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Long-term laboratory culture causes contrasting shifts in tolerance to two marine pollutants in copepods of the genus *Tigriopus*

Patrick Y. Sun[1] · **Helen B. Foley**[1] · **Leslie Wu**[1] · **Charlene Nguyen**[1] · **Shiven Chaudhry**[1] · **Vivien W. W. Bao**[2] · **Kenneth M. Y. Leung**[2,3] · **Suzanne Edmands**[1]

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Abstract Organismal chemical tolerance is often used to assess ecological risk and monitor water quality, yet tolerance can differ between field- and lab-raised organisms. In this study, we examined how tolerance to copper (Cu) and tributyltin oxide (TBTO) in two species of marine copepods, *Tigriopus japonicus* and *T. californicus*, changed across generations under benign laboratory culture (in the absence of pre-exposure to chemicals). Both copepod species exhibited similar chemical-specific changes in tolerance, with laboratory maintenance resulting in increased Cu tolerance and decreased TBTO tolerance. To assess potential factors underlying these patterns, chemical tolerance was measured in conjunction with candidate environmental variables (temperature, UV radiation, diet type, and starvation). The largest chemical-specific effect was found for starvation, which decreased TBTO tolerance but had no effect on Cu tolerance. Understanding how chemical-specific tolerance can change in the laboratory will be critical in strengthening bioassays and their applications for environmental protection and chemical management.

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✉ Patrick Y. Sun
patricys@usc.edu

[1] Department of Biological Science and Wrigley Institute for Environmental Studies, University of Southern California, 3616 Trousdale PKWY STE 107, Los Angeles, CA 90089, USA

[2] The Swire Institute of Marine Science and School of Biological Sciences, The University of Hong Kong, Pokfulam, Hong Kong, China

[3] State Key Laboratory in Marine Pollution, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, China

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Introduction

Toxicological bioassays are an important tool used to establish regulatory practices and ecological risk assessments, and are used internationally as a standard for toxicity testing (Keddy et al. 1995; Power and Boumphrey 2004; Pandard et al. 2006). Bioassays usually employ laboratory-cultured organisms to make inferences on how natural populations would respond. Laboratory-cultured organisms offer many advantages such as consistent availability, standardizing sample population across multiple projects, and elimination of environmental acclimatization effects. However, there is potential for differences in chemical tolerance to develop between field and laboratory populations (Berthet et al. 2011). As a result, extrapolating experimental findings from the laboratory to the field may not be appropriate in all settings (Nowak et al. 2008; Woods et al. 1989). Although risk assessments do incorporate safety factors to account for uncertainties, how these safety factors are determined is hotly debated (Konietzka et al. 2014). The aims of this study are to measure changes in chemical tolerance across generations in laboratory conditions, to assess candidate factors responsible for those changes, and to contribute to the application of more appropriate safety factors that will account for dynamic chemical tolerances. This is important because lab-induced change in chemical tolerances will lead to inappropriate extrapolation of effect thresholds (e.g., predicted no effect concentration, PNEC, or lethal median concentration, LC_{50}). On one hand, this could lead to costly environmental damage if the laboratory organisms were more tolerant resulting in underestimated laboratory-derived

PNEC. On the other hand, if the laboratory organisms used to derive PNEC were more sensitive, then that would lead to an overestimation and unnecessarily higher cost for pollution control and cleanup.

Stress tolerance has been shown to shift in the laboratory due to either physiological processes such as acclimation or genetic processes such as adaptation. A decrease in stress tolerance could be attributed to a loss of acclimations gained while in the field. For example, in the absence of natural levels of zinc, *Daphnia* clones gradually lost their zinc tolerance in the laboratory (Muyssen et al. 2002). Decreased tolerance has also been attributed to loss of genetic diversity. Low genetic diversity, which laboratory populations often face due to small population size and inbreeding, has been shown to decrease starvation tolerance (Stewart et al. 1982) as well as resistance to contamination (Barata et al. 2000). Tolerance can also be reduced by evolutionary tradeoffs, where selection improves one trait at the cost of another (Stearns 1989). As an illustration, a laboratory population of *Drosophila* that evolved earlier fertility had a tradeoff of lower desiccation tolerance (Hoffmann et al. 2001). These examples illustrate that there can be significant loss of tolerance in laboratory populations.

However, stress tolerance can also increase in the laboratory. Physiological acclimation has been shown to increase tolerance following a pre-exposure to sublethal levels of trace metals (LeBlanc 1982; Kwok et al. 2009; Sun et al. 2014) and salinity (Wu et al. 2014). Likewise, genetic adaptation can increase tolerance through multiple generation of exposure to stressors such as temperature (Kelly et al. 2012). Further, unexpected increases in stress tolerance can also occur in a laboratory setting through indirect selection. One example is seen when *Drosophila* raised in ambient temperature (25 °C) developed higher cold tolerance (Condon et al. 2015). Yet, the rate of change in chemical tolerance and the conditions responsible are not well characterized. Tolerance can change gradually over the course of several generations or abruptly after a short period in laboratory culture, driven by the differences between the field and laboratory conditions.

