Acclimation and adaptation to common marine pollutants in the copepod Tigriopus californicus

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HIGHLIGHTS

• Multigenerational exposure to Cu generates a response consistent with acclimation.
• Phenotypic response to long term Cu exposure mirror short term Cu exposures.
• Multigenerational exposure to TBTO generates a response consistent with adaptation.
• Results from bioassay depend on test organism’s history of exposure.

ABSTRACT

Establishing water quality criteria using bioassays is complicated by variation in chemical tolerance between populations. Two major contributors to this variation are acclimation and adaptation, which are both linked to exposure history, but differ in how long their effects are maintained. Our study examines how tolerance changes over multiple generations of exposure to two common marine pollutants, copper (Cu) and tributyltin oxide (TBTO), in a sexually reproducing marine copepod, Tigriopus californicus. Lines of T. californicus were chronically exposed to sub-lethal levels of Cu and TBTO for 12 generations followed by a recovery period of 3 generations in seawater control conditions. At each generation, the average number of offspring produced and survived to 28 d was determined and used as the metric of tolerance. Lines exposed to Cu and TBTO showed an overall increase in tolerance over time. Increased Cu tolerance arose by generation 3 in the chronically exposed lines and was lost after 3 generations in seawater control conditions. Increased TBTO tolerance was detected at generation 7 and was maintained even after 3 generations in seawater control conditions. It was concluded from this study that tolerance to Cu is consistent with acclimation, a quick gain and loss of tolerance. In contrast, TBTO tolerance is consistent with adaptation, in which onset of tolerance was delayed relative to an acclimation response and maintained in the absence of exposure. These findings illustrate that consideration of exposure history is necessary when using bioassays to measure chemical tolerance.

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1. Introduction

The use of bioassays to determine water quality criteria is complicated by intraspecific variation in chemical tolerance. In aquatic environments, wild populations collected from sites with past or present contamination are often found to be more tolerant to those contaminants than wild populations from uncontaminated regions (Lopes et al., 2005; Agra et al., 2010; Agra et al., 2011). Similarly, strains maintained in the lab for extended periods may also have sensitivities that do not reflect tolerances of wild populations. Major contributors to inter-population variation in chemical sensitivity include physiological acclimation and genetic adaptation. If a population is responding through acclimation then even brief change to stress exposure can dramatically alter its ability to respond (Kwok et al., 2009). In contrast, a population adapted to a particular contaminant is generally expected to maintain its tolerance regardless of its current condition. As a result, differences in toxicant sensitivity may remain even after several generations of maintenance under common laboratory conditions. Whether differences in sensitivity are due to acclimation or adaptation should be a central concern for understanding how sensitivity can differ and change over time between populations.

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Acclimation is a plastic physiological change driven by an external factor that can occur within a single generation at the level of an individual. As a plastic response, effects of acclimation can be quickly gained when challenged with a condition and lost in the absence of the condition. In the past, offspring were not generally expected to inherit an acclimation response gained by their parents (e.g. LeBlanc, 1982; Kwok et al., 2009). However, acclimation responses could be inherited as transgenerational epigenetic effects such as maternal effects (Wolf and Wade, 2009) and methylation patterns (Vandegehuchte et al., 2009; Verhoeven et al., 2010), but these effects are not consistently inherited and are often limited to one generation (Youngson and Whitelaw, 2008). With acclimation, even short-term physical conditions in laboratory cultures can substantially alter results of toxicity tests. In contrast, adaptation is a slower evolutionary response that occurs at the population level with selection for genetically fit individuals better suited to their local environment. As a result, these individuals will pass on their genes disproportionately to the next generation (Klerks and Weis, 1987). Once the selective force is removed, tolerance is maintained for different lengths of time depending on the strength of selection against the adapted individuals in the absence of the stressor.

