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ECOTOXICOLOGICAL EVALUATION OF OCTACHLOROSTYRENE IN FOURTH INSTAR LARVAE OF *CHIRONOMUS RIPARIUS* (DIPTERA, CHIRONOMIDAE)

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Abstract—The ecotoxicological effects of octachlorostyrene (OCS) on the aquatic larvae of the nonbiting midges *Chironomus riparius* (Diptera, Chironomidae) were investigated by evaluating their responses in various biological organizations, from the molecular and biochemical to the ecophysiological levels. The results of the 24-h lethality test of OCS on *C. riparius* suggest that this compound has a low acute toxic potential with respect to this species. The OCS exhibited serious chronic toxicity in *C. riparius*, however, especially on the species' development (pupation and emergence failure), the sex ratio of the emerged adults (high female to male ratio), and the adults' reproduction (oviposition failure). As the basic premise that changes in the gene expression can be harnessed to diagnose the exposure and effects of environmental chemicals is currently receiving significant attention, the increase in the expression of the heat shock protein and hemoglobin genes suggests that altered molecular-level parameters could be interpreted as early warning biomarkers of chemical stress. The establishment of a causal relationship between molecular and organism or population indicators is important, however, from the ecotoxicological point of view, although it is difficult to perform. The experimental evidence generated in the present study, especially at the molecular and biochemical levels, is not sufficient to demonstrate a plausible mechanistic linkage among responses across biological levels. Had more parameters at the molecular and biochemical levels been investigated, the mechanistic linkage might have been better identified. A mechanistic study appears to be necessary to understand causal relationships between gene expression and higher-level consequences, such as development and reproduction. The results of the present study may constitute an important contribution to knowledge on the ecotoxicology of OCS in aquatic organisms, about which little data is available.

Keywords—*Chironomus riparius* Octachlorostyrene Ecotoxicity Multilevel biomarkers Biomonitoring

INTRODUCTION

Octachlorostyrene (OCS) is a byproduct of the manufacture of many chlorinated hydrocarbons and has been consistently found in fish and sediment samples collected near incineration areas or near potential point sources due to industrial discharges (www.epa.gov/glnpo/bnsdocs/98summ/ocs). The presence of OCS in the environment has been particularly puzzling, as this compound has never been a commercial product but is used only for experimental synthetic purposes. Despite its ubiquitous distribution in the environment, which accounts for the importance of OCS toxicity in aquatic ecosystems, little ecotoxicological information on this pollutant is available.

In the present study, which investigates the effects of OCS on aquatic ecosystem components, *Chironomus riparius* was selected as a model biological system for the ecotoxicological evaluation of OCS. The aquatic larvae of nonbiting midges (Diptera, Chironomidae) are globally distributed and comprise the most abundant group of insects found in freshwater ecosystems. They hold an important position in the aquatic food chain and are a major food source for fish and other vertebrates and for invertebrates [1]. Thus, they are used extensively to assess the acute and sublethal toxicity of contaminated sediments and water [2–6]. Taking into account the frequent occurrence of OCS in surface water and the ecological importance of *Chironomus* larvae in freshwater, research on the effects of OCS on *C. riparius* could provide valuable information for the biomonitoring or risk assessment of this compound in aquatic ecosystems.

The aim of the present study was to evaluate the ecotoxicological response of OCS in *C. riparius* using different biological responses that ranged from the molecular to the ecophysiological levels. The present study was designed as a midterm experiment under controlled laboratory conditions, using measured concentrations of OCS in test water. Responses at the molecular, biochemical, physiological, and population levels were investigated in OCS-exposed *C. riparius*. The DNA damage was measured to evaluate whether or not OCS induces genetic toxicity in *C. riparius*. To screen the OCS-induced gene expression profiling, an annealing control primer (ACP)–based polymerase chain reaction (PCR) was used. Then the expression of heat shock protein (HSP) and hemoglobin (Hb) genes was investigated, and oxidative stress–related parameters were studied as biochemical-level effects. Growth, development, and reproduction were studied as organism/population-level responses. The larval dry body weight was measured as a growth indicator, whereas successes in pupation and in adult emergence, as well as the total number of times of emergence, were examined as development descriptors. The emerged adult sex ratio was also studied to identify any po-

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tential difference in male and female susceptibility to this compound. The oviposition rate and the number of eggs per egg mass were used as parameters for reproduction.

MATERIALS AND METHODS

Organisms

Using an original strain provided by the Toxicology Research Center of the Korea Research Institute of Chemical Technology (Daejeon, Korea), *C. riparius* larvae were obtained from adults reared in a laboratory. The larvae, which were fed fish flakes (Tetramin®, Tetrawerke, Melle, Germany), were reared under a 16:8-h light:dark photoperiod at room temperature (20 ± 1°C) in a 2-L glass chamber that contained dechlorinated tap water and acid-washed sand with aeration.

Exposure conditions

Using groups of the fourth instar larvae collected in the rearing aquaria, the effects of the exposure of OCS (Wako Pure Chemical Industries, Osaka, Japan) were assessed. All the larvae used in the experiment originated from the same egg mass and were collected within the same period (30 d) after egg hatching to obtain the age-synchronized population. At the beginning of the experiment, 1 ml of an acetonic solution of OCS was added to the 1-L experimental tanks. The analytical grade of acetone (99.7%, Wako Pure Chemical Industries) was used in all the experiments. Afterward, 10 (in the acute toxicity and biomarker study) to 50 (in the chronic toxicity study) larvae were randomly introduced in each test aquarium. The exposure was carried out under constant temperature (20 ± 1°C), and a 16:8-h photoperiod was used in all the experiments. Three replicates were used in all the experiments.

Quantitative determination of octachlorostyrene in water

Concentrations of OCS in water were determined using the gas chromatography–mass spectrometry (GC-MS) method. Five mg/L of OCS was spiked in water at the beginning of the experiment. At 24, 48, 72, and 96 h after water spiking, 50 ml of water was collected from the experimental tank for the extraction and quantification of OCS. The extraction of OCS from the water sample was performed using a liquid–liquid extraction method, with hexane as the extraction solvent. The sample analysis was carried out with an Agilent GC-MS (Palo Alto, CA, USA) equipped with a DB-5MS fused-silica capillary column (30 m × 0.25 mm internal diameter, film thickness 0.25 μm, J&W Scientific, Folsom, CA, USA). To improve sensitivity, the selected ions monitoring mode was used, guided by specific ions of the individual chlorostyrene. Octachlorostyrene was identified via its retention time and specific ions and was quantified using the internal standard method.

