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Joint effects of temperature and copper exposure on developmental and gene-expression responses of the marine copepod *Tigriopus japonicus*

Adela Jing Li[1,2] · Racliffe Weng Seng Lai[3,4] · Guang-Jie Zhou[3] · Priscilla To Yan Leung[3] · Eddy Y. Zeng[5] · Kenneth Mei Yee Leung[3]

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Abstract

There is growing contamination of copper (Cu) in the marine environment, particularly after the ban of organotin compounds and the increase of the use of Cu-based antifouling paints. Although there are increasing research interests in temperature-dependent chemical toxicity to aquatic organisms, most existing studies focused on acute impacts of chemicals at high concentrations. This study aimed to investigate the interacting effect of temperature and copper exposure at environmentally relevant concentrations on survival and development in the marine copepod *Tigriopus japonicus* with a partial life-cycle toxicity test. Expressions of five stress response genes in the copepod, namely two glutathione S-transferases (GST-S and GST-O), two heat shock proteins (HSP70 and HSP90), and glutathione reductase (GR) were also investigated. The copepod's survival was significantly impaired at 15 °C after development to adult stage, while its developmental time reduced significantly with increasing temperature. Copper at the two environmentally relevant test concentrations had no significant impacts on these apical endpoints whereas the interaction between Cu and temperature was more significant in modulating gene expressions. GST-S, GST-O and HSP90 genes in copepods exposed to 100 µg Cu L^{-1} were significantly upregulated at 20 °C. At 32 °C, most genes were either insignificantly expressed or down-regulated, compared to the control, likely suggesting that thermal stress inhibited the copepod's antioxidative defense system. Overall, the results revealed that the joint Cu and thermal stresses have significantly elicited antioxidative system in the copepods. It clearly demonstrated the need for more fundamental studies about potential impacts of different environmental factors such as temperature on chemical toxicity under realistic scenario of marine pollution.

Keywords Trace metal · Multiple stressors · Chronic toxicity · Sub-lethal responses · Gene expression · Antioxidant responses

Introduction

Aquatic environments are the final sink of anthropogenic contaminants (Häder et al. 2020). Copper (Cu) is a

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✉ Racliffe Weng Seng Lai
wslai@um.edu.mo

✉ Kenneth Mei Yee Leung
kmyleung@cityu.edu.hk

[1] The Swire Institute of Marine Science and School of Biological Sciences, The University of Hong Kong, Pokfulam, Hong Kong 999077, China

[2] College of Natural Resources and Environment, South China Agricultural University, Guangzhou 510642, China

[3] State Key Laboratory of Marine Pollution and Department of Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong 999077, China

[4] Department of Civil and Environmental Engineering, Faculty of Science and Technology, University of Macau, Macau 999078, China

[5] School of Environment, Jinan University, Guangzhou 510632, China

ubiquitous pollutant in the marine environment with increasing application (Leal et al. 2018; Yan et al. 2010). In particular, after the ban of organotin-based antifouling paints in the early 1990s in many countries, there was growing application of Cu-based antifouling coatings on ship hulls as a replacement (Bao et al. 2011; Dafforn et al. 2011). Leaching rates of Cu from these coatings varied between 4.4 and 27.5 μg cm^{-2} day^{-1} and such release has become a major source of Cu pollution in coastal seawaters and sediments (Lagerström et al. 2020). Despite being an essential metal for many biological processes, excessive uptake of free Cu in the body could catalyse the formation of reactive hydroxyl radicals, leading to excessive oxidative stresses and interference to important cellular activities (Gaetke and Chow 2003). Toxicity of Cu has been associated with interrupted membrane stability, cellular dysfunction, metabolic non-homeostasis and can ultimately adversely affect biological performances such as heartbeat and feeding rate in marine invertebrates (Brown et al. 2004). The ecological risk of Cu requires further characterisation.