*Tigriopus californicus* is a harpacticoid copepod, native to supra-littoral tidepools ranging from Baja California to Alaska (Edmands, 2001). As a result of their wide distribution, different populations are likely exposed to a broad spectrum of environmental stresses allowing for local adaptation, which has been previously shown for temperature (Willett, 2010; Kelly et al., 2012; Schoville et al., 2012). These copepods are easily raised in the laboratory and have a fast generation time (minimum of 23 d, Burton, 1987), making multi-generational experiments feasible. *Tigriopus* spp. respond to a variety of relevant environmental toxicants through standard toxicity end points such as LC₅₀ (Lee et al., 2007) life-history traits (Kwok et al., 2008; Lee et al., 2008; Kwok et al., 2009), and through gene expression (See et al., 2008; Ki et al., 2009). These characteristics make *Tigriopus* a tractable marine model for ecotoxicological studies (Raisuddin et al., 2007).

The copepod *T. californicus* mates and reproduces year round. During mating, adult males clasp virgin females with their antennules and mate guard until the female is sexually mature. Males have multiple mating events whereas females mate only once, relying on the original spermatophore to fertilize multiple clutches (Burton, 1985). Individual lifespan may be up to 95 d and females may produce up to 20 egg clutches in their lifetime. The number of offspring produced in each clutch varies from less than 10 to more than 100 nauplii (larvae) (Vittor, 1971). The average generation time of *T. californicus* in our experiment was roughly 30 d.

In this study, two common marine pollutants were used: tributyltin (TBT) and copper (Cu). TBT was a common additive in anti-fouling paints, and was found to be toxic even at low concentrations (Leung et al., 2001). As a result, the International Maritime Organization has banned its use in antifouling paints globally since 2008 (IMO 2001). Despite the ban, TBT is still found in many marine environments at concentrations on par or higher than that used in our experiment partly due to terrestrial input and long residence time in sediments (Dziek et al., 2002; Arambarrí et al., 2003; Bhosle et al., 2004; Burton et al., 2005; dos Santos et al., 2010). As a known endocrine disrupting chemical, TBT may be responsible for major changes to population composition and reproductive success (Horiguchi et al., 1997; Huang et al., 2006; Huang et al., 2010) making TBT an ideal chemical for multigenerational effect studies. This study uses the TBT compound tributyltin oxide (TBO).

Cu is a ubiquitous ion and a micronutrient, but it can be toxic at high concentrations (White and Rainbow, 1985). The average dissolved Cu concentration in the surface waters of the Southern California Bight was found to be relatively low, 0.09 ± 0.06 μg L⁻¹ (mean ± standard deviation) (Smail et al., 2012). However, as a main ingredient of common antifouling paints used worldwide, Cu has become a major contaminant in regions of high boat traffic. A comprehensive review assessing the impact of Cu based antifouling paint examined 13 different studies of California bays, harbors, and marinas from 1974 to 2002 and documented high Cu concentrations in sediments ranging from 22 to 647 mg kg⁻¹ (California Department of Pesticide Regulation, 2005). Dissolved concentrations in the water column in two local bays, San Diego Bay (Sanders, 2005) and Newport Bay (Orange County Coastkeeper, 2007), have been shown to be above the California Toxic Rule standards of 3.1 μg L⁻¹ for chronic exposure.

The aim of this study was to examine the time course of development and loss of tolerance to Cu and TBT within a marine invertebrate. The experimental design was structured to examine tolerance holistically through a multi-generation life-cycle test. An additional goal was to determine whether these patterns of tolerance were consistent with either acclimation or adaptation. To accomplish this we tracked the onset of tolerance in *T. californicus* through multiple generations of exposure, as well as the status of tolerance post-exposure.

