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RESEARCH ARTICLE

# Variation in tolerance to common marine pollutants among different populations in two species of the marine copepod *Tigriopus*

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**Abstract** Geographical variation in chemical tolerance within a species can significantly influence results of whole animal bioassays, yet a literature survey showed that the majority of articles using bioassays did not provide detail on the original field collection site of their test specimens confounding the ability for accurate replication and comparison of results. Biological variation as a result of population-specific tolerance, if not addressed, can be misinterpreted as experimental error. Our studies of two marine copepod species, *Tigriopus japonicus* and *Tigriopus californicus*, found significant intra- and inter-specific variation in tolerance to copper and tributyltin. Because both species tolerate copper concentrations orders of magnitude higher than those found in coastal waters, difference in copper tolerance may be a by-product of adaptation to other stressors such as high temperature. Controlling for inter-population tolerance variation will greatly strengthen the application of bioassays in chemical toxicity tests.

**Keywords** Copper · Tributyltin · *Tigriopus japonicus* · *Tigriopus californicus* · Bioassay

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## Introduction

Bioassays are widely used in environmental risk assessment (ERA). A common approach is to use the response of a sentinel species as a biomarker of stressors like chemical contaminants. Bioassays have proven to be an effective and powerful ecotoxicological tool in assessing environmental impact of chemical contaminants. However, ERA can be complicated by variation in chemical tolerance within a species (Berthet et al. 2011). Distinct populations could have unique chemical tolerances tailored by their specific environments. Variation in other environmental factors could also generate variation in chemical tolerance between populations and lead to misleading ERA results.

Controlling for potential variation in chemical tolerance among populations is not widely applied in ecotoxicology. The majority of ecotoxicology publications lack collection site details. Even scarcer are GPS coordinates for accurate resampling of a particular population. Without a well-defined collection location, researchers may encounter challenges in replication and interpretation of bioassay data associated with significant inter-population variation in chemical tolerance. A literature survey (Fig. 1) found that the majority of test species used in toxicity bioassays used had no details regarding their original collection site. Only 11 % of all test species surveyed had the ideal level of detail for their collection site(s), which included location along with GPS coordinates, while 24 % of test species had only listed a collection site by name. In total, 35 % of test species surveyed provided some level of detail on the original collection site. An average of 1.4 different test species per article indicates that underreporting of collection site detail is not isolated to a few articles using a large number of types of test species but rather a wider practice. Although this small survey does not encompass the entirety of toxicity research, these results still provide strong

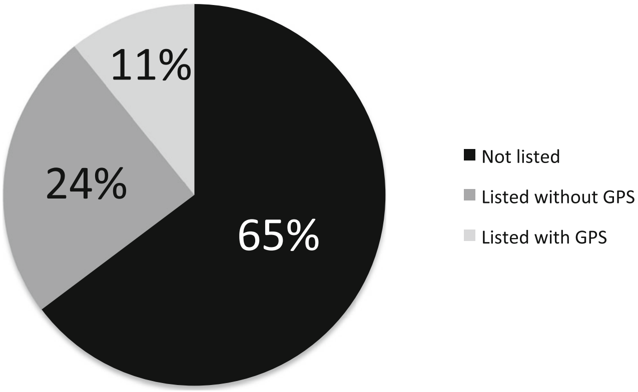

**Fig. 1** Ten years of research articles (Jan 2004–August 2014) were surveyed on the Thomson Reuters' Web of Science search engine using the search term "ecotoxicology bioassay." Species used for bioassays in each article were placed into one of three categories based on the collection information listed in the article. Results show the percentage of sets of test species in each category (94 articles, 139 sets of test species, mean of 1.4 sets of test species per article). Only bioassays using animals (i.e., multicellular, non-plant, non-fungal organisms) were considered in the survey

evidence that collection site information is frequently not reported, as only 35 % of the articles included details on the original collection site.

Variation in chemical tolerance has been found in species commonly used in ERA. Various crustaceans are known to have significant variation in their degree of chemical tolerance with and without direct chemical exposure. A population of *Daphnia longispina* collected from a copper (Cu)-contaminated site had higher Cu tolerance than populations from uncontaminated sites (Agra et al. 2009). Different populations within a species also have significant variation in copper (Cu), zinc, and cadmium tolerance without any previously documented exposure (Sarabia et al. 2002; Lukkari et al. 2004). The current study surveyed populations in two species of the marine crustacean *Tigriopus*, a genus of intertidal marine copepods that have been proposed as a model system for ecotoxicolgical studies such as characterization of stress response and pollution monitoring (Raisuddin et al. 2007). As an additional benefit, *Tigriopus* populations are globally distributed and span a very wide range of genetic distances, enabling population comparisons across a broad spectrum of geographic and evolutionary divergence (Edmands 2001; Ki et al. 2009; Peterson et al. 2013). This facilitates analysis of chemical tolerance for geographical trends in variation, such as the one seen for temperature (Willett 2010), which would give insight into the potential cause of variation. Our study compared two congeners, *Tigriopus japonicus* (*Tj*) and *T. californicus* (*Tc*), and five conspecifics (i.e., populations) within each species. The two species are closely related: no diagnostic phenotypic differences have been reported, and phylogenetic reconstruction based on ribosomal DNA (Ki et al. 2009) and mitochondrial DNA (Peterson et al. 2013) did not resolve the species into monophyletic clades. A study of phenotypic variation in *Tc* did not detect diagnostic differences among populations, but did show a pattern of larger body size and faster development rate in more northern populations (Edmands and Harrison 2003).

