Single and mixed exposure to cadmium and mercury in Drosophila melanogaster: Molecular responses and impact on post-embryonic development

Laëtitia Frat, Thomas Chertemps, Élise Pesce, Françoise Bozzolan, Matthieu Dacher, Rosario Planelló, Óscar Herrero, Lola Llorente, Didier Moers, David Siaussat

Ecotoxicology and Environmental Safety 220 (2021) 112377

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Single and mixed exposure to cadmium and mercury in *Drosophila melanogaster*: Molecular responses and impact on post-embryonic development

Laëtitia Frat [a,b], Thomas Chertemps [b], Elise Pesce [b], Françoise Bozzolan [b], Matthieu Dacher [b,c], Rosario Planelló [d], Oscar Herrero [d], Lola Llorente [d], Didier Moers [a], David Siaussat [b,*,1]

[a] Syndicat Intercommunal pour l'Assainissement de la Région de Pontoise (SIARP), 95000 Pontoise, France
[b] Sorbonne Université, INRA, CNRS, IRD, UPEC, Institut d'Ecologie et des Sciences de l'Environnement de Paris (iEES-Paris), F75005 Paris, France
[c] Sorbonne Université, INRA, CNRS, IRD, UPEC, Institut d'Ecologie et des Sciences de l'Environnement de Paris (iEES-Paris), 78026 Versailles, France
[d] Biology and Environmental Toxicology Group, Faculty of Sciences, Universidad Nacional de Educación a Distancia (UNED), Paseo de la Senda del Rey 9, 28040 Madrid, Spain

ARTICLE INFO

Edited by: Professor Bing Yan

Keywords:
Heavy metals
Mixture
Wastewater
Insect
Sublethal concentration
Gene expression
qPCR

ABSTRACT

Heavy metals, like many other chemical elements, are naturally present in the environment; however, the concentrations of these metals in various environmental matrices have increased through their intensive use in many human activities (such as industry, mining and agriculture). Among the heavy metals, cadmium (Cd) and mercury (Hg) induce a wide variety of defects in animals. While the effects of these heavy metals have been widely documented, a single exposure paradigm is typically used. Few studies have focused on evaluating combined exposure to these metals. However, in the environment, animals are confronted with a plethora of substances simultaneously; thus, the presence and origin of such substances must be determined to reduce the sources of contamination. Using the model of the fruit fly *Drosophila melanogaster*, for which many tools are readily available, we investigated how different concentrations of Cd and Hg in single and combined exposures impact post-embryonic development. In parallel, we evaluated the extended expression pattern of 38 molecular targets used as potential biomarkers of exposure through qPCR. Our results showed that both metals caused developmental delays and mortality in dose-dependent responses. Both metals were able to deregulate genes involved in hormonal control, general stress, and oxidative stress. Importantly, we confirmed synergistic interactions between Cd and Hg. Our results indicate the importance of assessing several biomarkers and their kinetics in mixtures. *Drosophila* represents a useful model for monitoring the toxicity of substances in polluted environments.

1. Introduction

Heavy metals and many others chemical elements are naturally present in environment, with cadmium (Cd) and mercury (Hg) being particularly widespread (Wu et al., 2016). These two metals are intensively used in several human activities, including non-ferrous metal refining and household waste incinerators, resulting in their being discharged in industrial waste and wastewater; consequently, their concentrations are high in various environmental matrices (Caballero-Gallardo et al., 2016; Wu et al., 2016). Cd and Hg are toxic compounds that induce a wide variety of defects in animals. Depending on their mode of action and route of exposure, Cd and Hg can bioaccumulate, induce protein denaturation, disrupt essential enzymes (e.g., DNA methyltransferase), and even damage cell membranes by oxidative stress. These molecular effects disrupt critical functions, including locomotion, digestive activity, and the nervous system (Genchi et al., 2020). Over the last 10 years, Cd and Hg have also been identified as potential endocrine disruptors (EDCs) in vertebrate and invertebrate models, altering the expression of hormone receptor genes and circulating hormone levels, along with disrupting the production in the endocrine gland (Iavicoli et al., 2009; Planelló et al., 2010; Rana, 2014). Yet, the analysis of heavy metals impacts needs refining to

* Correspondence to: Institut d'Ecologie et Sciences de l'Environnement de Paris – Département Ecologie Sensorielle.
E-mail address: david.siaussat@sorbonne-universite.fr (D. Siaussat).
[1] Present address: Sorbonne Université, 4 place Jussieu, Campus Pierre etMarie Curie – Tours 44-45, 3ème étage, boite courrier 237, 75005 Paris, France.

https://doi.org/10.1016/j.ecoenv.2021.112377
Received 23 January 2021; Received in revised form 22 May 2021; Accepted 24 May 2021
Available online 27 May 2021

incorporate complex environmental matrices, which constitute mixtures of pollutants often at moderate to low concentrations. For instance, wastewater is subject to regulations in some countries, whereby toxicity must be evaluated to trace low concentrations of pollutants and their sources (European Community, 2000).

Bioassays are typically used to detect pollutants (Chapman, 1995), with the presence and toxicity of Cd and Hg being detected using single exposure paradigms (Rana, 2014; Rice et al., 2014). However, very few studies have examined the effect of combined exposure to these metals. Moreover, combined exposure is often difficult to interpret, because the effects are not predictable, potentially being synergistic, additive, or antagonistic depending on complex modified physicochemical parameters, such as bioavailability and affinity (Wu et al., 2016). Thus, the effects of these substances must be tested in mixtures to verify their traceability via currently known biomarkers. Furthermore, the biological impact of heavy metal exposure is usually examined through acute toxicity testing of a given biological model, using high concentrations to trigger a clear phenotype over a short observation time (typically 1 h to 3 days). However, these tests are not well suited for understanding the effects of moderate to low levels of pollutants. In such cases, chronic toxicity tests are more effective in detecting the deleterious effect of lower doses of pollutants. A longer observation time (most of the life cycle of the exposed organism) allows the effects of chronic or continuous exposure to low doses alone or in mixtures to be detected, reflecting the conditions found in complex matrices.

The fruit fly *Drosophila melanogaster* represents a relevant model for short and long –term toxicological studies. This model has many advantages, including high fecundity, short lifespan, ease of rearing, available genetic tools and databases, combined with a good knowledge of the biology, physiology, development and behavior of this species. Because this model offers many opportunities, it has been widely used for many decades to study various biological processes and human diseases (Masamitsu and Hideki, 2018). This model also provides many possibilities for assessing the biological impact of pollutants and environmental change (Atli, 2013; Carmona et al., 2008; Hu et al., 2019). In addition, several studies have been published on the two target metals and their effects at the molecular (Chen et al., 2016; Doğanlar et al., 2014) and organism (Chauhan et al., 2017; Hu et al., 2019) level. Furthermore, changes to both the anatomy and molecular composition must be evaluated to increase the sensitivity of the bioassay.