### 2. Materials and methods

#### 2.1. Test organism

*Californicus* were collected January 2010 from tide pools at Ocean Beach in San Diego (SD), California (32°45′3.18″N, 117°15′6.13″W) and maintained in the lab in filtered autoclaved seawater (FASW) for 2 generations before establishment of multi-generational exposures. To obtain FASW, seawater was collected from the University of Southern California’s Wrigley Marine Science Center (WMSC) located in a marine reserve on Catalina Island, CA, filtered through 37 μm mesh and autoclaved. Using specimen collected from SD, exposure lines were established and maintained at 20 °C with 12 h: 12 h light: dark photoperiod. *T. californicus* was maintained on a diet of 0.04 g TetraMin (Tetra Holding, Inc., USA) and 0.04 g Spirulina (Nutraceutical Science Institute, USA). The three lines S – seawater control (i.e., FASW only), C – Cu chronic exposure (13.74 μg Cu L⁻¹, prepared in FASW), and T – TBT (0.15 μg TBT L⁻¹, prepared in FASW) chronic exposure used in this experiment were established from 500 copepod pairs taken equally from the original collection sample.

#### 2.2. Chemical preparation

The final Cu and TBO concentrations used in this study were calculated to be 10% of the LC₅₀ values from a preliminary study performed on two-week old juveniles. This concentration was chosen to limit mortality while still being potent enough to exert an observable effect. The Cu and TBT chronic solutions were used both for maintaining the multi-generation lines and in fitness assays. The Cu stock solution was prepared by diluting CuSO₄·5H₂O (Sigma) in nanopure water to a concentration of 1 g L⁻¹. The Cu stock solution was added to FASW for the chronic Cu solution of 13.74 μg L⁻¹ before use. The TBT stock solution was made by diluting bis (tributyltin) oxide (EMD, USA) with acetone (Macron Chemicals, USA) to a concentration of 0.004 g L⁻¹ and stored in the dark. The TBT chronic solution was prepared by adding TBO stock solution to FASW for a final concentration of 0.15 μg L⁻¹ before use. Both stock solutions were stored at 20 °C, and replaced monthly.

#### 2.3. Multi-generation selection experiment

Copepods were acclimated to laboratory conditions for 2 generations before the start of the multi-generational selection experi-
ment. Each line was started with 5 replicates of 100 clasped pairs per replicate and maintained for 14 generations (Fig. 1). For each generation, each line was divided into 5 new replicates. In the case of a major mortality event in one of the replicates, the remaining uncompromised replicates were used to seed the next generation. At the start of a new generation, a total of 100 clasped pairs were taken equally from each of the 5 original (or all remaining extant) replicates to establish a replicate for the next generation. This practice of mixing the replicates after every generation was done to maintain the effective population size \( N_e \) of the lines. \( N_e \) is the size of an ideal population that would undergo the same amount of random genetic drift as the actual population (Wright, 1931). Our goal was to maintain a relatively large \( N_e \) to limit the effect of genetic drift and maintain genetic variation for selection to act upon.

When copepodites (juvenile copepods) grew to a visible size, the parents were removed to avoid intergenerational breeding. When clasped pairs began to form, we randomly selected 500 clasped pairs that were then divided into 5 replicates to start the next generation. Each replicate was kept in a polyethylene container at 400 ml and fed once a week. A complete solution renewal was performed twice weekly. At generation 12, the \( T \) and \( C \) lines were moved into seawater control conditions and their accompanying name change was \( TS \) and \( CS \), respectively (Fig. 1).

2.4. Fitness assays

The fitness of each line was estimated by measuring reproductive output. Fitness assays were labeled with their respective line followed by \( x \) (FASW only), \( y \) (FASW + 13.74 \( \mu \)g L\(^{-1}\) Cu), or \( z \) (FASW + 0.15 \( \mu \)g L\(^{-1}\) TBTO) (Fig. 1). For example, \( Sx \) indicates a fitness assay established with individuals from the control line in FASW only. Each fitness assay was started with 1 clasped pair in an acid washed 30 mL Petri dish with 2 mg of food. A total of 25 replicates per line were incubated for 28 d in 20 °C with 12 h: 12 h light: dark photoperiod. At day 14, the adults were removed and the juvenile inhabitants of each dish were fed and dezionized \( H_2O \) was added to maintain original volume in case of evaporation. At 28 d, living copepods were counted in each fitness assay. For generations 3, 7, 12, and 15, each line had 3 sets of fitness assays, corresponding to each treatment (\( x \), \( y \), and \( z \) as indicated above). For the remaining generations, one set of fitness assays was done in its native conditions (e.g. \( Sx \) – control line in FASW only).