This study focused on two marine contaminants, copper (Cu) and tributyltin (TBT), each with different relationships with *Tigriopus*. At low concentrations, Cu is an essential micronutrient that serves key biological functions such as reversibly binding oxygen in hemocyanins in many invertebrates, but Cu is toxic at high concentrations (Philips and Rainbow 1993; Kwok and Leung 2005). As a result, Cu is one of the main antifouling paint additives in major use since the ban of TBT as an antifouling additive (Kwok et al. 2008). As a synthetic organotin compound, TBT does not occur naturally. This compound has been widely used as an antifouling biocide and a wood preservative only since the 1960s (Champ and Seligman 1996). Though there has been a ban on TBT as an antifouling additive, adopted in 2001 by the International Maritime Organization, it is still commonly found in coastal marine environments as a result of long retention in sediments and terrestrial input (Díez et al. 2002; Burton et al. 2005; Santos et al. 2010). There are several TBT salts that are commonly used; however, this study focuses on bis(tributyltin) oxide (TBTO). *Tigriopus* has a long-standing relationship with Cu evident by its incorporation of Cu as a micronutrient, while TBT serves no known biological function and has only been relatively recently introduced into marine environments.

The aim of the study was to test for differences in chemical tolerance due to long-standing differences between species and populations. Variation in chemical tolerance within a species can be attributed to either genetic or physiological changes. Genetic changes occur at the level of a population and result from changes in a population's genetic composition over the course of several generations. A physiological change occurs at the level of an individual and can develop quickly, such as within an organism's lifetime (Qiu and Qian 1999). There are examples of delayed effects of toxicants on individuals, even if exposed as embryos (Heintz et al. 2000; Weis 2014). To eliminate any short-term physiological changes, this experiment adopted the common laboratory practice of acclimating field-collected specimen for one generation in the lab prior to toxicity testing to eliminate any short-term physiological changes.

Initial results showed chemical tolerance far exceeding chemical concentrations the copepods are believed to experience, prompting the hypothesis that other environmental stressors may indirectly select for increased chemical tolerance. There are many candidate stressors in intertidal habitats including temperature, salinity, pH, dissolved oxygen, and UV radiation. As a preliminary test of the hypothesis, we conducted additional studies on temperature tolerance. High temperature stress is known to be a particularly important factor in limiting the tidal zonation and biogeographic range of intertidal organisms (Somero 2010; Helmuth et al. 2006). Further, *Tc* populations show strong differentiation in high-temperature tolerance (Willett 2010; Kelly et al. 2012), suggesting that temperature may be an important selective factor. Our study found differences in chemical tolerance among the geographically dispersed CA populations (maximum of ~640 km between sites), as well as among the more geographically proximate HK populations (maximum of ~34 km between sites). Even over short geographic distances, populations may still experience different thermal regimes due to differences in intertidal rock type, topography, and shading. Still, it is certainly true that other environmental factors (such as radiation, salinity, pH, and DO) may also play a role, especially in explaining microgeographic differences in tolerance.

The overarching goal of this study was to test for geographical variation in pollution tolerance, a pattern that would have important implications for how bioassays should be used to inform policy and regulation. Management practices based on test groups with high chemical tolerance would be detrimental to the native flora and fauna that might be more sensitive. Also, natural biological variation in tolerance could be misinterpreted as experimental error, thereby falsely eroding confidence in the accuracy of bioassays. Temperature tolerance was also examined in to identify patterns between pollution tolerance and another environmental stressor that could be contributing to variation in pollution tolerance. Unrecognized variation in tolerance among test specimens could lead to misinterpretation of toxicity results and establishment of inappropriate management practices.

## Materials and methods

### Specimen collection and maintenance

Five *Tj* populations were collected from Hong Kong in November 2011 and transported to the University of Southern California (USC), USA. The sites of collection were Gold Coast (22° 22′ 47″, 113° 58′ 48″) (GC), Ma Wan (22° 21′ 0″, 114° 3′ 36″) (MW), Shek O (22° 13′ 48″, 114° 15′ 36″) (SKO), Stanley (22° 13′ 11″, 114° 13′ 11″) (Stan), and Cape D'Aguilar (22° 12′ 36″, 114° 15′ 36″) (CDA) (Fig. 2). Five populations of *Tc* were collected from California, USA, in November 2011. The collection sites were Santa Cruz (36° 57′ 0″, −122° 2′ 59″) (SC), Santa Cruz Island (34° 1′ 12″, −119° 40′ 48″) (SCI), Leo Carrillo (34° 2′ 23″, −118° 56′ 23″) (LC), Laguna Beach (33° 32′ 23″, −117° 47′ 24″) (LB), and San Diego (32° 45′ 0″, −117° 15′ 36″) (SD) (Fig. 3). A population collected from Catalina Island (33° 27′ 0″, −118° 29′ 24″) (CAT) was used only for the heat stress assay.