There are growing concerns regarding if environmental factors, such as changes in temperature could affect the vulnerability of organisms and consequently the toxicity of chemical contaminants to them (Lai et al. 2016; Noyes et al. 2009; Zhou et al. 2014). Some studies have investigated temperature-induced changes of chemical toxicity to organisms (e.g., Brown et al. 2017; Li et al. 2022; Verheyen et al. 2019). However, the joint toxic response and mechanisms have not been fully understood. Temperature-dependent chemical toxicity to aquatic organisms could be chemical-specific (Mao et al. 2019) and species-specific (Li et al. 2014; Pereira et al. 2017). Besides, existing studies mostly concentrated on acute adverse impacts of chemicals at high concentrations, while organisms in the environment are typically exposed to much lower concentrations of the chemicals throughout their daily activities (Seeland et al. 2013).

Copepoda is the second largest crustacean taxa and represents 70% of the ocean biomass (Raisuddin et al. 2007). The test copepod *Tigriopus japonicus* resides in supralittoral rocky shore pools and has a broad distribution along Western Pacific regions such as Japan, South Korea, and China (Raisuddin et al. 2007). They have a short life cycle and distinct life stages, and are crucial primary consumers in the marine food web (OECD 2011; Raisuddin et al. 2007). A set of stress-associated genes and their corresponding enzymes have been identified in *T. japonicus*, providing a concrete basis for detecting early stresses faced by the copepod. For example, upon exposure to xenobiotics, glutathione reductase (GR) could reduce glutathione disulphide to glutathione, which could then conjugate with the xenobiotics by glutathione S-transferases (GSTs) to generate water soluble products for easier elimination of the xenobiotics during phase II detoxification (Park et al. 2019; Wong et al. 2020). Induction of heat shock proteins (HSPs) is also deemed a physiologically protective and genetically conserved response to various environmental stressors (Park and Lee 2021). All these have made *T. japonicus* an ideal model organism for ecotoxicity testing.

We hypothesized that variation in temperature could affect the chronic toxicity of copper at environmentally relevant concentrations towards the marine copepod *T. japonicus*. Apart from mortality, multiple sub-lethal endpoints were monitored, including developmental time from nauplius to copepodite and adult stages, as well as modulation in stress response genes. This study will provide fundamental and essential knowledge regarding how an ecologically-important marine crustacean responds to the concerted challenges posed by copper and temperature under an environmentally realistic scenario.

Materials and methods

Test species and chemical preparation

The intertidal harpacticoid copepods *T. japonicus* were collected from supralittoral rock pools in the Cape D'Aguilar Marine Reserve, Hong Kong. The range of diurnal temperature variations was 26–34 °C and 15–19 °C for summer and winter, respectively in the region where the copepods were collected for the current study (Chan 2000). They were acclimated for at least one month before experimentation under laboratory conditions in filtered artificial seawater (FASW; salinity: 33 ± 0.5‰, using sea salt of Instant Ocean and filtered through 0.45 μm cellulose nitrate membrane). The acclimation conditions are as follows: temperature: 23–25 °C, pH: 8.1–8.4; light-dark cycle 12: 12 h with light density around 1500 lux. The alga *Tetraselmis suecica* (about 10^6 cells L^{-1}) was used as the food for the copepod.

A stock solution of 1 g Cu L^{-1} was prepared in Millipore water using copper sulphate pentahydrate (≥ 99.5%; BDH Chemicals Ltd. Pode, England) and further diluted in FASW to obtain the working solutions. The sampled working solution was digested with 2% HNO_3, followed by measurement with the Inductively Coupled Plasma-Optical Emission Spectroscopy (PerkinElmer ICP Optima 8300). Measured Cu concentrations in the working solutions were >80% of the nominal concentrations within 48 h during the semi-static waterborne exposure.

Chronic toxicity test

Prior to the experimentation, gravid females were randomly selected, and the newly hatched nauplii (< 24-h) were immediately allocated to individual wells of the 12-well

Table 1 Sequences of specific primer pairs used in quantitative real-time PCR analyses