Thus, this study investigated the impact of Cd and Hg alone and in combination on the post-embryonic development. First, we evaluated the effect of each metal alone on the extended expression pattern of 38 molecular targets *of D. melanogaster* to identify relevant biomarkers during development and for gene expression. Then, we tested the co-exposure of metals, to assess the impact on biomarkers observed during single exposure and their interactions. We used concentrations starting from low doses (defined as regulatory threshold in French wastewater networks) and higher doses. For mixtures, we focused on the low doses that are regularly encountered in wastewater. Finally, we tested the potential of this model for use as a chronic bioassay to analyze the impact of water polluted by Cd and Hg alone or in mixture.

2. Material and methods

2.1. Selection of concentrations

The soluble forms of Cd and Hg (cadmium chloride, methylmercury (II) chloride, Sigma Aldrich France) were used. We started with a lower concentration that corresponded to (and did not exceed) the threshold concentration in wastewater, so as not to contaminate natural aquatic environments after treatment by the wastewater treatment plant (ORF, 1998). Then, we tested higher concentrations based on the threshold value. Four concentrations per metal were tested for Cd (0.2 = Threshold value; 2; 20 and 200 mg/L) and Hg (0.05 = Threshold value; 0.5; 5 and 50 mg/L) (Table 1, left part). For the co-exposure experiments, we mixed the two lowest concentrations of each pollutant (0.2 and 2 mg Cd/L and 0.05 and 0.5 mg Hg/L) to evaluate potential cocktail effects, and because these are more likely found in the matrices of interest (Table 1, right part).

2.2. Insect rearing and experimental procedure for monitoring post-embryonic development

The Canton-S strain of *D. melanogaster* was reared on standard *Drosophila* medium (cornmeal, agar, sugar, yeast and water) at 25 °C ± 1 °C, 60–80% relative humidity and 12:12 h dark/light cycle. Eggs were collected after 20 h of oviposition on 1% agar supplemented with grape juice and sugar (2%). About 45–50 eggs were transferred to vials containing 2 g Nutri Fly Instant medium (GENESEE, France) rehydrated with 8 mL metal-contaminated solution in water (corresponding to day 0 of the experiment). Flies were exposed by contact and ingestion to Cd and Hg, either as single exposure (Table 1, right part) or co-exposure (Table 1, left part). For the control condition and the 12 treated conditions, mortality rates during development, mean duration of larval and pupal stage, and sex-ratio were recorded. we specially focused on larval development, as it is often more disrupted by metals compared to the pupal stage (Abnoos et al., 2013; Akins et al., 1992). We expressed our daily data of newly formed pupae as percent larva-pupa transition and larvae that reached the pupal stage. For each experimental condition, five biological replicates were performed representing a minimum total of 225 eggs initially deposited in control and metal-contaminated tubes.

2.3. Molecular analysis: gene expression with RT-qPCR

2.3.1. Total RNA extraction and cDNA synthesis

About 10 larvae from the control and exposed Cd and Hg single and co-exposure groups (Table 1, conditions with asterisk) were collected per biological replicate 4 days after egg laying. The larvae were stored at −80 °C until total RNA extraction using the TRIzol method (Invitrogen, Carlsbad, CA, USA) coupled with the RNAeasy extraction kit (Qiagen,

Table 1
Test conditions of *Drosophila melanogaster*. The experiments took place in two phases: (1) single exposure, and (2) co-exposure (gray background, arrows indicate the selected concentration). Asterisks indicate the conditions used to measure gene expression levels.

Single exposure (1)		Co-exposure (2)
Cadmium ($CdCl_2$)	Mercury (CH_3HgCl)	0.2 + 0.05*
0.2*	0.05*	0.2 + 0.5*
2*	0.5	2 + 0.05*
20	5*	2 + 0.5*
200	50	

USA), according manufacturers protocols. RNA quality was controlled by spectrophotometry (BioPhotometer, Eppendorf, Hamburg, Germany). DNase I Treatment (Roche, USA) was implemented following the manufacturer's instructions. cDNA was synthesized from 5 μg RNA with Superscript II reverse transcriptase (Invitrogen) following the manufacturer's protocol. For each experimental condition, three biological replicates were performed.

2.3.2. RT- qPCR

Five genes were first tested as putative housekeeping genes (*actin5C, αTub84, zw, rpS20,* and *pgk*) by Bestkeeper analysis (Pfaffl et al., 2004). *Pgk* was used as the reference gene, as it had the most stable expression among the experimental groups. Forward and reverse primers (*EcR, USP, met, ERR, BR-C, HR3, dib, cyp18a1, jheh1, hsf, hsp22, hsp23, hsp40, hsp68, hsp70, hsp83, hsc70, mtna, mtnb, TI, imd, def, p38, mef2, sirt1, keap1, cnc, cat, phgpx, sod, debcl, creba, cpr,* and *mdr49*) were designed from Flybase using AmplifX software (Supplementary Data Table S1). In cases where genes were transcribed to several isoforms, specific primers were designed in a shared region, allowing the accurate amplification of all isoforms. PCR reactions were performed on the LightCycler480 Real-Time PCR Detection System (Roche Applied Science, France), adapted from Bigot et al. (2012). Each reaction consisted of 5 μL Absolute Blue SYBR Green Fluor (Thermo Scientific, Waltham, MA, USA), 4 μL of cDNA (6.25 ng/μL) and 0.5 μL of each primer (10 μM). The PCR program consisted of an initial step at 95 °C for 5 min, followed by 50 cycles, consisting of 10 s at 95 °C, 15 s at 60 °C, and 15 s at 72 °C. Under these conditions, a single and discrete peak was detected for all primers tested after melting curve analysis, and all primers gave efficiencies of 85–105%. Each run included a fivefold dilution series, candidate genes, reference gene, and negative control. Each condition was evaluated in triplicate. The mean Ct value (cycle threshold) of each triplicate reaction was used to normalize the candidate gene expression level to the geometric mean of *pgk* level in Q-Gene software (Simon, 2003). Expression levels are summarized on a heatmap built on Past 3.14 Software. The raw data were normalized to the housekeeping gene *pgk*, centered to the control group, and were transformed to a logarithmic base 2 scale to emphasize variation between control and treated conditions.