All populations were maintained in 37 μm filtered autoclaved seawater (FASW) at 20 °C with a 12-h light/12-h dark photoperiod for one full generation before testing. Long-term 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 0.04 g TetraMin (Tetra Holding, Inc., USA) and 0.04 g Spirulina (Nutraceutical Science Institute, USA). In acute toxicity tests and heat stress assays, only adult males were used, because males are generally more sensitive to stress than females (Raisuddin et al. 2007; Kelly et al. 2012; P. Sun, unpublished data). Additionally, female tolerance to stress potentially fluctuates depending on whether they have been mated or stage of their egg sac. Females pass on pigmentation and other materially derived components to their offspring (Raisuddin et al. 2007), which potentially can increase their susceptibility to stress right after an egg sac is released or increase their tolerance of a portion of a chemical stressor is maternally deposited in the eggs decreasing the body burden on the female.

### Acute toxicity tests

Median lethal concentration ($LC_{50}$) assays were conducted for 96 h without feeding or solution renewal in FASW. The assays had three replicates of ten males for a total of 30 individuals per concentration. Mortality was assigned to individuals that did not respond to gentle prodding. The range of Cu and TBTO concentrations used were based on preliminary $LC_{50}$ values, which were obtained from pilot range-finding tests (data not shown). 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 concentrations used for *Tj* were 0, 127, 255, 509, 1018, 2036, and 3054 μg $L^{-1}$ Cu. The concentrations used for *Tc* were 0, 127, 255, 509, 1018, and 2036 μg $L^{-1}$ Cu. The TBTO stock solution was made by diluting bis(tributyltin) oxide (EMD, USA) with acetone to a concentration of 0.1 g $L^{-1}$. The concentrations used for both *Tj* and *Tc* were acetone control (i.e., 0), 20, 40, 60, 80, and 120 μg $L^{-1}$ TBTO. The amount of acetone in all treatment solutions was $\leq 1.2 \times 10^{-6}$ *v*/*v*.

### Heat stress assays

Thermal tolerance was measured by proportion of mortality at each temperature. Each temperature test had a 2-h ramp up from ambient temperature and a 1-h hold at the specific temperature followed by a 1-h recovery period at 20 °C before assessment. Temperatures for the heat stress assay were 37.3, 38.5, 39.5, 41.5, and 42.5 °C. Not all populations produced enough males for the additional heat stress assays and were therefore omitted from these assays. An additional population, CAT was a smaller collection, which lacked sufficient males

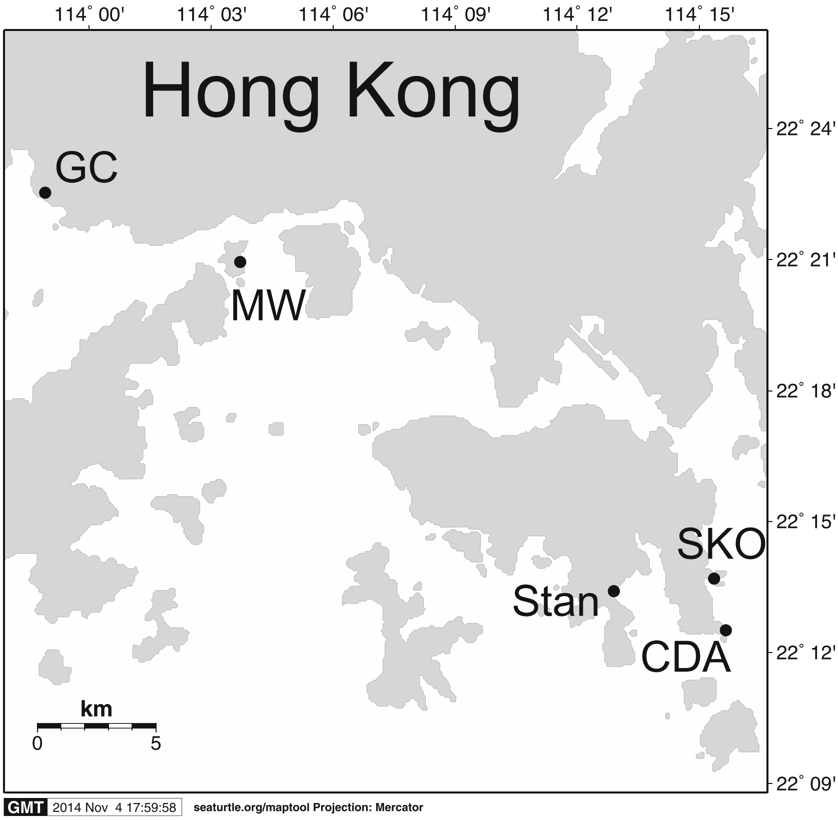

**Fig. 2** Map of Hong Kong territory with the sampling locations denoted by their respective population abbreviations, Gold Coast (*GC*), Ma Wan (*MW*), Shek O (*SKO*), Stanley (*Stan*), and Cape D'Aguilar (*CDA*)

