Supplemental Material

Teratogenic, bioenergetic, and behavioral effects of exposure to total particulate matter on early development of zebrafish (*Danio rerio*) are not mimicked by nicotine

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Experimental set-up (TPM)

The partial composition of TPM is summarized in Table S1. The nicotine equivalents concentration in experimental TPM treatments (i.e. $TPM_{0.4}$ and $TPM_{1.4}$) was calculated based on the known nicotine concentration in stock TPM solution as per Labstat International. For example, to generate $TPM_{1.4}$ the stock solution was diluted 1000x, thereby reducing the nicotine concentration from 1379 μg/mL to 1.4 μg/mL.

The number of larvae and replicate dishes used for each endpoint is summarized in Table S2. The study consisted of Part I – general physiological parameters, o-Dianisidine staining, angiogenesis, and antioxidant markers, Part II – bioenergetics, and Part III – larval behavior.

In Part I, each experimental group had 8 replicate dishes with 15 embryos in each. Mortality, hatching, and deformities were assessed in each of the 8 replicate dishes and averaged. Spontaneous contractions at 24 hpf and heart rate at 56 hpf were assessed in 4 randomly chosen embryos/larvae from 3 randomly chosen Petri dishes for each of the experimental groups and averaged in each case. Pericardial area and CYP1a activity were assessed at 72 hpf in one of the Petri dishes. At the end of the experiment each Petri dish was assigned to a different assay, including hemoglobin analysis, angiogenesis, enzyme activities, and glutathione levels. Larvae from 1 Petri dishes were also anesthetized and visualized under the microscope to determine larval length and eye diameter. Larval mass was obtained during the collection of larvae for antioxidant markers assays into pre-weighed 1.5 mL Eppendorf tubes. The experiment was repeated on at least 3 separate zebrafish cohorts, such that each cohort constituted an n-value of 1.

In Part II, each experimental group had 3 replicate dishes with 15 embryos in each. At 36 hpf 7 pairs of embryos were randomly selected from the 3 Petri dishes for the bioenergetic

assessment (2 embryos per well, 7 wells per treatment). The experiment was repeated on at least 3 separate zebrafish cohorts, such that each cohort constituted an n-value of 1.

In Part III, each experimental group had 3 replicate dishes with 15 embryos in each. At 96 hpf the larvae were screened for deformities, transferred to a beaker containing ~30 mL fresh (and TPM-free) Danieau. At 144 hpf the larvae were randomly transferred to a 96-well plate (1 embryos per well, ~30-40 wells per treatment) to conduct behavioral assessments. The experiment was repeated on at least 3 separate zebrafish cohorts, such that each cohort constituted an n-value of 1.

Experimental set-up (nicotine)

In subsequent experiments exposures to nicotine alone were carried out in a similar fashion as described for TPM exposures. Briefly, nicotine was prepared in DMSO and the embryos were exposed to 0.4, and 1.4 μg/mL nicotine, corresponding to the nicotine content in $TPM_{0.4}$, $TPM_{1.4}$; the treatments are abbreviated $Nic_{0.4}$ and $Nic_{1.4}$, respectively. On the day of the exposure, a fresh stock solution of 5 mg nicotine/mL was prepared in DMSO, adjusting for the difference in molecular weights of nicotine hydrogen tartrate salt (462.41 g/mol) and nicotine (162.23 g/mol). To prepare $Nic_{0.4}$, 12 μL of stock solution was combined with 138 μL DMSO and 9850 μL of embryo medium; one milliliter of that solution was then added to the appropriate Petri dishes, containing 15 embryos and 14 mL of embryo medium to yield a final volume of 15 mL. To prepare $Nic_{1.4}$, 42 μL of stock solution was combined with 108 μL DMSO and 9850 μL of embryo medium; one milliliter of that solution was then added to the appropriate Petri dishes, containing 15 embryos and 14 mL of embryo medium to yield a final volume of 15 mL. Multiple endpoints were assessed as described for TPM.