Acute toxicity test

Groups of 10 larvae were exposed to four concentrations of OCS and its related compounds, such as 2-chlorostyrene, 3-chlorostyrene, 4-chlorostyrene, and 2,6-chlorostyrene, whereas the other group was made the control group. The acute toxicity was determined after 24 h of exposure, using the death of individuals as an end point. The log-probit transformation of the data was used to estimate the 24-h 10% of lethal concentration, median lethal concentration, and 90% of lethal concentration values and the corresponding 95% confidence intervals.

Comet assay

A total of 10 larvae were collected from the control and experimental tanks 24 h after treatment and were pooled for a comet assay. The larvae were placed in 1 ml of phosphate-buffered saline that contained 20-mM ethylenediaminetetraacetic acid and 10% dimethylsulfoxide, and they were disintegrated mechanically by pressing them against gauze. A cell suspension was precipitated by vortexing and then immediately mixed with 100 μl of 1% low-melting-point agarose for use in the comet assay. Then an alkaline comet assay was performed, as described by Singh et al. [7]. As the positive control group, 100-mM hydrogen peroxide (H_2O_2) was used.

RNA extraction

A total of 10 larvae were collected from the control and experimental tanks 24 h after treatment and were pooled for PCR analysis. The larvae were homogenized in 700 μl of a Trizol® reagent (Molecular Research Center, Cincinnati, OH, USA), and the RNA was isolated according to the manufacturer's standard protocol.

Annealing control primer–based GeneFishing™ polymerase chain reaction

Differentially expressed genes (DEGs) were screened using the ACP-based PCR method [8] with GeneFishing DEG kits (Seegene, Seoul, South Korea), according to the manufacturer's instructions [9]. The DNA sequencing was performed by Macrogen (Seoul, South Korea) on up-regulated DEGs by OCS exposure, identified by ACP-PCR. The DNA of DEGs was extracted from the agarose gel using the Geneclean® II kit, a Glassmilk gel extraction kit (Q-BIOgene, Cambridge, UK). The DNA sequence of each gene was confirmed by comparing it with the sequences in GenBank® (www.ncbi.nlm.nih.gov/Genbank/; National Institutes of Health, Rockville, MD, USA). The primer sequence of each gene was designed using the Primer3® program (primer3.sourceforge.net; Massachusetts Institute of Technology, Cambridge, MA, USA). The synthesis of the primers for five up-regulated DEGs was performed by Bioneer (Seoul, Korea).

Gene expression analysis

For the reverse transcription–polymerase chain reaction (RT-PCR), a two-step method was employed with RT premix and PCR premix kits (Bioneer). Before the RT, 2 μg of total RNA and a random hexamer (Promega, Madison, WI, USA) were denatured at 70°C for 5 min and then rapidly cooled on ice. These solutions were added to the RT premix kits, with the RT conducted at 42°C for 60 min and at 94°C for 5 min. These templates were then added to the PCR premix kit, which contained HSP70 and heat shock cognate 70 (HSC70); the five Hb open reading frames, which were named HbA, HbB, HbC, HbD, and HbE; and the actin primers. The primers were designed based on the sequences retrieved from GenBank (Table S1; http://dx.doi.org/10.1897/07-219.S1). To optimize the semiquantitative PCR conditions, a cycle number test was conducted for each gene prior to the main experiment (data not shown). All the PCR experiments were conducted using an optimized cycle number and a template amount. Finally, actin messenger RNA (mRNA) normalized the expression of each gene level. Using a Peltier Thermal Cycler-100™ (MJ Research, Lincoln, MA, USA), 30 cycles of PCR were conducted by denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C

for 7 min. The PCR products were separated via electrophoresis on a 1.5% agarose gel (Promega) and visualized with ethidium bromide (Bioneer). All the tests were repeated at least three times, and the relative densities of each band were determined with the aid of an image analyzer, a gel documentation system (TFX-20.M ultraviolet transilluminator, Vilber Lourmat TFX-20.M, Marne la Vallee, France), and a Kodak 1D 3.6 camera (Kodak EDAS 290, Rochester, NY, USA).

Antioxidant enzyme activities and lipid peroxidation measurement

A total of 10 larvae were collected from the control and experimental tanks 24 h after treatment and were pooled for enzyme activity measurements. The larvae were homogenized in 2.5 ml of a tris-ethylenediaminetetraacetic acid buffer (40 mM, pH 7.8; Sigma-Aldrich, St. Louis, MO, USA) using a Potter-Elvehjem homogenizer (Wheaton Science, Milliville, PA, USA). The crude homogenate was centrifuged for 15 min at 500 *g* (4°C), and the supernatant was centrifuged for 30 min at 12,000 *g* (4°C). The resulting supernatant (postmitochondrial fraction) was used to measure the enzyme activities. The rate of H_2O_2 disappearance (measured at 240 nm) was used to quantify the catalase (CAT) activity [10]. The total peroxidase (Px) activity was measured using a guaiacol test [11]. The enzymatic activities were calculated relative to the measured protein content of the extracts using the Bradford method [12]. Malondialdehyde (MDA) was measured via spectrophotemetry using thiobarbituric acid reactive substances, as in the study of Ohkawa et al. [13].

Body weight measurement

The water content and the dry body weight of 10 larvae that were measured 48 h after the commencement of their exposure. Their fresh weight was immediately measured. Their larval dry weight was evaluated after the larvae were placed at 105°C for 24 h. The weighing was performed to the nearest 0.1 mg.

Adult emergence and oviposition

To measure the adult emergence rate, 50 of fourth instar larvae were introduced at the beginning of the experiment. The emerging adults were retained using wood cages covered with steel wire mesh until the emergence was completed in the control and experimental aquaria. The number of emerged adults from each vessel was counted and their sexes were determined as endpoints of the toxicity tests. The two sexes were easily distinguished by the form and length of their antennae and abdominal terminalia. In addition, the dead pupae were counted and the time to completion of their emergence was investigated. For the reproduction parameters, the numbers of egg masses oviposited by the emerged adults and the numbers of eggs per egg mass in the control and OCS-treated vessels were counted. Every 2 d, 50 mg of Tetramin fish food flakes was supplied to each aquarium. The test solutions were not renewed. All the data were recorded at daily intervals.

