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

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Supplementary Material

1. Introduction

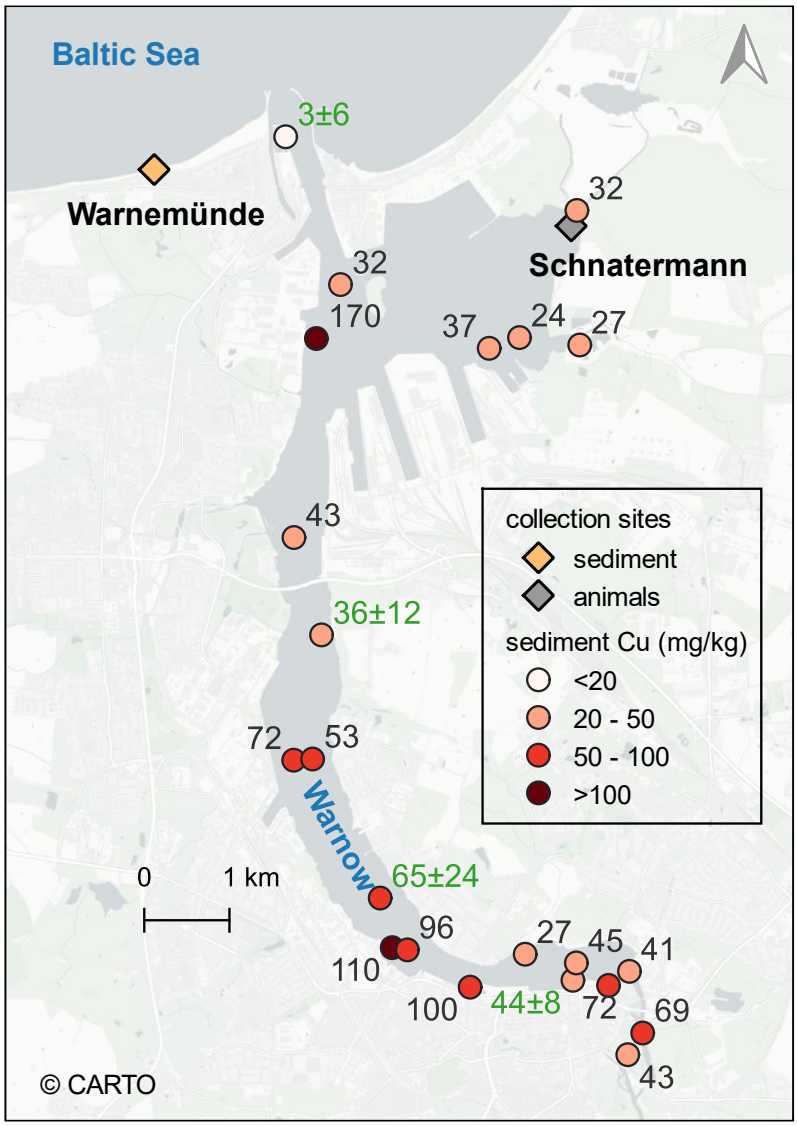

Figure S1. Collection sites of sediment and animals for the experiments and monitoring sites for Cu content in surface sediments (<2 mm) of the Warnow estuary, Germany. Data from our own analyses of samples collected by Rönspieß et al. (2020) in 2017 (*M*±*SD*, in

green) and from the state database (LUNG, 2023) during 2002-2013. Basemap from https://carto.com/.

2. Materials and methods

2.5. Cu analyses

Cu was extracted from 200 mg of the homogenized sediment samples by adding 10 mL of 0.5 M HCl and shaking for 1 h at room temperature. The 0.45 µm-filtered solutions were diluted 50-fold with 2% v/v HNO_3. Pore water samples were diluted 25-fold with 2% HNO_3. Dried worm homogenates (55-300 mg) were digested with 3 mL of HNO_3 and 1 mL of $HClO_4$ in closed Teflon vessels at 180°C for 12 h. After evaporating the acids on a heated block at ~180°C and fuming three times with 6 M HCl, 20 mL of 2% HNO_3 was added to the near-dry residues. Rhodium was added to all samples as an internal standard. ICP-MS was performed with external calibration, using helium as a collision gas to minimize molecular interferences (Dellwig et al., 2019; Lagerström et al., 2013). For measurements of sediment samples, the trueness and precision based on the international reference material SGR-1b (USGS) were 1.6 – 2.1% and 1.4 – 1.5%, respectively. For measurements of pore water samples, the trueness and precision based on the Atlantic seawater (Ocean Scientific International, UK) spiked with 4.8 µg L^{-1} of Cu were 1.9 – 2.8% and 0.4 – 1.1%, respectively. For measurements of worm samples, the trueness and precision based on the international reference material SGR-1b were 2.7% and 0.2%, respectively.

