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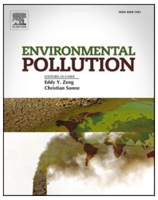

Hot and heavy: Responses of ragworms (*Hediste diversicolor*) to copper-spiked sediments and elevated temperature☆

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ABSTRACT

Sediment contamination and seawater warming are two major stressors to macrobenthos in estuaries. However, little is known about their combined effects on infaunal organisms. Here we investigated the responses of an estuarine polychaete *Hediste diversicolor* to metal-contaminated sediment and increased temperature. Ragworms were exposed to sediments spiked with 10 and 20 mg kg^{-1} of copper at 12 and 20 °C for three weeks. No considerable changes were observed in the expression of genes related to copper homeostasis and in the accumulation of oxidative stress damage. Dicarbonyl stress was attenuated by warming exposure. Whole-body energy reserves in the form of carbohydrates, lipids and proteins were little affected, but the energy consumption rate increased with copper exposure and elevated temperature, indicating higher basal maintenance costs of ragworms. The combined effects of copper and warming exposures were mostly additive, with copper being a weak stressor and warming a more potent stressor. These results were replicable, as confirmed by two independent experiments of similar settings conducted at two different months of the year. This study suggests the higher sensitivity of energy-related biomarkers and the need to search for more conserved molecular markers of metal exposure in *H. diversicolor*.

1. Introduction

Estuarine ecosystems have changed rapidly in recent decades due to many anthropogenic and climatic pressures (Cloern et al., 2016; Mitchell et al., 2015). The introduction of hazardous substances is considered one of the greatest threats to estuarine communities (Borgwardt et al., 2019). Elevated water temperature due to climate change is also a major stressor, given its direct effects on the physiology of organisms and its common association with unfavorable events such as harmful algal blooms and hypoxia (Harley et al., 2006; Kimmerer and Weaver, 2013). Understanding how estuarine biota, living in inherently highly variable environment (Elliott and Quintino, 2007), responds to these additional stressors is therefore critical to maintaining estuarine ecosystem functions and services (Barbier et al., 2011; O'Brien et al., 2019).

Among estuarine species, macrobenthos is an ecologically important but highly vulnerable group (Pinto et al., 2009), often exposed to sediment-deposited contaminants, such as trace heavy metals (Brady et al., 2015; Wang and Fisher, 1999). Although some metals such as zinc (Zn) and copper (Cu) have essential functions (Festa and Thiele, 2011; Maret, 2013), their excessive amounts are toxic (Brix et al., 2022; Eisler, 1998; Rainbow, 2002). Metal toxicity is influenced by temperature, with increased temperature often enhancing metal uptake and accumulation, but concurrently facilitating detoxification and repair processes (Cairns et al., 1975). Higher temperature may also reduce the oxygen and energy supply required to offset the elevated maintenance costs due to metal exposure (Sokolova and Lannig, 2008). Because of these possible interactions, the combined effects of metal contamination and warming may be more or less extreme than the additive expectation of the individual effects, often referred to as synergism and antagonism, respectively (Crain et al., 2008; Folt et al., 1999; Piggott et al., 2015).

While several studies have examined the combined effects of metal

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contamination and warming on macrobenthic epifauna such as mussels and corals (Biscéré et al., 2017; Boukadida et al., 2016; da Silva Fonseca et al., 2019; Parry and Pipe, 2004; Tracy et al., 2020), similar research on infauna in contaminated sediments is lacking. In this study, we investigated the bioenergetic and stress responses of a benthic polychaete, ragworms *Hediste diversicolor* to Cu exposure and elevated temperature. Ragworms are widely used in ecotoxicological research because of their easy collection and maintenance, broad ecological niche, as well as burrowing behavior and diverse feeding modes that increase contaminant exposure (Scaps, 2002; Silva et al., 2020). Cu was chosen because of its often elevated levels in modern estuarine sediments, with reported contents ranging from tens to hundreds of mg kg^{-1} dry mass (Glasby et al., 2004; Müller and Heininger, 1999; Szefer et al., 2009; Wetzel et al., 2013). The increased use of Cu in antifouling paints to replace tributyltin over the last two decades also makes it of particular concern (Brooks and Waldock, 2009; Comber et al., 2022). Our preliminary analysis of surface sediments from the Warnow estuary, Germany showed a median Cu content of ~44 mg kg^{-1} (Fig. S1, LUNG, 2023; Rönspieß et al., 2020). Thus, we exposed ragworms to uncontaminated sediments and sediments spiked with 10 and 20 mg kg^{-1} of Cu at 12 and 20 °C. The temperature difference was chosen to mimic the warming scenario with surface temperatures in northern Europe projected to increase by ~3 °C by the mid-21st century (SSP5-8.5, 1995–2014 baseline, Carvalho et al., 2021; IPCC, 2021), and to account for daily temperature variations (~5 °C within a month). To assess the potential bioenergetic disruption from Cu and warming exposures, we measured whole-body levels of energy reserves (carbohydrates, lipids, and proteins) and mitochondrial aerobic capacity (electron transport system activity) and calculated the worm's cellular energy allocation (Verslycke et al., 2004). Potential cellular toxicity and molecular stress responses were evaluated using indices of dicarbonyl stress (methylglyoxal level), oxidative damage (malondialdehyde and protein carbonyl levels), and mRNA expression levels of genes encoding Cu transporters and metal-binding proteins (*ATP7A*, *CCS*, *MTS*, and *MTL*), metal stress-protective proteins (*GSTO1*, *HSP70MAJ*, and *HSP70MIN*), and a hemoglobin linker chain (*HBL2*) (Green Etxabe et al., 2021; McQuillan et al., 2014). To examine the research replicability (Romero, 2019), we conducted two experiments with similar setups in two different months of the year.

2. Materials and methods

2.1. Sediment collection and characterization

Surface sediment (upper 10 cm) was collected in December 2020 from a beach in Warnemünde, Germany (54.1787, 12.0666) with no point sources of pollution nearby (Fig. S1). Wet sediment was sieved through a 1-mm mesh to remove larger particles and organisms. The sediment was oven-dried at 60 °C for 96 h and stored at room temperature.

Physicochemical properties of wet sediment were measured using conventional procedures (Blake, 1965; Davies, 1974; Robertson et al., 1984; Verardo et al., 1990). The sediment had a bulk density of 1.49 g cm^{-3}, a water content of 17.9% w/w, a total organic matter content of 0.18% w/w, and a C:N mass ratio of 36.9. The sediment was composed of 2.5% mud, 0.2% very fine sand, 34.7% fine sand, 60.4% medium sand, and 2.2% coarse sand (Wentworth, 1922) with a median grain size of 409 μm.

2.2. Animal collection

Ragworms *Hediste* (*Nereis*) *diversicolor* were collected in January and March 2021 near Schnatermann, Germany (54.1728, 12.1414), a contaminated area due to intense port activity (Fig. S1, Abraham et al., 2017). Worms were sieved out of the sediment and transported in seawater-filled plastic drums to the laboratory. They were transferred to 20-L plastic trays pre-filled with the dried sediment (6 cm thick) and aerated artificial seawater (Pro-Reef, Tropic Marin, Germany) at 12 ± 0.5 °C and salinity 15 ± 0.25, which approximated the average annual conditions of their habitat. The worms were fed on alternate days with fish food (TetraMin Flakes, Tetra, Germany) and the overlying seawater was changed daily during a 10-day acclimation period to assist depuration of any pre-existing Cu burdens in the worms (Ozoh, 1994).

