

Supplementary Information for

Marine pollutant tributyltin affects DNA methylation and fitness of banded

murex (*Hexaplex trunculus*) populations

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Material and methods

Sampling and sample processing

Table S1. Total number of banded murex (*H. trunculus*) individuals collected in the Adriatic Sea and processed in each analysis.

Site	N total (♀ + ♂)	N morphometry (♀ + ♂)	N imposex (♀)	N OTCs (♀)	N biomarkers (♀ + ♂)	N MSAP (♀ + ♂)
Split-harbour	48 (33 + 15)	48	33	10	6	30
Vranjic	77 (29 + 48)	77	29	8	10	30
Trogir	60 (24 + 36)	60	24	10	10	30
Split-Špinut	59 (31 + 28)	59	31	10	10	30
Ston	86 (31 + 55)	86	31	10	8	30
Marina	88 (36 + 52)	88	36	10	10	30
Čiovo	54 (31 + 23)	54	31	9	10	30
Total	472 (215 + 257)	472	215	67	64	210

Biomarkers activity measurements

For protein extraction, the digestive gland tissue of 6–10 individuals per population was homogenized in an MM300 TissueLyser (Qiagen-Retsch) with steel balls in 1.2 mL of a 50 mM potassium phosphate buffer (pH 7.0) with 0.1 mM of EDTA. The homogenate was centrifuged at $10000 \times g$ for 12 min at 4 °C. The supernatant was collected and used for biomarker activity measurements.

For enzyme assays, supernatants were diluted with an extraction buffer 1:5 (v/v). All measurements were performed with a SPECORD 40 spectrophotometer (Analytic Jena AG). Catalase (CAT) activity was assayed by measuring the decrease in absorbance at 240 nm ($\epsilon = 36 \text{ mM}^{-1}\text{cm}^{-1}$) [31]. The reaction mixture consisted of a 50 mM potassium phosphate buffer (pH 7.0), 10 mM of H_2O_2 , and 50 μL of the sample. CAT activity is expressed in units per mg of protein, where one unit is the amount of enzyme necessary for the hydrolysis of 1 mol of H_2O_2 per minute at 25 °C and pH 7.0. Glutathione reductase (GR) activity was determined via NADPH oxidation at 340 nm ($\epsilon = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$) [32]. The reaction mixture contained a 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM of EDTA, 0.2 mM of NADPH, 0.5 mM of GSSG, and 100 μL of the sample. GR activity is expressed in units per mg of protein, where one unit is the amount of enzyme necessary for the oxidation of 1 nmol of NADPH per minute at 25 °C and pH 7.0. Glutathione S-transferase (GST) activity was assayed by measuring the decrease in absorbance at 340 nm ($\epsilon = 9.6 \text{ mM}^{-1}\text{cm}^{-1}$), as previously described [33] with slight modifications. The reaction mixture consisted of a 50 mM

potassium phosphate buffer (pH 6.5), 0.1 mM of EDTA, 1 mM of reduced glutathione (GSH), 50 μ L of the extract, and 500 μ L of 1.5 mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol (final concentration of 0.5 mM). GST activity is expressed as nmol of conjugated glutathione per minute per mg of protein. Acetylcholinesterase (AChE) activity was assayed by measuring the decrease in absorbance at 412 nm ($\epsilon = 0.07 \text{ mM}^{-1}\text{cm}^{-1}$), as previously described [34]. The reaction mixture consisted of a 100 mM potassium phosphate buffer (pH 7.4), 0.1 mM of EDTA, 8 mM of 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), 8 mM of acetylcholine, and 50 μ L of the sample. AChE activity is expressed as nmol of thiocholine released per minute per mg of protein. For carbonyl quantification, a dinitrophenylhydrazine (DNPH) reaction was used [35]. Briefly, 200 μ L of supernatants diluted to 1:1 (v/v) with an extraction buffer were combined with 300 μ L of 10 mM DNPH in 2 M of HCl. As a blind sample, 200 μ L of an extract mixed with 2 M of HCl (300 μ L) was used. After 1 h of incubation at room temperature (samples were mixed every 15 min), the proteins were precipitated with 500 μ L of cold 10% (w/v) trichloroacetic acid, and the pellets were washed three times with 500 μ L of ethanol:ethylacetate (1:1; v:v) and centrifuged at 12000 \times g for 10 min at 4 $^{\circ}$ C to remove the excess reagent. The precipitated proteins were dissolved in 6 M of urea in a 20 mM potassium phosphate buffer (pH 2.4), and the absorption at 370 nm was measured. Protein recovery was estimated for each sample by measuring the absorption at 280 nm. Carbonyl contents were calculated using a molar absorption coefficient for aliphatic hydrazones ($\epsilon = 22 \text{ mM}^{-1} \text{ cm}^{-1}$) and is expressed in μ mol per mg of protein. The level of lipid peroxidation was indirectly determined as the formation of malondialdehyde (MDA) in a reaction with thiobarbituric acid (TBA), as previously described [36] with slight modifications. Homogenates (150 μ L) were mixed with 450 μ L of cold 10% trichloroacetic acid (TCA) to precipitate proteins. The precipitate was pelleted by centrifugation (10000 \times g for 15 min at 4 $^{\circ}$ C), and 550 μ L of the supernatant was reacted with an equal volume of 0.7% (w/v) TBA in 10% TCA. After heating at 90 $^{\circ}$ C for 15 min, the mixture was cooled in an ice bath. The absorbance of the supernatant was measured at 532 nm, and correction for unspecific turbidity was conducted by subtracting the absorbance at 600 nm. As a blind sample, 0.7% TBA in 10% TCA was used. The content of lipid peroxides was calculated using a molar absorption coefficient ($\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$) and is expressed as μ mol per mg of protein. The total protein