2.6. Colorimetric assays

Briefly, carbohydrate content in the cytoplasmic fraction of homogenates expressed as glucose equivalents, was determined by the reaction with phenol-sulfuric acid forming a 492 nm absorbing product (Masuko et al., 2005). Lipid content in the chloroform-methanol extraction of homogenates (Folch et al., 1957) was assessed by the sulfo-phospho-vanillin reaction producing a 490 nm absorbing compound (Van Handel, 1985). Protein content in the cytoplasmic fraction expressed as bovine serum albumin equivalents, was quantified by the Bradford assay yielding a 595 nm absorbing protein-dye complex (Bradford, 1976). The level of methylglyoxal (MGO) in the cytoplasmic fraction was evaluated by the reaction with Girard's reagent T in borax solution that produces a disubstituted compound measurable at 325 nm using MGO standards (Mitchel and Birnboim, 1977). Malondialdehyde (MDA) level in the homogenates was measured by the reaction with thiobarbituric acid forming a product measurable at 530 nm with the extinction coefficient of 156 mM^{-1} cm^{-1} (Buege and Aust, 1978). Protein carbonyl (PC) level was determined by the reaction with 2,4-dinitrophenylhydrazine (DNPH) that forms protein-dinitrophenylhydrazone measurable at 370 nm using the extinction coefficient of 22 mM^{-1} cm^{-1} (Levine et al., 1990). The activity of mitochondrial electron transport system (ETS) expressed as oxygen consumption equivalents, was assessed by the NAD(P)H-dependent reduction of iodonitrotetrazolium chloride (INT) to formazan monitorable at 490 nm using the extinction coefficient of 15.9 mM^{-1} cm^{-1} (De Coen and Janssen, 1997). All assays were performed using a microplate reader (SpectraMax iD3, Molecular Devices, Germany).

2.7. Quantitative reverse transcription PCR (RT-qPCR)

The anterior part was lysed in TRI Reagent (Zymo Research, USA) in a FastPrep-24 homogenizer (MP Biomedicals, Germany) for RNA extraction. DNA was removed (TURBO DNA-free Kit, Thermo Fisher Scientific) and cDNA was synthesized from 1 µg of total RNA (RevertAid RT Reverse Transcription Kit, Thermo Fisher Scientific) in a thermal cycler (peqSTAR 96 Universal Gradient, PEQLAB Biotechnologie, Germany). Transcript levels were determined by quantitative PCR (Biozym Blue S'Green qPCR Kit Separate ROX, Biozym Scientific, Germany) in a StepOnePlus System (Thermo Fisher Scientific). Post-amplification melting curve analyses were conducted to confirm the specificity of the PCR product. Gene-specific amplification efficiencies (E) were computed using a cDNA standard dilution series (Pfaffl, 2001).

Table S1. Permutation tests for the effects of Cu, temperature (T), and their interaction (int) on the expression (quantification cycle) of reference genes in *Hediste diversicolor* at the end of the exposure in the Mar experiment[1].

reference gene	Factor	df_{num}	F	p
GAPDH	Cu	2	0.69	0.70
	T	1	1.08	0.38
	int	2	0.64	0.70
HIS3	Cu	2	0.24	0.80
	T	1	2.04	0.18
	int	2	0.96	0.40

[1] df_{den} are not available for linear mixed-effects models (Bates, Fri May 19 22:40:27 CEST 2006).