2.3. Sediment spiking

Copper (II) chloride ($CuCl_2 \cdot 2H_2O$, CAS 10125-13-0, Carl Roth, Germany) was used to prepare Cu-spiked sediments (Green Etxabe et al., 2021; Hutchins et al., 2009; Ward et al., 2015). Stock solutions of $CuCl_2$ were made in deionized water and diluted to the desired Cu masses in 125-mL water volumes in 800-mL glass beakers. The beakers were orbitally shaken for 90 min (Dual-Action Shaker KL 2, Edmund Bühler, Germany) and 500 g of the dried sediment was gradually added to obtain a 6-cm sediment thickness. Nominal Cu contents were 0 (control, no Cu salt addition), 10, and 20 mg kg^{-1} dry sediment. After 24 h settling without pH adjustment, the thin layer of overlying water was gently removed using syringes and replaced by 500 mL of artificial seawater (salinity 15). The beakers were kept in the dark at 12 ± 0.5 °C for one week until exposures.

2.4. Cu and warming exposures

Two two-factor experiments were conducted with the ragworms collected in January (Jan experiment) and March (Mar experiment). In each experiment, worms were randomly assigned to one of six exposure groups corresponding to the combinations of three Cu contents (0, 10, and 20 mg kg^{-1}) and two temperatures, i.e., 12 °C (control) and 20 °C (elevated). Five beakers were used for each group. The Jan experiment had three worms per beaker (wet mass 298 ± 160 mg) and the Mar experiment had four worms per beaker (wet mass 299 ± 137 mg). All beakers were kept in an environmental room at 12 ± 0.5 °C for three weeks, which is the common exposure duration for ragworms in toxicological experiments (Buffet et al., 2013; Fernandes et al., 2006; Mouneyrac et al., 2003; Zhou et al., 2003). Beakers receiving warming treatment were placed in a circulating water tub equipped with an aquarium heater (HT 75, Tetra). Temperature was set to gradually increase from 12 to 20 °C during the first week (to avoid excessive thermal stress) and remain stable at 20 ± 0.5 °C during the following two weeks. The overlying water was aerated to maintain high dissolved oxygen levels (>9 mg L^{-1} at 12 °C and >8 mg L^{-1} at 20 °C, pH > 8). The worms were fed daily with fish food (TetraMin Granules, Tetra) and overlying seawater was renewed once a week to avoid nitrate buildup. Dead worms and unconsumed food were promptly removed from the sediment surface to maintain the water quality.

After the three-week exposure, sediment and pore water were sampled in each beaker to measure Cu concentrations ($n = 5$). Surface sediments (~10 g, upper 1 cm) were freeze-dried for 24 h (Alpha 1–4 LSCplus, Martin Christ Gefriertrocknungsanlagen, Germany), homogenized using a porcelain mortar and pestle, and stored at room temperature for analysis of total Cu. Pore waters (~10 mL) were extracted from the bottom sediments using rhizon samplers (0.15-μm pore size, 19.21.23 F, Rhizosphere Research Products, Netherlands, Seeberg-Elverfeldt et al., 2005), acidified with high-purity HNO_3 (to 2% v/v), and stored at 4 °C for analysis of dissolved Cu. Worms were sieved out of the sediment, rinsed with seawater, shock-frozen in liquid nitrogen and stored at −80 °C to measure biomarker responses ($n = 15$ and 20 in the Jan and Mar experiments, respectively). After that, the remaining samples of worms from each beaker were pooled, freeze-dried for 72 h, and stored at room temperature for analysis of total Cu body burden ($n = 2$).

2.5. Cu analyses

Cu concentrations in sediments, pore waters, and worms were measured using inductively coupled plasma mass spectrometry (ICP-MS, iCAP Q, Thermo Fisher Scientific, Germany) following protocols described elsewhere (Dellwig et al., 2019; Lagerström et al., 2013). Details are given in the Supplementary Material.

2.6. Colorimetric assays

Whole worms were homogenized in ice-cold buffer (0.1 M Tris-HCl pH 8.5, 153 μM $MgSO_4$, 0.2% w/v Triton X-100, and 0.1 mM phenylmethylsulfonyl fluoride) using Potter-Elvehjem glass-Teflon homogenizers. The levels of carbohydrates, lipids, proteins, methylglyoxal (MGO), malondialdehyde (MDA) and protein carbonyls (PC) were measured with colorimetric end-point assays (Bradford, 1976; Buege and Aust, 1978; Folch et al., 1957; Levine et al., 1990; Masuko et al., 2005; Mitchel and Birnboim, 1977; Van Handel, 1985). Mitochondrial electron transport system (ETS) activity was measured kinetically at 25 °C (De Coen and Janssen, 1997). Details are given in the Supplementary Material.

ETS activity was corrected for the exposure temperatures (12 and 20 °C) using the Q_{10} temperature coefficient of 2.0 previously reported for *H. diversicolor* (Galasso et al., 2018). Contents of carbohydrates, lipids, and proteins were converted to energy equivalents using the specific enthalpy of combustion of 17.5, 39.5, and 24 J mg^{-1}, respectively (Gnaiger, 1983) and summed up to obtain the total available energy (Ea). Energy consumption rate (Ecr) was calculated from the ETS activity using the oxyenthalpic equivalent of 484 kJ mol^{-1} O_2 and cellular energy allocation (CEA) as an energy budget index was computed as the Ea/Ecr ratio (Verslycke et al., 2004).

2.7. Quantitative reverse transcription PCR (RT-qPCR)

Transcript levels of the target genes associated with metal-induced stress (Green Etxabe et al., 2021; McQuillan et al., 2014) and the reference genes *GAPDH* and *HIS3* were determined in the anterior part of ragworms, including the head and a few segments, following RT-qPCR protocols described elsewhere (Falfushynska et al., 2019) with gene-specific primers (Table 1). The anterior part was chosen because this body region was highly responsive to Cu exposure (Bouraoui et al., 2015). The expression levels of the target genes were normalized against those of the reference genes (which were stable across the exposure groups, Table S1) using geometric averaging (Matz et al., 2013; Pfaffl, 2001; Vandesompele et al., 2002). Due to limited resources, RT-qPCR was only performed on worms from the Mar experiment ($n = 15$). Details on RNA extraction, cDNA synthesis, and qPCR are given in the Supplementary Material.