Staining

For hemoglobin analysis, the larvae were incubated in a solution of o-Dianisidine (0.6 mg/mL) containing 0.01 M sodium acetate (pH 4.5), 0.65% hydrogen peroxide, and 40% (v/v) ethanol in the dark for 15 min. For angiogenesis, the larvae were treated with pre-cooled acetone for 30 min at -20°C, rinsed twice with PBST for 5 min, and equilibrated three times with NTMT (in mM: 100 NaCl, 100 Tris, 50 $MgCl_2$, and 0.1% Tween-20) for 15 min at room temperature, and incubated in the staining solution (1 mL NTMT, 2.25 μL 4-nitroblue tetrazolium chloride (NBT; 75 mg/mL in 70% dimethylformamide), 1.75 μL 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; 50 mg/mL in dimethylformamide) for 15-30 min. The larvae were washed three times with PBST (in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, and Tween-20) 1 min each, and transferred to 50% glycerol for 5 min, then 87% glycerol, and visualized using a Nikon SMZ 1500 dissecting microscope equipped with DS-Fi1 digital camera. To quantify the vascular pattern in the brain the images were blindly scored as either 'normal' (all vessels are present, the correct pattern is apparent) or 'disrupted' (missing vessels and/or the correct pattern is absent) and analyzed for statistical significance.

Enzymes activities and glutathione concentrations

Larval extracts for antioxidant enzymes activities were prepared by sonication for 10 s using Ultrasonic Homogenizer (Model 3000, Biologics, Inc.) in 0.2 mL 50 mM potassium-phosphate buffer (KPB-50; pH 7.0) supplemented with a protease inhibitor cocktail (P8340, Sigma-Aldrich), followed by a 15 min centrifugation at 15,000 g at 4°C. The supernatants were collected into fresh tubes for the assays. Larval extracts for glutathione measurements were prepared by sonication as above in 0.2 mL 5% sulfosalicylic acid bubbled with argon gas for 20

min prior to use. The homogenates were centrifuged at 5000 g for 5 min at 4°C and the supernatants were collected into fresh tubes for the assays.

Bioenergetics

Several trials were conducted to assess the oxygen consumption rate (OCR), using the XFe24 Extracellular Flux Analyzer. The trials are summarized in Table S3 in the sequence that they were performed during the study. Briefly, embryos (36 hpf) were transferred to a XFe24 islet plate (2 embryos/well, 7 wells/treatment). Each well received 525 μL 65 ppm artificial seawater solution. Each measurement cycle consisted of 2 min intervals of mixing, waiting, and measurement periods. Basal OCR was measured over 8 cycles, prior to the injection of pharmacological agents: (i) the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (2.5 μM FCCP; represents maximal mitochondrial respiration), (ii) an inhibitor of the proton channel of ATP synthase oligomycin (9.4 μM Oligo; represents the OCR in absence of ATP synthesis), and (iii) the inhibitor of cytochrome c oxidase, sodium azide (6.25 mM NaN_3; represents non-mitochondrial respiration). FCCP and Oligo treatments were never performed in the same trial. Final OCRs were calculated by averaging the three lowest recorded values for basal, oligomycin, and NaN_3 treatments, and the three highest recorded values for the FCCP treatment for a given well per embryo. The following were calculated based on the obtained OCRs:

i. Total mitochondrial respiration = None – NaN_3,
ii. Maximal mitochondrial respiration = FCCP – NaN_3,
iii. Spare capacity = FCCP – None, and
iv. ATP synthesis = None – Oligo.

Note: Oligo treatment was not performed with nicotine, since no significant differences were observed with that treatment with TPM.

Larval Behavior

At 96 hpf the embryo medium was removed and fresh embryo medium was added to the Petri dish, the larvae were gently swirled, and the procedure was repeated once more. Larvae were then transferred into a 100 mL beaker containing 50 mL of fresh embryo medium and transported to Dr. Levin's laboratory, where they were kept under a 14:10 h light-dark cycle at 28°C in an incubator. At 144 hpf, larvae were randomly transferred to a 96-well plate (1 larvae/well, ~30 wells/treatment, in each of the 3 cohorts; Table S2) and allowed to acclimate in dark for 1 h before being transferred to a DanioVision™ observation chamber (Noldus Inc., Wageningen, The Netherlands). Swimming distance was monitored for 50 min in alternating 10 min dark ("0% illumination", <1 lux) and light ("100% illumination", 5,000 lux) periods, starting with a 10 min habituation period in dark. Larval motion was recorded at a sample rate of 30 times/second via a high speed infrared camera. Video data were analyzed by computer tracking software, EthoVision XT® (Noldus, Wageningen, The Netherlands), to calculate total distance moved for each individual larvae over the course of the trial.