3. Results

3.1. Cu concentrations

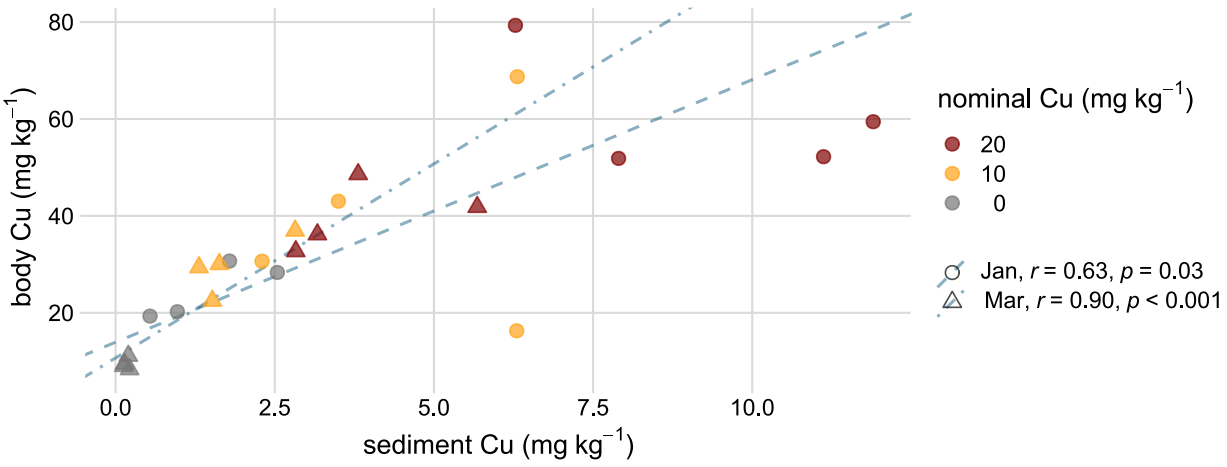

Figure S2. Correlation between total Cu contents in surface sediments and total Cu body burdens in worms at the end of the exposures in the Jan (circles) and Mar (triangles) experiments. Sediments were spiked with Cu at nominal contents of 0, 10, and 20 mg kg^{-1}. Standard deviation lines (Freedman et al., 2007), Pearson correlation and permutation *p*-values are given for each experiment.

Table S2. Permutation tests for the effects of nominal Cu content, exposure temperature (T), and their interaction (int) on 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 and Mar experiments[2].

Medium	Experiment	Factor	*F*	*p*
Sediment	Jan	Cu	37.10	**< 0.001**
		T	0.88	0.37

[2] df_{num} are the same as in Table S1, df_{den} = 24 for sediment and pore water and 6 for body.

Medium	Experiment	Factor	*F*	*p*
		int	0.51	0.62
Sediment	Mar	Cu	19.30	**< 0.001**
		T	0.13	0.74
		int	0.61	0.56
Pore water	Jan	Cu	43.16	**< 0.001**
		T	0.03	0.89
		int	0.02	0.98
Pore water	Mar	Cu	39.74	**< 0.001**
		T	0.54	0.50
		int	2.15	0.14
Body	Jan	Cu	8.50	**0.02**
		T	1.14	0.33
		int	3.16	0.11
Body	Mar	Cu	31.80	**0.002**
		T	1.48	0.28
		int	0.50	0.57

3.2. Survival

Table S3. Survival of *Hediste diversicolor* at the end of the exposures in the Jan and Mar experiments.

Experiment	Number of lives	12 °C	20 °C
Jan, *n* = 15	0 mg kg^{-1}	11	11
	10 mg kg^{-1}	11	8
	20 mg kg^{-1}	7	9
Mar, *n* = 20	0 mg kg^{-1}	18	17
	10 mg kg^{-1}	16	15
	20 mg kg^{-1}	18	17

Table S4. Permutation tests for the effects of Cu, temperature (T), and their interaction (int) on the survival of *Hediste diversicolor* at the end of the exposures in the Jan and Mar experiments[3].

[3] df_{num} are the same as in Table S1, df_{den} are not available for linear mixed-effects models (Bates, Fri May 19 22:40:27 CEST 2006).

Experiment	Factor	*F*	*p*
Jan	Cu	1.22	0.29
	T	0.04	0.91
	int	0.84	0.51
Mar	Cu	0.93	0.29
	T	0.57	0.32
	int	0.01	0.97