2.8. Data analyses

The effects of Cu-spiked sediments, elevated temperature, and their interaction on experimental outcomes were evaluated by permutation tests for multi-factor analysis of variance (Anderson and Braak, 2003; Howell, 2015; Manly, 2007), which is the recommended approach for analyzing randomized experiments (Ernst, 2004; Ludbrook and Dudley, 1998). Specifically, linear models (LMs), binomial generalized linear mixed-effects models (GLMMs) with logit link function, and linear mixed-effects models (LMMs, Bates et al., 2015) were used for the measured concentrations of Cu, worm survival, and biomarkers, respectively, with nominal Cu content and temperature as interacting fixed effects. Experimental beaker was added as a random intercept in GLMMs and LMMs to account for the potential non-independence of worms within each beaker (Colegrave and Ruxton, 2018). The wet mass of worms was added as a covariate in LMMs to control for possible influences of size on biomarker responses (Durou et al., 2005; Stomperudhaugen et al., 2009). The *F*-statistics from these models and their recalculated values under 1999 permutations were used to compute the *p*-values. The test results were reported in the language of evidence (Muff et al., 2022), in which evidence against the null hypotheses was considered very strong, strong, moderate, or weak when $p \leq 0.001, 0.01, 0.05$, or 0.1, respectively. Multiple comparisons tests were not used in this study (Kozak and Powers, 2017).

We computed Glass's delta effect size (Glass et al., 1981; Lakens, 2013) for the effects of Cu and warming exposures on biomarker responses compared with the shared control condition (0 mg kg^{-1} of Cu at 12 °C). Glass's delta (Δ) is a measure of the standardized difference between means and was used to determine whether an interaction is synergistic or antagonistic (Piggott et al., 2015). To assess the similarity in the responses of biomarkers, we calculated Spearman correlation and performed agglomerative hierarchical clustering using the correlation-based distance ($\sqrt{1-|r_s|}$) and average linkage (Chen et al., 2023).

All analyses were implemented in R v4.3.0 (R Core Team, 2023) using the *peramo* package v0.1.3 for permutation tests (Pham et al., 2022) and the *mbRes* package v0.1.7 for effect size calculation (Pham and Sokolova, 2023).

3. Results

3.1. Cu concentrations

Mean total Cu contents in the surface sediments in experimental beakers after the three-week exposure were 1.0, 3.8, and 7.5 mg kg^{-1} for the Jan experiment and 1.0, 1.6, and 3.8 mg kg^{-1} for the Mar experiment, corresponding to the nominal Cu contents of 0, 10, and 20 mg kg^{-1}, respectively. Respective mean dissolved Cu concentrations in the

Table 1
Primer sequences used for the amplification of the target and reference genes in *Hediste diversicolor*.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	NCBI accession number
ATP7A	CTACGAGAAGCCACGAGTCC	TCTCCAGGGACCACTTTCAG	–
CCS	AGCAGTTGGAGTCAGCAGGT	TGCCAGCTCTCCGTATTTCT	–
MTS	CATTGCACTGGGAATGTTTG	CATCACAGCATTGGATGGAC	–
MTL	GGAGCTTCTGTTTCCTGTGC	TCACAAATCCAGCACCATGT	–
GSTO1	CATCGCAGATTGAGGATTCA	TGTCCCTATGCCCAGAGAAC	–
HSP70MAJ	TTTCTGGCCTGAATGTCTTGCGTA	AGAGCGTTTCGTTCTCCTCCTACT	KX271712.1
HSP70MIN	ATTTGATGAAGCCTCTGTGCAATC	TTCTTCTCCGCCTTGTATTCAACT	KX271711.1
HBL2	GCTCGCTCATGGGATAATAACAAC	TCTCTGAAACTAACAGAGCACGAG	D58413.1
GAPDH	CATCCATGACCATCCTCAGCAA	GTTGTCATCAAACCCTCAACGATT	KX284894.1
HIS3	GTGAGATCCGTCGTTACCAGAAA	CAAGTCAGTCTTGAAGTCCTGGG	LC380659.1

Gene symbols: *ATP7A* – ATPase copper transporting alpha; *CCS* – copper chaperone for superoxide dismutase; *MTS* – Cd/Se metallothionein (small protein); *MTL* – atypical metallothionein-like protein (large protein); *GSTO1* – glutathione *S*-transferase omega 1; *HSP70MAJ* – 70 kDa heat shock protein major form; *HSP70MIN* – 70 kDa heat shock protein minor form; *HBL2* – linker chain L2 of the giant extracellular hemoglobin; *GAPDH* – glyceraldehyde-3-phosphate dehydrogenase; *HIS3* – histone H3. Primer sequences for *ATP7A*, *CCS*, *MTS*, *MTL*, and *GSTO1* were retrieved from McQuillan et al. (2014).

pore waters of bottom sediments were 20.1, 183.1, 565.4 μg L^{-1} for the Jan experiment and 14.2, 195.9, and 337.1 μg L^{-1} for the Mar experiment. Respective mean total Cu burdens in worms were 24.6, 39.7, and 60.7 mg kg^{-1} for the Jan experiment and 9.5, 29.7, and 39.8 mg kg^{-1} for the Mar experiment. Cu levels in the sediments, pore waters, and worms were correlated (Fig. 1 and S2), determined by the nominal Cu contents but not affected by the exposure temperature ($p > 0.1$, Table S2).

3.2. Survival

At the end of the exposures, worm survival in six groups was 63.3% ($SD = 11.7\%$) in the Jan experiment and 84.2% ($SD = 5.8\%$) in the Mar experiment (Table S3). There was no evidence that Cu and warming exposures affected the survival ($p > 0.1$, Table S4).

3.3. Energy reserves

In the Jan experiment, there was no evidence that Cu exposure and temperature altered the carbohydrate content (Fig. 2a). Moderate evidence for the effect of temperature on carbohydrate content was found in the Mar experiment, in which worms exposed to 20 °C had lower carbohydrate levels than those at 12 °C. There was no evidence that Cu and warming exposures affected the lipid and protein contents (Figs. S3a and b).

In the Jan experiment, there was no evidence that Cu exposure and temperature affected the Ea (Fig. 2b). The Mar experiment showed moderate evidence for the effect of temperature, in which worms exposed to warming had lower Ea than those at control temperature.

3.4. Mitochondrial aerobic capacity and energy budget

Both experiments showed very strong evidence for the impact of temperature on mitochondrial ETS activity, in which worms exposed to 20 °C had elevated ETS activity compared with those at 12 °C (Fig. 2c). The effect of Cu on ETS activity was found with strong and weak evidence in the Jan and Mar experiments, respectively, in which worms exposed to Cu-spiked sediments had higher ETS activity than those in the control sediment.

Both experiments showed very strong evidence for the effect of temperature on CEA, in which worms exposed to 20 °C had misbalanced energy budget compared with those at 12 °C (Fig. 2d). Strong and weak evidence for the impact of Cu was found in the Jan and Mar experiments, respectively, in which worms exposed to Cu-spiked sediments had lower CEA than those in the control sediment.

3.5. Dicarbonyl and oxidative stress

Very strong and strong evidence was found for the effect of temperature on MGO level in the Jan and Mar experiments, respectively, in which worms at 20 °C had lower MGO levels than those at 12 °C (Fig. 2e). No evidence was found for the impact of Cu on MGO level. There was no evidence that Cu and temperature affected MDA and PC levels in both experiments (Figs. S3c and d).

3.6. Molecular markers of metal exposure and stress

In the Mar experiment, strong evidence was found for the effect of interaction between Cu and temperature on the expression level of *ATP7A* (Fig. 3a), in which warming led to an increase in the *ATP7A* transcript level in worms exposed to 20 mg kg^{-1} of Cu.