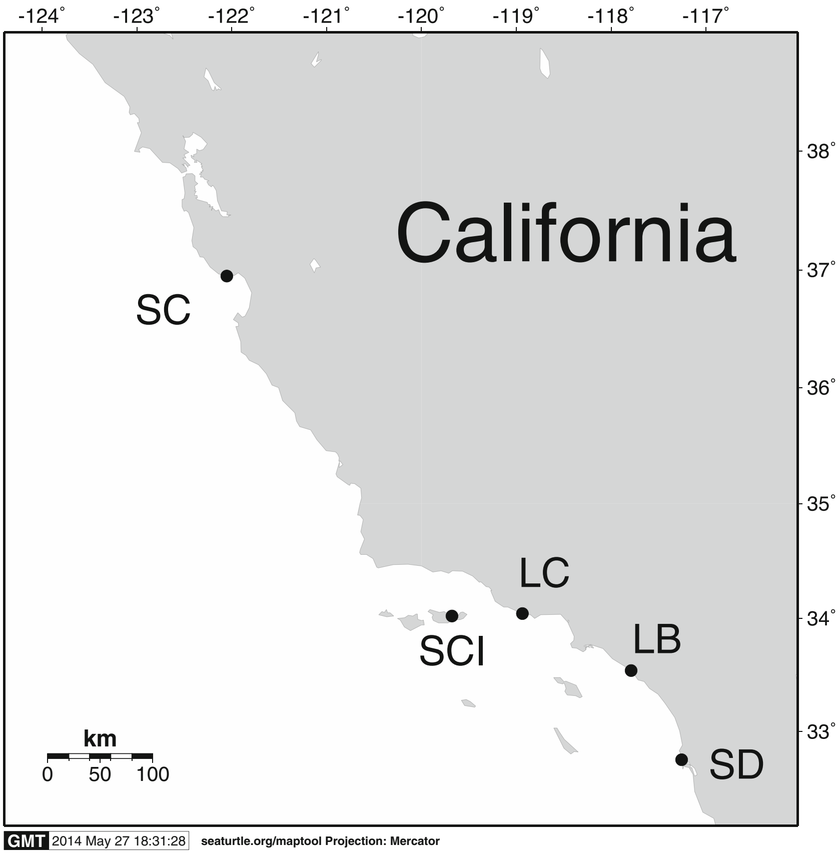

**Fig. 3** Map of the California coastline with the sampling locations denoted by their respective population abbreviations, Santa Cruz (*SC*), Santa Cruz Island (*SCI*), Leo Carrillo (*LC*), Laguna Beach (*LB*), and San Diego (*SD*)

for the toxicity tests, but did have enough males for the heat stress assays. The number of copepods tested at each temperature was limited by the number of males in each population available at the time of testing. A total of four populations were tested, two *Tj* populations (SKO and GC) and two *Tc* populations (LB and CAT). For temperatures 37.3 and 38.5 °C, 16 males were used per population. For temperatures 39.5 and 41.5 °C, 24 males were used. In 42.5 °C, eight males were used. For all tests, males were individually deposited into wells of a 96-well plate with 200 μL filtered seawater and sealed to reduce evaporation during heating, Peltier Thermocycler (PTC-200, MJ Research, USA).

## Data analysis

Based on the mortality data obtained from the acute toxicity assays, Trimmed Spearman–Karber Program v. 1.5 obtained from the US Environmental Protection Agency was used to calculate $LC_{50}$ values and their respective 95 % confidence intervals (95 %CI). $LC_{50}$ values were determined to be significantly different if their 95 %CI did not overlap. This method of comparison has been shown to be very conservative, often only able to identify highly significant differences (Wheeler et al. 2006). However, this method is appropriate for this study because we sought to find extreme differences between populations. Chi-squared tests were used to determine whether mortality in the heat stress assays differed significantly among the four tested populations.