Table S1. List of the major constituents in the total particulate matter (TPM) obtained from 3R4F Kentucky Reference Cigarette. The experimental concentrations were prepared by diluting the stock solution in embryo medium to yield $TPM_{0.4}$ and $TPM_{1.4}$, respectively (see footnotes for details).

Tobacco constituent[a]	Stock	$TPM_{0.4}$[b]	$TPM_{1.4}$[c]
Nicotine	1379	0.414	1.379
N-Nitrosamines			
Nitrosonornicotine (NNN)	156	0.047	0.156
Nitrosoanatabine (NAT)	205	0.062	0.205
Nitrosoanabasine (NAB)	28.6	0.009	0.029
4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK)	151	0.045	0.151
Polycyclic aromatic hydrocarbons (PAHs)			
Naphthalene	463	0.139	0.463
1-methylnaphthalene	645	0.194	0.645
2-methylnaphthalene	572	0.172	0.572
Acenaphthylene	195	0.059	0.195
Acenaphthene	73.2	0.022	0.073
Fluorene	279	0.084	0.279
Phenanthrene	163	0.049	0.163
Anthracene	65	0.019	0.065
Fluoranthene	100	0.030	0.100
Pyrene	64.7	0.019	0.065
Benzo[*a*]anthracene	26.3	0.008	0.026
Chrysene	30.3	0.009	0.030
Benzo[*b*]fluoranthene	9.8	0.003	0.010
Benzo[*k*]fluoranthene	3.52	0.001	0.004
Benzo[*e*]pyrene	6.96	0.002	0.007
Benzo[*a*]pyrene	11.2	0.003	0.011
Perylene	1.24	0.0004	0.001
Indeno[*1,2,3-cd*]pyrene	3.36	0.001	0.003
Benzo[*g,h,i*]perylene	2.8	0.0008	0.003

[a] Concentrations are reported in μg/mL for nicotine and ng/mL for the rest of the constituents.

[b] $TPM_{0.4}$ was prepared by combining 45 μL of stock TPM solution with 105 μL DMSO and 9850 μL of embryo medium; one milliliter of that solution was then dispensed into appropriate Petri dishes, containing 15 embryos and 14 mL of embryo medium to yield a final volume of 15 mL.

[c] $TPM_{1.4}$ was prepared by combining 150 μL of stock TPM solution with 9850 μL of embryo medium; one milliliter of that solution was then dispensed into appropriate Petri dishes, containing 15 embryos and 14 mL of embryo medium to yield a final volume of 15 mL.

Table S2. Summary of the number of larvae and Petri dishes used for each cohort throughout the study.

	Petri dishes [a]	Embryos/larvae per Petri dish [b]	Total number of embryos/larvae [c]
Part I			
Mortality	8	15[d]	120
Hatching	8	15[d]	120
Spontaneous contractions	3	4	12
Heart rate	3	4	12
Deformities	8	15[d]	120
Pericardial area and CYP1a activity	1	15[d]	15
Hemoglobin analysis	1	15[d]	15
Angiogenesis	1	15[d]	15
Antioxidant enzymes	1	15[d]	15
Glutathione levels	1	15[d]	15
Larval length and eye diameter	1	15[d]	15
Larval mass	2	15[d,e]	n/a[e]
Part II	3	4-6[f]	14
Part III	3	30-40	90-120

[a] Petri dishes assessed for a given endpoint per experimental group in each experiment.
[b] Larvae per each Petri dish assessed for a given endpoint per experimental group in each experiment.
[c] Total number of larvae assessed for a given endpoint per experimental group in each experiment.
[d] For simplicity the initial number of embryos at the beginning of each experiment is indicated. See Section 3.1 of the main article for details on mortality.
[e] Larval mass was obtained by collecting larvae for antioxidant enzymes and glutathione assays into pre-weighed Eppendorf tubes.
[f] Seven pairs of embryos were randomly selected from the three Petri dishes.
'n/a' refers to 'not applicable'

Table S3. Summary of the trials performed using the XFe24 Extracellular Flux Analyzer to measure oxygen consumption rate (OCR). The OCR was measured in the absence (None) or presence of pharmacological agents carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), oligomycin (Oligo), and sodium azide (NaN_3). See the Bioenergetics section above as well as in the Materials and Methods section for more details.