content was determined with the Bradford method [37]. Values for each biomarker were normalized based on the protein content in each sample. A PCA of biomarker data was performed using R studio version 1.0.136. ANOVA and Tukey post-hoc tests were applied to PC1 and PC2 scores to define significance in population separation using the “aov” and “TukeyHSD” functions, respectively, in R studio ($p \leq 0.05$).

Results

Biomarkers

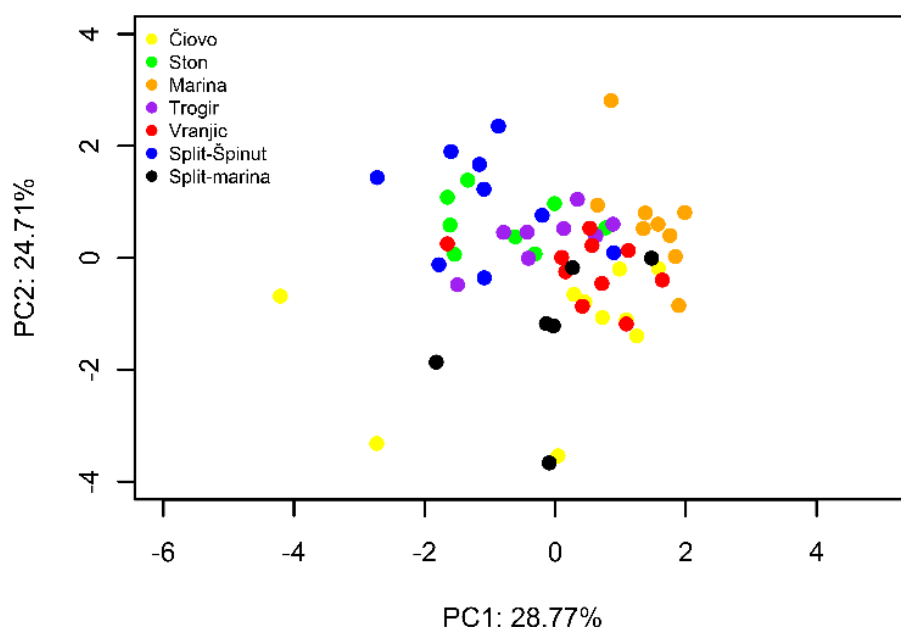


Figure S1. Principal component analysis (PCA) of *H. trunculus* biomarker responses. In PC1, only the intermediately polluted site of Marina was significantly separated from the lowly and highly polluted sites; ANOVA and Tukey post-hoc test, $p \leq 0.05$; In PC2, there was no significant separation based on pollution intensity; ANOVA and Tukey post-hoc test, $p \leq 0.05$.