Data analysis

The data passed the normality test and the equal variance test. Statistical differences between the control and the treated larvae were examined using variation analysis with Dunnett's multiple comparison test. A parametric Pearson test was conducted to study correlations among the parameters. All the statistical tests were performed using SPSS® 12.0KO (SPSS, Chicago, IL, USA).

Table 1. Concentrations of octachlorostyrene (OCS) in water measured by gas chromatography–mass spectrometry

Time (h)	OCS (mg/L)
0	5[a]
24	0.021 ± 0.001
48	0.023 ± 0.000
72	0.020 ± 0.001
96	0.017 ± 0.001

[a] Nominal value.

RESULTS

Acute toxicity and residue analysis

Prior to the main experiment, a solvent control test was conducted to compare the responses of the nonsolvent control group and the solvent control group, which indicated that acetone did not provoke any significant effect in all the experiments (data not shown). The controls presented in this section of the present study were solvent controls.

An acute toxicity study was conducted not only on OCS but also on its related compounds to compare the sensitivity of OCS with that of related compounds. The 24-h median lethal concentration of OCS-related compounds, such as 2-chlorostyrene, 3-chlorostyrene, 4-chlorostyrene, and 2,6-chlorostyrene, in the fourth instar larvae of *C. riparius* was estimated (Table S2; http://dx.doi.org/10.1897/07-219.S1). The order of acute toxicity in *C. riparius* after 24 h of exposure was 2,6-chlorostyrene > 2-chlorostyrene > 3-chlorostyrene, 4-chlorostyrene ≫ OCS. The median lethal concentration could not be estimated for OCS, because up to 5 mg/L, OCS did not cause mortality among the *C. riparius* larvae and over 5 mg/L, it was not soluble. An ecotoxicity study, using multilevel biological responses, was conducted on OCS using three concentrations: 0.05, 0.5, and 5 mg/L, with 0 mg/L for the control group.

Residues of OCS were detected in water within 96 h using the GC-MS method (Table 1). The extraction efficiency of the method used for OCS analysis was approximately 92 to 102%, and the detection limit was 0.08 μg/L. The nominal concentration of OCS in the test water at the beginning of the kinetics experiment was 5 mg/L, the highest concentration for an ecotoxicity study. The results of the 96-h-long study show that the OCS concentrations decreased dramatically in the first 24 h and that less than 1% of the initial amount remained after 24 h and remained so until the end of the experiment.

Molecular- and biochemical-level markers

Damage to DNA, particularly DNA strand breaks, was measured using a single-cell gel electrophoresis (comet) assay to evaluate the genotoxicity of OCS in *C. riparius* (Fig. 1). Both the tail moment and the olive tail moment, which are widely used parameters of DNA damage using comet assay, did not increase significantly at any tested concentration of OCS.

To detect genes that are expressed differentially via OCS exposure in *C. riparius* larvae, the ACP-based PCR technique was used (Fig. S1; http://dx.doi.org/10.1897/07-219.S1). Using 20 ACPs, six DEGs were identified. Five DEGs were up-regulated, and one DEG was down-regulated. Direct sequencing was conducted on the five up-regulated DEGs. Using basic

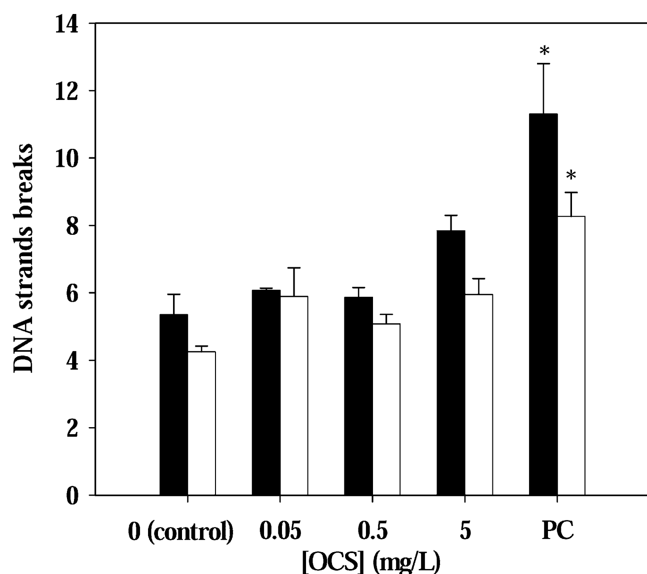


Fig. 1. Damage to DNA measured in the fourth instar larvae of *Chironomus riparius* exposed to 0 (control group), 0.05, 0.5, and 5 mg/L of octachlorostyrene (OCS) for 24 h using single-cell gel electrophoresis ($n = 3$, mean ± standard error of mean, $*p < 0.05$). PC = positive control; ■ = tail moment; □ = olive tail moment.

local alignment search-tool searches, the expressed sequence tags did not show any similarity with known genes or with expressed sequence tags of other species in the GenBank in terms of their significant sequence. The five sequences were thus submitted to GenBank as DEGs via OCS exposure in *C. riparius* (Table 2).

Since all the DEGs revealed by ACP-PCR turned out to be unknown genes, the effects of OCS on *Chironomus* gene expression were subsequently investigated in the most well-known and well-studied genes in *Chironomus*, the Hb and HSP genes (Fig. 2). The HSP and Hb genes were selected because HSP is the gene most frequently used as a nonspecific pollution marker in aquatic environment monitoring and Hb is considered a potential species-specific biomarker in this species. The expression of both the HSP70 and the HSC70 genes increased in the OCS-treated larvae. Among the Hb genes, the increase in the expression of the HbB and HbD genes was most significant. At the highest level of OCS (5 mg/L), the increase in the expression of all five Hb open reading frames studied was observed.

As biochemical parameters, oxidative stress–related parameters were measured in the fourth instar larvae of *C. riparius* exposed to OCS for 24 h (Fig. 3). Peroxidase activity increased in all the OCS-exposed larvae, whereas an increase in CAT activity and MDA was observed in 0.5 and 0.05 mg/L of OCS-treated larvae, respectively.

Organism- and population-level indicators

The OCS-induced physiological-level effect was investigated by measuring the fresh body weight and the dry body weight of the *C. riparius* larvae 48 h after their treatment (Table S3; http://dx.doi.org/10.1897/07-219.S1). As a growth indicator, the fresh body weight and the dry body weight of the larvae did not change upon OCS exposure.