This study used two species of marine copepods, *Tigriopus japonicus* and *T. californicus*, which both inhabit supralittoral tide pools. Tolerance to two antifouling paint additives, copper (Cu) and tributyltin oxide (TBTO), was measured. Although TBTO has been banned for marine uses (IMO 2001), it was extensively used before its ban and is still found in marine environments through terrestrial runoff and legacy contamination (Díez et al. 2002; Burton et al. 2005; Santos et al. 2010) as well as illegal uses (Ho et al. 2016). Since the ban on TBTO, Cu has taken its place as the most common antifouling additive. Additionally, these two chemicals represent two broad categories of marine pollutants. TBTO is a man-made lipophilic compound that is highly toxic even at low concentrations (Champ and Seligman 1996), whereas Cu is a naturally occurring trace metal, but especially toxic to marine invertebrates at higher concentrations (Philips and Rainbow 1993). Another point of difference between these two chemicals is that these *Tigriopus* species have been shown to acclimate to Cu and develop Cu tolerance in the laboratory (Kwok et al. 2009, Sun et al. 2014), while they consistently exhibited relatively higher sensitivity to TBTO with no acclimation-like response (Bao et al. 2011; Sun et al. 2014).

This study tracked chemical tolerance across three generations in the laboratory, immediately following collection from the field. The goal was to determine how chemical tolerance can change across several generations under benign laboratory conditions (culturing was done in the absence of chemical exposure). To assess factors that might alter tolerance in lab-raised animals, chemical tolerance was also measured in combination with several environmental factors that may differ between field and laboratory environments. Sublethal levels of environmental stressors were chosen to test the threshold of physiological response in these copepods. It was hypothesized that these additional stressors would reduce chemical tolerance by siphoning off resources used in chemical defense (Calow 1991). Ultraviolet irradiation is one of the tested stressors because it is believed to be a constant stressor based on the general topography of tide pools which offer little to no shelter against direct exposure. Temperature stress is another common stressor in high-tide pool environments, yet closely regulated in the laboratory. Two types of temperature regimens were applied to mimic constant vs. variable heat stress. Diet types were chosen to contrast a standard laboratory diet of dried food with a more natural diet of live algae. Finally, a starvation treatment was tested to mimic conditions likely to occur in the field.

Methods

Specimen collection and maintenance

Four *Tigriopus japonicus* populations were collected from Hong Kong in November 2011 and transported to the University of Southern California, USA. The sites of collection in Hong Kong were Gold Coast (22° 22′ 47″, 113° 58′ 48″), Shek O (22° 13′ 48″, 114° 15′ 36″), Stanley (22° 13′ 11″, 114° 13′ 11″), and Cape D'Aguilar (22° 12′ 36″, 114° 15′ 36″) (CDA). Five populations of *Tigriopus californicus* were collected from California, USA, in November 2011. The collection sites were Santa Cruz (36° 57′ 0″, − 122° 2′ 59″), Santa Cruz Island (34° 1′ 12″, − 119° 40′ 48″), Leo Carrillo (34° 2′ 23″, − 118° 56′ 23″), Laguna Beach (33° 32′ 23″, − 117° 47′ 24″) (LB), and San Diego (32° 45′ 0″, − 117° 15′ 36″) (SD). Populations were acclimated to the laboratory for 1 week before toxicity testing.

All populations were maintained in 37-μm filtered autoclaved seawater (FASW) at 20 °C with a 12-h light/12-h

dark photoperiod for the duration of the study. Seawater was collected from the Wrigley Marine Science Center on Catalina Island, which is situated within a marine reserve. Salinity was adjusted as needed to approximately 35 ppt with deionized water. After autoclaving, seawater was cooled overnight in an air-permeable container. Cu and TBTO concentrations in seawater were tested (Sun 2016) and levels were found to be lower than the allowable threshold stated in the National Recommended Water Quality Criteria set by the United States Environmental Protection Agency. Cu concentrations were similar to previous reports of coastal seawater from this region, the Southern California Bight (Smail et al. 2012). TBTO was not detected (testing done by ALS Environmental, USA). Cultures were maintained in Nalgene containers at a volume of 400 mL with a complete solution renewal and feeding performed once a week. Cultures were fed a dry diet, which consisted of 0.04 g ground TetraMin fish food (Tetra Holding, Inc., USA) and 0.04 g ground Spirulina cyanobacteria (Nutraceutical Science Institute, USA). These food rations are sufficient to be considered ad libitum. Each new generation started with three containers per population with 100 pre-copulatory pairs each for a total of 300 pairs per population. A generation is defined as the time it takes a pre-copulatory pair (a male clasping a virgin female) to produce sexually mature offspring. In the experiment, the estimated generation time is 42 days, which is slightly higher than previous estimates because pre-copulatory pairs are formed with sexually immature females and require additional time to reach maturity and produce offspring. At 14 days post establishment of a new generation, all adult copepods were removed to maintain discrete generations. This husbandry protocol has been successfully used to maintain multiple copepod populations (Sun et al. 2015) and multiple consecutive generations (Sun et al. 2014).