Average daily maximum temperature near collection sites was obtained from the NOAA National Climatic Data Center, Global Summary of the Day (www.ncdc.noaa.gov) and correlated with each population's copper $LC_{50}$ (Fig. 4). For all sites, the climate station was chosen based on proximity to the collection site and whether it had the recorded temperature data for the 12 months prior to collection, December 2010 to November 2011. For the California sites, the following climate stations with the respective collections were used: #998173 (SC), #723910 (LC), #722910 (SCI), #722970 (LB), and #994018 (SD). For Hong Kong, only one station had the appropriate data, #450070 (GC). Mean daily maximum temperature was analyzed at each station using R (R Core Development Team. 2013) and package "MASS" (Venables and Ripley 2002). A linear regression analysis was done comparing temperature data and Cu $LC_{50}$ data of all corresponding populations and a separate regression analysis compared sites within CA only. Temperature data from coastal climate stations was chosen over ocean temperature obtained from moorings for two reasons. First, we believe temperature in tide pools are better reflected by air temperature than ocean temperature. This is due to their small volumes, position in the supralittoral zone, and inconsistent rehydration mainly through sea spray rather than inundation by wave action, which decreases the influence of incoming seawater on tide pool temperature. Second, the location of moorings was sparse relative to coastal climate stations. In several cases, this resulted in mooring sites being at a much greater distance from the collection site than the nearest coastal station.

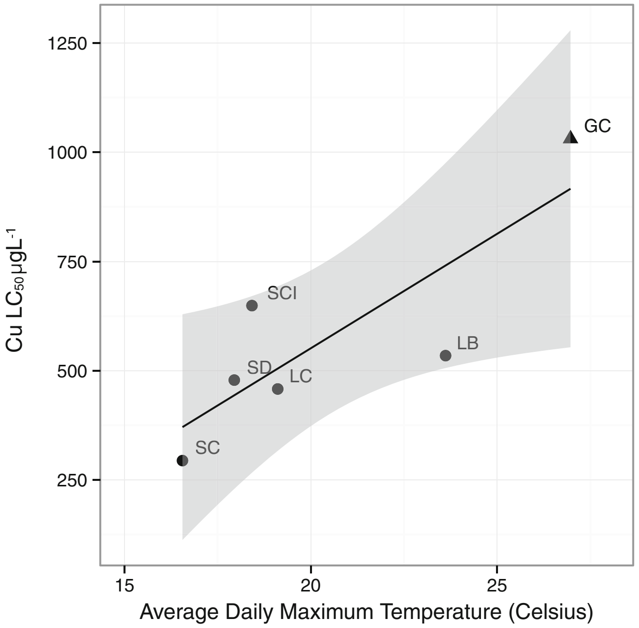

**Fig. 4** A linear regression between average daily maximum temperature and Cu tolerance. Temperature data was obtained from NOAA National Climatic Data Center, Global Summary of the Day (www.ncdc.noaa.gov) from December 2010 to November 2011 at climate stations near five Californian sites, Santa Cruz (*SC*), San Diego (*SD*), Santa Cruz Island (*SCI*), Leo Carrillo (*LC*), and Laguna Beach (*LB*) and one climate station near the Hong Kong site, Gold Coast (*GC*). The *shaded region* indicates the 95 % confidence interval for the linear model fit. $R^2=0.6072$, F-statistic=14.91, p=0.004796

## Results

In the acute toxicity tests, the control treatments experienced >99.99 and 100 % survival for *Tj* and *Tc*, respectively. There is a clear segregation of $LC_{50}$ values between species and variation in tolerance within each species for Cu. Populations of *Tj* show an overall higher tolerance to Cu than its congener *Tc* (Fig. 5). The average $LC_{50}$ value for *Tj* is 3.9 times higher than that for *Tc*. The most tolerant *Tj* population, SKO, has an $LC_{50}$ value 2.4 times higher than the least tolerant *Tj* population, GC. The most tolerant *Tc* population, SCI, has an $LC_{50}$ value 2.2 times higher than the least tolerant *Tc* population, SC. Though there is a large numerical difference in $LC_{50}$ values between the two species, the pattern of tolerance within each species is very similar. There is roughly a twofold difference between the most and least tolerant population.

Tolerance to TBTO is not as distinct as Cu tolerance between the copepod species. On average $LC_{50}$ values are 1.3 times higher in *Tj* than *Tc* (Fig. 6). The mean $LC_{50}$ values of