2.5. Data analysis

Student’s t-tests were used to compare the results from fitness assays of the treated lines (C and \( T \)) to the control line (\( S \)). To assess temporal changes within treatments, linear regression was used to analyze fitness for generations 2–12 for each line in its native condition. To correct for temporal changes in laboratory conditions, linear regression was also done for proportional deviation of \( C \) in its native condition against \( S \) and similarly done for \( T \) in its native condition against \( S \).

3. Results

Linear regression analyses (Table 1) showed a temporal increase in tolerance for bioassays that were pre-exposed to Cu (\( Cy \)) and TBTO (\( Tz \)), but there was no temporal change in tolerance for the control line (\( Sx \)). Regressions focused on generations 2–12. generation 1 was omitted because all lines were taken directly from SD laboratory cultures and had yet to be exposed. Generations 13–15 were also omitted due to absence of exposure to Cu and TBTO in the \( Cy \) and \( Tz \) bioassays, respectively. For the control treatment (\( Sx \)) there was no overall directional change in average offspring produced across generations 2–12. For the Cu treatment there was a marginally significant increase in Cy over time and a highly significant increase in proportional deviation from the control [(\( Cy – Sx \))/Sx] over time. For the TBTO treatment there was no significant change in \( Tz \) over time, but proportional deviation from the control [(\( Tz – Sx \))/Sx] showed a highly significant increase.

Results for Cu showed a temporary gain in tolerance that was lost after animals were transferred to control conditions (Fig. 2). A comparison of the average number of offspring at the 28 d counts in Cu exposure showed a significant difference for all \( S \) and \( C \) line comparisons, but not for the \( S \) and \( C \) line comparison. There was a significant difference in the average number of offspring produced between the \( S \) line and \( C \) line in generations 3, 7, and 12 with a higher mean in the \( C \) line for all three generations. By generation 15 the \( C \) line had been moved into control conditions with an accompanying name change to the \( CS \) line. The comparison

Fig. 1. Experimental design for multi-generation selection experiment. The SD square represents the initial sample collected from San Diego, California. Each subsequent square represents a generation for a particular line. The seawater (\( S \)) line was maintained for 14 generations and the copper sulfate (\( C \)) and TBTO (\( T \)) lines were maintained for 11 generations. After 11 generations, the \( C \) and \( T \) lines were transferred to seawater (\( CS \) and \( TS \)) for the final three generations. Each circle represents fitness assays for a total of 25 copepod pairs (male plus virgin female), with each pair housed in its own Petri dish. Fitness assays are annotated with either \( x \), \( y \), or \( z \) to signify seawater, Cu, or TBTO treatments, respectively.
between the S line and CS line showed no significant difference in estimated fitness. Results for TBTO showed a gain in tolerance, which was maintained after animals were transferred to control conditions (Fig. 3). There was no significant difference in mean number of offspring between the S and T fitness assays exposed to TBTO for generation 3. Generations 7 and 12 showed a significant increase of the average number of offspring in T fitness assays compared to S fitness assays. The average number of offspring between S and TS fitness assays remained significantly different at generation 15.

4. Discussion

4.1. Inter- and intra-generational variation

The regression analysis showed the average number of offspring in Sx (the S line fitness assays in FASW) did not have directional changes for generations 2–12 (Table 1). This demonstrates that the directional changes in the contaminant treatments were not due to overall temporal changes in laboratory conditions. Temporal variation in Sx with no overall directional change is likely due to several factors such as nutrient composition of seawater and ambient temperature in the lab. Nevertheless, within generation variation is expected to be minimal due to consistency in seawater batches and exposure to ambient temperature in the lab among the three lines at a given time.

