Identification of metabolic pathways in *Daphnia magna* explaining hormetic effects of selective serotonin reuptake inhibitors and 4-nonylphenol using transcriptomic and phenotypic responses.

Bruno Campos[1],Natàlia Garcia-Reyero[2], Claudia Rivetti[1], Lynn Escalon[3], Tanwir Habib[4], Romà Tauler[1], Stefan Tsakovski[5], Benjamín Piña[1], Carlos Barata [1]*.

[1]Institute of Environmental Assessment and Water Research (IDÆA-CSIC), Jordi Girona 18, 08034, Barcelona, Spain.

[2]Institute for Genomics, Biocomputing and Biotechnology, Mississippi State University, Starkville, MS 39759, USA

[3]U.S. Army Engineer Research and Development Center, Environmental Laboratory 3909 Halls Ferry Road, Vicksburg, MS 39180, USA

[4]Badger Technical Services, 12500 San Pedro Avenue, Suite 450, San Antonio, TX 78216, USA

[5]Department of Analytical Chemistry, Faculty of Chemistry, Sofia University, James Bourchier Blvd, 1164 Sofia, Bulgaria

*Address correspondence to Carlos Barata, Institute of Environmental Assessment and Water Research (IDAEA-CSIC), Jordi Girona 18, 08034 Barcelona, Spain. Telephone: +34-93-4006100. Fax: +34-93-2045904. E-mail: cbmqam@cid.csic.es

Table of content

METHODS

Chemicals

4-nonylphenol (CAS No 104-40-5, PESTANAL® analytical grade standard, 98.4% purity, Riedel-de-Haen, Germany); fluoxetine hydrochloride (CAS-No 56296-78-7; analytical standard, purity 100%) Sigma-Aldricht, USA), and fluvoxamine maleate (CAS-No 61718-82-9, analytical standard, purity 100%) were purchased from Sigma-Aldrich (USA/Netherlands). All other chemicals were analytical grade and were obtained from Merck (Germany).

Experimental animals. Individual or bulk cultures of 10 animals/L were maintained in ASTM hard synthetic water as described in Barata and Baird [1]. Individual or bulk cultures were fed daily with *Chorella vulgaris* Beijerinck ($5x10^5$ cells/mL, respectively, corresponding to 1.8 µg C/mL). *C. vulgaris* was grown axenically in Jaworski/Euglena gracilis 1: 1 medium (CCAP, 1989). The culture medium was changed every day, and neonates were removed within 24 h. Photoperiod was set to 14h light: 10h dark cycle and temperature at 20 ± 1°C.

Experiment 2: Ten gravid females were separately exposed to the same concentrations of 4-nonylphenol (20, 60 µg/L), fluvoxamine (7 µg/L) and fluoxetine (40 µg/L) described in experiment 1 using a food ration of 5 x 10^5 cells/mL of *C. vulgaris.* Likewise in experiment 1 all contaminants were dosed in the carrier acetone (0.1 mL/L) and a solvent control treatments of acetone (0.1 mL/L) was also included for baseline comparison of transcriptomic responses. Experiments started with 8-9 day old gravid females, which were exposed during three consecutive broods to the studied chemicals (10-14 days). Cultures of 100 to 150 individuals (< 24 h old neonates) were initiated and maintained in bulk cultures as described above. Within 24 h of deposition of the first clutch into the brood chamber, single females were removed and randomly

assigned to each treatment. The first batch of neonates (hatching within the first 48-72 h) was always discarded and not evaluated, as these animals were not exposed to the tested chemicals during their entire developmental period. Thus, only neonates from the second, third and fourth broods were counted for assessing effects on total offspring production. Just after releasing their fourth clutch into the brood pouch, eggs were gently flushed from the brood pouch and females were immediately flash- frozen in liquid N2 and preserved at -80 oC until RNA extraction. This protocol, by using only de-brooded females excludes the contribution of developing embryos on transcriptomic responses. Furthermore, the use of de-brooded adults in the first hours of their intermolt instar ensured measurement of transcriptomic responses of all the studied females at the beggining of the intermolt cycle, thus minimizing undesirable variation of gene transcription patters within females across the molt cycle [3].