Gene	Direction	Specific primers (5'→3')
18 S (reference gene)	F	TCGGGCTGTCTCGTTGGTGATTC
	R	TGCCACAGTCGACAGTTGATAGG
GR	F	CCATGACGGACAGAAAGCAGATGAC
	R	CTCCCATCTTGATGGCAACTCC
GST-O	F	CGGTCCATTGCATGGTTTGATG
	R	CGTTCAAACCATGGCCAAATAG
GST-S	F	ATGACTGGATTCGGATTTGGAC
	R	GGCGTTTGGTCACATATTCGG
HSP70	F	AATTCGCCGACAAGCTCAAG
	R	CATACCTCCGGGCATTCCAC
HSP90	F	CATTCTCAACGCATCCACC
	R	AGTTCGGGCATCTCTTCGG

sterile tissue culture plate (Falcon, France) with 2 mL test solution or seawater in each well for exposure. No temperature acclimation was performed for newly hatched nauplii due to their fast development, particularly under higher temperatures. Four common environmental temperatures (15, 20, 25 and 32 °C) at their habitats were tested and were precisely controlled using a water bath (± 0.1 °C; Grant, UK). There were two chemical concentrations: 20 µg Cu L^{-1} (an environmentally relevant maximum concentration: Wang et al. 2003) and 100 µg Cu L^{-1} (a higher concentration representing a worst-case scenario: Li et al. 2014), in addition to an FASW control. Each of the three replicates contained 10 nauplii, i.e., an experiment of 4 temperatures × 3 Cu concentrations × 3 replicates × 10 nauplii = 360 nauplii in total. Test conditions were identical to those applied in the acclimation period except for varied test temperatures. During the exposure, *T. suecica* (10^6 cells L^{-1}) was provided as food and the test solution was renewed every 48 h. The toxicity endpoints included mortality, the developmental time from nauplius to copepodite stage 1 (N-C) and developmental time from nauplius to adult stage (N-A). Developmental stage of the copepods was determined as described in Raisuddin et al. (2007). The endpoints were monitored once every 24 h, and until all survived copepods turned into adults.

Gene expression analyses

To investigate the gene expression patterns and toxic mechanisms of the copepods upon exposure to different combinations of temperature and waterborne Cu concentration, another 24 h acute exposure experiment was performed using randomly selected adult *T. japonicus* (Park et al. 2017). The selected genes include glutathione reductase (GR), two glutathione S-transferases (GSTs, GST-S and GST-O), and two heat shock proteins (HSPs, HSP70 and HSP90). The gene of 18 S rRNA was used as a reference in this study because it was commonly used as the final reference gene in previous studies with *T. japonicus* (Kim et al. 2014; Han et al. 2018a and 2018b). Their primer sequences are listed in Table 1. These genes have been commonly applied in toxicological studies using *T. japonicus* and proved to be sensitive indicators of temperature and chemical stresses (Han et al. 2018a and 2018b; Lee et al. 2019; Wong et al. 2020).

Prior to the exposure, the copepods were acclimated from the original culture temperature (23–25 °C) to 15, 20, and 32 °C, respectively, at a thermal change rate of 1 °C/h and were further acclimated under each test temperature for 72 h. Consistent with the chronic toxicity test, two copper concentrations (20 and 100 µg Cu L^{-1}) and a FASW control were used. There were four replicates for each treatment and each replicate contained around 50 copepods. Approximately 2400 copepods were used, i.e., an experiment of 4 temperatures × 3 Cu concentrations × 4 replicates × 50 copepods.

After exposure of 24 h, the copepods in each replicate were fixed by 500 µL RNA*later* (RNA*later*™ RNA Stabilization Reagent, QIAGEN, Hilden, Germany) for quantitative real-time polymerase chain reaction (RT-qPCR). The samples were kept in 4 °C overnight before storage in −20 °C. Total RNA was extracted from each sample using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) and treated with DNase I (DNase-Free DNase Set, QIAGEN, Hilden, Germany), according to the manufacturer's instruction. The extracted RNA of each sample was then transcribed to cDNA using the High-Capacity RNA-to-cDNA™ Kit (Invitrogen, Grand Land, USA). The amplification reactions with SYBR green (iQ™ *SYBR*® green supermix, Bio-Rad, USA) fluorescence dye were performed in duplicate on a CFX96™ Real-Time System (Bio-Rad, USA). The universal thermal cycler protocol was adopted as follows: 95 °C/3 min; 40 cycles of 95 °C/10 s, 60 °C/30 s. To confirm the amplification of specific products, melt curve analysis was measured: 10 s

