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Identification of Metabolic Pathways in *Daphnia magna* Explaining Hormetic Effects of Selective Serotonin Reuptake Inhibitors and 4-Nonylphenol Using Transcriptomic and Phenotypic Responses

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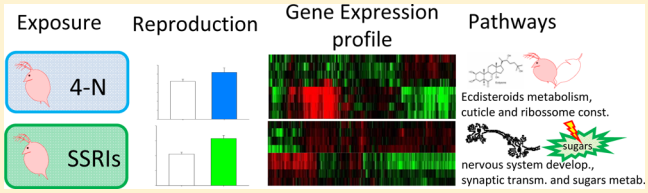
Supporting Information

ABSTRACT: The molecular mechanisms explaining hormetic effects of selective serotonin reuptake inhibitors (SSRIs) and 4-nonylphenol in *Daphnia magna* reproduction were studied in juveniles and adults. Transcriptome analyses showed changes in mRNA levels for 1796 genes in juveniles and 1214 genes in adults (out of 15 000 total probes) exposed to two SSRIs (fluoxetine and fluvoxamine) or to 4-nonylphenol. Functional annotation of affected genes was improved by assuming the annotations of putatively homologous *Drosophila* genes. Self-organizing map analysis and partial least-square regression coupled with selectivity ratio procedures analyses allowed to define groups of genes with specific responses to the different treatments. Differentially expressed genes were analyzed for functional enrichment using Gene Ontology and Kyoto Encyclopaedia of Genes and Genomes databases. Serotonin metabolism, neuronal developmental processes, and carbohydrates and lipid metabolism functional categories appeared as selectively affected by SSRI treatment, whereas 4-nonylphenol deregulated genes from the carbohydrate metabolism and the ecdysone regulatory pathway. These changes in functional and metabolic pathways are consistent with previously reported SSRIs and 4-nonylphenol hormetic effects in *D. magna*, including a decrease in reserve carbohydrates and an increase in respiratory metabolism.



INTRODUCTION

There is increasing evidence that the presence of many emerging pollutants in aquatic ecosystems may have detrimental effects on aquatic biota.[1] In the past decade substantial research has been conducted evaluating toxic effects of pharmaceuticals and other emerging pollutants across nonvertebrate species.[2,3] Among emerging pollutants, those that may act as putative endocrine disruptors in invertebrate species, for example altering moulting and reproductive processes, are of special concern.[4] The industrial detergent degradation product 4-nonylphenol and the pharmaceutical group of Selective Serotonin Reuptake Inhibitors (SSRIs) had hormetic consistent effects (i.e., increased) on offspring production and/or juvenile developmental rates in *Daphnia magna* at low concentrations (i.e., 3−15 μg/L), inhibiting those responses at higher concentrations (i.e., 100−125 μg/L).[5−7] Surveys in United States have reported levels of 12−540 ng/L of fluoxetine in surface waters and effluents,[8] but total concentrations of SSRIs in aquatic systems were measured in the range of 840 ng/L[9] to 3.2 μg/L.[10] Nonylphenol levels in surface waters as high as 8 μg/L have been reported in surface waters.[11] Therefore the above-mentioned hormetic effects occurred at environmental relevant concentrations.

SSRIs switch life-history responses toward higher food levels at limiting food conditions: juveniles develop faster, reach maturity earlier, and females produce more offspring.[12] Such hormetic effects, however, have a fitness cost: females exposed to SSRIs have enhanced glycogen and aerobic metabolism, which translates into a lower tolerance to starvation at low oxygen levels, a common situation in the field.[12] Exposure of *D. magna* adult females to low concentrations of 4-nonylphenol increases reproduction and decreases offspring size.[5,13] These effects are maladaptive in *D. magna*, since smaller offspring take longer to grow, reach a smaller adult size, and produce less eggs at maturity.[14]

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The mechanisms of action of SSRIs affecting phenotypic responses of *D. magna* exposed to SSRIs were analyzed by studying effects on levels of lipids, carbohydrate, proteins, oxygen consumption rates, survival, and offspring production after exposure to SSRIs alone or with a serotonin antagonist.[12] The results from this analysis showed that SSRIs act in a similar way in *D. magna* than in humans by increasing serotoninergic activity, but in doing so they alter physiological processes such as increased glycogen and aerobic metabolism. At high doses 4-nonylphenol causes embryo arrest in *D. magna*, an effect that according to LeBlanc et al.[13] may be related to altered metabolism of endogenous steroids (such as ecdysone), resulting in perturbations in their provision to the newly produced eggs.