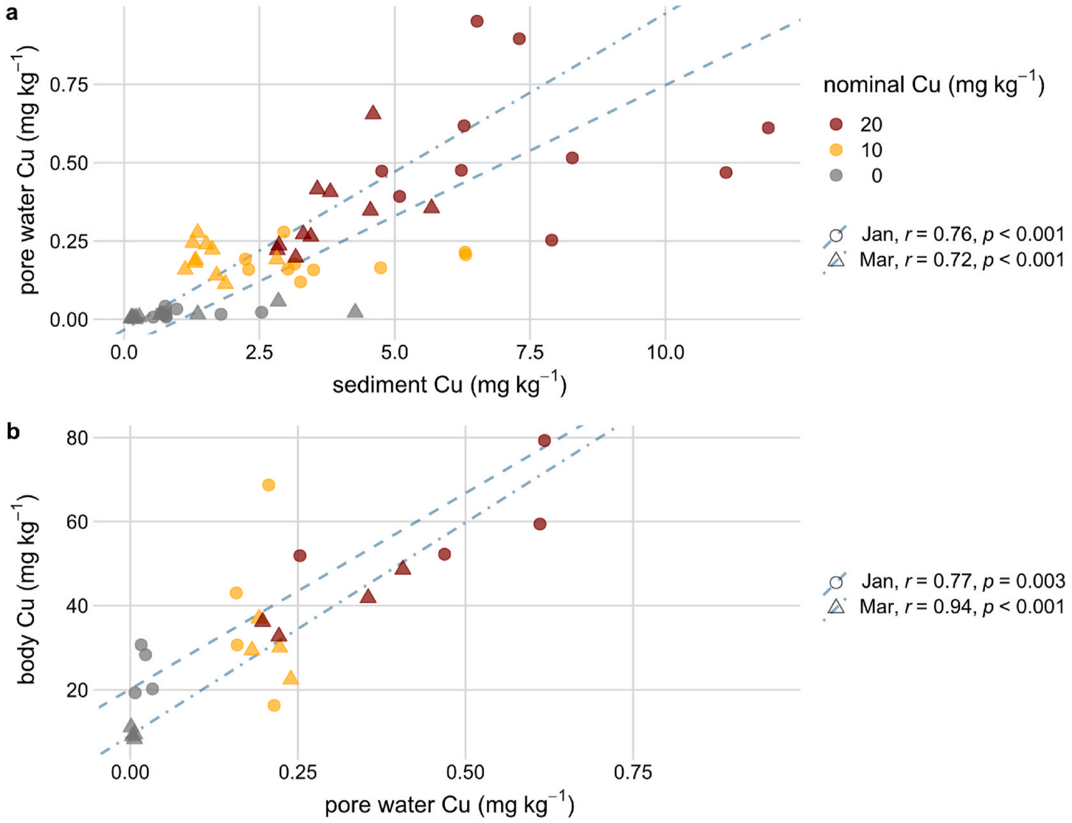


Fig. 1. Correlation between total Cu contents in surface sediments, dissolved Cu concentrations in pore waters of bottom sediments, and total Cu body burdens in worms at the end of the exposures in the Jan (circles) and Mar (triangles) experiments. Sediments were spiked with Cu at nominal contents of 0, 10, and 20 mg kg^{-1}. Standard deviation lines (Freedman et al., 2007), Pearson correlation and permutation p-values are given for each experiment.

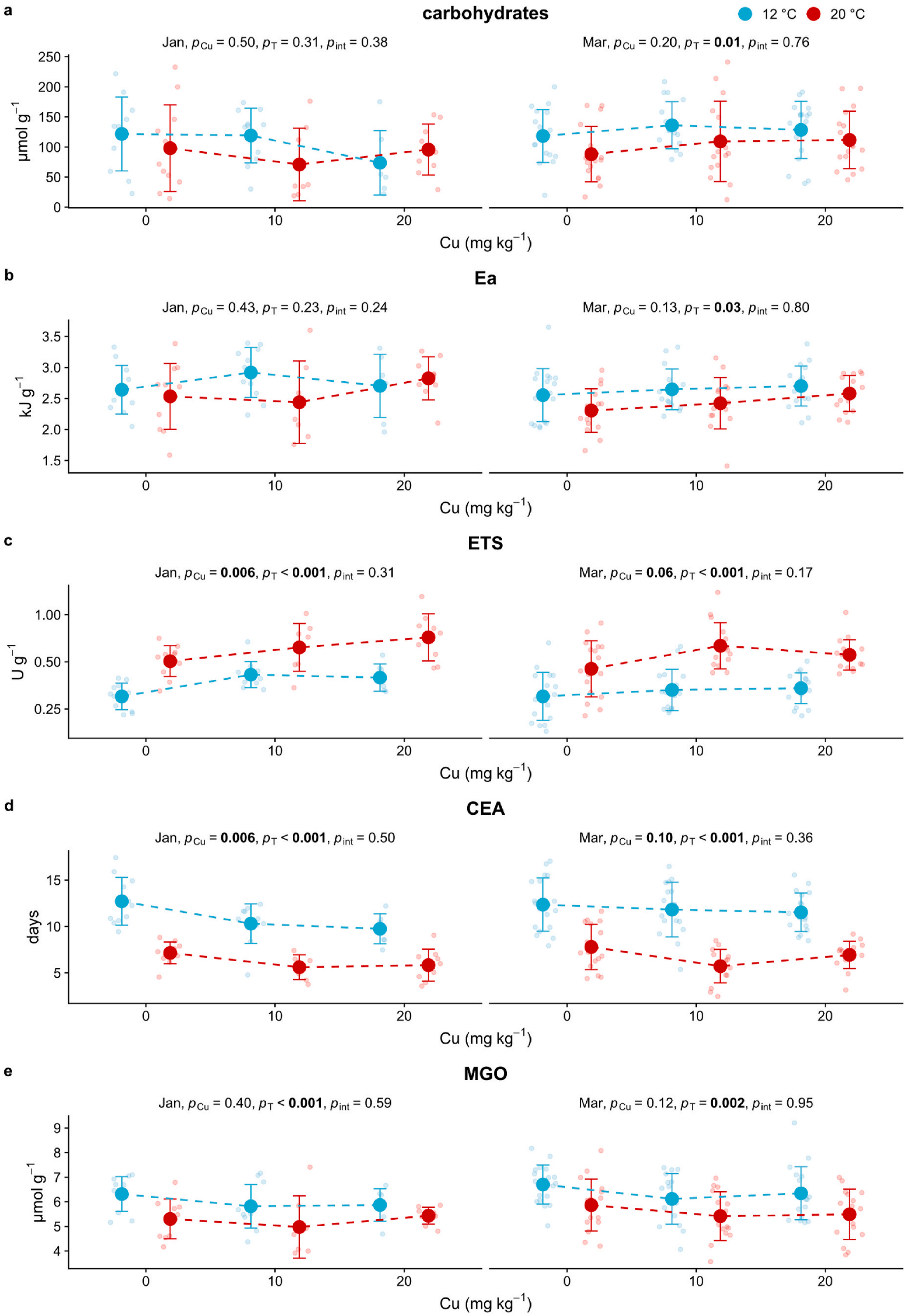


Fig. 2. Effects of Cu-spiked sediments and elevated temperature on carbohydrate content, total available energy (Ea), mitochondrial electron transport system (ETS) activity, cellular energy allocation (CEA), and methylglyoxal (MGO) level of *Hediste diversicolor* in the Jan and Mar experiments. Data are presented with individual observations and mean ± standard deviation. Permutation *p*-values are given for Cu, temperature (T), and their interaction (int). *F*-statistics are given in Table S5.