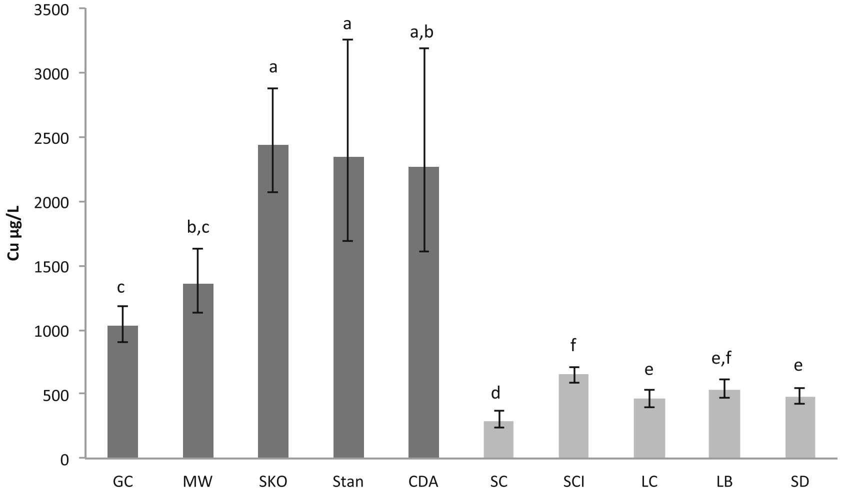

**Fig. 5** Cu median lethal concentration ($LC_{50}$, low/high 95 %CI) of *T. japonicus* in *dark gray* and *T. californicus* in *light gray*. Both species are arranged from highest to lowest latitude. *Error bars* denote 95 %CI. *Letters above each bar* indicate grouping. Populations with *two allocated letters* have 95 %CI that span two groups. GC (1030.8, 903.54/1178.42 μg $L^{-1}$ Cu), MW (1356.58, 1132.6/1626.37 μg $L^{-1}$ Cu), SKO (2438.28, 2069.23/2876.05 μg $L^{-1}$ Cu), Stan (2344.11, 1690/3255.28 μg $L^{-1}$ Cu), CDA (2265.21, 1608.55/3189.11 μg $L^{-1}$ Cu), SC (294.24, 239.25/363.96 μg $L^{-1}$ Cu), SCI (649.02, 587.94/715.19 μg $L^{-1}$ Cu), LC (458.13, 397.05/529.4), LB (534.49, 470.86/608.3 μg $L^{-1}$ Cu), SD (478.49, 422.5/544.67 μg $L^{-1}$ Cu)

the majority of *Tj* and *Tc* populations overlap with a population from the other species with the exception of the most tolerant (Stan) and least tolerant population (SCI).

Thermal tolerance was found to be significantly different between the two species. Differences in survivorship within each species across all temperatures, *Tj* ($\chi^2<4.60$, $p>0.05$) and *Tc* ($\chi^2<3.08$, $p>0.05$), were not significant (Fig. 7). However, heat stress survivability between *Tj* and *Tc* was significantly different across treatments ($\chi^2>22.42$, $p<0.001$). For five CA sites and one Hong Kong site, a linear model for maximum daily air temperature near collection sites and Cu tolerance of the resident copepod population was found to be positive and significant ($R^2=0.6072$, $F$-statistic=14.91, $p=0.004796$). Among only the five CA sites, the linear regression between site temperature and Cu tolerance was not significantly better than the null model ($R^2=0.03989$, $F$-statistic=0.1246, $p=0.7474$).

## Discussion

The results of the literature survey showed that the majority of test species considered did not have details regarding where they were originally collected. Our results clearly show

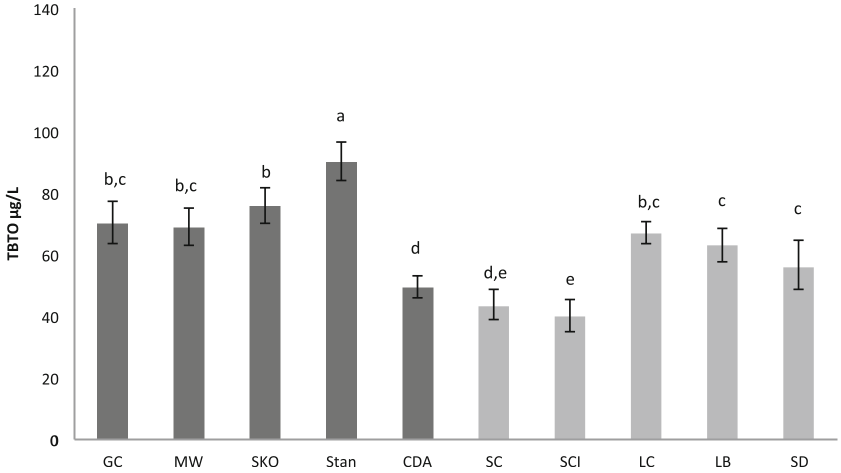

**Fig. 6** TBTO median lethal concentration ($LC_{50}$, low/high 95 %CI) of *T. japonicus* in *dark gray* and *T. californicus* in *light gray*. Both species are arranged from highest to lowest latitude. *Error bars* denote 95 %CI. *Letters above each bar* indicate grouping. Populations with *two allocated letters* have 95 %CI that span two groups. GC (70.02, 63.58/77.12), MA (68.82, 62.98/75.2), SKO (75.69, 70.11/81.72), Stan (90.07, 84.06/96.50), CDA (49.28, 45.68/53.16), SC (43.47, 38.9/48.59), SCI (39.71, 34.78/45.33), LC (66.92, 63.41/70.64), LB (62.75, 57.65/68.30), SD (56.06, 48.56/64.72)