The above-mentioned studies support the view that 4-nonylphenol and SSRIs increase offspring production through different mechanisms of action.[5,12] SSRIs affect life-history responses of both juveniles and adults, having important metabolic effects that in adults result in increased carbohydrate and aerobic metabolism. Sublethal effects of 4-nonylphenol have only been observed in adults and probably are related to altered metabolism of endogenous steroids like ecdysone.[13]

The water flea *D. magna* is possibly the invertebrate system most used in toxicology and experimental ecology and together with its close relative *Daphnia pulex* is used as a model for environmental genomics research.[15] The *D. pulex* genome has been fully sequenced, and about 50% of its genome is annotated;[16] thus that of its close relative *D. magna*, despite of being incomplete, may benefit from the former. Nevertheless, gene annotation available for the *D. pulex* genome was insufficient to aid functional analysis; thus additional annotation for functional enrichment and identification of gene networks and metabolic pathways can be performed using available bioinformatic tools such as Blast, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG).[17−20]

The main objective of this work was the study of molecular mechanisms explaining the hormetic effects observed in our two previous studies.[5,12] This was accomplished using transcriptional stress responses of *D. magna* juveniles and adults exposed to sublethal doses of 4-nonylphenol and the SSRIs fluoxetine and fluvoxamine. We aimed to identify gene pathways that characterized observed physiological and phenotypic responses to these contaminants. To this effect, we used a custom microarray that has been successfully used to assess mechanisms of action of different pollutants in *D. magna*.[21] Differentially transcribed genes were related to effects observed on offspring production of females chronically exposed to these pollutants using partial least-square regression coupled with selectivity ratio procedures. Existing information from *D. pulex* and *Drosophila melanogaster* genomes was used to improve the currently poor annotation of *D. magna* genes and to facilitate the functional analyses of the observed transcriptomic changes.

EXPERIMENTAL SECTION

Chemicals. Analytical grade standards of 4-nonylphenol, fluvoxamine (FV), and fluoxetine (FX) were used in this study. Details can be found in the Supporting Information, experimental section.

Analytical Chemistry. Methods and measured concentrations of the studied chemicals in adult reproduction tests were reported elsewhere [5] and in all cases were within 90% of nominal values, indicating that degradation/adsorption/uptake to experimental vessels/animals were negligible.

Experimental Animals. All experiments were performed using a well-characterized single clone of *D. magna* (Clone F), maintained indefinitely as pure parthenogenetic cultures.[22] Culture conditions are described in Supporting Information, experimental section.

Experimental Design. Juveniles and adult females from two distinct assay-experiments were considered for transcriptomic studies. Assays with adult females were conducted in a previous study[5] and are briefly described in Experiment 2. Selected exposure levels of the studied compounds include those treatments that in a previous study[5] affected most reproduction responses: for SSRIs, 7 μg/L of FV and 40 μg/L of FX; for 4-nonylphenol 20 and 60 μg/L.

Experiment 1. Experiment 1 consisted of 72 h exposures of juveniles to the SSRIs: FV (7 μg/L), FX (40 μg/L), and the industrial detergent 4-nonylphenol (20, 60 μg/L). Groups of 20 juveniles (less than 24 h old) were exposed to selected exposure levels in 0.5 L of ASTM hard water with food present (5×10^5 cells/mL of *C. vulgaris*) during two consecutive moults (about 3 days at 20 °C and high food levels).[23] Longer exposure periods were not performed as juveniles enter the adolescent instar where ovaries start to develop.[24] Chemicals were added using acetone as a carrier (0.1 mL/L). A carrier control of 0.1 mL/L was also used to serve as a baseline comparison for transcriptomic responses. Treatments were quadruplicated. At the end of exposure the 20 juveniles of each treatment per replicate were collected, pooled in an eppendorf vial, flash-frozen in liquid N_2, and preserved at −80 °C until RNA extraction.

Experiment 2. Adult reproduction tests were performed to determine the effects of the chemicals of interest on transcriptomic responses and reproduction rates on adult stages.[12] Experiments started with 8−9 day old gravid females, which were exposed during three consecutive broods to the studied chemicals (10−14 days). 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 N_2 and preserved at −80 °C until RNA extraction. As in Experiment 1, a carrier control of 0.1 mL/L was used to serve as a baseline comparison of transcriptomic responses. A full description of these experiments can be found in the Experimental Section, Supporting Information.

RNA Extraction. Total RNA was isolated from the samples using Trizol reagent (Invitrogen, Carlsbad, USA) following the manufacturer's protocol and quantified in a NanoDrop D-1000 Spectrophotometer (NanoDrop Technologies, Delaware, DE). RNA quality was checked in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). The samples that did not show degradation were used for microarray analysis.