2.4. Statistical analysis

Statistical analyses were performed using R 3.5.1 and R Studio 1.1.456 (R Core Team, 2017) and GraphPad Prism 6.01 (GraphPad Software, Inc.). The Shapiro–Wilk test was used to check the normality of the data. Mortality rates and sex-ratio of treated groups were analyzed using a Chi-square test to allow comparison with the control group. Larval developmental duration was analyzed with a Gehan–-Breslow–Wilcoxon test to compare developmental curves, and by one-way ANOVA to compare the global duration per stage. The Gehan-Breslow-Wilcoxon method was used to weight the early points, allowing discriminating effects at early larval-pupal transition, as we could only observe pupal molts (not larval molts) (Hazra, 2017). Gene Expression levels were analyzed using ANOVA with a permutation test followed by a Scheffe post-hoc test. Synergy or antagonism between Cd and Hg was analyzed following Ritz et al. (2021), using the delta method in R. Among the two currently models used (Loewe and Bliss; Tang et al., 2015), we chose the Bliss independence criterion with the initial assumption that, if there was an interaction, the effects would be multiplied. We only evaluated the interactive effects of mixtures using 0.2, 2 mg Cd/L and 0.05 mg Hg/L, since we had available references for single exposure. P-values lower than 5% were considered significant (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

3. Results

3.1. Impact of heavy metals on post-embryonic development

At the lowest concentrations tested (0.2–2 mg Cd/L and 0.05–0.5 mg Hg/L), mono-exposure to metals had no significant effect on the survival of individuals compared to the control group (Table 2). The highest Cd concentrations (20 mg/L and 200 mg/L) induced 99.04% and 100% larval mortality, respectively. Of the two highest Hg concentrations, 5 mg/L significantly increased larval mortality (10% compared to the control group), and resulted in the death of almost all larvae during the pupal stage, while 50 mg/L resulted in 100% larval mortality, similar to that of Cd (Table 2). Overall, the duration of the pupal stage was not affected by metal exposure. The time required to complete metamorphosis ranged from 4.53 to 5.04 days (Table 2). Conversely, the mean time to reach the pupal stage was affected after exposure to high metal concentrations. At 20 mg Cd/L, this mean time increased significantly, and reached 9.1 days after egg-laying, whereas it was about 6 days for the control group (Table 2). Similarly, at 5 mg Hg/L, this mean time increased to 7.5 days. Looking at the daily percentage of larval-pupal transition, significantly different percentages were obtained for 2 mg Cd/L and 0.5 mg Hg/L compared to the control group (Table 2), indicating that metals induced a developmental delay of a few hours.

During daily monitoring, an unusual phenotype was observed for the 5 mg Hg/L exposure. Indeed, hatching adults died rapidly, with their wings still folded and their abdomen not yet pigmented in contrast to the control group (data not shown). Many flies died during the pupal stage as "black pupa." Also, around 4% of adults failed to hatch and remained trapped in the pupal exuvia (data not shown). In comparison, no flies remained trapped in pupa in the control group. To date, few studies mentioned developmental abnormalities or such morphological changes following metal exposure.

Subsequently, we tested the impact of co-exposures to Cd and Hg at the selected low concentrations (Table 1). We assumed that simultaneous exposure to these metals would have stronger effects, due to possible cocktail effects; thus, we evaluated the lowest concentrations (Table 1). Combinations of 0.2–2 mg Cd/L with 0.05–0.5 mg Hg/L did not affect animal survival when compared to the control group (Table 2). Furthermore, the mean duration of pupal and larval stages did not change after exposure (Table 2). Interestingly, the daily cumulative proportion of larval-pupal transitions was affected by co-exposure to 2 mg Cd/L and 0.5 mg Hg/L in the same manner as that observed with single exposure to these concentrations (Table 2). The combination of 0.5 mg Hg/L and the lowest Cd concentration (i.e. 0.2 mg/L) also induced a delay. The other combinations were not significantly different from the control group.

Finally, sex-ratio remained unchanged between males and females in single and co-exposure groups (Table 2).

3.2. Impact of heavy metals on gene expression levels

We focused on the lowest concentrations more likely to be detected in wastewater, and that corresponded to sublethal concentration ranges (Table 1, right part). Molecular analysis consisted of measuring the expression of 38 genes involved in various signaling pathways: hormone signaling pathway, heat shock proteins (HSPs) signaling pathway, metallothioneines (MTs) signaling pathway, and cell stress signaling pathway. Overall, the results clearly differed between the single and co-exposure groups (Fig. 1). The raw values of gene expression and folding are presented in Table S2. Specifically, Cd modulated gene expression more than Hg. During co-exposure, the interaction between Cd and Hg was consistently synergistic based on Bliss criterion. The statistical results for interaction analyses are presented in Table S3.

3.2.1. Hormone signaling pathway

In larvae exposed to Cd (i.e., 0.2 and 2 mg/L) and 5 mg Hg/L, *EcR*

Table 2
Summary results of metals under single and co-exposure conditions on the developmental time, mortality, and sex ratio of *D. melanogaster* (mean ± standard deviation). C = control; NC = not calculable. P-values lower than 5% were considered significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

		C	0.2	2	20	200	0.05	0.5	5	50	0.2	0.2	2	2	mg Cd/L mg Hg/L
Mortality	Larval stages	13.88% *(±2.48)*	14.83% *(±5.93)*	14.23% *(±5.25)*	99.04% ***(±1.76)	100%	11.74% *(±7.32)*	11.80% *(±10.03)*	21.03% *** *(±20.95)*	100%	9.33% *(±4.27)*	*(±6.29)*	12.44% *(±10.26)*	9.33% *(±4.82)*	
	Pupal stage	1.81% *(±3.24)*	2.97% *(±4.55)*	1.94% *(±3.45)*	0.60% *(±35.19)*	*NC*	1.94% *(±2.61)*	1.06% *(±2.10)*	77.18% ***(±4.02)	*NC*	1.78% *(±5.82)*	0%	2.22% *(±4.08)*	0.44% *(±1.06)*	
Developmental time															
Mean duration of larval stages *(days)*		5.94 *(±0.17)*	5.98 *(±0.21)*	6.06 *(±0.18)*	9.13*** *(±0.96)*	*NC*	5.99 *(±0.21)*	6.21 *(±0.20)*	7.47*** *(±0.46)*	*NC*	5.94 *(±0.11)*	6.13 *(±0.07)*	6.01 *(±0.13)*	6.06 *(±0.04)*	
Mean duration of pupal stage *(days)*		5.02 *(±0.08)*	5.01 *(±0.16)*	4.96 *(±0.18)*	4.88 *(±0.35)*	*NC*	5.00 *(±0.19)*	4.80 *(±0.20)*	4.53 *(±0.02)*	*NC*	5.06 *(±0.01)*	4.87 *(±0.07)*	4.98 *(±0.12)*	4.94 *(±0.04)*	
Percentages larval-pupal transition *(day after egg-laying)*				*	***			***	***			***		***	
	Day 5	10.51% *(±12.32)*	10.83% *(±16.45)*	4.68% *(±7.65)*	0%	*NC*	11.11% *(±13.19)*	1.20% *(±1.89)*	0%	*NC*	8.82% *(±4.95)*	0.98% *(±3.37)*	5.58% *(±9.01)*	1.96% *(±4.26)*	
	Day 6	84.94% *(±11.31)*	81.24% *(±9.54)*	85.37% *(±11.78)*	0%	*NC*	79.53% *(±11.22)*	78.90% *(±17.57)*	21.71% *(±24.72)*	*NC*	88.73% *(±3.74)*	85.85% *(±6.81)*	88.32% *(±7.30)*	89.70% *(±6.16)*	
	Day 7	4.38% *(±1.45)*	6.96% *(±1.73)*	9.35% *(±1.24)*	0%	*NC*	8.63% *(±1.44)*	18.01% *(±2.51)*	68.75% *(±13.42)*	*NC*	2.45% *(±0)*	12.68% *(±0)*	6.09% *(±0)*	8.33% *(±0)*	
	Day 8	0.18% *(±0)*	0.77% *(±0.64)*	0.60% *(±0)*	0%	*NC*	0.58% *(±0.59)*	1.89% *(±0)*	7.89% *(±4.99)*	*NC*		0.48% *(±0)*			
	Day 9		0.19% *(±0)*		87.5% *(±16.67)*	*NC*	0%		0.99% *(±3.44)*	*NC*					
	Day 10				12.5% *(±0)*	*NC*	0.14% *(±0.59)*		0.33% *(±1.72)*	*NC*					
	Day 11					*NC*			0.33% *(±0)*	*NC*					
Sex-ratio	Male	50.84% *(±6.64)*	50.51% *(±8.46)*	52.87% *(±6.91)*	*NC*	*NC*	46.54% *(±7.43)*	52.76% *(±8.05)*	*NC*	*NC*	51.41% *(±7.88)*	50.76% *(±10.22)*	48.86% *(±6.41)*	51.04% *(±5.42)*	
	Female	49.16%	49.49%	47.13%	*NC*	*NC*	53.46%	47.24%	*NC*	*NC*	48.58%	49.24%	51.14%	48.96%	