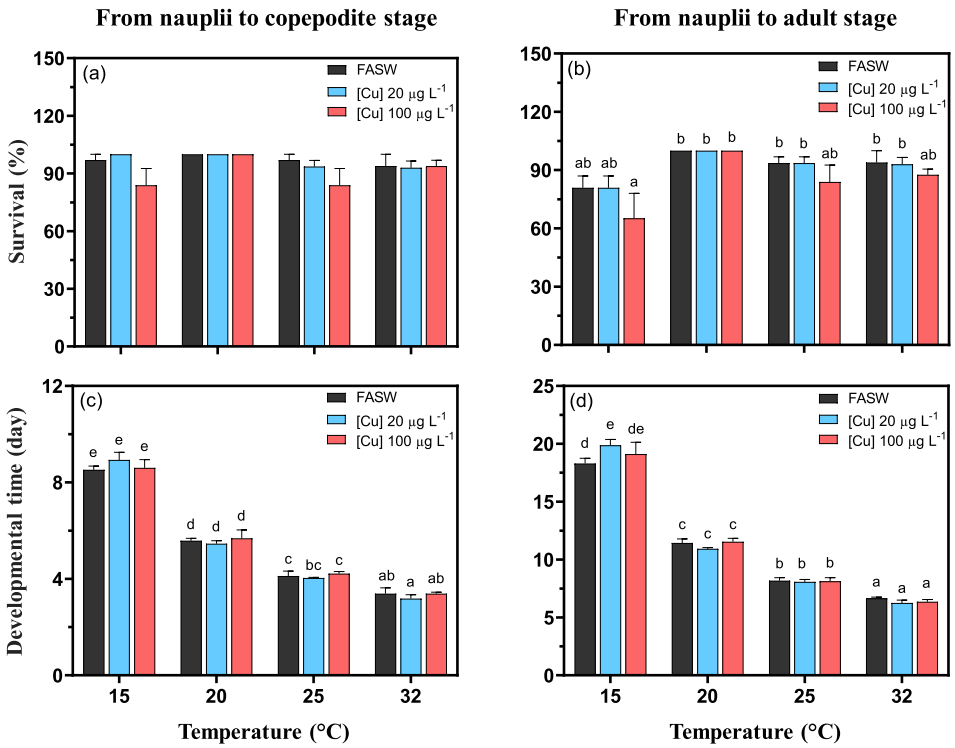

Fig. 1 Toxicological responses of *Tigriopus japonicus* upon the interacting effects of temperatures (15, 20, 25, and 32 °C) and Cu treatments (FASW control, 20 and 100 μg Cu L^{-1}): **a** Survival during development from nauplius to copepodite stage 1, **b** Survival during development from nauplius to adult stage, **c** Developmental time from nauplius to copepodite stage 1 and (**d**) Developmental time from nauplius to adult stage. Bars are expressed with mean ± 1 SEM ($n = 3$). Different letters represent significantly different means ($p < 0.05$, Student-Newman-Keuls test, one-way ANOVA)

preheating at 95 °C, then heating up gradually from 65 to 95 °C by 0.5 °C and reading the plate in 5 s at each step. Threshold cycle (C_T) values were recorded to evaluate the relative expressions of the target genes by $2^{-\Delta\Delta C_T}$ method relative to the reference gene 18 S rRNA.

Data analysis

Data analyses were performed using SPSS version 23 (SPSS, Chicago, USA). Two-way analysis of variance (ANOVA) using temperature and Cu treatments as fixed factors was applied to infer the effect of each factor alone and their interaction. To compare the difference among individual treatments, one-way ANOVA with *post-hoc* Student-Newman-Keuls (SNK) test was performed. Variation in the gene expressions of the copepods was also evaluated using a multivariate Principal Coordinates Analysis (PCO) and Permutational Multivariate Analysis of Variance (PERMANOVA, PRIMER v7 with PERMANOVA+). Pseudo-*F* and *p*-value were used for statistical significance interference (Clarke and Gorley 2015).