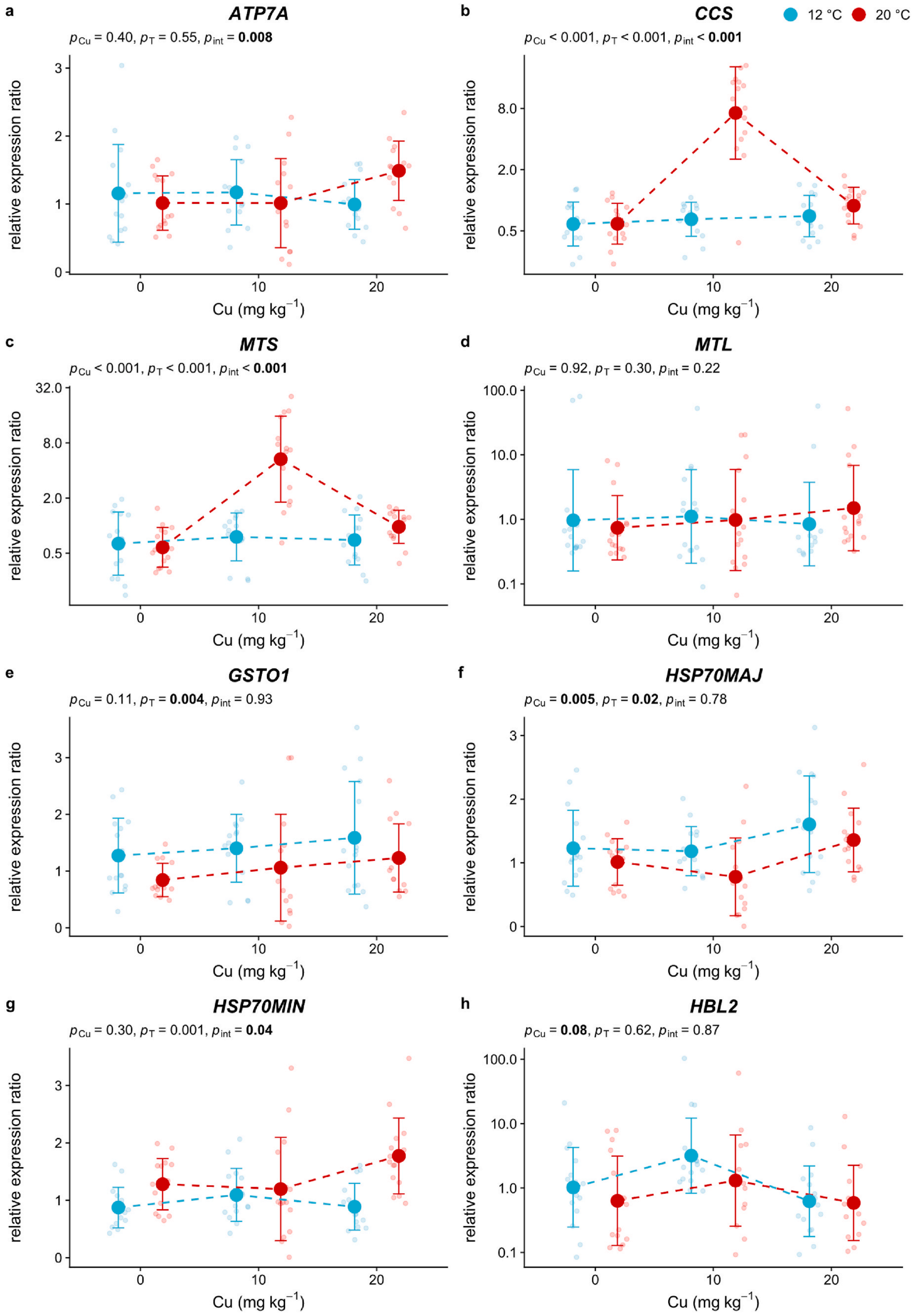


Fig. 3. Effects of Cu-spiked sediments and elevated temperature on mRNA expression levels of *ATP7A*, *CCS*, *MTS*, *MTL*, *GSTO1*, *HSP70MAJ*, *HSP70MIN*, and *HBL2* in the anterior part of *Hediste diversicolor* in the Mar experiment. Data are presented with individual observations and mean ± standard deviation. Permutation *p*-values are given for Cu, temperature (T), and their interaction (int). *F*-statistics are given in Table S5.

Very strong evidence was found for the interaction effect on the expression levels of *CCS* and *MTS* (Fig. 3b and c). The *CCS* and *MTS* transcript levels were strongly upregulated at the elevated temperature in worms exposed to 10 mg kg^{-1} of Cu. There was no evidence that Cu and warming exposures altered the expression level of *MTL* (Fig. 3d).

Strong evidence was found for the effect of temperature on the expression level of *GSTO1* (Fig. 3e), in which worms exposed to warming had downregulated *GSTO1* transcript levels compared with those at 12 °C.