Multi-generational population tolerance survey

The standard protocol for the multi-generational population tolerance survey consisted of a contaminant exposure for 96 h without feeding or solution renewal in FASW. The environmental chamber (Thermo Scientific Precision Incubator) was set to a 12-h light/12-h dark photoperiod and held at a constant 20 °C. Only male copepods were used because males are generally less stress tolerant than females (Willett 2010; Kelly et al. 2012), and because female stress tolerance may fluctuate due to maternal transfer during egg development (Raisuddin et al. 2007). Maternal transfer can either increase a female's susceptibility to stress following the release of eggs or increase their tolerance as they pass on a portion of the chemical stressor to the egg sac relieving their own body burden.

Toxicity assays had three replicates per chemical concentration. Each replicate had 10 male animals. Each replicate consisted of a 30-mL petri dish with an approximate volume of 15 mL. Mortality was assigned to copepods that failed to respond to physical stimulation (Bao et al. 2011). Initial observations were made on a light table and confirmed with a dissecting microscope. The range of Cu and TBTO concentrations used was based on preliminary estimates of median lethal concentration (LC_{50}), which were obtained from pilot range-finding tests. Each LC_{50} used seven concentrations, including a control, but the actual concentrations were adjusted each generation for each population due to changing chemical tolerance. The generation 0 (F0) survey began in November 2011, immediately after collection and each consecutive generation survey followed approximately 42 days after the preceding generation. The first acute toxicity assay was done within a week of collection.

The Cu stock solution was made with $CuSO_4 \cdot 5H_2O$ (Sigma) in nanopure water to a concentration of 1 g L^{-1} and serially diluted to obtain the target concentration. The TBTO stock solution was made by diluting bis(tributyltin) oxide (EMD, USA) with acetone (purity > 99.5%, Macron Chemicals, USA) to a concentration of 0.1 g L^{-1}. The amount of acetone in all treatment solutions including the acetone control for TBTO toxicity assays was $\leq 1.2 \times 10^{-6}$ *v*/*v*. This study aimed to test if multiple generations of laboratory culture would alter chemical tolerance and to assess effects of environmental variables that may be different between field and laboratory environments. It was not intended to generate toxicity endpoints for regulatory uses. Even though nominal chemical concentrations are not ideal, the practicality of verifying each exposure concentration of our multi-species, multi-population, and multi-generation assays was prohibitive. Nominal concentrations were considered adequate for the purpose of testing overall temporal patterns of chemical tolerance.

Tolerance of each population was measured by LC_{50} calculated by the Trimmed Spearman–Karber method (TSKM). Generalized linear models (GLM) were also used because of their effectiveness to determine LC_{50} values by inclusion of extreme response probabilities of 0 and 1 (Kerr and Meador 1996). TBTO LC_{50} values were calculated using TSKM (Trimmed Spearman–Karber Program v. 1.5, US Environmental Protection Agency). While TSKM is an older method, it fares well in comparison to more recent approaches. A study by Hamilton (1980) showed that the confidence intervals derived by TSKM performed as well as a maximum likelihood estimator (MLE) and under heavy-tailed tolerance distributions (a distribution characterized by large values), TSKM was more accurate than MLE. Cu LC_{50} values were calculated using GLM due to the abundance of extreme response probabilities. GLM was calculated with a binomial distribution using the R programing language (R Core Development Team 2013) and package "MASS" (Venables and Ripley 2002). Significant differences between

generations were inferred from non-overlapping confidence intervals, which has been shown to be a highly conservative approach (Wheeler et al. 2006), which is ideal for this study because the goal of these experiments was to identify extreme changes in chemical tolerance.

Environmental stress treatments

All environmental stress treatments used SD (San Diego) copepods. Due to the requirement of large numbers of specimens for each environmental stress assay, large long-term freely breeding cultures of overlapping generations were established. This is in contrast to the smaller discrete generations maintained during the multi-generational population tolerance survey. These stress treatments were conducted from July 2012 to August 2013. All toxicity assays following environmental stress were conducted using the standard protocol listed above. LC_{50} values were calculated using the TSKM and significance was determined according to the method outlined in Wheeler et al. (2006).

Ultraviolet irradiation treatment

UV assays were done using the SD population after approximately 21 generations under lab conditions. Males from the SD population were placed in an open Nalgene container with a FASW volume of 400 mL exposed to UV-B at 305 nm for 2 h. The 2-h time point was chosen based on field data showing that the peak duration of many environmental stressors, such as peak UV or maximum daily temperature, was approximately 2 h (unpublished data). Food was withheld during the treatment. UV exposure was administered with a UV lamp with an intensity of 0.9 mW/cm^2 (Cole-Parmer, 6 W/115 V/60 Hz/0.16 A). A control group of SD males was kept in identical conditions, but without UV. The absence of UV was confirmed with a UV A/B Light Meter (Sper Scientific, USA). No mortality was measured in UV treatments. There was a resting period of 1 h between UV exposure and toxicity testing.