Validation of Microarray results by qRT-PCR

Quantities of 1µg were retrotranscribed to cDNA using First Strand cDNA Synthesis Kit Roche® (Germany) and stored at -20ºC. Aliquotes of 10ng were used to quantify specific transcripts in Lightcycler® 480 Real Time PCR System (Roche, Germany) using Lightcycler 480 SYBR Green I Master® (Roche, Germany). Relative abundance values of all genes were calculated from the second derivative of their respective amplification curve, Cp values calculated by technical triplicates. Cp values of target genes were compared to the corresponding reference gene [4].

FIGURES AND FIGURE LEGENDS

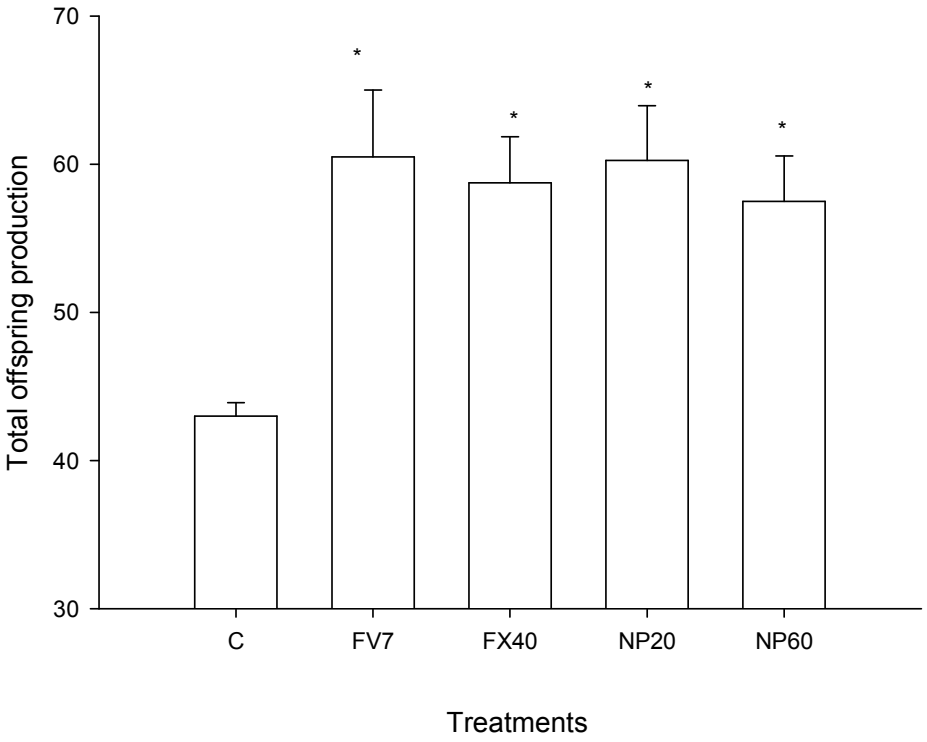

Fig S1. Total offspring production (Mean SE, N=4) of the four selected females exposed to the studied SSRI and 4-nonylphenol treatments. C, FV10, FX40, N20, N60 are solvent control, fluovoxamine at 7 µ/L, fluoxetine at 40 µ/L, 4-nonylphenol at 20 µ/L and 60 µ/L, respectively. Asterisks indicate significant differences from solvent controls following ANOVA and Dunnett's comparison tests.