Results and discussion

Effects on survival and developmental time

Most of FASW controls and treatments had > 80% of survival when reaching the copepodite and adult stages. No mortality was found at 20 °C (Fig. 1a, b). The decline of survival only became significant under 15 °C during the developmental stage from nauplius to adult copepod (Temperature $F_{3,24} = 9.309$, $p = 0.0003$, Fig. 1b, Tables S1 and S2). There was also a general decline of copepod survival at 100 μg Cu L^{-1} for all temperatures except for 20 °C (Fig. 1a, b). This suggested that temperature deviations from the optimal range of the copepods may increase their vulnerability to the copper (Koch et al. 2017; Li et al. 2014; Zhou et al. 2014). However, it should be noted that the effect of Cu and its interaction with temperature towards the copepod survival was not significant in this study ($p > 0.05$, Tables S1 and 2).

Different from survival, the developmental time of *T. japonicus* consistently decreased with increasing temperature from 15 to 32 °C (N-C: Temperature $F_{3,24} = 337.8$, $p < 0.0001$ and N-A: Temperature $F_{3,24} = 554.6$, $p < 0.0001$; Fig. 1c, d, Tables S3 and 4). Decreasing developmental time with increasing temperatures was also observed in two other copepods *Eodiaptomus japonicus* (Liu et al. 2014) and *Eurytemora affinis* (Karlsson et al. 2018). This is likely due to the influence of temperature in controlling the respiration, metabolic rate and physiology of ectothermic copepods (Heine et al. 2019). For *T. japonicus*, they would enter a dormant state at low temperatures with reduced metabolic rate, whereas they would have higher active metabolic activities and faster growth at higher temperatures (Lai et al. 2020; Li et al. 2014; Raisuddin et al. 2007). Meanwhile, Cu had no significant impacts on the