Diet treatments

Diet assays were done after approximately 28 generations under laboratory conditions. Copepods were raised in two different diet treatments for one entire generation before toxicity tests. The dry diet is our standard laboratory diet of 0.04 g of TetraMin and 0.04 g Spirulina per 400 mL replicate, and the animals were fed once a week. The live algae diet consisted of 160 mL *Platymonas* with a cell count of approximately 1,000,000/mL and the copepods were fed once a week. Cell counts were done on a hemocytometer (Bright-Line, USA) and calculated with the Palmer–Maloney cell count formula. Populations were established with 300 pre-copulatory pairs per biological replicate, with two biological replicates per diet. After 14 days, all adults were removed to limit cannibalism. Once mature, adult males were collected and used for toxicity assays.

Starvation assays

Starvation assays conducted after approximately 11 generations in the lab. Starvation was induced by isolating males for 1 week without food in FASW. There were a total of five replicates with 200 males per replicate. Cultures were maintained using the same husbandry protocol as in the population survey with solution renewal once a week.

Temperature treatments

Temperature assays were done after approximately 18 generations in the lab. The first temperature treatment was the cycling temperature treatment, which had environmental conditions cycle between 20 °C in the dark for 12 h and 28 °C in the light for 12 h per day for an entire generation. This treatment was based on results from Kelly et al. (2012) which showed a cyclic temperature treatment with a maximum temperature of 28 °C which was the warmest achievable temperature that had an impact (generated a temporary increase in thermal tolerance) without resulting in significant mortality. Pre-copulatory pairs were placed in the cyclic treatment, removed 14 days post culture establishment and the resulting males were harvested once fully mature for toxicity tests. Solution renewal and feeding schedule followed the standard protocol. Cultures were started with 300 pre-copulatory pairs from the SD population in Nalgene containers with a FASW volume of 400 mL. The cyclic temperature treatment had a total of five replicates.

The constant high-temperature stress treatment at 32 °C consisted of a daily 12-h light and 12-h dark cycle for 4 days. Preliminary trials with the San Diego population showed that a treatment of 32 °C for 4 days was the highest temperature and duration combination that resulted in no significant mortality. Males were collected from four replicates for this trial because at the time of the assay, the final replicate did not have enough individuals. The control males were maintained at 20 °C for 4 days in their own separate containers.

Results

Multi-generational population tolerance survey

Chemical tolerance was shown to vary across the three generations (i.e., from F0 to F2) under laboratory conditions in the two marine copepod species, with a general pattern of increase in Cu tolerance over time and decrease in TBTO tolerance

over time (Fig. 1). Eight of the nine populations (i.e., all but LB) had an increase in Cu tolerance. In five populations (two for *T. californicus* and three for *T. japonicus*), there were significant consecutive increases in Cu tolerance. In contrast, TBTO tolerance significantly decreased across seven of the nine tested populations (i.e., all but LC and CDA) during the experimental period. In two *T. japonicus* populations, there were decreases in TBTO tolerance in each consecutive generation under the laboratory setting.

Ultraviolet radiation

UV exposure before toxicity assays significantly decreased tolerance to both chemicals in all replicates, as determined by non-overlapping confidence intervals (Fig. 2).

Diet assays

Different diet treatments influenced chemical tolerance (Fig. 3). In both of the Cu replicates and one of the TBTO replicates, copepods that fed a dry diet (TetraMin and Spirulina) exhibited higher tolerance than those that fed live algae (*Platymonas*). The same trend was found in the second TBTO replicate but confidence intervals could not be calculated for one of the treatments due to an excess of extreme values (no mortality or complete mortality).

Starvation assay

It was shown that withholding food for 1 week had no significant effect on Cu tolerance in any of the replicates, but significantly decreased TBTO tolerance in four replicates (Fig. 4). The same trend was found in the fifth TBTO replicate but confidence intervals for one treatment are missing due to extreme mortality values.

Temperature

A cycling temperature treatment of 12 h:12 h 20 °C:28 °C over the course of an entire generation produced limited effects on chemical tolerance. For Cu (Fig. 5a), there was a trend toward higher tolerance under the cycling treatment, but only one of the five replicates showed a significant effect. For TBTO (Fig. 5b), there was also a trend toward higher tolerance in the cycling treatment, but no significant effects were found and several replicates had missing confidence intervals.

A constant temperature treatment at 32 °C for 4 days prior to toxicity assays produced mixed results with the majority of comparisons resulting in non-significant differences (Fig. 6). The one exception was a Cu replicate in which the high-temperature treatment reduced tolerance.

Discussion

Multi-generational population tolerance survey

This study examined the temporal component of chemical tolerance changes under typical laboratory conditions, where environmental parameters are held at mild and constant levels. Our previous work has shown differences in chemical tolerance among populations of *Tigriopus* (Sun et al. 2015). Other studies have shown tolerance of animal decreases as they are propagated in the laboratory (Stewart et al. 1982; Barata et al.