The effects of OCS on the kinetics of emergence in the fourth instar larvae of *C. riparius* were studied as development parameters (Fig. 4A). The total adult emergence rate dramatically decreased by 5 mg/L upon OCS exposure (~67%). Two

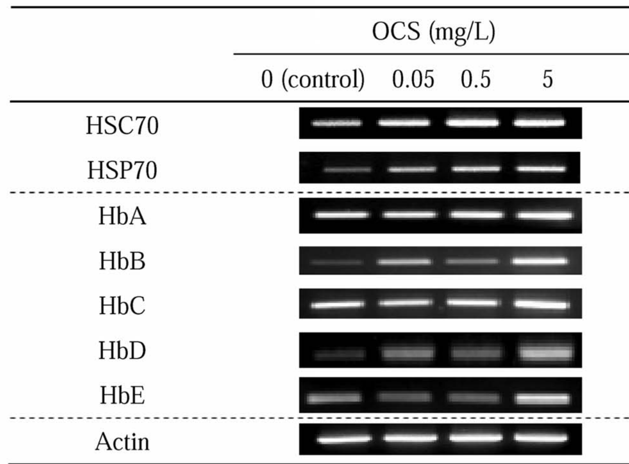
Table 2. Sequences submitted to GenBank as differentially expressed genes (DEGs) by octachlorostyrene (OCS) exposure in *Chironomus riparius*

DEG 1 (up-1)	
Locus	EU096096 635 base pairs mRNA linear INV September 16, 2007
Definition	*C. riparius* DEG 1 by OCS-exposure mRNA sequence
Accession	EU096096
Version	EU096096.1 GI:157091293
DEG 2 (up-2)	
Locus	EU096097 654 base pairs mRNA linear INV September 16, 2007
Definition	*C. riparius* DEG 2 by OCS-exposure mRNA sequence
Accession	EU096097
Version	EU096097.1 GI:157091294
DEG 3 (up-3)	
Locus	EU096098 509 base pairs mRNA linear INV September 16, 2007
Definition	*C. riparius* DEG 3 by OCS-exposure mRNA sequence
Accession	EU096098
Version	EU096098.1 GI:157091295
DEG 4 (up-4)	
Locus	EU096099 282 base pairs mRNA linear INV September 16, 2007
Definition	*C. riparius* DEG 4 by OCS-exposure mRNA sequence
Accession	EU096099
Version	EU096099.1 GI:157091296
DEG 5 (up-5)	
Locus	EU096100 516 base pairs mRNA linear INV September 16, 2007
Definition	*C. riparius* DEG 5 by OCS-exposure mRNA sequence
Accession	EU096100
Version	EU096100.1 GI:157091297

low concentrations of OCS (0.05 and 0.5 mg/L) showed approximately 13 to 18% lower emergence rates than the control, but these were not statistically significant. At the highest concentration of OCS (5 mg/L), 20 d after the beginning of the experiment, the average number of live individuals was only 15 out of the 50 initially introduced larvae, which means that almost 70% of the mortality occurred in the *C. riparius* population that had been exposed to 5 mg/L of OCS. The time to the completion of the emergence was 13, 15, 14, and 15 d after the beginning of the experiment for the control group and 0.05, 0.5, and 5 mg/L after OCS exposure, respectively. The pupation and emergence rates were studied in OCS-exposed larvae of *C. riparius* (Fig. 4B). At high concentration (5 mg/L of OCS), the pupation and emergence rates decreased with statistical significance. The degree of failure was more significant when counted from the larva to the adult (73%) than when counted from the pupa to the adult (38%).

Among the emerged adults, the males and females were identified to verify whether or not they had any difference in susceptibility to OCS exposure (Table 3). A serious alteration was observed in the sex ratio of the emerged adults at 5 mg/L of OCS exposure; a high female to male ratio was observed relative to the control group.

The effects of OCS on *Chironomus* reproduction were investigated by counting the number of egg masses oviposited by the emerged adults and the number of eggs per egg mass. The total numbers of egg masses were 12, 4, 8, and 0 at the control group and at 0.05, 0.5, and 5 mg/L of OCS exposure, respectively. The average numbers of eggs per egg mass were 438.6, 397.3, and 370 in the control group and after 0.05 and 0.5 mg/L of OCS exposure, respectively.



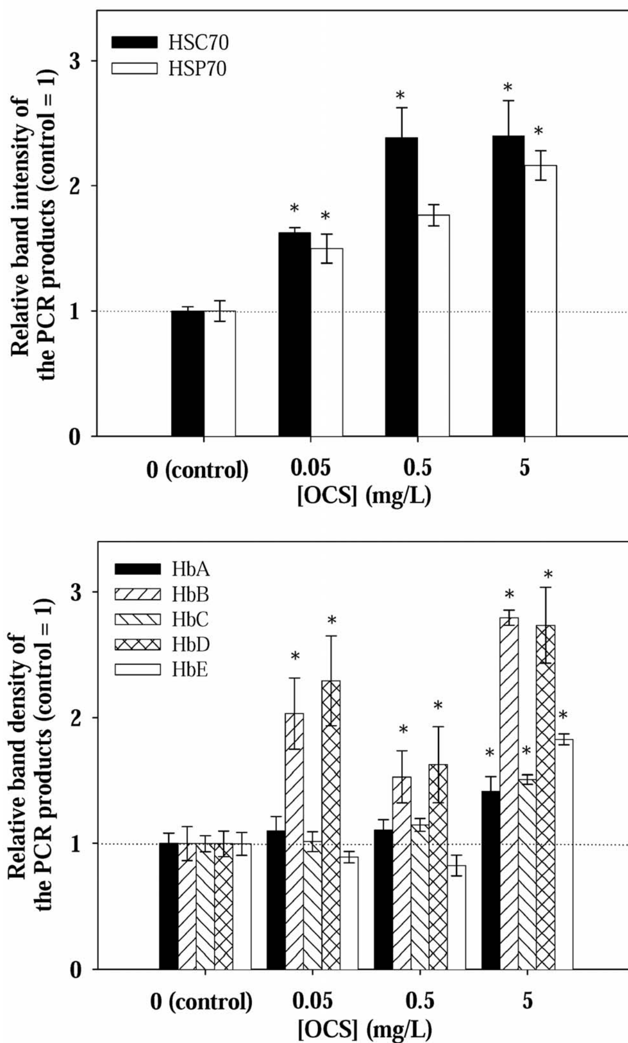


Fig. 2. Expression of heat shock protein (HSP) and hemoglobin (Hb) genes in the fourth instar larvae of *Chironomus riparius* exposed to 0 (control group), 0.05, 0.5, and 5 mg/L of octachlorostyrene (OCS) for 24 h (**A**). Both HSPs and Hbs are expressed in densitometric values normalized using actin messenger RNA (**B**) ($n = 3$, mean ± standard error of mean, $*p < 0.05$). HSC70 = heat shock cognate 70; PCR = polymerase chain reaction.