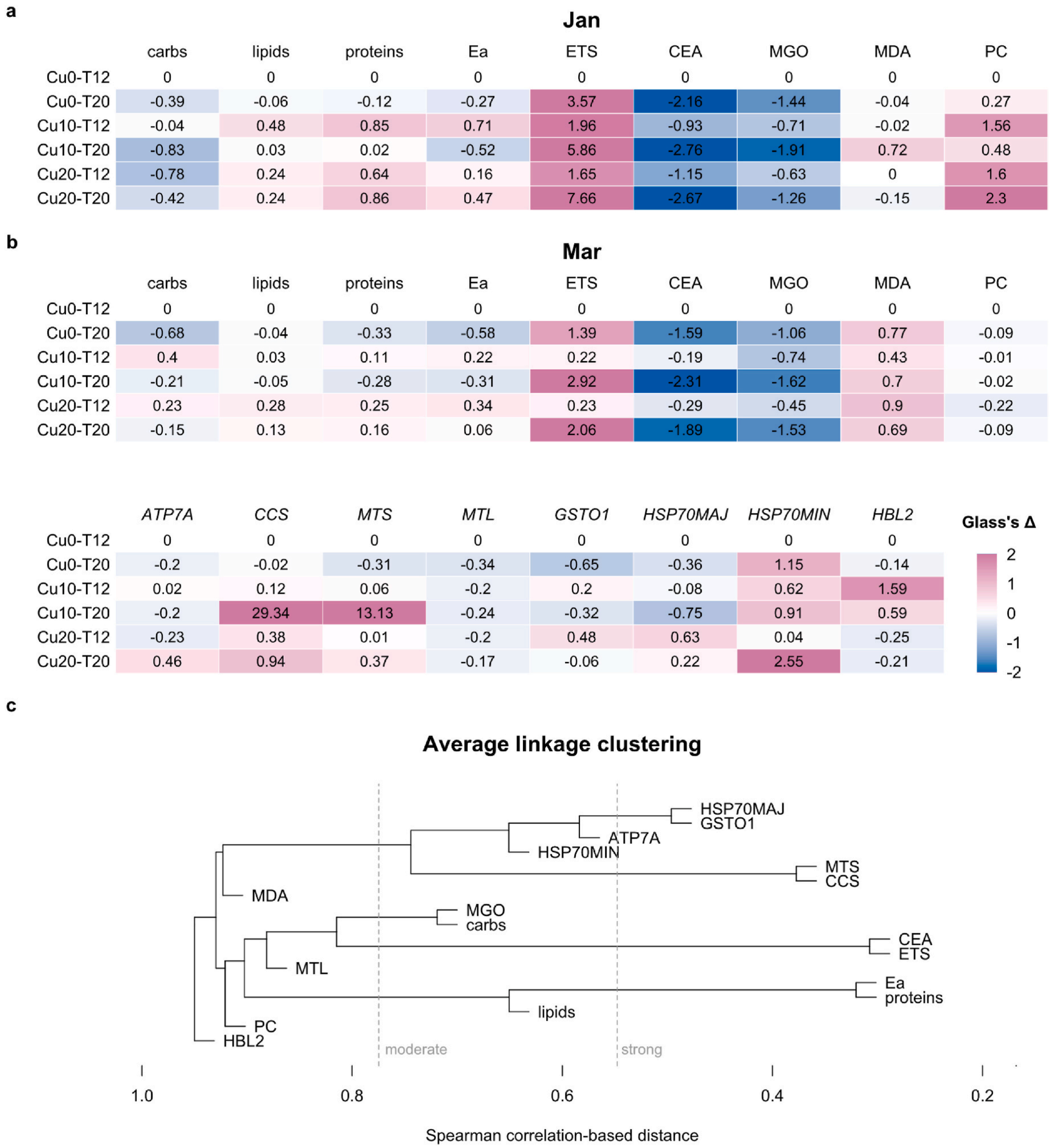
There was strong and moderate evidence for the impacts of Cu and temperature, respectively, on the expression level of *HSP70MAJ* (Fig. 3f). Worms exposed to 10 and 20 mg kg^{-1} of Cu, respectively, showed downregulation and upregulation of *HSP70MAJ* expression compared with those in the control sediment, while worms exposed to warming showed the inhibition of *HSP70MAJ* expression compared with those at control temperature.

Moderate evidence was found for the interaction effect on the expression level of *HSP70MIN* (Fig. 3g), in which warming led to a greater increase in the *HSP70MIN* transcript level in worms exposed to 20 mg kg^{-1} of Cu than in those exposed to other Cu levels.

There was weak evidence for the effect of Cu on the expression level of *HBL2* (Fig. 3h), in which *HBL2* transcript levels were highest in worms exposed to 10 mg kg^{-1} of Cu and lowest in those exposed to 20 mg kg^{-1} of Cu.

3.7. *Patterns of biomarker responses*

There was a lack of evidence for the effect of the Cu × temperature



a

Jan

	carbs	lipids	proteins	Ea	ETS	CEA	MGO	MDA	PC
Cu0-T12	0	0	0	0	0	0	0	0	0
Cu0-T20	-0.39	-0.06	-0.12	-0.27	3.57	-2.16	-1.44	-0.04	0.27
Cu10-T12	-0.04	0.48	0.85	0.71	1.96	-0.93	-0.71	-0.02	1.56
Cu10-T20	-0.83	0.03	0.02	-0.52	5.86	-2.76	-1.91	0.72	0.48
Cu20-T12	-0.78	0.24	0.64	0.16	1.65	-1.15	-0.63	0	1.6
Cu20-T20	-0.42	0.24	0.86	0.47	7.66	-2.67	-1.26	-0.15	2.3

b

Mar

	carbs	lipids	proteins	Ea	ETS	CEA	MGO	MDA	PC
Cu0-T12	0	0	0	0	0	0	0	0	0
Cu0-T20	-0.68	-0.04	-0.33	-0.58	1.39	-1.59	-1.06	0.77	-0.09
Cu10-T12	0.4	0.03	0.11	0.22	0.22	-0.19	-0.74	0.43	-0.01
Cu10-T20	-0.21	-0.05	-0.28	-0.31	2.92	-2.31	-1.62	0.7	-0.02
Cu20-T12	0.23	0.28	0.25	0.34	0.23	-0.29	-0.45	0.9	-0.22
Cu20-T20	-0.15	0.13	0.16	0.06	2.06	-1.89	-1.53	0.69	-0.09

	ATP7A	*CCS*	*MTS*	*MTL*	*GSTO1*	*HSP70MAJ*	*HSP70MIN*	*HBL2*
Cu0-T12	0	0	0	0	0	0	0	0
Cu0-T20	-0.2	-0.02	-0.31	-0.34	-0.65	-0.36	1.15	-0.14
Cu10-T12	0.02	0.12	0.06	-0.2	0.2	-0.08	0.62	1.59
Cu10-T20	-0.2	29.34	13.13	-0.24	-0.32	-0.75	0.91	0.59
Cu20-T12	-0.23	0.38	0.01	-0.2	0.48	0.63	0.04	-0.25
Cu20-T20	0.46	0.94	0.37	-0.17	-0.06	0.22	2.55	-0.21

Fig. 4. Summary of biomarker responses of *Hediste diversicolor* to Cu-spiked sediments and elevated temperature with Glass's delta effect size (a, b) and correlation-based clustering (c). Glass's delta (Δ) was calculated against the responses in the shared control condition (0 mg kg^{-1} of Cu at 12 °C) and is 0 for this group. Smaller distances in the cluster analysis imply stronger correlations among biomarker responses, and dashed lines indicate thresholds for correlation strength (Akoglu, 2018). Exposure groups: Cu0, Cu10, and Cu20 for 0, 10, and 20 mg kg^{-1} of Cu and T10 and T20 for 12 and 20 °C, respectively.

interaction on bioenergetic and cellular responses in both experiments ($p > 0.1$, Fig. 2 and S3). Interaction effects, however, were found with stronger evidence in some molecular markers including *ATP7A*, *CCS*, *MTS* and *HSP70MIN* (Fig. 3). In these cases, the effects of the combined Cu (either 10 or 20 mg kg^{-1}) and warming exposures were more extreme than the total effects of the separate exposures, suggesting synergistic interactions (Fig. 4b). Both experiments also showed that ETS, CEA, and MGO were more affected than other studied biomarkers as indicated by notably large effect sizes (Fig. 4a and b).