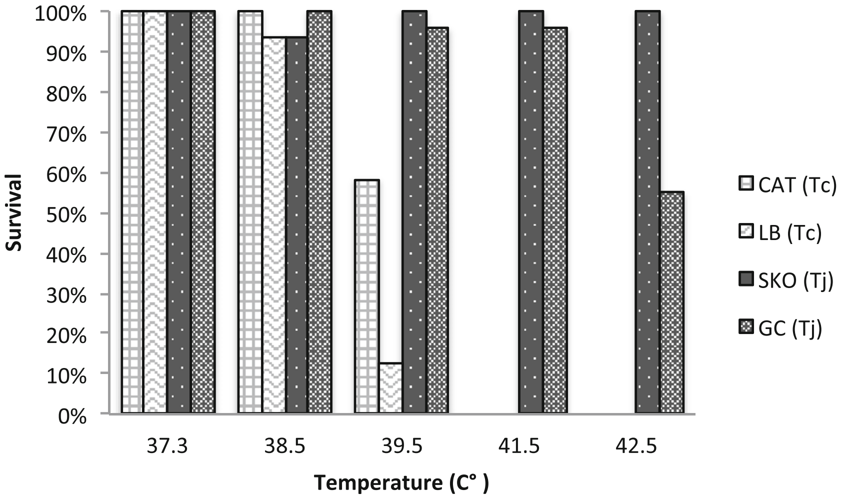

**Fig. 7** Percent survival following higher temperature stress in two *T. californicus* populations, CAT and LB, and two *T. japonicus* populations, SKO and GC, over five temperatures. *T. californicus* is shown by the *lighter patterns* while *T. japonicus* is shown by the *darker patterns*

significant variation in tolerance of Cu and TBTO among different populations of the same species of *Tigriopus* toward Cu and TBTO. However, it has been shown that *Tigriopus* generally has low variability and high repeatability in measurements of acute and life cycle toxicities within the same population (Kwok et al. 2008). Inter- and intra-population differences in chemical tolerance are not unique to *Tigriopus*. Some of the strongest examples come from the ecotoxicological model system, *Daphnia*. Differences to Cu tolerance within populations have been observed in *Daphnia longispina* with differences as great as 7× between the most sensitive and most tolerant clonal lineages (Martins et al. 2007). Differences between species also have been found in *Daphnia* (Winner and Farrell 1976). Providing accurate collection details will, therefore, allow for accurate interpretation of bioassay results when comparing two different populations or allow for accurate resampling of a single population for appropriate comparisons between experiments.

Among all populations assessed, Cu tolerance is higher in *Tj* than *Tc* without any overlap in tolerance between the two species. Higher Cu tolerance can be attributed to either acclimation, a physiological plastic response that can occur within an individual's lifetime, or adaptation, an adaptive heritable response that occurs at the population level over generations (Berthet et al. 2011; Mouneyrac et al. 2011). Both *Tj* and *Tc* have been shown to acclimate to Cu (Kwok et al. 2009; Sun et al. 2014). However, the current study controlled for potential acclimation to different field conditions by maintaining all populations in the lab for one full generation (about 21 days) before testing. Additionally, laboratory cultures of *Tigriopus* did not show a heritable adaptive response (but only phenotypic plasticity) after three generations of exposure (Kwok et al. 2009) or a longer 12 generations of exposure (Sun et al. 2014). Although adaption to Cu could have occurred over longer periods, such as hundreds of generations, Cu tolerance in *Tigriopus* does not appear to be a direct adaptation to elevated Cu exposure. This is because dissolved Cu concentrations in the coastal waters have orders of magnitude lower than the Cu $LC_{50}$ values obtained in this study. Cu tolerance seems to be an exaptation, a trait that has evolved for one role but subsequently has come to fill another role (Gould and Vrba 1982).

Measured levels of dissolved Cu in the surface waters adjacent to these *Tigriopus* populations are relatively low compared to their Cu tolerance. The average Cu $LC_{50}$ value is 31, 000 and 5000 times greater than coastal Cu concentrations for *Tj* and *Tc*, respectively. Surface water in the South China Sea had a measured concentration of 0.06 $\mu g\ L^{-1}$ (Wen et al. 2006). A close examination of the shelf waters near Hong Kong showed dissolved Cu concentrations between 0.02 and 0.06 $\mu g\ L^{-1}$ (Wang et al. 2012). Similarly, around the coast of California dissolved Cu concentrations were relatively low, $0.09 \pm 0.06\ \mu g\ L^{-1}$ (mean±standard deviation) (Smail et al. 2012). Preliminary results (Sun et al., unpublished data) show that dissolved Cu in Californian tide pools was comparable to coastal concentrations. Chemical tolerance within species does not appear to correlate with general pollution levels. For example, of the five CA sites, SCI is the furthest from ports and might be expected to be the most pristine location, and yet, it has the highest copper tolerance. As a result, the variation in Cu tolerance in *Tigriopus* does not appear to be a direct adaptation to Cu exposure.