Microarrays. Custom *D. magna* 15 000 probe microarrays (GLP13761) were purchased from Agilent (Palo Alto, CA, USA). A total of four replicates per treatment were used for both juveniles and adult exposures. A sample of 1 μg of total RNA was used for all hybridizations, and cDNA synthesis, cRNA labeling, amplification, and hybridizations were performed following the manufacture's kits and protocols (Quick Amp labeling kit; Agilent, Palo Alto, CA). The Agilent one-color Microarray Based Gene Expression Analysis v6.5 was used for microarray hybridizations according to the manufacturer's recommendations. An Agilent high-resolution C microarray scanner was used to scan microarray images. Data were

resolved from microarray images using Agilent Feature Extraction software v10.7. Raw microarray data from this study have been deposited at the Gene Expression Omnibus Web site (www.ncbi.nlm.nih.gov/geo/) with accession number GSE45053.

Validation of Microarray Results by qPCR. Microarray results were validated with real-time quantitative polymerase chain reaction (qPCR). We selected six significantly deregulated genes from different pathways/gene families: lipids metabolism (thiolase), Krebs cycle (aconitase—acon, isocytrate dehydrogenase—idh, ATP citrate lyase—ATPCL), tryptophan metabolism (dopamine decarboxylase—Ddc), and nucleotide metabolism (fumarilacetoacetase—Faa). The gene G3PDH (glyceraldehyde 3-phosphate dehydrogenase) was used as an internal control. For each of these genes primers were designed with Primer Quest (IDT Technologies, Coralville, IA) and are listed in Table S1 in the Supporting Information. qPCR was performed according to manufacturer's protocols described in the experimental section, Supporting Information.

Gene Expression Analysis. Microarray data were analyzed using GeneSpring GX v4.0 software (Agilent). Data were normalized using quantile normalization and baseline transformation to the median of all samples. Differentially transcribed genes of *D. magna* individuals exposed to 4-nonylphenol, and SSRIs from that of carrier controls (hereafter named as controls) were analyzed using one way ANOVA followed by Dunnett's pos-hoc comparison tests and filtered based on expression levels ($p < 0.05$; cutoff 1.5 fold change). A list of differentially transcribed genes for each of the treatments can be found in Table S2 (Supporting Information).

Transcriptomic patterns of differentially expressed genes of juveniles and adults were analyzed using the Multi-Experiment viewer MeV4 software,[25] both by hierarchical clustering (sites) and self-organizing map (SOM) analysis (features), and the standard normalization protocol. Furthermore, the assays performed with single adult females (Experiment 2), allowed to establish possible correlations between individual transcriptomic profiles and the observed effects of both SSRIs and 4-nonylphenol treatments on *D. magna* reproduction responses, and hence the identification of putative gene regulatory pathways affecting these reproduction responses. Partial least-squares regression (PLS) was used to investigate the correlations between transcriptome data (x variables) and total offspring production (y variable) under both SSRIs and 4-nonylphenol treatments. Since both treatments affected fecundity similarly (increased offspring production) but likely through different molecular mechanisms, SSRIs and 4-nonylphenol treatments were analyzed separately by PLS-SR. Genes contributing more to the prediction of the reproduction responses by the proposed PLS model were selected using the selectivity ratio (SR).[26] This variable selection method minimizes the effect of uncorrelated x variance sources. Those genes (x-variables) with SR values higher than the mean were considered to be the most relevant for explaining the y responses related to treatments. Prior to data analysis, x and y variables were mean-centered to correct for their offset. The number of PLS-SR components was finally set at two, according to cross validation by the leaving-one-out prediction errors' criteria.[27] PLS-SR analyses were performed using the PLS Toolbox version 6.5, eigenvector Research Inc. (Manson, WA) running under Matlab 7.4 computer environment (MathWorks, Natick, MA).

Genes included in the clusters identified by SOM and PLS-SR analyses were annotated using the recent update of the custom microarray, which incorporates *D. pulex* sequence annotations available at the Fleabase.[21] Further annotation was performed by identifying putative homologous *Drosophila melanogaster* genes using BLAST at Flybase server (http://flybase.org/blast/). Annotated genes were ascribed to functional Gene Ontology (GO terms) and to metabolic pathways using the AmiGO! Term Enrichment tool (http://www.geneontology.org/) and the Kyoto Encyclopaedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/kegg2.html) databases, respectively.

RESULTS

Phenotypic Responses. There was no mortality during juvenile exposures. Selected females for transcriptomic analyses produced significantly ($P < 0.01$; $F_{4,15} = 5.02$) more offspring than controls in all treatments (offspring production results are depicted in Figure S1, Supporting Information).