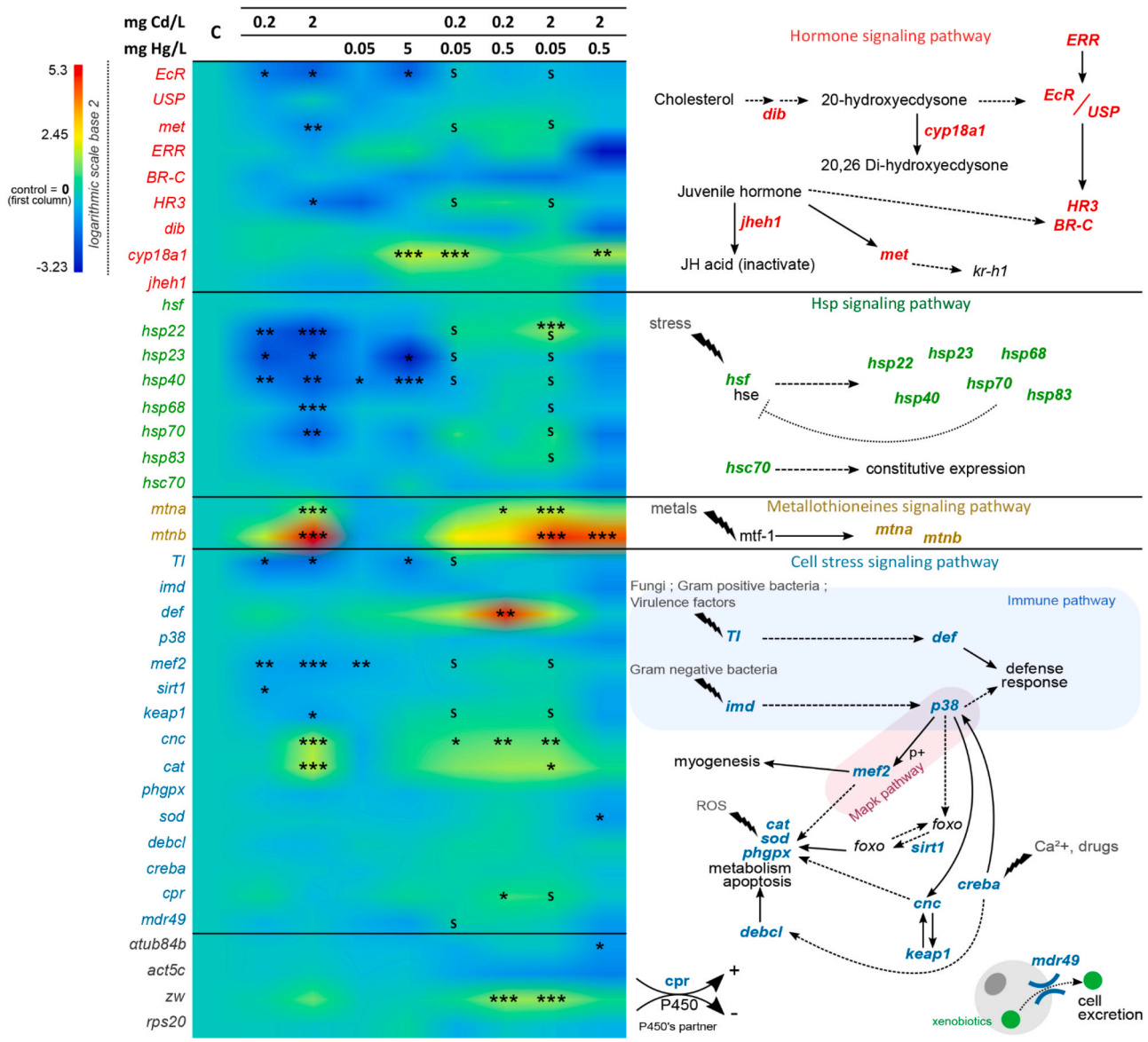

Fig. 1. Heat map representation of changes to expression levels in the third instar larvae of *D. melanogaster* after exposure to heavy metals. The map summarizes the mean normalized expression (MNE) with respect to the *pgk* gene (n = 9, three biological replicates with three technical replicates for each) of 38 genes involved in various metabolic pathways at different Cd and Hg concentrations under single and combined exposure. MNEs are centered to the control group (control = 1) and transformed to a logarithmic base 2 scale (log(control) = 0). Correspondence table between log value and fold value is provided in Supplementary Data S1. Color codes represent fold changes. Gene names are colored according to the signaling pathways represented on the right. Dotted arrows indicate an indirect interaction, while full arrows indicate a direct interaction between genes. C = controls. Asterisks (*) indicate significant differences among MNE in comparison to the control group (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Significant results indicating an interaction according to the Bliss criterion are represented by S (synergy). Bliss criterion values and P-values are presented in Supplementary Data S2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

expression significantly decreased by 2.5–3-fold compared to the control (Fig. 1). The *met* and *HR3* transcripts also decreased at 2 mg/L Cd. While the expression of these genes was not affected in larvae exposed to 5 mg/L Hg, a 3.5-fold increase of cytochrome p450 18a1 (*cyp18a1*) transcript was observed. During co-exposure, only cyp18a1 expression significantly increased in larvae exposed to 0.2 mg Cd/L + 0.05 mg Hg/L and 2 mg Cd/L + 0.5 mg Hg/L. Interactions were detected by statistical analysis for three genes (*EcR*, *met*, *HR3*). The interactions were synergistic based on the Bliss criterion.