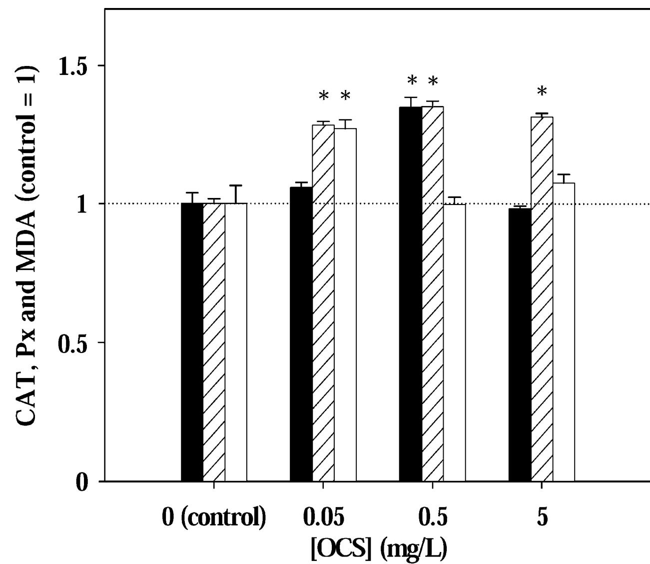


Fig. 3. Catalase (CAT) and peroxidase (Px) activities and malondialdehyde (MDA) measured in the fourth instar larvae of *Chironomus riparius* exposed to 0 (control group), 0.05, 0.5, and 5 mg/L of octachlorostyrene (OCS) for 24 h ($n = 3$, mean ± standard error of mean, $*p < 0.05$). ■ = CAT; ▨ = Px; □ = MDA.

To identify correlations between the molecular- or biochemical-level and the organism- or population-level effects, Pearson correlation tests were performed on the multilevel parameters studied (Table 4). Statistically significant positive correlations were observed between the exposure concentration of OCS and the DNA damage and gene expression (HbA, HbC, and HbE), whereas a negative correlation was observed between the OCS and the emergence rate and reproduction parameters (number of eggs per egg mass). Consequently, a negative correlation was observed between the Hb gene expression and the emergence rate and reproduction parameters.

DISCUSSION

The present study examines the ecotoxicity of OCS on *C. riparius* in various biological organizations, from the molecular and biochemical to the ecophysiological levels. The results of a 24-h lethality test of OCS and its related compounds on *C. riparius* suggest that, among the tested chemicals, OCS has the lowest acute toxic potential with respect to this species. A previous study with a bacteria bioassay also showed that OCS was not considered acutely toxic, but the long-lasting environmental impact of chlorinated styrenes is unclear [14].

A chemical analysis revealed that 24 h after exposure the concentration of OCS decreased dramatically in water. This phenomenon may suggest the rapid transfer of OCS from water to sediment or biota, which was highly probable as this chemical is known to have a high partition coefficient—log octanol-water partition coefficient (K_{OW}) = 6.29 (toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB). Indeed, the occurrence of OCS in sediment or in the tissues of aquatic organisms, including fish, was frequently reported in aquatic environments [15–17]. From the results of the present study and the physicochemical property of this compound (i.e., high lipid solubility), long-term bioaccumulation of OCS in *Chironomus* may be expected. Had OCS concentrations in *Chironomus* (internal dose) been measured, this could be better explained. Despite the associated difficulties and limitations, the use of the internal body concentration of OCS may have distinct advan-

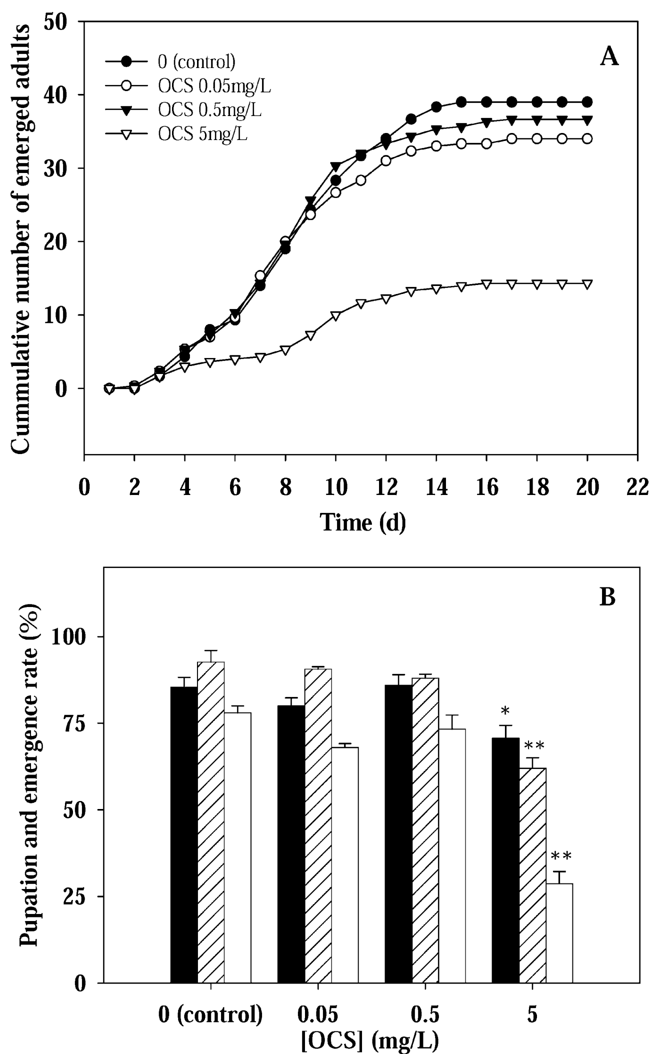


Fig. 4. Kinetics of cumulative adult emergence (**A**) and pupation and adult emergence rate (**B**), expressed as a percentage of the total number of larvae introduced at the beginning of the experiment, measured in 0 (control group), 0.05, 0.5, and 5 mg/L of octachlorostyrene (OCS)-exposed *Chironomus riparius* ($n = 3$, mean ± standard error of mean, $*p < 0.05$). (**B**) ■ = pupation; ▨ = emergence (from pupa); □ = emergence (from larva).

tages over the use of environmental concentrations as a toxicological index. In terms of toxicity, bioaccumulation data can be explained more meaningfully and can make a better connection between the accumulated dose and the toxicological effect, thus permitting better interpretation of the hazard associated with complex exposure routes [18].