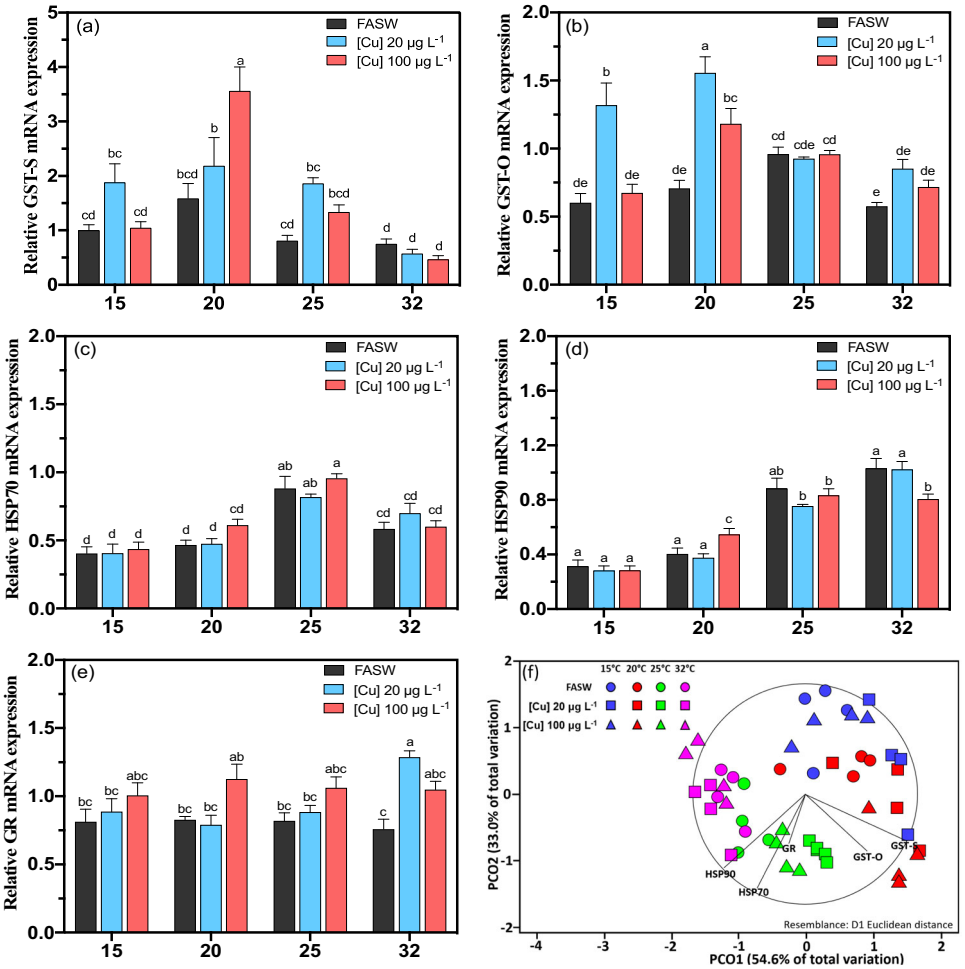

Fig. 2 Relative levels of expression (mean ± 1 SEM, $n = 4$) of (**a**) GST-S gene, (**b**) GST-O gene, (**c**) HSP70 gene, (**d**) HSP90 gene and (**e**) GR gene of the adult copepod *Tigriopus japonicus* upon the interacting effects of temperatures (15, 20, 25 and 32 °C) and Cu treatments (FASW control, 20 and 100 µg Cu L^{-1}). Bars with different letters represent significantly different means ($p < 0.05$, Student-Newman-Keuls test, One-way ANOVA). (**f**) Multivariate statistical analysis on the expression of the genes. The overlaid vector plot is established based on Eigenvectors indicating the relative significance of each parameter (based on its length) for leading to the separation of the various treatment groups

developmental time of the copepods (Fig. 1c, d, Tables S3 and 4). This might be because the significant impact of temperature had been dominant and overshadowed the toxicity of Cu, which was dosed at relatively low concentrations in this study.

Gene expression patterns under temperature and Cu interactions

Gene expressions of GSTs, GR and HSPs have been commonly investigated in toxicological studies, serving as sensitive indicators of xenobiotic stresses posed to *T. japonicus* (Lee et al. 2019; Wong et al. 2020). Among the five test genes, GST-S had the highest activation whereas GST-O had a smaller one with a comparable pattern (Fig. 2a and b). As a crucial superfamily of multifunctional enzymes for detoxification and antioxidation, GSTs have diversified isomers with distinct specificity across different species and chemicals, while GST-S is one of the most dominant GSTs in *T. japonicus* (Park et al. 2019). Lee et al. (2008) investigated the impacts of several trace metals, including copper, towards GST gene expression in *T. japonicus*. They found that GST-S was the most significantly expressed gene for all exposed metals whereas GST-O also had relatively higher expression in copper exposure than in some other metals, suggesting their apparent sensitivity to copper exposure. Although GSTs are known for detoxifying organic xenobiotics, their role as a more general antioxidant to fight against ROS has also been proposed (Wang and Wang 2010; Lauritano et al. 2021). A clear correlation between ROS and GST expression level was observed in *T. japonicus* upon exposure to different metals (Park et al. 2019). A similar elevation of GST was observed in other copepod (Ensibi and Yahia 2017) and other invertebrates (Magesky and Pelletier 2018). Taken together, it is broadly accepted that GST could be used as one of the indicators of oxidative stress in metal exposure.

The expression of both GST genes was generally most up-regulated under 20 °C while Cu interacted significantly with temperature and affected the gene expression pattern (GST-S: Interaction $F_{6,36} = 6.088$, $p = 0.0002$; GST-O: Interaction $F_{6,36} = 7.684$, $p < 0.0001$, Fig. 2a, b, Tables S5 and S6). At 15 and 20 °C, Cu at 100 µg Cu L^{-1} often resulted in lower expression compared to 20 µg Cu L^{-1},

except for GST-S under 20 °C. Such a suppression may be related to deteriorated defence systems under increased chemical stresses at higher chemical concentrations (Lee et al. 2008). The continuous down-regulation of the two genes with the increasing temperature above 20 °C in the control may also be a reflection of suppressed gene expression at a temperature close to upper thermal limit of the copepods (Low et al. 2018; Smolina et al. 2016). Alternatively, Almroth et al. (2015) proposed that the warmer temperature that matches with the optimal enzyme working temperature may enhance the enzymatic activity and their effectiveness in the organism, and hence *de novo* synthesis of the enzyme would reduce.