3.2.2. Heat-shock proteins signaling pathway

Single exposure to Cd induced the down-regulation of almost all tested HSPs genes, especially at 2 mg/L (Fig. 1). Compared to the control group, the expression of *hsp22*, *hsp23,* and *hsp40* decreased by 2.5–5-fold for both Cd concentrations (0.2 and 2 mg/L), whereas the highest dose raised *hsp68* and *hsp70* expression by up to 2.5-fold. For single Hg exposure, we recorded the 2-fold down-regulation of *hsp40* at both concentrations (0.05 and 5 mg/L) and a strong decrease (about 10-fold) of *hsp23* at the highest dose. In contrast, co-exposure to Cd and Hg did not affect these heat-shock genes, except for *hsp22*, which significantly increased (2.5-fold) in larvae exposed to 2 mg Cd/L and 0.5 mg Hg/L. However, synergy between Cd and Hg existed for most of the studied genes, especially when mixtures contained 2 mg Cd/L.

3.2.3. Metallothioneins signaling pathway

MTs were the most strongly deregulated genes by the two metals,

particularly Cd. In larvae exposed to 2 mg Cd/L, *mtna* and *mtnb*, transcripts increased from 2.8 to 39-fold compared to the control (Fig. 1). For larvae exposed to 0.2 mg Cd/L there was no statistical difference with the control, but a 3-fold increase in *mtnb* expression was recorded in treated versus untreated larvae. No change in the expression of metallothionein genes was detected after Hg exposure. However, these genes were clearly overexpressed in response to the co-presence of the two metals. *mtna* levels increased by 1.6–2.8-fold, and while *mtnb* levels increased by 5–24-fold (Fig. 1) compared to the control group. However, the Bliss criterion showed no interaction for these genes.

3.2.4. Cellular stress signaling pathway

The transcription activity of the *Toll* receptor (*Tl*) decreased by 3.4-at 0.2 mg Cd/L and 2.7-fold with 5 mg Hg /L. The interaction between metals was positive (synergy) based on the Bliss criterion for the mixture with 0.2 mg Cd/L + 0.05 mg Hg/L. The expression of other genes involved in the immune pathway did not change on single exposure (i.e., *imd, p38, def*). Only *def* was upregulated with 0.2 Cd/L + 0.5 (30 -fold). A significant 0.4-fold decrease in the expression of the transcription factor *mef2* involved in the Mapk pathway was detected for both Cd concentrations and 0.05 mg Hg/L. The immune and Mapk pathways were involved in the regulation of gene expression of detoxification actors, such as *sirt1* and the *cnc/keap1* pair, for which significant differences in expression levels were detected (down-regulation of *sirt1* at 0.2 mg Cd/L, down-regulation of *keap1* with up-regulation of *cnc* at 2 mg Cd/L). *Cnc* was also up-regulated during co-exposure, except for 2 mg Cd/L + 0.5 mg Hg/L. Based on the Bliss criterion, interactions between Cd and Hg were synergistic for the mef2 and keap1 genes (Fig. 1). Among the genes encoding detoxification enzymes that were tested, only catalase (*cat*), super oxide dismutase (*sod*), and cytochrome P450 reductase genes were significantly modulated (up-regulation of *cat* at 2 mg Cd/L and at 2 mg Cd/L + 0.05 mg Hg/L, down-regulation of *sod* at 2 mg Cd/L + 0. 5 mg Hg/L, up-regulation of *cpr* at 0.2 mg Cd/L + 0.5 mg Hg/L.

Only metal co-exposure affected genes originally chosen as putative housekeeping genes (down-regulation of *αtub84b* at 2 mg Cd/L + 0.5 mg Hg/L, up-regulation of *zw* at 0.2 mg Cd/L + 0.5 mg Hg/L and 2 mg Cd/L + 0.05 mg Hg/L), but with no interactions based on the Bliss criterion.

4. Discussion

To develop a biological test to detect metal pollution in water, we used *Drosophila melanogaster* as a simple and robust model of exposure. The simple addition of contaminated water in the culture medium without additional treatment (i.e., no heating, filtration, or dilution) allowed the impact of Cd and Hg on the development and physiology of the animals to be directly measured. Besides the fact that it is an easy-to-use model, existing knowledge on its biology makes it easy to observe modifications of the known phenotypes and the expression of key genes.

We recorded a dose-dependent response of *Drosophila* to heavy metals, supporting previous reports on other insect species for similar ranges of concentrations (Al-Momani and Massadeh, 2005; Rodrigues et al., 2013; Zhan et al., 2017). No mortality was recorded at low doses close to the regulatory threshold value, or for co-exposure to lower doses. Thus, the selected model was not too sensitive to heavy metal pollution, with the dose-dependent response being consistent with the regulatory values of toxicity. Our observation protocol over a life cycle allowed delayed toxic effects to be documented. Indeed, Hg caused both early larval stage mortality and pupal mortality, as previously described for *Chironomus riparius* and *Musca domestica* (Azevedo-Pereira and Soares, 2010; Raina et al., 2001). Thus, in addition to direct toxic effects at high doses, long-term effects could disturb physiological and functional processes. This phenomenon was confirmed by our molecular biology results, as several days of exposure significantly modified the expression levels of genes contributing to vital biological functions.

Developmental delays of a few hours to several days were also recorded for exposure to 2 mg Cd/L and 0.5 mg Hg/L separately and 2 mg Cd/L + 0.05 mg Hg/L combined (Table 2). These delays may be explained by heavy metals affecting gut structures and functioning, and disrupting food digestion and absorption, leading to impaired growth and even death (Maryanski et al., 2002; Vlahović et al., 2001; Wu et al., 2016). These two metals also act as EDCs, as demonstrated by our results showing that they disrupted several crucial regulators of the hormonal signaling pathway. This included the *EcR* the ecdysone receptor or *HR3*, which is a gene of the genetic cascade induced by ecdysteroid in insects, or methoprene-tolerant (*met*), which is involved in regulation by juvenile hormones (Fig. 1)(Jindra et al., 2013; Nakagawa and Sonobe, 2016). Modification of the expression of these hormones alters effector genes, which are responsible for various developmental and physiological processes (Aviles et al., 2019). For example, *cyp18a1* increased after larvae were exposed to 5 mg Hg/L in our study. This gene codes an enzyme that contributes to the metamorphosis of *D. melanogaster*, and is essential for inactivating steroid hormones that regulate correct development and survival of this insect (Guittard et al., 2010).

In our study, Cd and Hg exhibited specific transcriptional profiles for the selected genes, depending on exposure scenario (Fig. 2). Cd specifically modulated more genes compared to Hg (Figs. 2 & S1). This phenomenon might be explained by Cd being less toxic than Hg. At an equivalent concentration (for example 0.2 mg of Cd/L and 0.5 mg/L), deleterious effects were already recorded for Hg (Table 2), reflecting its higher toxicity. Independently of this difference in the number of genes with modulated expression, our results identified several interesting biomarkers of Hg, Cd, and Cd + Hg exposure (Fig. 2). However, one specific marker for each condition was not found. The set of biomarkers and their general profiles were the most informative and representative of any given exposure condition.