3.3-6. Energy reserves-Molecular markers of metal exposure and stress

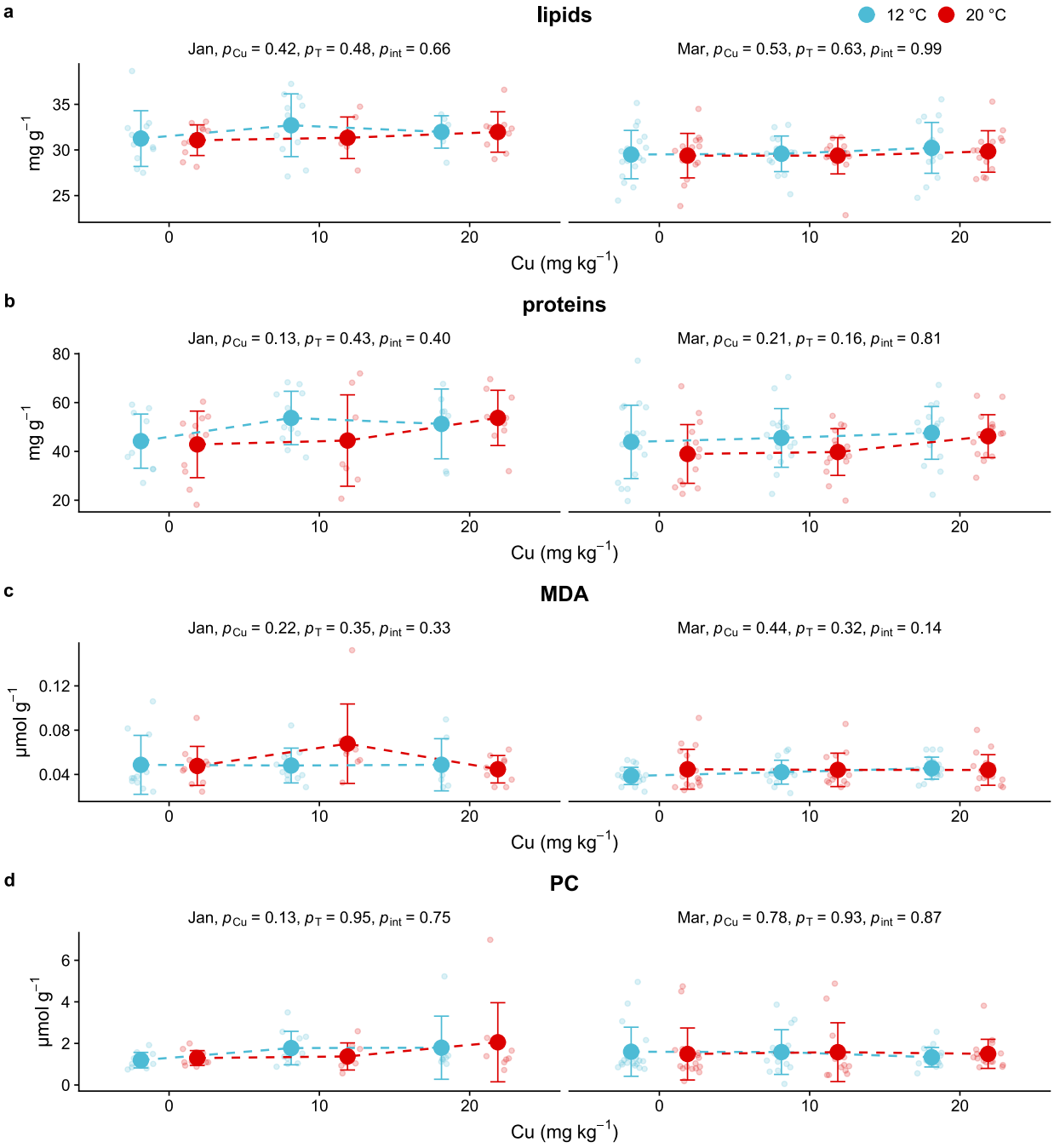

Figure S3. Effects of Cu-spiked sediments and elevated temperature on lipid and protein contents and malondialdehyde (MDA) and protein carbonyl (PC) levels of *Hediste diversicolor* in the Jan and Mar experiments. Data are presented with individual

observations and mean ± standard deviation. Permutation *p*-values are given for Cu, temperature (T), and their interaction (int). *F*-statistics are provided in Table S5.

Table S5. *F*-statistics in permutation tests for the effects of Cu, temperature (T), and their interaction (int) on the biomarker responses of *Hediste diversicolor* at the end of the exposures in the Jan and Mar experiments[4].

Biomarker	Experiment	Cu	T	int
carb	Jan	0.84	1.18	1.03
	Mar	1.58	6.37	0.26
lipid	Jan	0.88	0.52	0.44
	Mar	0.65	0.23	0.01
protein	Jan	2.36	0.65	0.98
	Mar	1.68	2.32	0.21
Ea	Jan	0.81	1.50	1.56
	Mar	2.26	5.84	0.24
ETS	Jan	7.33	39.08	1.26
	Mar	3.33	53.05	1.96
CEA	Jan	8.41	97.07	0.74
	Mar	2.67	112.72	1.07
MGO	Jan	0.89	12.88	0.51
	Mar	2.42	15.78	0.06
MDA	Jan	1.14	0.77	0.95
	Mar	0.80	0.99	2.19
PC	Jan	2.08	0.01	0.38
	Mar	0.23	0.01	0.12
ATP7A	Mar	0.84	0.34	4.00
CCS	Mar	29.97	31.92	30.21
MTS	Mar	14.99	14.89	14.72
MTL	Mar	0.07	0.94	1.13
GSTO1	Mar	1.79	6.15	0.06
HSP70MAJ	Mar	6.61	6.16	0.24
HSP70MIN	Mar	1.30	12.29	3.32
HBL2	Mar	2.10	0.46	0.37

[4] df_{num} are the same as in Table S1, df_{den} are not available for linear mixed-effects models (Bates, Fri May 19 22:40:27 CEST 2006).

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