	Trial	Pharmacological agent			
		None	FCCP	Oligo	NaN_3
TPM	1	●	●		
	2	●	●		●
	3	●		●	
	4	●		●	
	5	●	●		●
	6	●		●	●
	7	●	●		●
Nicotine	8	●	●		●
	9	●	●		●
	10	●	●		●

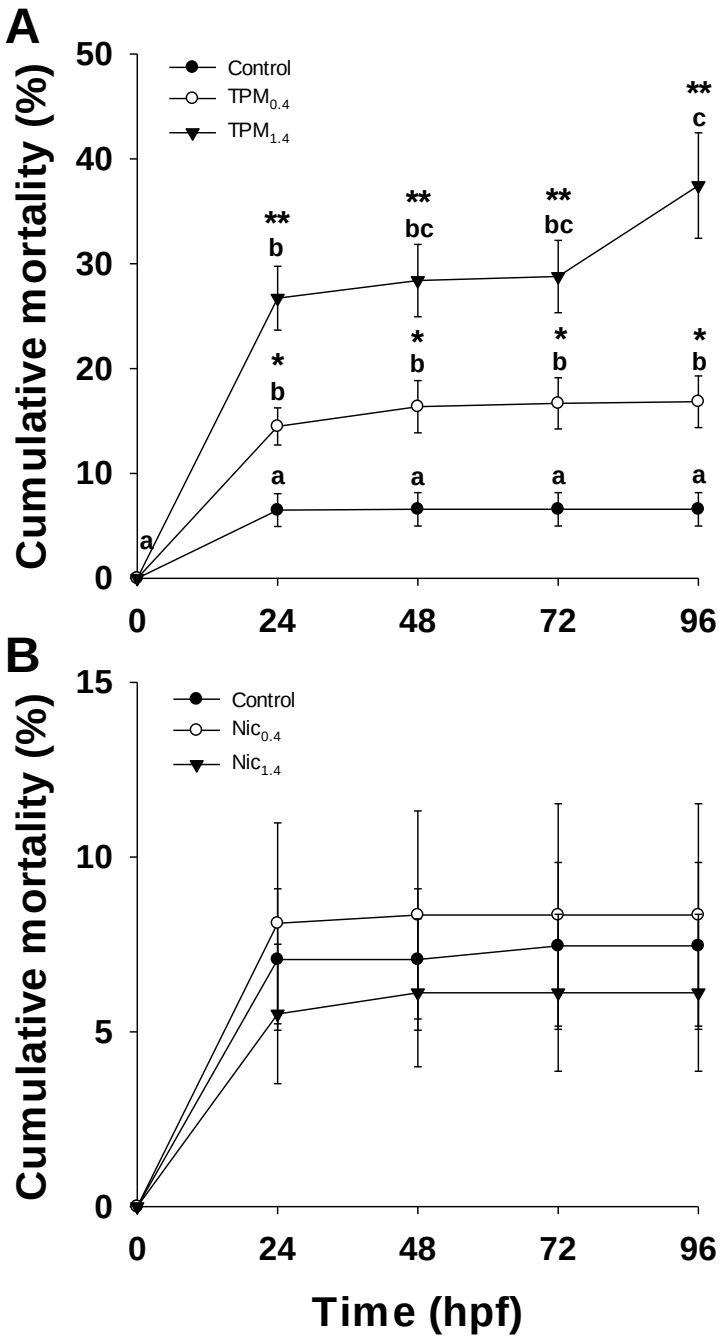

Figure S1. Cumulative mortality in zebrafish exposed to TPM (A) or nicotine (B). Means + SEM (n = 4-8) are displayed. Two-way ANOVA with a post-hoc Tukey method was used to assess statistical differences. The lower case letters indicate significant differences between time points within the same treatment, asterisk (*) indicates a significant difference from the control group within the same time point, and double asterisk (**) indicates a significant difference from both the control and $TPM_{0.4}$ within the same time point. No significant differences were observed with nicotine.