Although the ecological relevance of DNA damage in single cells within some tissues of some individual organisms is difficult to assess, the sensitive detection of DNA damage in wildlife species is necessary, as pollutant-induced DNA damage may influence the genetic constitution of populations [19]. Octachlorostyrene may not exert any genotoxic effect on *C. riparius*, given that the DNA strand breaks did not increase in the *C. riparius* exposed to any studied concentration of this compound. As previously reported in a bacterial bioassay, OCS did not exhibit any form of mutagenicity [14].

Environmental contaminants may induce the expression of certain genes in an organism. Depending on the severity and duration of exposure to the contaminant, the expression of certain genes may be linked to short-term toxicological re-

Table 3. Emergence of male and female adults measured in control and octachlorostyrene (OCS)-exposed *Chironomus riparius*[a]

OCS (mg/L)	Male (%)	Female (%)
0 (control)	41.399 ± 7.324	58.6 ± 7.324
0.05	54.040 ± 6.312	45.960 ± 6.312
0.5	50.122 ± 1.312	49.878 ± 1.312
5	24.753 ± 3.380*	75.250 ± 3.380*

[a] Results are expressed as a percentage of the total number of larvae introduced at the beginning of the experiment ($n = 3$, mean ± standard error of mean, $*p < 0.05$).

sponses that impact individual fitness (i.e., survival and reproduction). The basic premise that changes in gene expression can be harnessed to diagnose exposure to and effects of environmental chemicals is currently receiving significant attention. The recent sequencing of the entire genome on environmentally relevant aquatic organisms (i.e., *Daphnia pulex*) will heighten the use of these toxicity test organisms in molecular-level bioassessments. Recently, several studies have focused on the responses to chemical stressors at the molecular level in aquatic invertebrates [20–22]. A gene expression analysis was conducted to identify a biomarker of OCS exposure. For the screening of OCS-induced gene expression profiling, the ACP-based PCR system was used, which facilitates the identification of DEGs from samples that display low mRNA levels without generating false positives [23]. Due to the availability of little genetic information on *Chironomus*, the results of the sequence analyses of the DEGs revealed that expressed sequence tags had no significant sequence similarity with the genes or expressed sequence tags available in GenBank, which led the authors to submit them to GenBank. The ACP-PCR results of the present study were obtained, however, from only 20 pairs of random primers. Had more pairs of primers been tested, known genes might have been revealed. The sequences identified from this experiment could contribute to knowledge on the *C. riparius* genome, about which little information is available. A detailed investigation of the submitted DEGs, such as characterization of their functions and dose–response relationship, will be the subject of further research.

Heat shock proteins are the most frequently studied genes of aquatic invertebrates [24,25]. Although researchers have studied the induction and accumulation of HSPs after the invertebrates have been exposed to various pollutants, there has been only limited application of HSPs to environmental biomonitoring [20,26,27]. The increase in the HSP70 gene expression upon OCS exposure was expected, as HSP70 is known to be inducible by various environmental stressors. In contrast to HSP70, HSC70 is known to be constitutively expressed and not inducible by environmental stressors [28]. Yet the expression of the HSC70 genes increased in response to OCS exposure, which was also observed in the authors' previous study with various environmental pollutants [22]. Given the responsiveness to even minor assaults, the expression of HSP70 and HSC70 may prove useful as a molecular indicator of nonspecific chemical toxicity in the *Chironomus* species.

One of the main particularities of *Chironomus* is possession of Hb during the larval stage. Considering the potential of *Chironomus* larvae to be a biomonitoring species and the physiological particularities of their respiratory pigment, the expression of Hb genes has considerable potential as a species-specific biomarker for environmental monitoring in *Chironomus*, which has been investigated in previous studies [22,29].

Table 4. Coefficients of correlation[a] between ecophysiological and molecular/biochemical parameters investigated in octachlorostyrene (OCS)-exposed *Chironomus riparius*[b]

	Development					Reproduction	
	PR	EP	EL	EMA	EFA	NOE	NOEE
OCS	−0.903 (0.097)	−0.998 (0.002)**	−0.980 (0.020)*	−0.898 (0.102)	0.898 (0.102)	−0.777 (0.223)	−0.994 (0.006)**
PR		0.908 (0.092)	0.967 (0.033)*	0.743 (0.257)	−0.743 (0.257)	0.911 (0.089)	0.931 (0.069)
EP			0.984 (0.016)*	0.873 (0.127)	−0.873 (0.127)	0.804 (0.196)	0.998 (0.002)**
EL				0.828 (0.172)	−0.828 (0.172)	0.876 (0.124)	0.993 (0.007)**
EMA					−1.000 (0.000)**	0.457 (0.543)	0.847 (0.153)
EFA						−0.457 (0.543)	−0.847 (0.153)
TNE							0.842 (0.158)
NOEE							
FBW							
DBW							
CAT							
Px							
MDA							
TM							
OTM							
HSC70							
HSP70							
HbA							
HbB							
HbC							
HbD							

[a] Pearson correlation test; p value in parentheses. Statistically significant $*p < 0.05$, $**p < 0.01$.

[b] PR = pupation rate; EP = emergence from pupa; EL = emergence from larva; EMA = emergence of male adults; EFA = emergence of female adults; TNE = total number of egg mass; NOEE = number of eggs per egg mass; FBW = fresh body weight; DBW = dry body weight; CAT = catalase; Px = peroxidase; MDA = malondialdehyde; TM = tail moment; OTM = olive tail moment; HSC70 = heat shock cognate 70; HSP70 = heat shock protein 70; HbA = hemoglobin A; HbB = hemoglobin B; HbC = hemoglobin C; HbD = hemoglobin D; HbE = hemoglobin E.