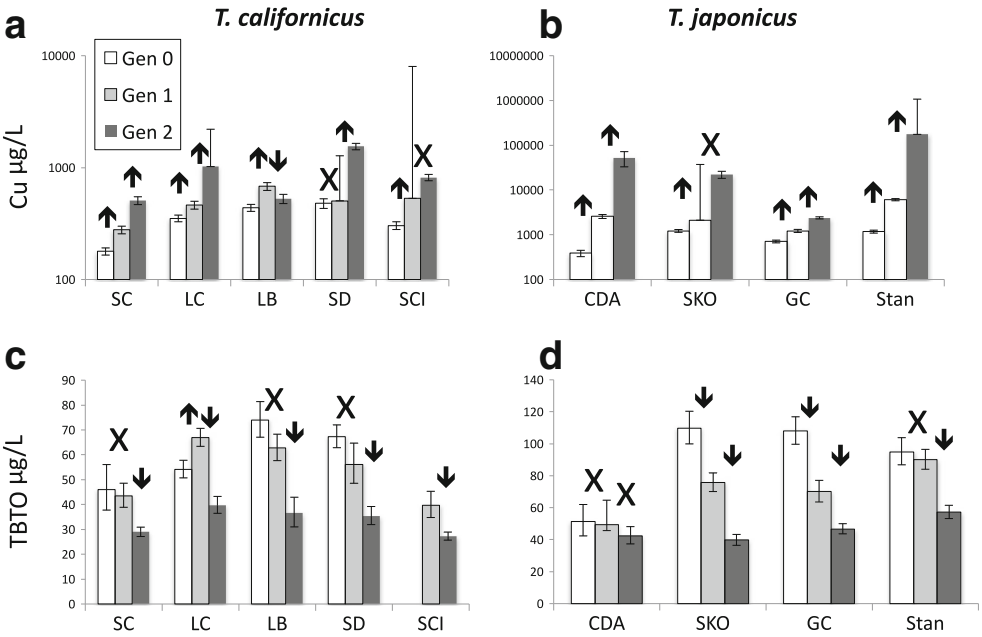

Fig. 1 Multi-generational population tolerance survey of Cu (**a**–**b**) and TBTO (**c**–**d**) LC_{50} with *Tigriopus californicus* (**a**, **c**) and *Tigriopus japonicus* (**b**, **d**). Cu values are log10 transformed. Non-overlapping confidence intervals were used to determine significant differences between generation 0 (field-collected) and generation 1, as well as between generation 1 and generation 2. Upward arrow indicates a significant increase from one generation to the next, downward arrow indicates a significant decrease from one generation to the next, and X indicates no significant change between generations

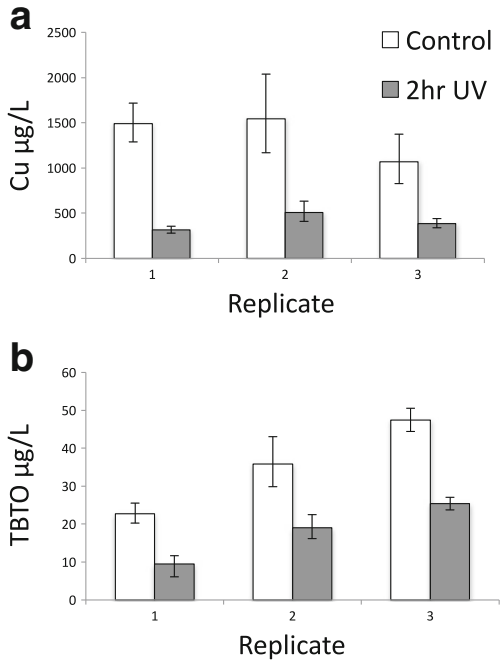

Fig. 2 Consecutive ultraviolet irradiation and contaminant exposure assay. LC50 values for copper (**a**) and TBTO (**b**), with error bars showing 95% CI. LC50 assays were conducted at 20 °C for 96 h on animals from one of two treatments: (1) control: constant 20 °C or (2) UV: ultraviolet exposure for 2 h prior to toxicity tests. The UV treatment reduced tolerance to both Cu and TBTO