The C line had a marginally significant intergenerational change seen as an increase in offspring number for Cy over the course of the experiment with a highly significant increase for Cy as a proportional deviation from the Sx. By comparing the proportional deviation of Cy from Sx we are able to control for temporal changes in laboratory conditions that may mask changes caused by chronic exposure.
For the T line there was no significant intergenerational change for the average number of offspring in Tz. However there was a highly significant change for Tz as a proportional deviation from Sx, suggesting that the average number of offspring produced in Tz is increasing with successive generations. The pattern of increasing mean of Cy and Tz points to a gradual gain of tolerance to Cu and TBTO, respectively.

4.2. Acclimation to copper

Our results showed that tolerance to Cu was rapidly gained in lines that had a pre-exposure to Cu, but also rapidly lost once Cu exposure was removed (Fig. 2). This pattern of tolerance is consistent with physiological acclimation. The increase in the average number of offspring for Cy (Table 1) can be explained by a multi-generational acclimation, which has also been documented in *Daphnia magna* (LeBlanc 1982; Bossuyt and Janssen, 2003). The observations in these studies would explain a steady increase in Cu tolerance as each generation became better acclimated to Cu. In line with acclimation, once *D. magna* was removed from Cu, they completely lost their tolerance.

We made similar observations for *T. californicus* (Fig. 2). By generation 3, the C line had a significantly increased mean number of offspring compared to the control line when both were exposed to Cu, suggesting the development of Cu tolerance in the C line. Our data also show that even with chronic Cu exposure up to generation 12, Cu tolerance remained a plastic response that was quickly lost when the C line was moved into clean conditions. This finding is consistent with other studies on Cu acclimation. Kwok et al. (2009) observed that a single generation of exposure to Cu significantly increased Cu tolerance in a congener, *T. japonicus*, which was then lost when the tolerant lines' offspring were raised in the absence of Cu. Similarly, a study by LeBlanc (1982) found that *D. magna* had gained tolerance to Cu within a single generation of exposure and lost the acquired tolerance in the next generation after being moved into clean conditions. These shorter multi-generational experiments with smaller population sizes have shown Cu response to be a strictly plastic response. One of the goals of our experiment was to increase exposure time and genetic variation in order to augment the ability of selection to act on an adaptive response to Cu. However, our findings for long term exposure mirror the shorter multi-generational experiments that used smaller population sizes.

4.3. Adaptation to tributyltin oxide

In contrast, comparisons between the T and S lines are consistent with genetic adaptation. This is marked by a slow gain in tolerance and maintenance of tolerance in the absence of TBTO exposure (Fig. 3). Observed differences between the TS line and S line after both were maintained under the same condition for 3 generations is consistent with a genetically based difference (Klerks and Weis, 1987). The delay in development of tolerance to TBTO is likely a result of the time needed for selection to act on the population. Under this scenario, genotypes with greater fitness to TBTO gradually increased in frequency as they out-produced other genotypes. Although the actual mechanism(s) responsible for TBTO tolerance in *T. californicus* is unknown, we speculate that maintenance of TBTO tolerance could be due to a tolerance mechanism with a low energetic cost, which in turn elicits a weak selective response. This result would explain how TBTO tolerance is maintained in the population in the absence of TBTO and how it would be maintained until lost to genetic drift or broken up through sexual recombination. Maternally and grand-maternally transferred cytoplasmic components can be ruled out as possible sources of TBTO tolerance because of the 3 generations the T line spent in clean conditions before assessment. Maternal effects, which should be induced after one generation of toxicant exposure, also fail to explain the slow gain in tolerance. The TBTO concentration used was a strong enough selective force to generate a response consistent with a genetic adaptation by the 7th generation of exposure. Our data show that chronic exposure to a low concentration of TBTO can elicit a response consistent with adaptation in *T. californicus* within a few generations of exposure.