Some biomarkers showed strong correlations ($|r_s| \geq 0.7$) in their responses (Fig. 4c). ETS activity and CEA were negatively correlated ($r_s = -0.91$), while Ea was positively correlated with the protein level ($r_s = 0.90$). Positive correlations existed between the expression levels of *CCS* and *MTS* ($r_s = 0.86$) and *GSTO1* and *HSP70MAJ* ($r_s = 0.75$). At the threshold distance corresponding to moderate correlations ($|r_s| \geq 0.4$), four clusters were identified, comprising the expression of *ATP7A*, *CCS*, *MTS*, *GSTO1*, *HSP70MAJ*, and *HSP70MIN* (1st cluster), carbohydrate and MGO levels (2nd cluster), ETS activity and CEA (3rd cluster), and Ea, proteins and lipids (4th cluster).

4. Discussion

4.1. Exposure conditions and Cu bioavailability

The sandy sediment in our study is not preferred by ragworms, which usually inhabit more muddy substrates (median grain size of ~50–225 μm, Van Colen et al., 2014). However, its limited fine grains and organic matter ensured low levels of contaminants that could mask the effects of spiked Cu (Simpson et al., 2004). Although the Cu content was not measured in the sediment before spiking, it should be as low as in the control sediment at the end of the exposures ($M = 1.0$ mg kg^{-1}). Because sandy sediments typically lack the main metal-binding solid phases (sulfides, iron hydroxides, and organic carbon, Seibert et al., 2019; Strom et al., 2011), we presume that a large fraction of the added Cu would have remained in the dissolved forms. Cu hydrolysis often causes the pH reduction in sediments after spiking, increasing the redox potential and favoring metals in dissolved phases (Hutchins et al., 2007). The lack of pH adjustment and the relatively short incubation time (Simpson et al., 2011) in our study could therefore explain the high Cu concentrations in pore waters (>100 μg L^{-1}). Cu levels in sediments and pore waters after spiking and during exposures could be higher than measured levels at the end, given the weekly renewal of overlying waters that likely removed copper from the beakers. Notably, the environmental Cu concentrations were generally lower in the Mar experiment than in the Jan experiment. This could be explained by the higher worm density in the Mar experiment, which enhanced bioturbation activity (François et al., 2002) and leakage of Cu into the overlying water. Furthermore, although elevated temperature often facilitates metal accumulation in particulate phases (Warren and Zimmerman, 1994), we found no effect of temperature on environmental Cu concentrations in either experiment, probably due to the mentioned shortage of sediment binding sites.

Given the low amount of sediment-bound Cu, we expect that Cu exposures in ragworms occurred primarily via the dissolved route (pore water, burrow water, and overlying water) rather than sediment ingestion (Simpson et al., 2011; Wang and Fisher, 1999). Because the artificial seawater used in our study did not have organic ligands that can form stable complexes with copper (II) (Paul et al., 2021; Waugh et al., 2022; Whitby et al., 2017), our experimental settings promoted the abundance of free Cu^{2+}, the most bioavailable copper species, in these water compartments. For *H. diversicolor*, the reported 96-h median lethal concentrations (LC$_{50}$) of Cu^{2+} in seawater without sediments vary from 125 μg L^{-1} (20 °C, salinity 20, Moreira et al., 2005) to 480 μg L^{-1} (7 °C, salinity 17.5, Jones et al., 1976) and >720 μg L^{-1} (12–22 °C, salinity 15.25, Ozoh, 1992). Thus, our observation that worm survival after three weeks was not affected by several hundred μg L^{-1} of Cu in pore waters seems surprising. A possible reason for the high tolerance of worms is that they have genetically adapted (Dinh et al., 2022) to the high contamination level in their habitat (Fig. S1, Abraham et al., 2017). Ozoh (1992) also found that in the presence of sandy sediment, the 96-h LC$_{50}$ of Cu^{2+} increased ~3–5 times to several thousand μg L^{-1}. The reduced toxic effects of Cu in sandy sediment could be related to the secreted mucus that worms used for lining their burrow galleries, which can act as a protective layer that adsorbs dissolved Cu and reduces its bioavailability (Fernandes et al., 2009; Geffard et al., 2005; Mouneyrac et al., 2003).

Consistent with previous studies (Amiard et al., 1987; Geffard et al., 2005; Zhou et al., 2003), we observed positive correlations between Cu concentrations in worms and their environment (especially dissolved Cu in pore waters). Worms in the Jan experiment generally had higher baseline Cu body burdens than those in the Mar experiment, likely due to temporal variations of contamination levels in the field (Aydin-Onen et al., 2015). Cu bioaccumulation was also evident, as Cu body burdens were ~10-fold and 100-fold higher than those in sediments and pore waters, respectively. We found no effect of temperature on Cu body burdens in both experiments, probably because this species has relatively constant metal bioaccumulation parameters across different populations and these parameters are insensitive to temperature (Kalman et al., 2010; Rainbow et al., 2009). Cu toxicity, however, depends on the metabolically available Cu rather than the total accumulated amount (Berthet et al., 2003; Rainbow, 2002).

4.2. Cu homeostasis under temperature effects

Due to the reducing environment in the cell, imported copper is predominantly Cu^{+}, which is toxic and must be strictly regulated (Nevitt et al., 2012; Turski and Thiele, 2009). Cu homeostasis was examined in our study via the mRNA expression levels of related genes (McQuillan et al., 2014) including *ATP7A*, *CCS*, *MTS*, *MTL*, and *GSTO1*. The Cd/Se metallothionein (MTS) and atypical metallothionein-like protein (MTL) help buffer excess Cu in the cytoplasm (Amiard et al., 2006). The active Cu transporter ATP7A delivers intracellular Cu to the Golgi apparatus for cuproprotein synthesis and exports excess Cu from the cell (Zeid et al., 2019). The chaperone CCS inserts Cu into superoxide dismutase, which is important in the defense against reactive oxygen species (Banci et al., 2009). The Phase II omega class glutathione *S*-transferase (GSTO1) conjugates the common Cu ligand glutathione and detoxifies electrophilic compounds formed during oxidative stress (Hayes and Strange, 2000). We found little alterations in the transcript levels of these genes in response to Cu exposure at normal temperature. Our observation is consistent with the previous studies in which ragworms exposed to sediments from certain Cu-contaminated sites showed no difference in gene expression compared with the reference sites (Breton and Prentiss, 2019; McQuillan et al., 2014). The metallothionein protein level and GST enzyme activity in ragworms were also not affected by metal exposure in several field and laboratory studies (Bouraoui et al., 2009; Poirier et al., 2006; Solé et al., 2009). These findings taken together suggest that Cu homeostasis-associated genes might not be sensitive markers of Cu stress (Green Etxabe et al., 2021).

At the elevated temperature, the pattern of gene expression became more complicated with some synergistic effects. The combined exposure of 20 mg kg^{-1} of Cu and warming triggered a weak upregulation of *ATP7A*, suggesting an enhanced Cu efflux. Additionally, we detected a spike in the expression levels of *CCS* and *MTS* in the combined exposure of 10 mg kg^{-1} of Cu and warming. These unexpected non-monotonic responses to Cu might imply that at the 10-mg kg^{-1} exposure the accumulated intracellular Cu necessitated CCS and MTS synthesis (e.g., to prevent oxidative stress), whereas at the 20-mg kg^{-1} exposure, the cells' higher Cu efflux reduced that need. Notably, warming led to the downregulation of *GSTO1* regardless of Cu exposure. Although the exact mechanisms remain unknown, the reduction of GST enzyme activity in response to elevated temperature has been documented in other species

(Dorts et al., 2012; Madeira et al., 2013).