The OCS-induced Hb gene expression observed in the present study suggests that Hb may be a target molecule of OCS exposure and/or can be involved in the toxicity of this compound. A good supply of oxygen may help in the active and rapid removal of toxic compounds by accelerating metabolic reactions. To elucidate this mechanism, however, further studies are needed with a broad range of chemicals.

Oxidative stress–related toxicities due to environmental contaminants have been studied in many aquatic organisms [30–32]. The authors' previous studies have shown that enzymatic radical scavengers, including superoxide dismutase (SOD), are abundant in *C. riparius* larvae and that an early response of antioxidant enzymes, including SOD, could be considered biomarkers in *C. riparius* [4,33]. Catalase and Px are the main H_2O_2-scavenging enzymes, which are produced by SOD activity for the dismutation of $O_2^{\circ -}$ into H_2O_2 and O_2. According to a previous report, *Chironomus* hemolymph contains approximately 90% of total Px activity, and Hb content and Px activity are positively correlated [12], which suggest that *Chironomus* Px seems to play a more vital role than CAT in scavenging H_2O_2. This may partially explain why the response of CAT was not sensitive enough to be activated at the lowest and highest levels of OCS (0.05 and 5 mg/L). In the present study, however, the prime enzyme in defense of oxidative stress, i.e., SOD activity, was not measured. Had SOD activity been measured, the response of CAT and Px activity might have been explained more deeply. It has been reported that oxidative stress may not be accompanied by an increase in MDA content, which is an index of oxidative damage in lipids [34]. It can be hypothesized that OCS is a highly lipophilic compound and will be incorporated in the membrane lipids of organelles, which can be translated as the low level of lipid peroxidation (therefore, less MDA). Hence, for cell homeostasis, this compound may not affect membrane integrity. The experimental evidence provided in the present study, such as the slight increase in the antioxidant enzyme activities and in lipid peroxidation, was not sufficient to fully explain the involvement of oxidative stress in OCS toxicity. This merely suggests that oxidative stress may not be the central cause of OCS toxicity in *Chironomus*. Nevertheless, a broad range of oxidative stress–related parameters, such as SOD and glutathione–Px activities, DNA and protein oxidation, and their physiological meanings, has to be investigated.

Development and reproduction seem to be more affected than growth by OCS exposure in *C. riparius*, as OCS did not perturb larval growth. Alteration of the development and reproduction was observed, however, with long-term OCS exposure. In the present study, OCS exhibited serious chronic toxicity in *C. riparius*, such as the failure in pupation and emergence and the sex ratio. The increased mortality 25 d after OCS exposure, without renewal of the test solution, also suggests that this compound may have a higher chronic toxicity potential. The decrease in the pupation and emergence rates, an indicator of animal development, at the highest OCS concentration suggests that the alteration of this parameter may be considered a consequence of a serious progression of the toxic effect. One of the most notable results of the present study is OCS-induced disturbance on the male to female adult ratio. It is widely accepted that sex-specific effects, effects on sexual differentiation, and/or alteration of sex ratios induced by a chemical compound hint at the potential of the substance to disturb hormonal processes [35]. Bogart suggested that sexual differentiation processes in animals, including in arthropods, are based on the ratio of male to female sex hormones [36]. In the insect endocrinological system, ecdysone is known to act both as a direct precursor of the active molting hormone 20-hydroxyecdysone and as a male sex steroid [37]. Octachlorostyrene may alter invertebrate sex hormone–related

Table 4. Extended

Growth		Oxidative stress			DNA damage	
FBW	DBW	CAT	Px	MDA	TM	OTM
0.802 (0.198)	0.557 (0.443)	−0.368 (0.632)	0.377 (0.623)	−0.0973 (0.903)	0.964 (0.036)*	0.554 (0.446)
−0.966 (0.034)*	−0.641 (0.359)	0.572 (0.428)	−0.378 (0.622)	−0.324 (0.676)	−0.957 (0.043)*	−0.763 (0.237)
−0.798 (0.202)	−0.519 (0.481)	0.334 (0.666)	−0.428 (0.572)	0.0698 (0.930)	−0.975 (0.025)*	−0.592 (0.408)
−0.883 (0.117)	−0.565 (0.435)	0.425 (0.575)	−0.437 (0.563)	−0.102 (0.898)	−0.993 (0.007)**	−0.690 (0.310)
−0.712 (0.288)	−0.762 (0.238)	0.545 (0.455)	0.0522 (0.948)	0.349 (0.651)	−0.763 (0.237)	−0.169 (0.831)
0.712 (0.288)	0.762 (0.238)	−0.545 (0.455)	−0.0521 (0.948)	−0.349 (0.651)	0.763 (0.237)	0.169 (0.831)
−0.819 (0.181)	−0.286 (0.714)	0.263 (0.737)	−0.701 (0.299)	−0.498 (0.502)	−0.916 (0.084)	−0.954 (0.046)*
−0.826 (0.174)	−0.517 (0.483)	0.350 (0.650)	−0.455 (0.545)	−0.000 (1.000)	−0.988 (0.012)*	−0.644 (0.356)
	0.780 (0.220)	−0.757 (0.243)	0.168 (0.832)	0.410 (0.590)	0.852 (0.148)	0.678 (0.322)
		−0.954 (0.046)*	−0.468 (0.532)	0.0531 (0.947)	0.469 (0.531)	0.0750 (0.925)
			0.499 (0.501)	−0.304 (0.696)	−0.338 (0.662)	−0.128 (0.872)
				0.289 (0.711)	0.541 (0.459)	0.781 (0.219)
					0.144 (0.856)	0.684 (0.316)
						0.754 (0.246)

pathways, as a strikingly serious alteration on the sex ratio was observed upon 5 mg/L of OCS exposure. To further understand this phenomenon, however, more experimental evidence, such as the results of a hormone analysis, is needed. Such a significant disturbance on the male to female adult ratio by a high level of OCS exposure suggests that this compound may provoke serious consequences on *Chironomus* reproduction and, in turn, its population. Complete inhibition of egg mass oviposition by 5 mg/L of OCS exposure and decrease in the average number of eggs per egg mass upon OCS exposure in a concentration-dependent manner provide experimental evidence to support this hypothesis. To validate an indicator of toxicity, an attempt was made to demonstrate the plausible mechanistic linkage between the alteration observed and an adverse effect on development and reproduction. The authors' data set established a negative correlation between Hb gene expression and adverse development and reproduction outcomes (emergence rate and number of eggs per egg mass), which could suggest the protective role of Hb toward OCS stress in *Chironomus*. The experimental evidence provided by the present study, however, especially at the molecular and biochemical levels, is not sufficient to demonstrate a causal relationship among responses across biological levels.