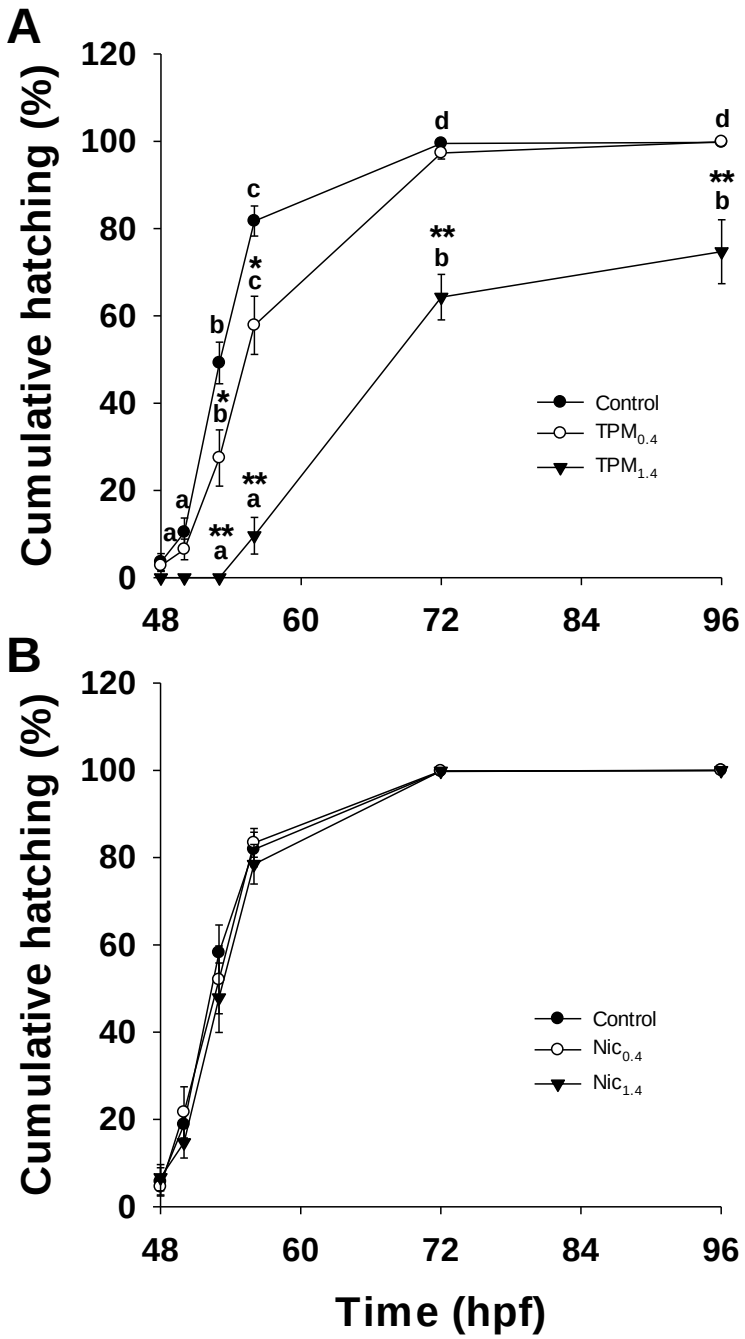

Figure S2. Cumulative hatching success in zebrafish exposed to TPM (A) or nicotine (B). Means + SEM (n = 4-8) are displayed. Two-way ANOVA with a post-hoc Tukey method was used to assess statistical differences. The lower case letters indicate significant differences between time points within the same treatment, asterisk (*) indicates a significant difference from the control group within the same time point, and double asterisk (**) indicates a significant difference from both the control and $TPM_{0.4}$ within the same time point. No significant differences were observed with nicotine.