Interestingly, we also reported a clear difference in response between single and combined exposure for lower concentrations close to environmental regulatory values. For example, we reported a decrease in

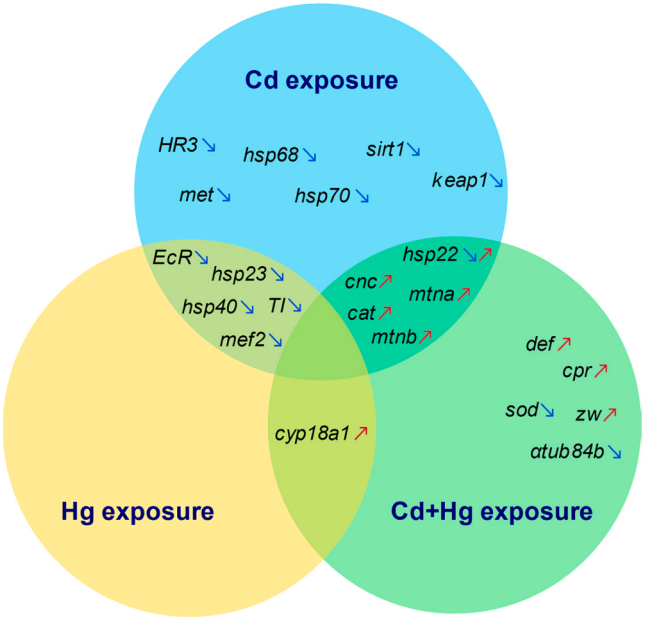

Fig. 2. Venn diagram summarizing the results of the qPCR analyses. The conditions were pooled into 3 groups: cadmium, mercury, and co-exposure to both metals. As soon as the transcription of a gene changes significantly, the gene is presented in the diagram. Blue arrows indicate down-regulation and red arrows indicate up-regulation. The presence of both arrows indicates that a gene underwent both types of transcriptional regulation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HSPs expression especially at the lower concentrations (Fig. 1), whereas co-exposure to Cd and Hg did not affect HSPs expression, except for hsp22, which significantly increased in some exposed larvae. Our profiles clearly differed from the induction of HSPs genes, supporting existing reports on insects exposed to heavy metals (Martínez-Paz et al., 2014; Zhang et al., 2015). The molecular and physiological response of individuals seemed to depend on the level of stress. At low concentrations, the response of exposed larvae was moderate, and embryonic development proceeded normally (Table 2 & Fig. 1). A slight increase in concentration of heavy metals altered the level of response (involving many more genes), and affected embryonic development. This type of dose-dependent molecular switch is consistent with the concept of the "growth-defense" trade-off that has been described in plants exposed to environmental stress (Huot et al., 2014). Specifically, there is a trade-off between defense mechanisms (e.g., HSPs detoxication enzymes) and growth mechanisms (e.g., energy production). Environmental stress has a cost to organisms by impacting normal physiological processes, such as development and reproduction. This concept has also been applied to some animals, such as *Microhyla fissipes* tadpoles, which exhibit a trade-off between protein synthesis and energy production under the sublethal concentrations of glyphosate (Wang et al., 2019).

Our results showed that mixtures of Cd/Hg globally have synergistic effects that could be more stressful to *Drosophila* than heavy metals alone. For instance, while stocks of HSP proteins were sufficient to cope with low and moderate Cd and Hg concentrations alone (causing HSPs expression levels to decline), restoration or increased expression of HSPs is needed in response to more severe stress. This phenomenon detected for MT proteins in our study, whereby MTs genes were up-regulated in the presence of metals, particularly Cd. MTs are involved in combating metal stress (Egli et al., 2006; Qiang et al., 2017). For instance, *mtna* expression significantly increased in response to the 0.2 mg Cd/L and 0.5 mg Hg/L mixture compared to 0.3 mg/L Cd alone, indicating that it generated more stress. Even though no statistical difference was reported, we recorded higher MTs expression in all mixtures.

This difference in responses between single and combined exposure was also recorded for genes involved in the immune pathway (e.g., *TI, def*) (Fig. 1). In addition to the direct impact of heavy metal exposure on gene expression (Ciacci et al., 2011; Taylor et al., 2013), indirect disruption occurs, impacting existing interactions between signaling pathways. This interaction was documented in *D. melanogaster* exposed to chromium (VI) (Pragya et al., 2015). Exposure to chromium causes the expression of humoral pathway receptors (*TI*) to decline, reducing resistance to bacterial infection (Pragya et al., 2015). In contrast, the overexpression of humoral immunity genes is beneficial to *Drosophila* through the humoral immunosuppressive effect induced by Cr(VI). Thus, the restoration or increased expression of the immune genes that we observed in the mixtures might cause inflammatory responses or trigger innate defenses to prevent microbial infection when an organism is weakened following metal exposure. With single exposure to low concentrations, expression did not change, or decreased, probably because cellular machinery can cope with stress, and does not require investment in immune pathways. This phenomenon was also observed for oxidative stress with the Mapk pathway.

The only exception was *cnc* (drosophila *Nrf2* ortholog), which activates gene expression to respond to oxidative stress, including catalase (*cat*), which is an oxidoreductase (Sykiotis and Bohmann, 2008) (Fig. 1). Expression increased with 2 mg Cd/L in single and combined exposure, as well as for combinations with lower Cd concentrations. In the same way as the gene encoding *cpr* is transcribed co-exposure induced NADPH–cytochrome P450 oxidoreductase to transfer electrons from NADPH to cytochrome P450 enzymes that catalyze the oxidative modification of organic compounds (Hovemann et al., 1997), probably in response to the higher toxicity of mixtures.

5. Conclusion

Through evaluating the impact of Cd and Hg exposure on *Drosophila melanogaster*, we identified several developmental and molecular biomarkers of single or combined exposure. Developmental time and mortality represented good parameters for analyzing the chronic toxicity of moderate and higher heavy metal concentrations. In comparison, gene expression appeared promising for analyzing low and moderate heavy metal concentrations, close to environmental regulatory values for wastewater. In addition, while Hg appeared more toxic than Cd, mixtures of low concentrations of both metals had a stronger effect on *D. melanogaster* compared to single exposure. This phenomenon was attributed to the synergistic effect of co-exposure. A "growth-defense" trade-off might exist, explaining the different responses across conditions. Analysis of the expression pattern of 38 genes revealed a specific transcriptional profile for each heavy metal, and for each co-exposure condition tested. Thus, it is necessary to conduct integrated studies combining different levels of observation. Overall, our results confirmed that *Drosophila melanogaster* is a useful model organism for toxicity bioassays under standard laboratory conditions. Further experiments with other classes of pollutants are required to challenge the potential use of this model in standardized laboratory bioassays.