Morphometry

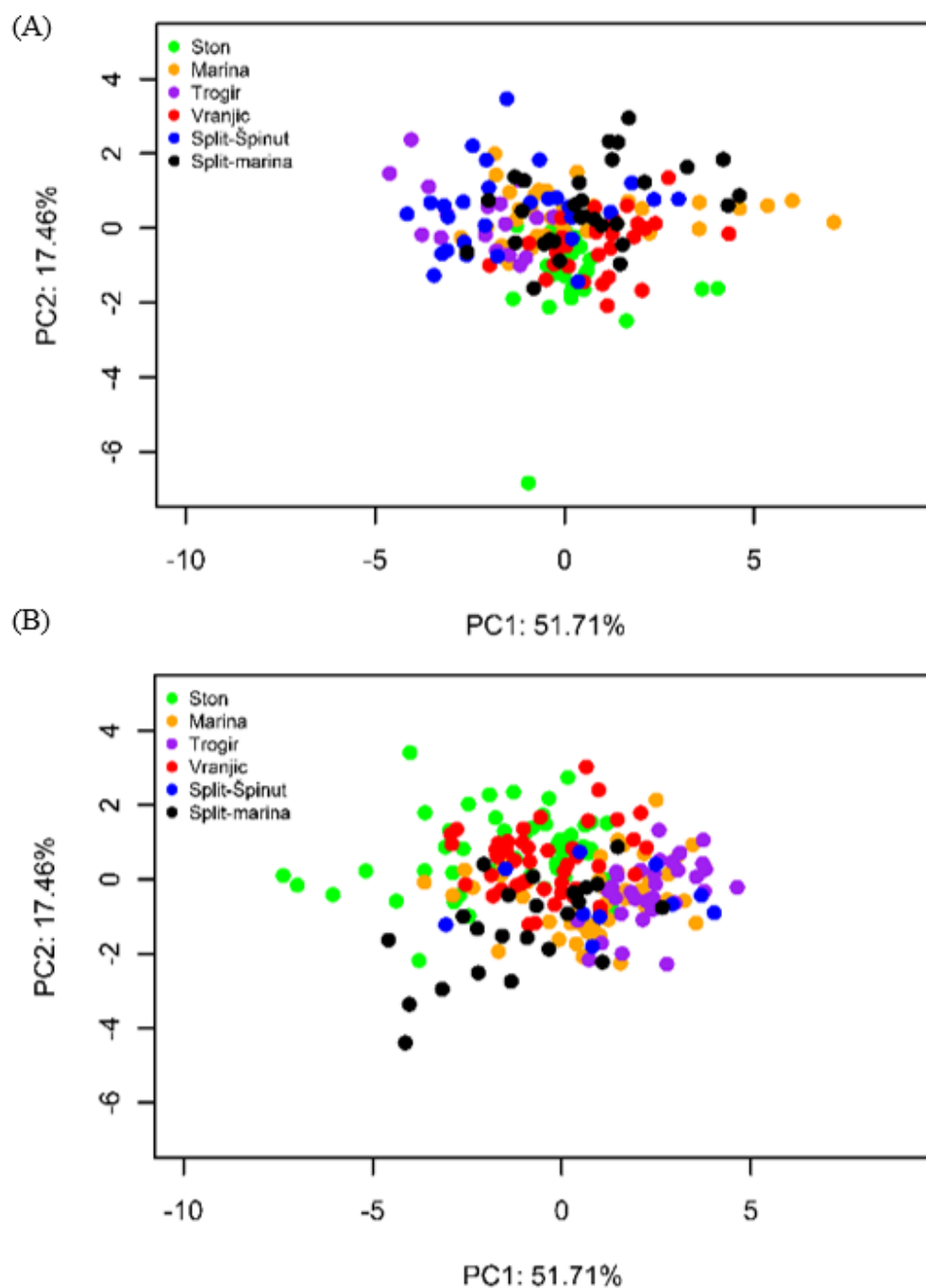


Figure S2. Principal component analysis (PCA) of *H. trunculus* morphometric measures. (A) Females: In PC1, there was a significant separation of snails originating from the highly polluted sites of Trogir and Split-Špinut from the other sites. In PC2, the lowly polluted site of Ston was significantly separated from the intermediately and highly polluted sites. (B) Males: In both PC1 and PC2, the lowly polluted site was significantly separated from the intermediately and highly polluted sites; ANOVA and Tukey post-hoc test, $p \leq 0.05$.