Transcriptome Analysis. Transcriptome responses of juveniles and adults of experiments 1 and 2 were analyzed separately. A total of 1796 and 1214 genes were identified as differentially transcribed in at least one of the chemical treatments, in juveniles and adults, respectively. From the above-mentioned genes, only 221 were annotated (see Table S2, Supporting Information for further information). Hierarchical clustering separated most biological replicates of SRRIs, 4-nonylphenol and control treatments on both the juvenile and adult transcriptomic data set (full clusters and heat maps are provided in Figures S2 and S3 of Supporting Information). The SOM analysis performed on the differentially transcribed genes in juveniles and adults revealed six clusters of significantly deregulated genes. For juveniles cluster 1 include 151 significantly ($P < 0.01$) up-regulated genes by both SSRI compounds (FX and FV). Clusters 2 and 3 included 210 and 456 FV down-regulated ($P < 0.01$) unique genes, respectively. For adults, cluster 4 included 32 genes significantly ($P < 0.01$) down-regulated by both SSRIs (FV and FX treatments), and cluster 5 included 86 genes significantly ($P < 0.01$) down-regulated by 4-nonylphenol treatments (20, 60 μg/L), whereas cluster 6 included 19 genes up-regulated by 4-nonylphenol treatments. SOM graphs together with the deregulated genes for each cluster are depicted in Figure S4 and Table S3 (Supporting Information). PLS analyses were performed separately for the different treatments considered [(control and SSRIs) and (control and 4-nonylphenol treatments)]. In each data set the 1214 deregulated gene fragments were selected as predictors ($\mathbf{X}$ matrix of predictor variables), and the total offspring production of the exposed females was used as the predicted variable ($\mathbf{Y}$ vector of predicted variables). The two components introduced in the PLS analysis explained around 80% of the total Y variance ($r^2 = 0.79$) for the prediction of total offspring production of females treated either with SSRIs (Figure 1A) or with 4-nonylphenol (Figure 1B). Following the established SR cutoff criteria, PLS analysis identified 272 and 331 genes differentially affected by SSRIs and 4-nonylphenol, respectively (Figure 1). These predictor genes formed two differentiated groups: one in close proximity with SSRIs or 4-nonylphenol y-treatment including genes that covaried positively with both treatments, and a second one close to control treatments and including genes inversely correlated with the two studied treatments. The final set of annotated genes differentially affected by SSRIs and 4-

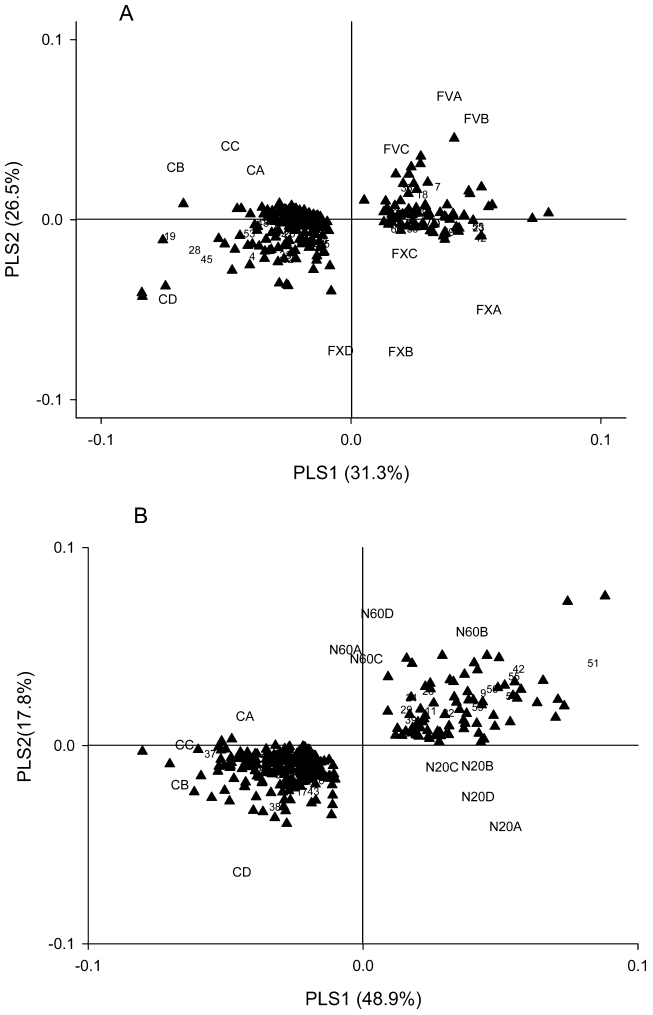

Figure 1. PLS-SR summary of results for the two different sample treatments (A, SSRIs; B, 4-nonylphenol) using differentially expressed genes as predictors (x) and total offspring production as response (y). First and second PLS biplots show selected gene loadings (triangles) and offspring production scores (sample identification letters). Only genes with a selectivity ratio above the mean were considered. Annotated genes are indicated by numbers described in the Supporting Information, Table S4. The four replicates are identified as A, B, C, and D.