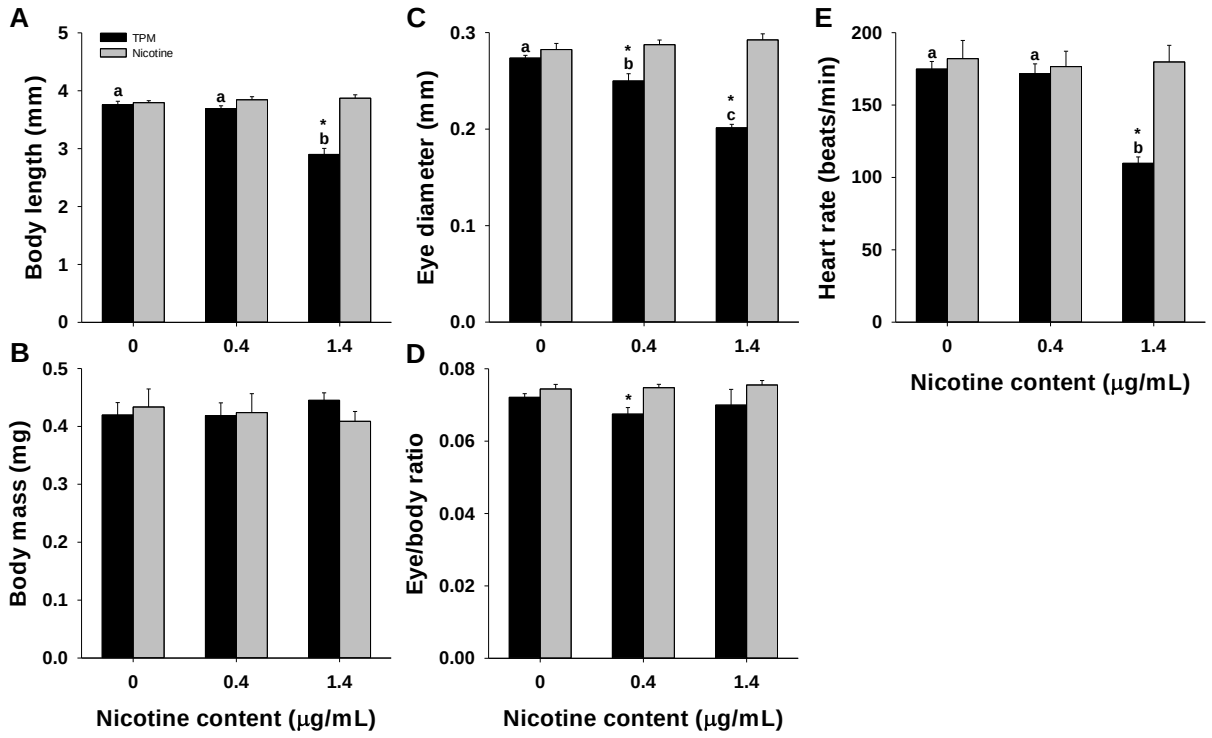

Figure S3. Various parameters that were assessed during TPM exposure: (A) larval body length at 96 hpf, (B) larval body mass at 96 hpf, (C) Eye diameter at 96 hpf, (D) Eye diameter normalized to body length, and (E) heart rate at 56 hpf. One-way ANOVA with a post-hoc Tukey method was used to assess statistical differences. The lower case letters indicate differences between treatments. In all cases Means + SEM (n = 4-8) are displayed. Note: the control group is referred to as '0 μg/mL'.