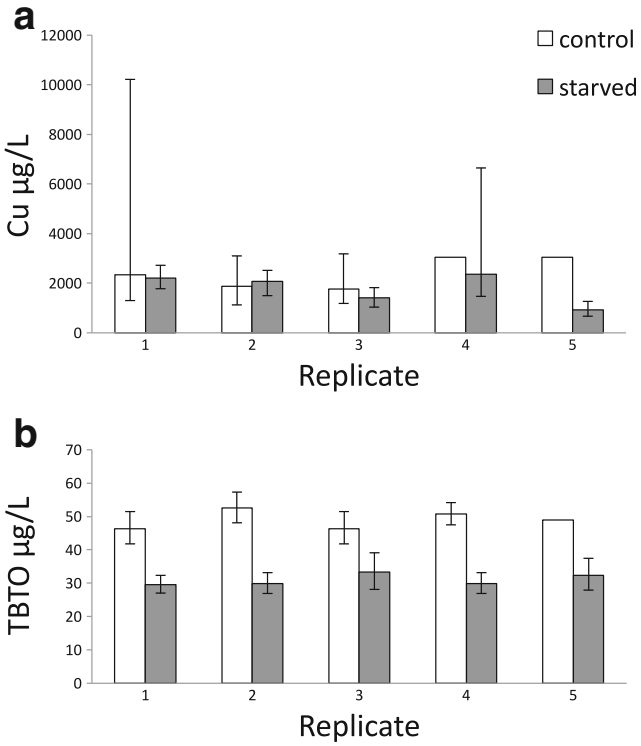

Fig. 4 Starvation assay. LC50 values for copper (**a**) and TBTO (**b**), with error bars showing 95% CI. LC50 assays were conducted at 20 °C for 96 h on animals from one of two treatments: (1) control: standard food conditions or (2) starved: food was withheld for 1 week before toxicity assays

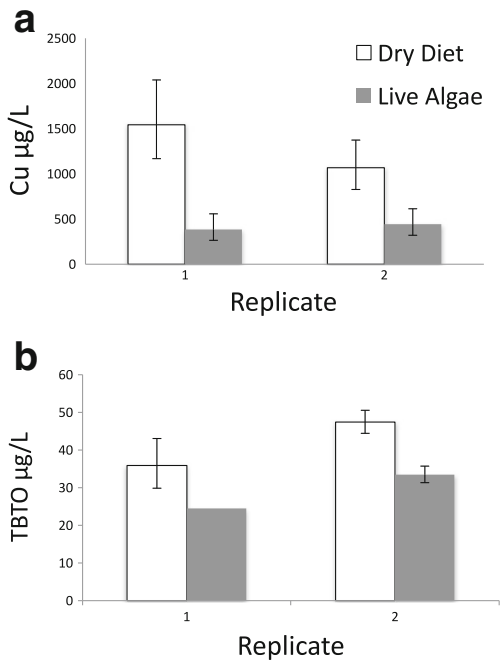

Fig. 3 Different diet treatments significantly impact chemical tolerance. LC50 values for copper (**a**) and TBTO (**b**), with error bars showing 95% CI. LC50 assays were conducted at 20 °C for 96 h. The dry diet treatment was comprised of TetraMin and Spirulina and the live algae diet consisted of *Platymonas* administered once a week. Treatment groups were raised from birth on these diets before toxicity assays

2000; Muyssen et al. 2002). In the current study, we found that TBTO tolerance decreased, which is in line with these previous studies. However, in contrast to the standard expectation, Cu tolerance was found to increase under benign laboratory conditions. The observation of contrasting shifts in chemical tolerance signifies a chemical-specific response to laboratory culturing. Additionally, the shift in chemical tolerance is similar in both species and across multiple divergent populations indicating that this may be a relatively conserved pattern.

Previous work has shown that with chronic sublethal exposure, adaptation to TBTO can occur, but not in Cu (Sun et al. 2014). The changes in chemical tolerance observed under benign conditions in the current study are not easily attributable to adaptation, since changes occurred after a single generation in the laboratory. These changes are more likely products of physiologically plastic processes.

The follow-up environmental assays were designed to elicit physiological responses and were therefore conducted within the span of a single generation under largely sublethal conditions. Although the environmental assays were done several generations after the initial population tolerance survey, the purpose of these follow-up assays was not to make direct comparisons but rather to identify potential candidate environmental stressors that could impact chemical tolerance. The