HSP70 and HSP90 had a relatively milder variation in their expressions, but their expression patterns were slightly different (Fig. 2c, d, Tables S7 and S8). The expression of both genes generally increased with increasing temperatures, except that the expression of HSP70 reduced at 32 °C. Han et al. (2018b) also found that HSP90 had a more significant and prolonged activation over time than HSP70 when the copepods were under thermal stress, which was consistent with our results. Both HSP70 and HSP90 are cytoplasmic chaperones that regulate protein folding processes such as refolding of misfolded and aggregated proteins due to thermal stress. Although their differential expressions were frequently observed, no solid conclusion has been drawn for the underlining mechanism and that may be species-specific (Rahlff et al. 2017). The impact of Cu towards the two HSP genes was less prominent compared to the one observed in GSTs and only interacted significantly in HSP90 (HSP70: Temperature $F_{3,36} = 44.16$, $p < 0.0001$; HSP90: Interaction $F_{6,36} = 3.945$, $p = 0.0039$, Tables S7 and S8). For HSP90, under 20 °C, the expression in 100 µg Cu L^{-1} was higher than the control and 20 µg Cu L^{-1} whereas at higher temperatures, it started to deplete with increasing Cu concentration, especially under 32 °C. Such reduction may be due to joint stress of high temperature and high Cu concentration, which supressed or deteriorated the antioxidant defence system as discussed above.

Different from other studied genes, temperature had no apparent effect on GR gene expression in copepods (Fig. 2e). However, Cu exposure constantly induced up-regulation of the GR gene, especially at 32 °C (Interaction $F_{6,36} = 3.700$, $p = 0.0058$, Table S9). A previous finding also suggested that GR was one of the key biochemical indicators in copepods when exposed to chemical stresses (Kim et al. 2014). At 32 °C, the reduced expression at 100 µg Cu L^{-1} might be due to over-stress of the defence system at the combination of high temperature and high Cu concentration.

Overall, although the effect of Cu at environmentally relevant concentrations towards the test apical endpoints of copepods was not apparent, the molecular analyses revealed significant interaction and disturbance by Cu under different temperatures, suggesting the copepods might have modulated their antioxidative strategies to resolve the joint chemical and thermal stresses. Accordingly, the multivariate analysis also revealed that the five evaluated genes were both temperature- and concentration-dependent (Fig. 2f and Table S10, Interaction pseudo $F_{6,36} = 5.817$, $p = 0.001$). It is noted that the Cu concentration dependent toxicity was often the most obvious at 20 °C while the gene expressions started to become insignificant and depleted with increasing temperature. This might be because *T. japonicus* had a most optimal living under this temperature and hence it could maximize its stress response genes to deal with the external stressor (i.e., Cu exposure in this study). Previous studies also revealed a decline in gene expressions at temperatures higher than the optimal thermal range of copepods *Calanus finmarchicus* and *Pseudodiaptomus annandalei* (Low et al. 2018; Smolina et al. 2016). Nevertheless, different biological species may have different gene responses along a temperature gradient (Han et al. 2020) and hence more studies are warranted. To obtain a more complete toxicological profile of temperature-dependent Cu toxicity, forthcoming studies should include more genes, such as metallothionein, that have been shown to be activated during metal exposure (Magesky and Pelletier 2018; Li et al. 2022). A transcriptomic analysis may also facilitate the understanding of the mechanistic molecular pathways.

Conclusions

The present study validated the hypothesis that variation in temperature could considerably affect the response to Cu in the marine copepod *T. japonicus*, especially through changes in gene expression. Regardless of the Cu concentration, the survival of *T. japonicus* during the developmental stage from nauplius to adult copepod was impaired at the lower temperature extreme (15 °C, representing the lowest seawater temperature in the marine environment of Hong Kong), while the developmental time was reduced with the increasing temperature. For the more sensitive molecular endpoints, there was a significantly perturbed expression profile among the five studied genes along the changes of temperature and Cu concentration. These results suggest that Cu at environmentally relevant concentrations may disturb the normal cellular functions and antioxidative strategies of the copepods, but such responses are found to be temperature-dependent, in particular at higher temperatures (25 and 32 °C).

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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