2013) showed that the smallest *Strongylocentrotus purpuratus* juveniles tested (5 to 14 mm) were more than 3 times more likely to be eaten by crabs than were larger juveniles, regardless of predator size. It has also been suggested that juvenile urchins may reach a threshold size at which the predation rate dramatically decreases (Menge & Sutherland 1976) or at which they may start feeding on macroalgae, thus substantially increasing their growth rate (Rowley 1990). Any delay in reaching this 'escape size' would therefore have a strong impact on a juvenile's chance of survival or growth, thus affecting population dynamics and adult abundance (Ebert 1983, Underwood & Fairweather 1989). Furthermore, if the observed negative growth was due to shrinkage, then juveniles may also be in poorer body condition, as was observed for shrinking *Helicidaris erythrogramma* (Constable 1993), and thus be less likely to survive in the field.

Very few studies have followed the impact of copper on long-lived benthic invertebrates for as long after settlement as in this study (6 wk post-settlement). Indeed, most studies on pollutants are typically either short-term assays (days) or end shortly after settlement. However, Ng & Keough (2003) reported a dramatic decrease in survival and growth following larval copper exposure in bryozoans that was visible only weeks to months post-settlement. In contrast, no carry-over effects were observed in growth or survival of juvenile sponges up to 6 mo after larval copper exposure (Cebrian & Uriz 2007).

In sea urchins, the early days post-settlement are arguably the lesser known, due to difficulties in finding highly cryptic, small individuals in the field and the time necessary to raise them from larvae in the laboratory. However, latent growth impairment following exposure to stressors during embryonic or larval development has been documented in various invertebrate species for low food availability (Miller & Emler 1999, Phillips 2002, 2004, Pechenik 2006), ocean acidification (Gobler & Talmage 2013, Lane et al. 2013) and other environmental stressors (Pechenik et al. 2001, Fischer & Phillips 2014). The mechanisms underlying latent and carry-over effects in marine invertebrates are not well understood, but Pechenik et al. (1998) suggested an impairment in gene transcription while Hoare et al. (1995) suggested that copper exposure could reduce larval lipid reserves, thus impairing growth post-settlement. In our study, the duration of exposure was only 2 d and it is unclear whether the above mechanisms could

have been triggered by such a short exposure. All latent effects reported occurred only at the highest copper concentration. However, this concentration is environmentally realistic and found in moderately polluted areas such as marinas or waters receiving urban runoffs near large cities (see Table S1.1 in Supplement 1).

Resistance hypothesis

Having been exposed to copper as larvae, juveniles were more vulnerable to a subsequent re-exposure than naive juveniles. Contrary to what would be expected if larvae had developed resistance, larvae appeared instead to have been damaged by the first copper exposure in ways that made them more vulnerable to a subsequent exposure. Mortality increased dramatically, in particular for those juveniles that had been exposed to a high copper concentration during early larval development. This is one of the first studies that demonstrate the effect of further exposure to a pollutant in later life stages in aquatic organisms. A recent study on cadmium in juvenile freshwater snails also showed an increase in sensitivity for those previously exposed to the same stressor during embryonic development (Kimberly & Salice 2014). In sea urchins, similar results have been observed for ocean acidification in *Strongylocentrotus droebachiensis*, where juveniles in elevated $p\text{CO}_2$ had a higher mortality rate only when they had also been raised in elevated $p\text{CO}_2$ as larvae (Dupont et al. 2013).

On the other hand, surviving juveniles that had been previously exposed to the highest concentration of copper as larvae had higher growth rates when re-exposed than all other groups, including naive juveniles that had never been exposed to copper before. Faster growth arising from larval exposure to the highest concentration of copper may be explained by selective juvenile mortality in favour of fast growing juveniles, or increased mortality after re-exposure may have decreased conspecific competition. The selective mortality hypothesis is supported by a decrease in size heterogeneity in treatments with exposure early during larval development, where juvenile mortality was the highest, but not treatments with exposure later during larval development.

Timing of larval exposure to copper

The difference between early and late timing of exposure was important for many, but not all vari-

ables. Juveniles exposed early during larval development were more vulnerable to a subsequent copper exposure than those exposed later. On the other hand, in the absence of further exposure, individuals exposed early during the larval stage seemed to fare slightly better, or similar, after settlement than those exposed late during larval stage. The effect of timing may also depend on the stressor, as Phillips & Shima (2006) found a greater vulnerability of younger larvae for sediment stress on *E. chloroticus*.

Surprisingly, young juveniles were more vulnerable than larvae to a first copper exposure. Indeed, a short pulse of the highest concentration of copper to naive juveniles directly impaired growth, while no direct effect of copper on larval size or survival was observed. Copper toxicity has never been assessed for young juvenile urchins. Comparison of sensitivity to stressors across life stages of the sea urchin usually includes all the stages of larval development until, or shortly after, metamorphosis, but not young juveniles (Kobayashi 1980, King & Riddle 2001).

These findings confirm that *E. chloroticus* is sensitive to copper during the whole larval stage as well as shortly after settlement. Therefore, punctuated pollution events such as runoff are likely to affect, to some extent, all larval and young juvenile cohorts.

CONCLUSIONS

Our results show that even a short pulse of copper during larval development may have long-lasting effects, especially on juvenile growth and resistance to further stress. When larvae are vulnerable to a stressor throughout their whole larval development, as was the case here, adult populations close to the source of larval stress not only produce few recruits but also have a low chance of acquiring recruits dispersed from populations further from the stressor source (Shima et al. 2010). Therefore, transient larval exposure, as in a river plume, may have a strong impact on recruitment and local population persistence. Decreased resistance to further stress is of particular importance as larvae exposed to pollution may settle in a polluted area; this is especially the case for species with low dispersal, or in semi-enclosed embayments where larvae are relatively contained. On the other hand, selective mortality after subsequent copper exposure might mitigate the impact at the population level, as only the fitter, faster growing juveniles would survive. This study highlights the importance of evaluating the long-term effects of toxicants. Because no strong impact

was seen until 8 d post-settlement, focusing only on the larval stage would have led to misleading conclusions about short copper exposure on early life stages of *E. chloroticus*.

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