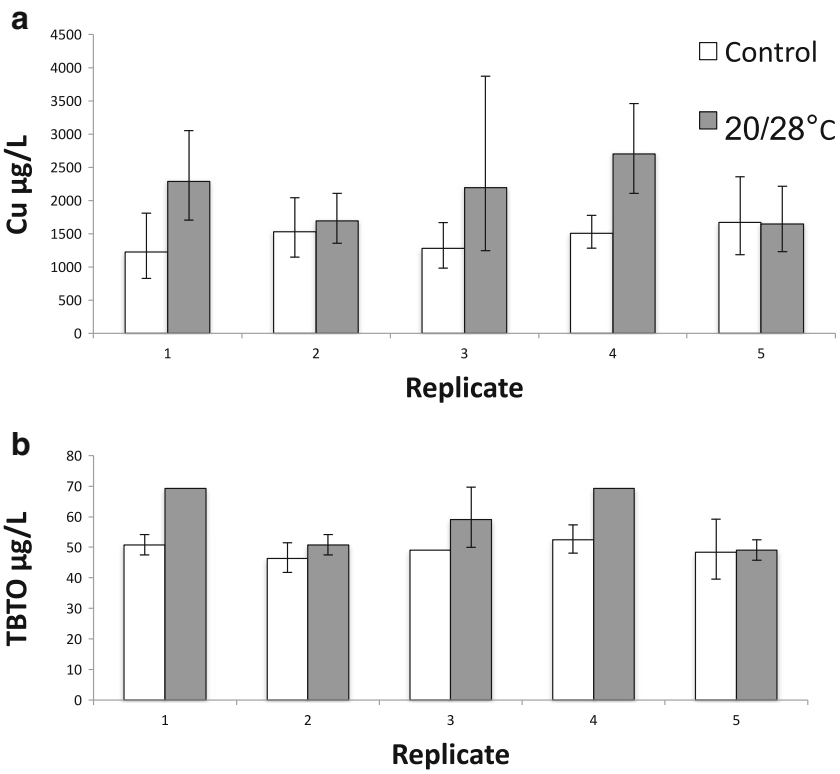

Fig. 5 Cycling temperature assay. LC50 values for copper (**a**) and TBTO (**b**), with error bars showing 95% CI. LC50 assays were conducted at 20 °C for 96 h on animals that had been maintained for an entire generation under one of two conditions: (1) control: stable 20 °C or (2) cycling temperature: a cyclic temperature treatment 20 °C:28 °C for 12 h:12 h daily

field is a complex mosaic of factors that would be incredibly difficult to replicate in the laboratory. However, by testing candidate factors, we can begin to understand the potential for individual stressors to contribute to tolerance changes.

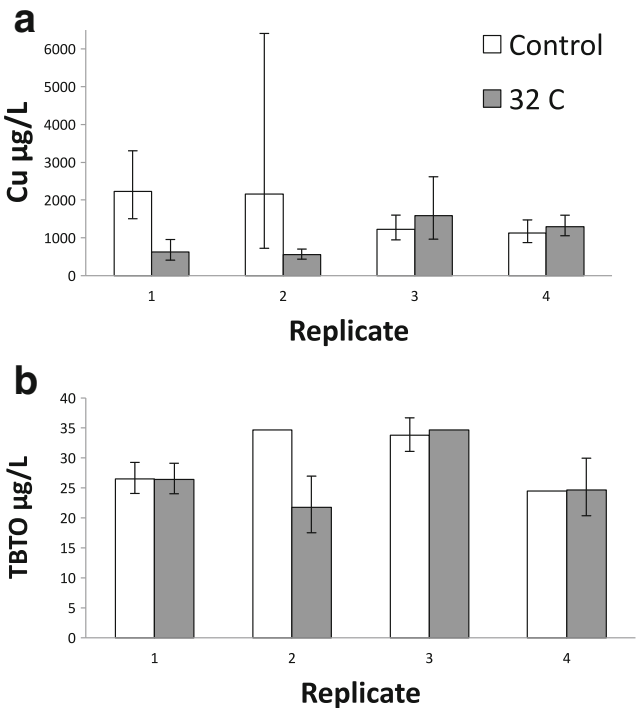

Fig. 6 Constant high-temperature stress assay. LC50 values for copper (**a**) and TBTO (**b**), with error bars showing 95% CI. LC50 assays were conducted at 20 °C for 96 h on animals from one of two treatments: (1) control: 20 °C or (2) temp: 32° for 4 h

Ultraviolet exposure

UV radiation decreased tolerance of the copepods to both chemicals. UV was chosen as a candidate stressor because it is a particularly strong and consistent influence in tide pool environments where these copepods can be found. Tide pools such as those inhabited by *Tigriopus* often have little to no permanent shade, which allows UV to be a daily presence in these environments. There is ample evidence of UV exerting a selective force on other organisms in the field (Rhode et al. 2001; Häder et al. 2007; Cockell and Blaustein 2013). Our results show that exposure to UV significantly reduced tolerance to both chemicals. Finite resources may be used to defend against UV in the field, which could in turn be reallocated in laboratory populations to mount a greater defense against a toxicity challenge. There seems to be a common mode of toxicity, namely oxidative stress, between UV and Cu and as a result, a potential for defense mechanisms to overlap is reasonable. An antioxidant, astaxanthin, found in copepods has been shown to be directly correlated with Cu tolerance as well as UV-B tolerance (Caramujo et al. 2012). However, because UV decreased tolerance to both chemicals, it does not seem likely to explain the chemical-specific shift observed in these copepods.

Diet treatments

The two diets were chosen based on their potential to represent two different types of diet regimes. The dried diet was composed roughly of dried fishmeal and dried cyanobacteria extract, which is a common dietary regime in the laboratory. In contrast, the live diet was a live culture of algae which is believed to be a much closer match to their natural diet (although no formal studies have been conducted what these particular copepods actually eat in the field). This is because these copepods inhabit splash pools that are irregularly inundated and any detritus that would simulate the dried diet would be an inconsistent nutrient source. On the other hand, photosynthetic microorganisms such as those delivered in the live algae diet are expected to be a constant nutrient source. Studies show a consistent presence of photosynthetic organisms in tide pools through the measurement of pH (Morris and Taylor 1983). During daylight hours, the pH is more basic because photosynthesis is offsetting CO_2 release from respiration, while during night hours, respiration continues without the abatement leading to an increase in pH.