In freshwater ecosystems, chemical pollution is frequently caused by a complex mixture of pollutants. This considerably increases the difficulty in predicting pollutant effects and emphasizes the need for studies on multiple biological endpoints to identify pertinent biomarkers. Simultaneous measurement of various biological parameters gives the opportunity to obtain data at different levels of biological organization and may help fully explain the effects of a toxicant on organisms. In addition, the determination of population-level parameters improves the interpretation of data collected at lower biological levels [38]. The multilevel biomarker approach, wherein different biological responses ranging from the molecular to the physiological are evaluated, is essential to determine the general health status of an organism in pollutant biomonitoring programs. Furthermore, it permits extrapolation of the relationship among responses at different levels of biological organization. As proven by many ecotoxicity studies, *Chironomus* seems to be a good biological model for this kind of approach.

In conclusion, a series of descriptors at different levels of biological organization (molecular, biochemical, and ecophysiological) were investigated in OCS-exposed *C. riparius* larvae. The most notable results of the present study showed that, although OCS has a low acute toxic potential, chronic exposure to this compound may have serious consequences on the *Chironomus* population through alteration of reproduction. The study showed that this compound significantly disturbed the male to female adult ratio and oviposition. To provide the mechanistic linkage between the molecular-level effect and the higher-level consequences, more biomarkers have to be simultaneously investigated.

SUPPORTING INFORMATION

Fig. S1. Screening of differentially expressed genes (DEGs) in octachlorostyrene (OCS)-exposed fourth instar larvae of *Chironomus riparius* by annealing control primer (ACP)–based polymerase chain reaction (PCR) method (1 = control; 2 = OCS 5 mg/L; arrows indicate DEG in OCS-treated larvae compared to control).

Table S1. Sequence of primers used in the amplification of heat shock protein, hemoglobin, and actin complementary DNA.

Table S2. Twenty-four-hour lethal concentration (24-h LC10, 50, and 90) of octachlorostyrene (OCS)-related compounds in the fourth instar larvae of *Chironomus riparius*.

Table S3. Fresh body weight (FBW) and dry body weight (DBW) measured in the fourth instar larvae of *Chironomus riparius* exposed to 0.05, 0.5, and 5 mg/L of octachlorostyrene (OCS) for 24 h ($n = 3$, mean ± standard error of mean).

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Table 4. Extended

	Gene expression						
	HSC70	HSP70	HbA	HbB	HbC	HbD	HbE
OCS	0.612 (0.388)	0.805 (0.195)	0.974 (0.026)*	0.836 (0.164)	0.981 (0.019)*	0.721 (0.279)	0.972 (0.028)*
PR	−0.451 (0.549)	−0.720 (0.280)	−0.928 (0.072)	−0.939 (0.061)	−0.834 (0.166)	−0.882 (0.118)	−0.913 (0.087)
EP	−0.649 (0.351)	−0.836 (0.164)	−0.985 (0.015)*	−0.859 (0.141)	−0.986 (0.014)*	−0.751 (0.249)	−0.960 (0.040)*
EL	−0.596 (0.404)	−0.819 (0.181)	−0.987 (0.013)*	−0.920 (0.080)	−0.947 (0.053)	−0.833 (0.167)	−0.956 (0.044)*
EMA	−0.279 (0.721)	−0.484 (0.516)	−0.776 (0.224)	−0.542 (0.458)	−0.840 (0.160)	−0.383 (0.617)	−0.950 (0.050)
EFA	0.279 (0.721)	0.484 (0.516)	0.776 (0.224)	0.542 (0.458)	0.840 (0.160)	0.383 (0.617)	0.950 (0.050)
TNE	−0.659 (0.341)	−0.853 (0.147)	−0.890 (0.110)	−0.995 (0.005)**	−0.761 (0.239)	−0.996 (0.004)**	−0.708 (0.292)
NOEE	−0.650 (0.350)	−0.846 (0.154)	−0.992 (0.008)**	−0.892 (0.108)	−0.977 (0.023)*	−0.794 (0.206)	−0.955 (0.045)*
FBW	0.207 (0.793)	0.519 (0.481)	0.805 (0.195)	0.844 (0.156)	0.689 (0.311)	0.794 (0.206)	0.869 (0.131)
DBW	−0.314 (0.686)	−0.0110 (0.989)	0.427 (0.573)	0.342 (0.658)	0.393 (0.607)	0.240 (0.760)	0.735 (0.265)
CAT	0.469 (0.531)	0.154 (0.846)	−0.274 (0.726)	−0.293 (0.707)	−0.181 (0.819)	−0.240 (0.760)	−0.566 (0.434)
Px	0.906 (0.094)	0.849 (0.151)	0.559 (0.441)	0.667 (0.333)	0.496 (0.504)	0.721 (0.279)	0.171 (0.829)
MDA	−0.0883 (0.912)	0.0617 (0.938)	0.0652 (0.935)	0.421 (0.579)	−0.174 (0.826)	0.561 (0.439)	−0.0747 (0.925)
TM	0.673 (0.327)	0.876 (0.124)	0.996 (0.004)**	0.951 (0.049)*	0.947 (0.053)	0.879 (0.121)	0.916 (0.084)
OTM	0.618 (0.382)	0.769 (0.231)	0.719 (0.281)	0.919 (0.081)	0.551 (0.449)	0.975 (0.025)	0.465 (0.535)
HSC70		0.944 (0.056)	0.718 (0.282)	0.667 (0.333)	0.742 (0.258)	0.644 (0.356)	0.410 (0.590)
HSP70			0.899 (0.101)	0.868 (0.132)	0.880 (0.120)	0.831 (0.169)	0.652 (0.348)
HbA				0.930 (0.070)	0.971 (0.029)*	0.849 (0.151)	0.913 (0.087)
HbB					0.818 (0.182)	0.983 (0.017)*	0.772 (0.228)
HbC						0.706 (0.294)	0.912 (0.089)
HbD							0.649 (0.351)

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