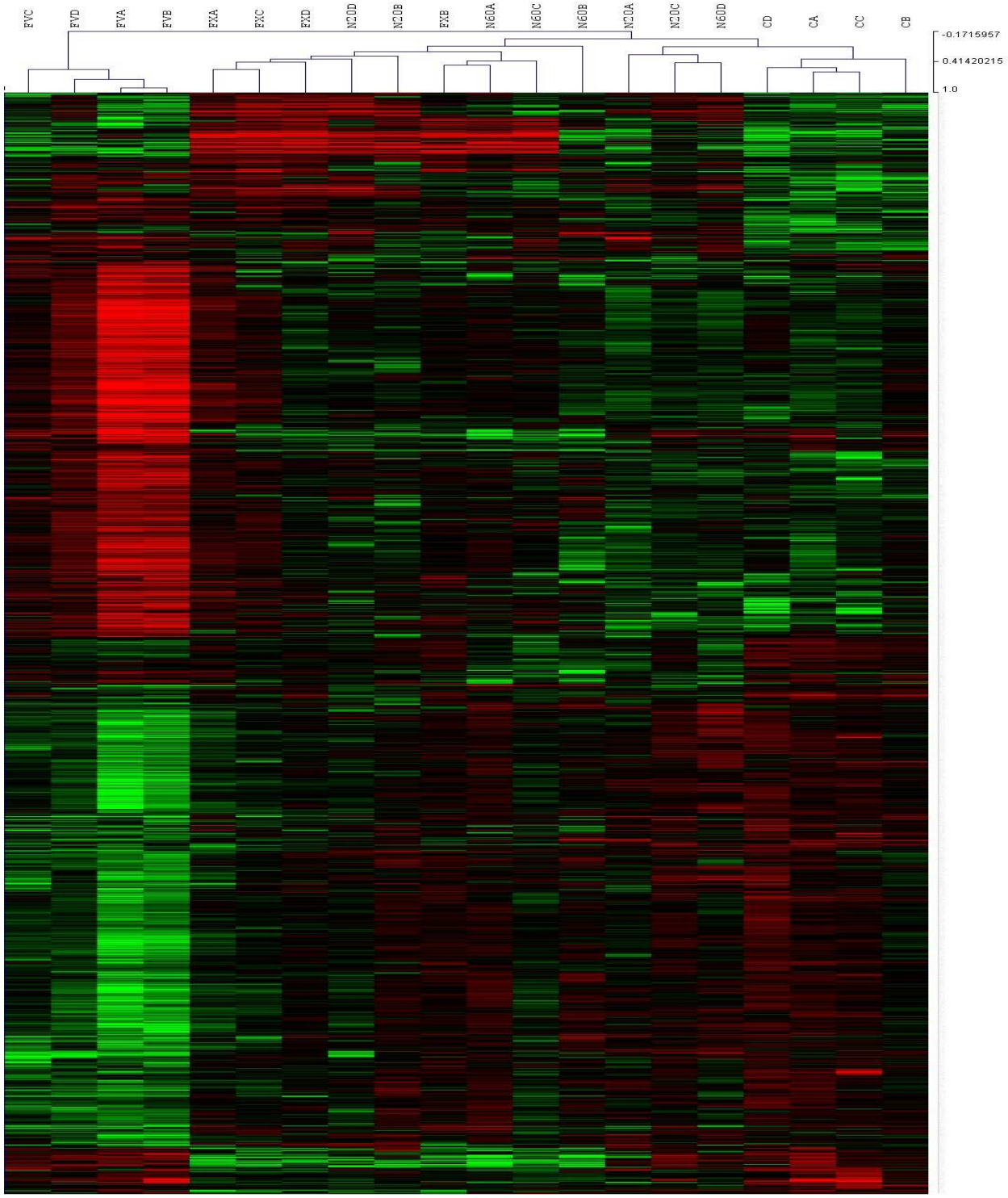

Fig S2. Heat map and hierarchical clustering (Pearson correlation) base on log2-ratios of the differentially transcribed genes in *Daphnia magna* juveniles across the experimental replicates for the SSRIs (FV, FX) and nonylphenol (N20, N60) treatments. The four replicates are identify as A,B,C,D. Gene transcripts in red and green are up and down regulated and those in black unchanged. Colour scale spans from -2.5 to 2.5.