We measured the expression of *HSP70MAJ*, *HSP70MIN*, and *HBL2* encoding the major and minor forms of stress-70 proteins (Sanders, 1990) and linker chain L2 of the giant extracellular hemoglobin (Suzuki et al., 1994) following the suggestion that they might be conserved biomarkers of metal stress in polychaetes (Green Etxabe et al., 2021). However, we only observed inconsistent or weak responses of *HSP70MAJ* and *HSP70MIN* to Cu exposure. Warming had opposite effects on the expression of two stress-70 forms (i.e., inhibited the major form and induced the minor form) implying their different roles during heat stress. *HBL2* also did not present an ideal marker of metal exposure given its induction at 10 mg kg^{-1} of Cu but downregulation at 20 mg kg^{-1} and its very variable expression.

4.3. Cellular effects of Cu and warming exposures

While the molecular markers failed to differentiate the effects of Cu, we found a clear elevation of mitochondrial ETS activity, a proxy of cellular energy demand correlated to the standard metabolic rate (Fanslow et al., 2001; Sokolova, 2021) in response to Cu and warming exposures. The increased ETS activity due to warming is unsurprising given the dependence of biochemical processes on temperature in ectothermic animals (Willmer et al., 2005). However, the enhanced ETS activity in worms exposed to Cu might suggest the higher cellular maintenance costs to regulate Cu homeostasis and alleviate its toxic effects. Increased ETS activity in ragworms has also been recorded following exposure to various contaminants including mercury, carbon nanomaterials, nanoplastics, pharmaceuticals, as well as acidified seawater (Bhuiyan et al., 2021; De Marchi et al., 2017, 2018; Freitas et al., 2016, 2017; Pires et al., 2016, 2022; Silva et al., 2022). We did not find a considerable accumulation of MDA and PC, which are markers of lipid and protein oxidation, respectively, indicating that Cu-induced oxidative stress did not exceed the antioxidant capacity of ragworms. The lack of oxidative stress damage in ragworms despite exposure to contaminants was also observed in many studies, implying the high tolerance of this species to oxidative stress (Buffet et al., 2011; Buffet et al., 2013; Durou et al., 2007; Nunes and Costa, 2019; Pires et al., 2022; Urban-Malinga et al., 2021, 2022).

Given the higher energy demand during Cu and warming exposures, one would expect the declines in energy reserves and total available energy (Ea) as found in some other studies on ragworms (De Marchi et al., 2018; Durou et al., 2005; Durou et al., 2007; Freitas et al., 2016, 2017; Pook et al., 2009). However, we found only a weak reduction of carbohydrate content and Ea in the Mar experiment in responses to elevated temperature, probably due to the utilization of readily available glucose to fuel cellular respiration (Pires et al., 2022). In general, the worms were able to sustain their energy storage under Cu and warming exposures. This finding is consistent with other research which suggests that worms living in contaminated environments can still have high levels of energy reserves when the food is abundant (Mouneyrac et al., 2006, 2010; Pires et al., 2022). In our case, worms were fed daily during the exposure and the aeration of overlying water and the shallow depth of sediments allowed high dissolved oxygen levels for aerobic respiration. These exposure conditions, therefore, facilitated the ATP supply, which can be used for cellular maintenance or stored in energy reserves (Sokolova et al., 2012; Sokolova, 2021).

Due to the little variations in Ea, the energy budget index CEA was mostly dependent on ETS activity as shown by their strong negative correlation. CEA can be roughly interpreted as the time that worms can survive after the energy influx is stopped and the worms begin to deplete their energy reserves at a constant rate. CEA therefore could be an ecologically relevant biomarker (De Coen and Janssen, 1997; Pook et al., 2009; Verslycke et al., 2004) indicating the susceptibility of worms to environmental stressors under field conditions. Both experiments showed that Cu exposure and warming reduced CEA, prompting potentially adverse impacts of Cu and warming at higher biological organization levels.

MGO was another biomarker strongly affected by warming. This reactive glycolytic by-product can form adducts with proteins, lipids, and nucleic acids, causing pathophysiological issues (Allaman et al., 2015). Increased glucose metabolism is expected to increase the formation of MGO (Rabbani et al., 2020), which could explain the correlation between MGO and carbohydrate content in our study. Surprisingly, we observed the MGO decrease in response to warming, probably due to the enhanced activity of the glyoxalase system that detoxifies MGO (Thornalley, 1990).

4.4. Combined effects and research replicability

Interactive (synergistic) effects of Cu and warming were only detected in some molecular responses. At the cellular level, the combined effects of Cu and warming were additive with temperature as a stronger driver. Little effects were found on energy reserves and oxidative stress damage, whereas impacts on the energy consumption rate, energy balance, and MGO were large. Notably, although both experiments were conducted at different times and with different pools of ragworms, they resulted in similar biomarker response patterns.

Previous studies on the combined effects of metal exposure and warming on macrobenthos showed both additive and non-additive effects (Biscéré et al., 2017; da Silva Fonseca et al., 2019; Martino et al., 2021; Tracy et al., 2020; Wang et al., 2016). Systematic reviews on the combined effects of multiple stressors on aquatic organisms demonstrated diverse patterns in the responses, influenced by various factors such as stressor identity, level of biological organizations, and evolutionary history of animals (Crain et al., 2008; Dinh et al., 2022; Morris et al., 2022). Mack et al. (2022) suggested that the detection of interactive effects is influenced by research design rather than reflecting actual ecological processes.

The lack of interaction between Cu exposure and elevated temperature on cellular responses in our study therefore could be explained by several reasons. For example, temperature did not affect the uptake and elimination of Cu in *H. diversicolor* (Kalman et al., 2010; Rainbow et al., 2009). The generally weak effect of Cu and the absence of other stressors such as food scarcity and hypoxia may also leave less room for the interactive effects of Cu and temperature on worm energy balance.

4.5. Conclusions and future directions

We found that Cu exposure and elevated temperature had little impact on the expression of genes related to Cu homeostasis. Our study highlights the need to look for more conserved molecular markers of metal exposure in estuarine polychaetes. By contrast, our data showed additive and replicable effects of Cu exposure and warming on the energy balance of ragworms. We suggest that energy metabolism can be a focus to explore the effects of multiple stressors, because energy-related biomarkers have higher sensitivity and higher predictive power for ecological consequences. This research also indicates that warming could be a potent stressor for infaunal species. A more realistic study design, such as the use of field-collected sediment with multiple contaminants in laboratory exposure, can also be applied to examine the combined effects of sediment contamination and warming.

Credit author statement

Duy Nghia Pham: Methodology, Software, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. **Julie Angelina Kopplin**: Methodology, Investigation, Data Curation, Writing - Review & Editing. **Olaf Dellwig**: Methodology, Validation, Investigation, Data Curation, Writing - Review & Editing. **Eugene P. Sokolov**: Methodology, Validation, Writing - Review & Editing, Supervision. **Inna M. Sokolova**: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Supervision, Project administration,

Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Research data are publicly available on Zenodo at https://doi.org/10.5281/zenodo.7978848.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.121964.

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