Adaptation to tributyltin in a midge *Chironomus riparius* showed a similar pattern of a slow gain of tolerance, where tolerance only appeared after 9 generations of exposure (Vogt et al., 2007). However, the main goal of this prior work was not to differentiate between adaptation and acclimation, therefore the study did not examine whether or not increased tolerance was maintained in the absence of tributyltin exposure. The observed tolerance could be due to adaptation, acclimation, or a combination of both. Showing that *T. californicus* maintains tolerance after being removed from TBTO exposure indicates that tolerance is consistent with a heritable genetic adaptation. These data also suggest that adaptation to TBTO concentrations equivalent to levels found in the field can occur in a laboratory setting in a relatively short amount of time.

4.4. Differences in tolerance

The cost of tolerance is likely the determining factor to whether tolerance is lost or maintained in the absence of contamination. Cu tolerance has been documented to carry a relatively high energetic cost (Lukasik and Laskowski, 2007; Kwok et al., 2009; Agra et al., 2010, 2011) which likely results in a strong selective pressure against maintaining expression of genes associated with Cu tolerance in control conditions. Cu levels in the supra-littoral tidepools may fluctuate widely, depending on various environmental parameters (e.g. rain events), so *T. californicus* may have undergone selection for a tolerance mechanism that could be switched on and off quickly to cope with the rapidly changing environment (i.e. plastic acclimation response). In contrast, TBTO tolerance may have a lower energetic cost, allowing it to be maintained in the absence of exposure. Alternatively, genotypes with TBTO tolerance could be maintained due to the absence of strong negative selection.

The tolerance mechanisms for Cu and TBTO are likely very different as a result of the different properties of each chemical, but there is overlap. A common defense, heat shock proteins are recruited with both Cu (Boone and Vijayan, 2002; Rhee et al., 2009; Guo et al., 2012) and tributyltin exposure (Cochrane et al., 1991; Oberdörster et al., 1998). Additionally, oxidative stress has also been traced to both Cu (Stohs and Bagchi, 1995; Rhee et al., 2011; Rhee et al., 2013) and tributyltin (Ishihara et al., 2012). An overlap in tolerance has been found in sediment bacteria that were originally selected for their tolerance to tributyltin, but were also found to be resistant to Cu (Pain and Cooney, 1998).

Different tolerance responses to these two toxicants could be explained in light of *T. californicus* evolutionary history of exposure. Cu is a naturally occurring heavy metal and a micronutrient in marine invertebrates (White and Rainbow, 1985; Hernández and Allende, 2008). *T. californicus* has had a long evolutionary history with Cu, evident in its requirement of Cu in critical proteins such as hemocyanins. This long existing relationship could have led to the evolution of an optimal response to variable Cu levels that can be quickly activated when Cu levels dramatically increase and deactivated in periods of low Cu concentrations to conserve energy. Unlike Cu, TBTO is solely an anthropogenically derived compound to which *T. californicus* has a comparatively shorter evolutionary relationship. Given the short period of interaction, we speculate that the majority of the original *T. californicus* population lack tolerance mechanisms to TBTO. The gain of TBTO tolerance in
a few generations suggests that the mechanism responsible for tolerance likely existed in a small subset of individuals within the population prior to the experiment. TBT tolerance only came into prominence in the population after prolonged TBT exposure led to selection of TBT-resistant genotypes.

4.5. Conclusion

We find that *T. californicus*’ multigenerational response to Cu is consistent with acclimation, while the multigenerational response to TBT is consistent with adaptation. Cu tolerance was lost quickly after a transfer to clean conditions, while TBT tolerance was maintained. A linear regression analysis showed that the observed increase in mean offspring production across generations was maintained. A linear regression analysis showed that the genetic basis of copper toxicity, deficiency, and metabolism. Am. J. Clin. Nutr. 88, 8355–8359.


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