Copepods raised on the dry diet had higher chemical tolerance than copepods raised on live algae. In other studies, different diets have been shown to alter amounts of astaxanthin (Caramujo et al. 2012) and other antioxidants (Gaetke and Chow 2003), which are known to influence Cu tolerance (Caramujo et al. 2012). However, preliminary data on astaxanthin concentrations in copepods raised on the two diets showed that copepods raised on live algae had higher concentrations of that carotenoid (unpublished data). With the dried diet, copepods could obtain higher concentrations of other antioxidants. The benefits of the dried diet could be due to a greater diversity in components. The dry diet contains animal protein (TetraMin contains fishmeal) as well as the photosynthetic cyanobacteria *Spirulina*, known as a source antioxidant including phycocyanin (Deng and Chow 2010).

Starvation assays

Results showed that a short period of starvation had no influence on Cu tolerance, but resulted in a substantial decrease in TBTO tolerance. This indicates that the reduction in TBTO tolerance in the laboratory could be attributed to dietary problems, despite our attempt to provide abundant food. This would be surprising for several reasons. First, *Tigriopus* is known to have extreme starvation resistance and can survive up to 90 days in autoclaved seawater (unpublished data), suggesting the presence of long-term energy stores including fat deposits such as those found in pelagic copepods (Lee et al. 2006). Second, Cu tolerance is known to be energetically demanding (Lukasik and Laskowski 2007), suggesting that the 1-week starvation treatment did not significantly reduce all energetically demanding biological functions. Third, there is little evidence that our standard feeding conditions lead to dietary problems, as we regularly maintain *Tigriopus* cultures for years using the same feeding conditions (e.g., Pritchard et al. 2013). Nevertheless, our results show that dietary restriction had a particularly negative impact on TBTO tolerance. This may be because TBTO mainly bioaccumulates in fat due to its lipophilic properties. Additionally, lipid stores can be adversely affected in the laboratory-cultured copepods by other factors such as body size even under replete conditions (Huho et al. 2007). Thus, under starved conditions, when an organism begins to metabolize its fat stores for energy, there is an increased exposure of more sensitive tissues to TBTO because there is less lipid to sequester TBTO. Additionally, metabolizing fat stores could release any inert TBTO stored in fat, thereby increasing exposure to TBTO and causing toxicity. The starvation effect may be further magnified by the fact that TBTO can also inhibit feeding and food assimilation rates (Mouneyrac et al. 2011). While our feeding strategy is sufficient to propagate laboratory cultures for years, these laboratory populations may have lower resource reserves than those in field populations. Ultimately, these results indicate that food availability has a chemical-specific impact on contaminant tolerance.

Temperature assays

The cycling temperature treatment produced trends toward higher chemical tolerance, suggesting a slight hardening effect. Similar hardening effects have been previously documented in *Drosophila* (Sejerkilde et al. 2003; Loeschcke and Hoffmann 2007). A temperature of 28 °C may be too low to induce significant stress. The average maximum summer temperature experienced in the SD habitat has recently been shown to be well above 28 °C (Leong et al. in press). Heat stress may have minimal effect on SD fitness at all temperatures below a small lethal window. Many intertidal organisms are already living at or close to their thermal limit (Somero 2010) and may have evolved the ability to maintain homeostasis until a tipping point very close to their lethal temperature. This would result in a very small thermal or temporal window to observe sublethal effects.

Constant heat stress for 4 days produced few changes in chemical tolerance. There is ample evidence that high temperature has exerted strong selective pressure on *Tigriopus* populations across its evolutionary history. *T. californicus* has been shown to be locally adapted to high-temperature stress and exhibits a strong cline in temperature tolerance (Willett 2010; Kelly et al. 2012). Likewise, it was discovered that in these copepods, there is a positive correlation between Cu tolerance and air temperature at collection site (Sun et al. 2015). However, these results show that constant heat stress had minimal effects on chemical tolerance. This again, may be

due to a very small thermal window where sublethal effects can be observed.

Conclusion

Tolerance to two common marine pollutants has been shown to change under benign laboratory conditions for two copepod species. Additionally, the direction of change is chemical specific, with Cu tolerance increasing and TBTO tolerance decreasing. This contradicts the expectation that organismal fitness will generally decline in the laboratory. A search for candidates to explain these changes revealed that chemical tolerance is significantly impacted by several environmental parameters. UV was shown to lower chemical tolerance while the standard laboratory diet was shown to increase chemical tolerance. These results suggest that laboratory populations, which are protected from UV and fed a protective diet, likely have certain chemical tolerances that are higher than their wild counterparts. This result, coupled with the observed increase in Cu tolerance under benign laboratory conditions, suggests that tolerance measurements derived from laboratory studies may drastically overestimate tolerance of wild populations in the field.

The exact mechanism responsible for the contrasting shifts in chemical tolerance remains elusive, but the present results demonstrate that chemical tolerance is highly labile. Understanding the pattern as well as the underlying cause of these changes will be critical for effective implementation of bioassays as a tool for environmental protection and chemical management.

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