CRediT authorship contribution statement

Laëtitia Frat: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft. **Thomas Chertemps:** Conceptualization, Methodology, Writing - review & editing. **Elise Pesce:** Investigation. **Françoise Bozzolan:** Methodology, Investigation. **Matthieu Dacher:** Formal analysis, Writing - review & editing. **Rosario Planelló:** Conceptualization, Methodology, Writing - review & editing. **Oscar Herrero:** Conceptualization, Methodology, Writing - review & editing. **Lola Llorente:** Methodology, Writing - review & editing. **Didier Moers:** Resources, Supervision, Funding acquisition. **David Siaussat:** Conceptualization, Methodology, Resources, Data curation, Writing - review & editing, Supervision, Project administration.

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.

Acknowledgements

We thank the Intercommunal Association for Wastewater in the Region of Pontoise (SIARP- FRANCE) and the Seine and Normandy Water Agency (FRANCE) for their financial support.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112377.

References

Abnoos, H., Fereidoni, M., Mahdavi-Shahri, N., Haddad, F., Jalal, R., 2013. Developmental study of mercury effects on the fruit fly (Drosophila melanogaster). Interdiscip. Toxicol. 6, 34–40. https://doi.org/10.2478/intox-2013-0007.

Akins, J.M., Schroeder, J.A., Browert, D.L., Aposhian, H.V., 1992. Evaluation of Drosophila-melanogaster as an alternative animal for studying the neurotoxicity of heavy-metals. Biometals 5, 111–120. https://doi.org/10.1007/BF01062222.

Al-Momani, F.A., Massadeh, A.M., 2005. Effect of different heavy-metal concentrations on Drosophila melanogaster larval growth and development. Biol. Trace Elem. Res. 108, 271–277. https://doi.org/10.1385/BTER:108:1-3:271.

Atli, E., 2013. The effects of three selected endocrine disrupting chemicals on the fecundity of fruit fly, Drosophila melanogaster. Bull. Environ. Contam. Toxicol. 91, 433–437. https://doi.org/10.1007/s00128-013-1083-7.

Aviles, A., Boulogne, I., Durand, N., Maria, A., Cordeiro, A., Bozzolan, F., Goutte, A., Alliot, F., Dacher, M., Renault, D., Maibeche, M., Siaussat, D., 2019. Effects of DEHP on post-embryonic development, nuclear receptor expression, metabolite and ecdysteroid concentrations of the moth Spodoptera littoralis. Chemosphere 215, 725–738. https://doi.org/10.1016/j.chemosphere.2018.10.102.

Azevedo-Pereira, H.M.V.S., Soares, A.M.V.M., 2010. Effects of mercury on growth, emergence, and behavior of Chironomus riparius Meigen (Diptera: Chironomidae). Arch. Environ. Contam. Toxicol. 59, 216–224. https://doi.org/10.1007/s00244-010-9482-9.

Bigot, L., Shaik, H.A., Bozzolan, F., Party, V., Lucas, P., Debernard, S., Siaussat, D., 2012. Peripheral regulation by ecdysteroids of olfactory responsiveness in male Egyptian cotton leaf worms, Spodoptera littoralis. Insect Biochem. Mol. Biol. 42, 22–31. https://doi.org/10.1016/j.ibmb.2011.10.003.

Caballero-Gallardo, K., Olivero-Verbel L., J., Freeman, J., 2016. Toxicogenomics to evaluate endocrine disrupting effects of environmental chemicals using the zebrafish model. Curr. Genom. 17, 515–527. https://doi.org/10.2174/1389202917666160513105959.

Carmona, E.R., Kossatz, E., Creus, A., Marcos, R., 2008. Genotoxic evaluation of two mercury compounds in the Drosophila wing spot test. Chemosphere 70, 1910–1914. https://doi.org/10.1016/j.chemosphere.2007.07.032.

Chapman, P.M., 1995. Bioassay testing for Australia as part of water quality assessment programmes. Aust. J. Ecol. 20, 7–19. https://doi.org/10.1111/j.1442-9993.1995.tb00517.x.

Chauhan, V., Srikumar, S., Aamer, S., Pandareesh, M.D., 2017. Methylmercury exposure induces sexual dysfunction in male and female Drosophila melanogaster. Int. J. Environ. Res. Public Health 14, 1108. https://doi.org/10.3390/ijerph14101108.

Chen, Z., Wu, X., Luo, H., Zhao, L., Ji, X., Qiao, X., Jin, Y., Liu, W., 2016. Acute exposure of mercury chloride stimulates the tissue regeneration program and reactive oxygen species production in the Drosophila midgut. Environ. Toxicol. Pharmacol. 41, 32–38. https://doi.org/10.1016/j.etap.2015.11.009.

Ciacci, C., Barmo, C., Fabbri, R., Canonico, B., Gallo, G., Canesi, L., 2011. Immunomodulation in Mytilus galloprovincialis by non-toxic doses of hexavalent chromium. Fish Shellfish Immunol. 31, 1026–1033. https://doi.org/10.1016/j.fsi.2011.09.002.

Doğanlar, Z.B., Doğanlar, O., Tabakçioğlu, K., 2014. Genotoxic effects of heavy metal mixture in Drosophila melanogaster: expressions of heat shock proteins, RAPD profiles and mitochondrial DNA sequence. Water Air Soil Pollut. 225. https://doi.org/10.1007/s11270-014-2104-9.

Egli, D., Domènech, J., Selvaraj, A., Balamurugan, K., Hua, H., Capdevila, M., Georgiev, O., Schaffner, W., Atrian, S., 2006. The four members of the Drosophila metallothionein family exhibit distinct yet overlapping roles in heavy metal homeostasis and detoxification. Genes Cells 11, 647–658. https://doi.org/10.1111/j.1365-2443.2006.00971.x.

European Community. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 Establishing a Framework for Community Action in the Field of Water Policy. 2000.

ORF, 1998. February 2nd Order 1998 Relating to Water Withdrawals and Consumption as Well as to Emissions of all Kinds from Installations Classified for the Protection of the Environment Subject to Authorization.

Genchi, G., Sinicropi, M., Graziantonio, L., Carocci, A., Catalano, A., 2020. The effects of cadmium toxicity. Int. J. Environ. Res. Public Health 17, 1–24. https://doi.org/10.3390/ijerph17113782.

Guittard, E., Blais, C., Maria, A., Parvy, J.-P., Pasricha, S., Lumb, C., Lafont, R., Daborn, P.J., Dauphin-Villemant, C., 2010. CYP18A1, a key enzyme of Drosophila steroid hormone inactivation, is essential for metamorphosis. Dev. Biol. 349, 35–45. https://doi.org/10.1016/j.ydbio.2010.09.023.

Hazra, A., 2017. Biostatistics series module 8: survival analysis. Indian J. Dermatol. 62, 251–257. https://doi.org/10.4103/ijd.IJD.

Hovemann, B.T., Sehlmeyer, F., Malz, J., 1997. Drosophila melanogaster NADPH-cytochrome P450 oxidoreductase: pronounced expression in antennae may be related to odorant clearance. Gene 189, 213–219. https://doi.org/10.1016/S0378-1119(96)00851-7.