The difference in Cu tolerance between *Tj* and *Tc* could be linked to variation in thermal tolerance. *Tj* populations have higher thermal tolerance and Cu tolerance compared to *Tc* populations. In support of this hypothesis, we found a significant positive correlation between temperature near a collection site and the Cu tolerance of the corresponding copepod population ($t=4.34$, $p<0.0025$). Defense mechanisms for thermal tolerance in theory could be used to respond to Cu stress because of overlapping toxicity pathways. For instance, both high temperature (Abele et al. 2002) and Cu (Rhee et al. 2013) have been shown to cause oxidative stress through the generation of reactive oxygen species (ROS). Adaptation in *Tj* for thermal tolerance could be co-opted and used to alleviate, at least in part, ROS toxicity generated by elevated Cu exposure. One of many candidate defense mechanisms includes heat shock proteins (hsp), which are recruited under both heat and Cu stress (Boone and Vijayan 2002; Rhee et al. 2009). We found thermal tolerance in *Tj* to be significantly higher than *Tc* (Fig. 7) with a significant correlation between average daily maximum temperature near collection site and Cu tolerance (Fig. 4). Furthermore, thermal tolerance of *Tc* has been shown to fall along a latitudinal gradient with northern populations being less thermally tolerant (Willett 2010). Consistent with this pattern, the current study found *Tj* from a lower latitude (22° 12′–22° 22′) has higher Cu tolerance than *Tc* from a higher latitude (33° 24′–36° 57′). These findings show that the most northern populations, SC and GC, had the lowest Cu tolerance. In addition, SC was shown to have lower *hsp* expression during heat stress than SD, a southern population (Schoville et al. 2012). However, a larger sample of populations with corresponding environmental data would be needed to support this hypothesis. Currently, only $LC_{50}$ values of a few populations are available for comparison. Whether there is a direct link between thermal tolerance and Cu tolerance remains to be seen.

TBTO tolerance exhibits a general pattern similar to that seen with Cu tolerance. The pattern of TBTO tolerance roughly mirrors Cu with *Tj* being more robust than *Tc*. This could be due to at least a partially shared toxicity pathway between the species, which elicit similar defense mechanisms. For instance, TBTO has also been known to cause oxidative stress (Ishihara et al. 2012; Katika et al. 2011). A general oxidative stress defense mechanism could be responding to both of these chemical exposures. Therefore, Cu and TBTO tolerance would correlate with an organism's ability to respond to oxidative stress as shown by our results. Differences in oxidative stress mechanisms and heat stress response could explain the general pattern of *Tj* being more robust than *Tc* as shown in the present results. However, comparisons of individual populations show a more complex picture.

Differences between individual populations point to additional chemical specific responses. For instance, CDA and SC have a similar level of tolerance to TBTO, yet their Cu tolerances are significantly different. This pattern of similar TBTO tolerance, but different Cu tolerance is exhibited by three other pairings between *Tj* and *Tc*. Differences are also found within species with SC and SCI having similar TBTO tolerance but significantly different Cu tolerance. Alternatively, there are populations such as SKO and Stan that have similar Cu tolerance but different TBTO tolerance. Some *Tc* populations also have higher TBTO tolerance than *Tj*, such as SD and CDA. The rank order of population tolerances for copper is not the same as the rank order of population tolerances for TBTO. These results show that there are finer differences in tolerance to Cu and TBTO within each species. Differences in chemical tolerance among *Tigriopus* populations cannot be explained solely by differences in a particular suite of defense mechanisms such as for oxidative stress, rather there are likely other factors that contribute to the overall chemical response in these species.

The literature survey of bioassays used over the span of 10 years revealed that the majority of articles do not include details regarding collection site. This could prove to be detrimental to ecotoxicological studies if test organisms collected from multiple populations have significant differences in chemical tolerance. Pooling of test organisms in this manner may introduce significant inter-population biological variation. This variation can be introduced as an exaptation that may be difficult to predict due to complex response mechanisms. Tolerance can also be chemical specific with populations being robust to one chemical, yet extremely sensitive to another. This hinders the ability to extrapolate inter-population comparisons from one chemical to another.

An effective way to control for the variation in chemical tolerance and to minimize errors in bioassays in ERA is to have a documented collection history. This would allow for consistency across bioassays and allow for accurate replication. Otherwise, biological variation may be mistakenly attributed to experimental error and erode the effectiveness of this valuable technique. Additionally, understanding how other environmental factors, such as temperature, influence chemical tolerance can give rise to patterns that provide general predictions on how chemical tolerance can change across different populations of a species. Bioassays remain a foundational assessment tool for the field of ecotoxicology and can be greatly strengthened by controlling for variation in chemical tolerance with proper details regarding the origin of test specimens.

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**Compliance with ethical standards** The authors assure that all procedures were performed in compliance with national and institutional guidelines for the protection of animal welfare.

**Conflict of interest** The authors declare that there is no conflict of interest.

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