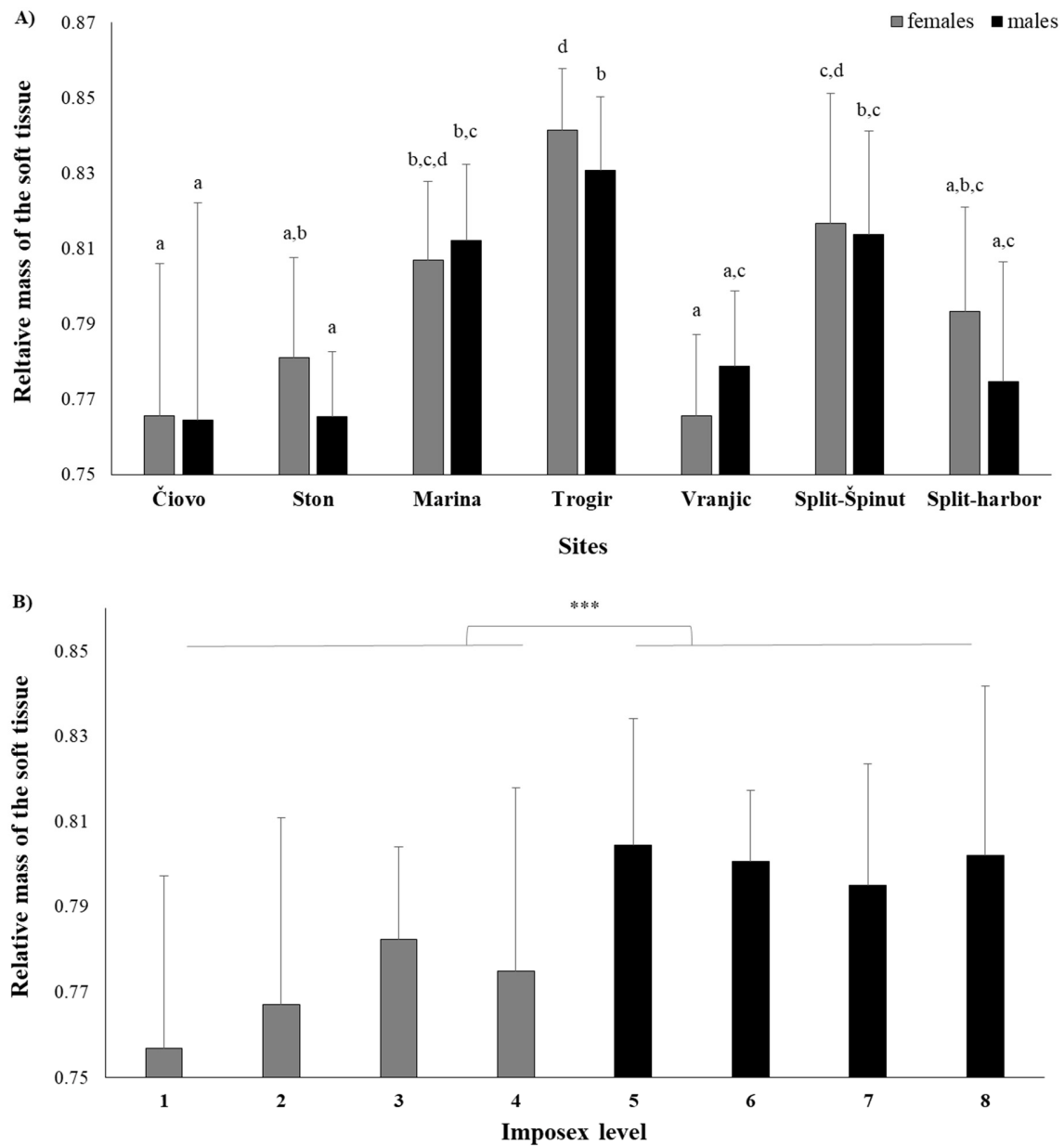


Figure S3. Relative mass of the soft tissue normalized on total animal length (log data); (A) per sampling site on females and males; (B) per imposex level across all populations. Letters relate to the significance between populations (for females and males separately) (ANOVA and Tukey post-hoc test, $p < 0.05$), and stars denote the significance between low (1-4) and high (5-8) imposex levels (ANOVA and Tukey post-hoc test, $p < 0.001$).

Epigenetic and genetic diversity

Table S2. Genetic diversity indices calculated for genetic (NML) and epigenetic (MSL) fragments. A_N —observed number of alleles; A_E —effective number of alleles; h —Nei's gene diversity; I —Shannon's information index.

Pollution level	Population	Genetic (NML)				Epigenetic (MSL)			
		A_N	A_E	h	I	A_N	A_E	h	I
Low	Čiovo	1.698	1.097	0.082	0.159	1.518	1.142	0.098	0.166
	Ston	1.395	1.052	0.044	0.085	1.501	1.130	0.088	0.151
Intermediate	Marina	1.581	1.074	0.062	0.123	1.558	1.165	0.109	0.182
High	Trogir	1.488	1.067	0.057	0.111	1.524	1.162	0.109	0.182
	Vranjic	1.419	1.057	0.048	0.095	1.597	1.160	0.108	0.184
	Split-Špinut	1.558	1.077	0.065	0.126	1.575	1.161	0.110	0.186
	Split-harbour	1.605	1.087	0.074	0.142	1.549	1.155	0.105	0.178

Table S3. Fragments putatively under selection (outliers) detected using Arlequin and Mcheza software: (A) epigenetic (MSL) fragments; (B) genetic (NML) fragments.

(A) Epigenetic (MSL) fragments

Fragment ID	Arlequin	Mcheza
S1B_164	+	-
S1G_41	+	-
S1G_227	+	-
S2G_176	+	+
S3B_145	+	+
S3B_161	-	+
S3G_2	+	-

(B) Genetic (NML) fragments

Fragment ID	Arlequin	Mcheza
S1G_53	+	+

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