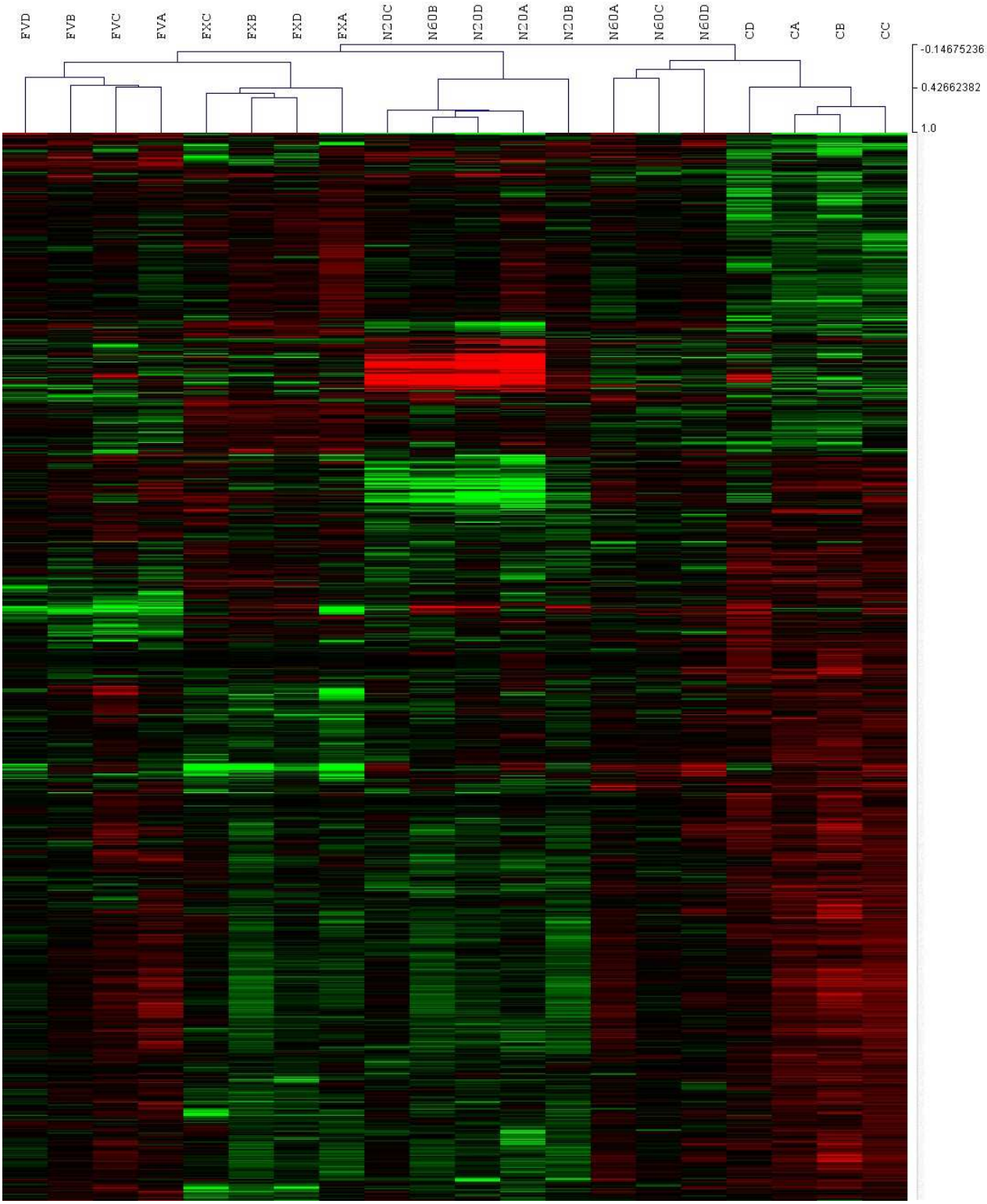

Fig S3. Heat map and hierarchical clustering (Pearson correlation) base on log2-ratios of the differentially transcribed genes in *Daphnia magna* adults across the experimental replicates for the SSRIs (FV, FX) and nonylphenol (N20, N60) treatments. The four replicates are identify as A,B,C,D. Gene transcripts in red and green are up and down regulated and those in black unchanged. Colour scale spans from -2.5 to 2.5.