Hu, X., Fu, W., Yang, X., Mu, Y., Gu, W., Zhang, M., 2019. Effects of cadmium on fecundity and defence ability of Drosophila melanogaster. Ecotoxicol. Environ. Saf. 171, 871–877. https://doi.org/10.1016/j.ecoenv.2019.01.029.

Huot, B., Yao, J., Montgomery, B.L., He, S.Y., 2014. Growth-defense tradeoffs in plants: a balancing act to optimize fitness. Mol. Plant 7, 1267–1287. https://doi.org/10.1093/mp/ssu049.

Iavicoli, I., Fontana, L., Bergamaschi, A., 2009. The effects of metals as endocrine disruptors. J. Toxicol. Environ. Health Part B 37–41. https://doi.org/10.1080/10937400902902062.

Jindra, M., Palli, S.R., Riddiford, L.M., 2013. The juvenile hormone signaling pathway in insect development. Annu. Rev. Entomol. 58, 181–204. https://doi.org/10.1146/annurev-ento-120811-153700.

Martínez-Paz, P., Morales, M., Martín, R., Martínez-Guitarte, J.L., Morcillo, G., 2014. Characterization of the small heat shock protein Hsp27 gene in Chironomus riparius (Diptera) and its expression profile in response to temperature changes and xenobiotic exposures. Cell Stress Chaperones 19, 529–540. https://doi.org/10.1007/s12192-013-0479-y.

Maryanski, M., Kramarz, P., Laskowski, R., Niklinska, M., 2002. Decreased energetic reserves, morphological changes and accumulation of metals in carabid beetles (Poecilus cupreus L.) exposed to zinc- or cadmium-contaminated food. Ecotoxicology 11, 127–139. https://doi.org/10.1023/A:1014425113481.

Masamitsu, Y., Hideki, Y., 2018. Drosophila as a modelorganism. In: Drosoph. Model. Hum. Dis., 1076, 1–10. ⟨https://doi.org/10.1007/978-981-13-0529-0⟩.

Nakagawa, Y., Sonobe, H., 2016. Ecdysteroids. In: Handbook of Hormones. Elsevier Inc, pp. 557–559. https://doi.org/10.1016/B978-0-12-801028-0.00485-2.

Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – excel-based tool using pair-wise correlations. Biotechnol. Lett. 26, 509–515. https://doi.org/10.1016/j.ejor.2016.09.013.

Planelló, R., Martínez-Guitarte, J.L., Morcillo, G., 2010. Effect of acute exposure to cadmium on the expression of heat-shock and hormone-nuclear receptor genes in the aquatic midge Chironomus riparius. Sci. Total Environ. 408, 1598–1603. https://doi.org/10.1016/j.scitotenv.2010.01.004.

Pragya, P., Schukla, A., Murthy, R., Abdin, M., Chowdhuri, D.K., 2015. Characterization of the effect of Cr(VI) on humoral innate immunity using Drosophila melanogaster. Environ. Toxicol. 30 (11), 1285–1296. https://doi.org/10.1002/tox.21999.

Qiang, W., Huang, Y., Wan, Z., Zhou, B., 2017. Metal-metal interaction mediates the iron induction of Drosophila MtnB. Biochem. Biophys. Res. Commun. 487, 646–652. https://doi.org/10.1016/j.bbrc.2017.04.109.

Raina, R.M., Pawar, P., Sharma, R.N., 2001. Developmental inhibition and reproductive potential impairment in Musca domestica L. by heavy metals. Indian J. Exp. Biol. 39, 78–81.

Rana, S.V.S., 2014. Perspectives in endocrine toxicity of heavy metals - a review. Biol. Trace Elem. Res. 160, 1–14. https://doi.org/10.1007/s12011-014-0023-7.

Rice, K.M., Walker, E.M., Wu, M., Gillette, C., Blough, E.R., 2014. Environmental mercury and its toxic effects. J. Prev. Med Public Health 47, 74–83. https://doi.org/10.3961/jpmph.2014.47.2.74.

Ritz, C., Streibig, J.C., Kniss, A., 2021. How to use statistics to claim antagonism and synergism from binary mixture experiments. Pest Manag. Sci. https://doi.org/10.1002/ps.6348.

Rodrigues, N.R., Nunes, M.E.M., Silva, D.G.C., Zemolin, A.P.P., Meinerz, D.F., Cruz, L.C., Pereira, A.B., Rocha, J.B.T., Posser, T., Franco, J.L., 2013. Is the lobster cockroach Nauphoeta cinerea a valuable model for evaluating mercury induced oxidative stress? Chemosphere 92, 1177–1182. https://doi.org/10.1016/j.chemosphere.2013.01.084.

Simon, P., 2003. Q-Gene: processing quantitative real-time RT-PCR data. Bioinformatics 19, 1439–1440. https://doi.org/10.1093/bioinformatics/btg157.

Sykiotis, G.P., Bohmann, D., 2008. Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in Drosophila. Dev. Cell 14, 76–85. https://doi.org/10.1016/j.devcel.2007.12.002.

Tang, J., Wennerberg, K., Aittokallio, T., 2015. What is synergy? The Saariselkä agreement revisited. Front. Pharmacol. 6, 1–5. https://doi.org/10.3389/fphar.2015.00181.

Taylor, D.A., Thompson, E.L., Nair, S.V., Raftos, D.A., 2013. Differential effects of metal contamination on the transcript expression of immune- and stress-response genes in the Sydney Rock oyster, Saccostrea glomerata. Environ. Pollut. 178, 65–71. https://doi.org/10.1016/j.envpol.2013.02.027.

Vlahović, M., Ilijin, L., Lazarević, J., 2001. Acute effect of cadmium on larval growth of Lymantria dispar L. Ekologija 36, 131–137.

Wang, X., Chang, L., Zhao, T., Liu, L., Zhang, M., Li, C., Xie, F., Jiang, J., Zhu, W., 2019. Metabolic switch in energy metabolism mediates the sublethal effects induced by glyphosate-based herbicide on tadpoles of a farmland frog Microhyla fissipes. Ecotoxicol. Environ. Saf. 186, 109794 https://doi.org/10.1016/j.ecoenv.2019.109794.

Wu, X., Cobbina, S.J., Mao, G., Xu, H., Zhang, Z., Yang, L., 2016. A review of toxicity and mechanisms of individual and mixtures of heavy metals in the environment. Environ. Sci. Pollut. Res. 23, 8244–8259. https://doi.org/10.1007/s11356-016-6333-x.

Zhan, H., Zhang, J., Chen, Z., Huang, Y., Ruuhola, T., Yang, S., 2017. Effects of Cd^{2+} exposure on key life history traits and activities of four metabolic enzymes in Helicoverpa armigera (Lepidopteran: Noctuidae). Chem. Ecol. 33, 325–338. https://doi.org/10.1080/02757540.2017.1299714.

Zhang, Y., Liu, Y., Zhang, J., Guo, Y., Ma, E., 2015. Molecular cloning and mRNA expression of heat shock protein genes and their response to cadmium stress in the grasshopper Oxya chinensis. PLoS One 10, 1–16. https://doi.org/10.1371/journal.pone.0131244.