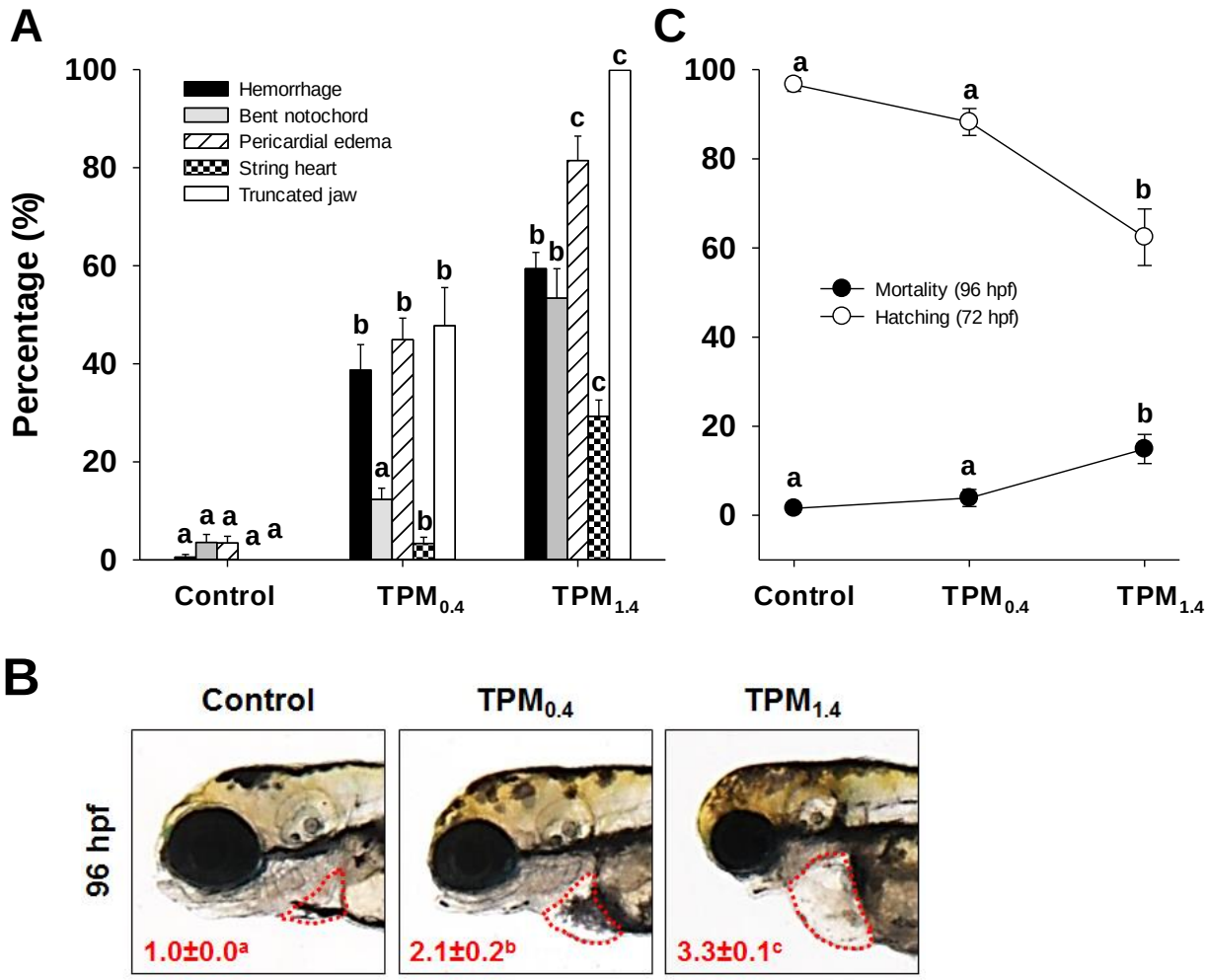

Figure S4. Summary of endpoints that were assessed in embryos that were exposed to $TPM_{0.4}$ and $TPM_{1.4}$ starting at 24 hpf. A. Incidence of deformities in 72 hpf zebrafish larvae exposed to TPM. Means + SEM (n = 3) are displayed. One-way ANOVA with a post hoc Tukey method was used to assess statistical differences. The lower case letters indicate differences between treatments within each type of deformity. B. Pericardial area in 96 hpf zebrafish larvae exposed to TPM. The numbers in red indicate mean ± SEM (n = 3) pericardial area as fold change. One-way ANOVA with a post hoc Tukey method was used to assess statistical differences as indicated by lower case letters. C. Closed circles represent cumulative mortality at 96 hpf (total percentage of dead embryos/larvae) and open circles represent cumulative hatching at 72 hpf (total percentage of hatched embryos). Means + SEM (n = 3) are displayed. One-way ANOVA with a post hoc Tukey method was used to assess statistical differences.