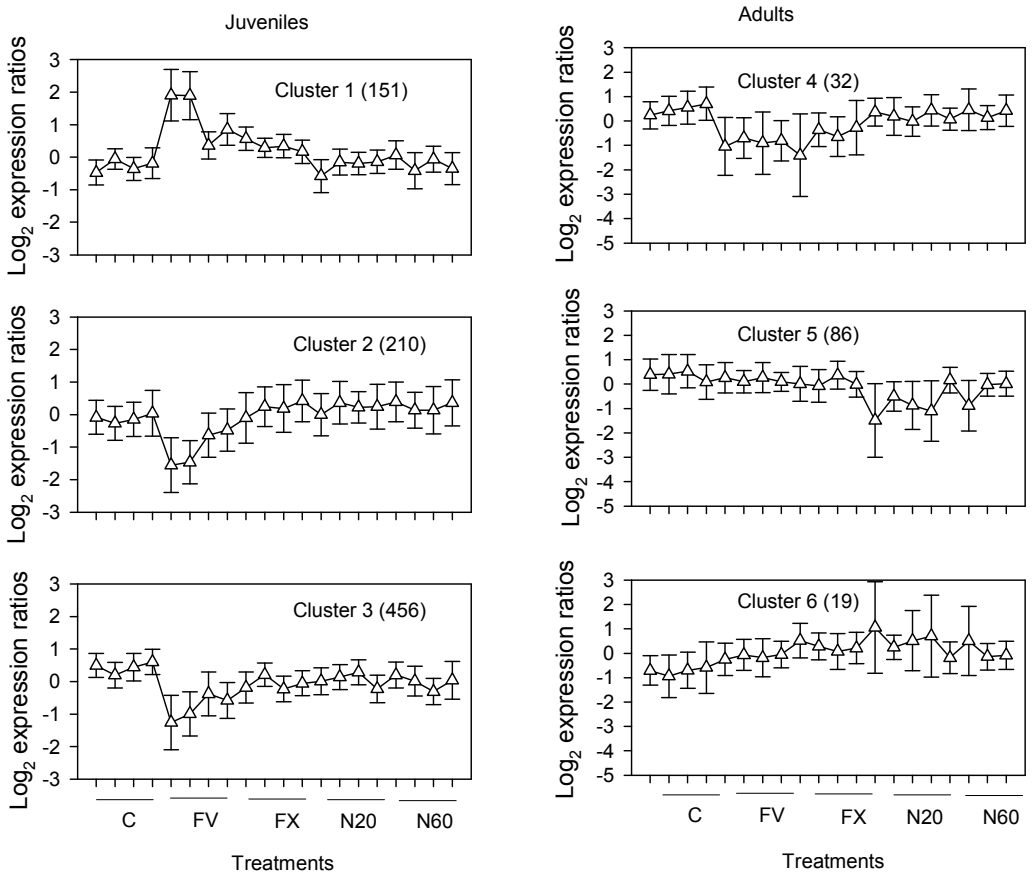

Fig S4. Self Organizing Maps (SOM) of gene transcription responses of juveniles and adults across the experimental replicates for SSRIs (fluvoxamine, FV; fluoxetine, FX) and 4-nonylphenol (N20, N60) treatments. Results are depicted as Mean ± SD of log2-ratios. Number of de-regulated genes are depicted between parenthesis.

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