nonylphenol treatments (35 and 31 genes, respectively) are identified by numbers in Figure 1. Correlation plots between the log2 of fold change ratios of the selected annotated and non annotated genes with the total offspring production are given in Table S4 of Supporting Information.

Results from qPCR confirmed the fold change ratios of mRNA levels observed with the microarray, both in magnitude and direction, for the six selected genes (Figure 2) with a Pearson correlation coefficient of 0.83 ($P < 0.01$, $N = 33$).

GO analyses were performed on annotated genes from SOM and PLS-SR clusters (Table 1, further details of GO terms and the name of involved genes are depicted in Table S5, Supporting Information). Juveniles appeared as selectively more affected by SSRIs treatments than adults, showing the highest amount of annotated genes grouped into GO term ($P < 0.05$; 151 genes in total, Table 1). Adult SOM and PLS-SR clusters affected by SSRIs included 40 genes that could be adscribed to a specific GO term (Table 1). 4-Nonylphenol only affected adult SOM and PLS-SR clusters, which included 38 genes with a specific GO term assignation (Table 1). The GO terms with the highest number of deregulated genes were related to three parental GO categories: developmental processes, structural constituents, and binding (Table 1). Developmental processes were only affected by SSRIs. Other processes only affected by SSRIs were synaptic vesicle transport, localization, and response to stimuli. Structural constituents, binding, and catalytic activity related GO categories were shared by both SSRIs and 4-nonylphenol treatments although most of the deregulated genes and sub-GO categories belonged to SSRI treatments. The only unique GO category affected by 4-nonylphenol was lipid binding.

Metabolic pathway analyses of deregulated genes (>1.5 fold) in adult females exposed chronically to SSRIs and 4-nonylphenol indicated that 20 of these genes regulated metabolic pathways (Figure 3). More specifically, SRRIs and 4-nonylphenol deregulated 12 and 7 genes from metabolism of carbohydrates and lipids and Krebs cycle, respectively. Further details of KEGG analyses are in Supporting Information (Table S6, Supporting Information).

DISCUSSION

In this study we tested the hypothesis that low concentrations of SSRIs and 4-nonylphenol affected offspring production and/or juvenile developmental rates though different mechanisms of action. Our hypothesis was tested by combining two developmental stages, juveniles and adults, exposed during two (3 days) and three (10 days) consecutive instars, respectively. Although in absolute terms juvenile exposures were much shorter than that of adults, both schemes included physiologically equivalent periods (i.e., include 2−3 instars).[28] Previous results also indicate that sublethal effects on juvenile developmental rates of SSRIs occurred at similar concentrations than those of adults.[12] Therefore, we consider that juvenile and adult stages were chronically exposed to equivalent concentrations and periods in our experiments.

Hierarchical clustering clearly grouped replicated samples of SSRIs, 4-nonylphenol, and control by treatment in both juvenile and adult exposures, providing the first evidence that these contaminant families induced specific and unique transcriptional patterns. The number of deregulated genes and significant GO terms obtained for juveniles exposed to all three chemicals was higher than that of adults, which supports the hypothesis that short-term exposures may be more suited to assess differentially altered transcriptomic responses. This was particularly evident for the SOM clusters obtained for adults that only involved 17 annotated genes, whereas those of juveniles include 151 ones (Table 1). Alternatively, the number of deregulated genes could be a direct consequence of previously reported phenotypic effects. Previous work indicated that at the studied exposure levels FV affected juvenile developmental rates whereas FX and 4-nonylphenol did not.[5] This is consistent with the apparent stronger effect of FV on the juveniles' transcriptome, both in terms of number of deregulated genes and of the number of significantly enriched GO terms (Figures S2 and S3 of SI). Reproduction was similarly affected by the three chemicals at all exposure levels, and so was the number of annotated genes and enriched GO terms defined by both SOM and PLS-SR analyses (7 and 10 genes in SOM clusters for SSRIs and 4-nonylphenol, respectively; 35 and 32 genes selected by PLS-SR for SSRIs and 4-nonylphenol, respectively; Table 1).

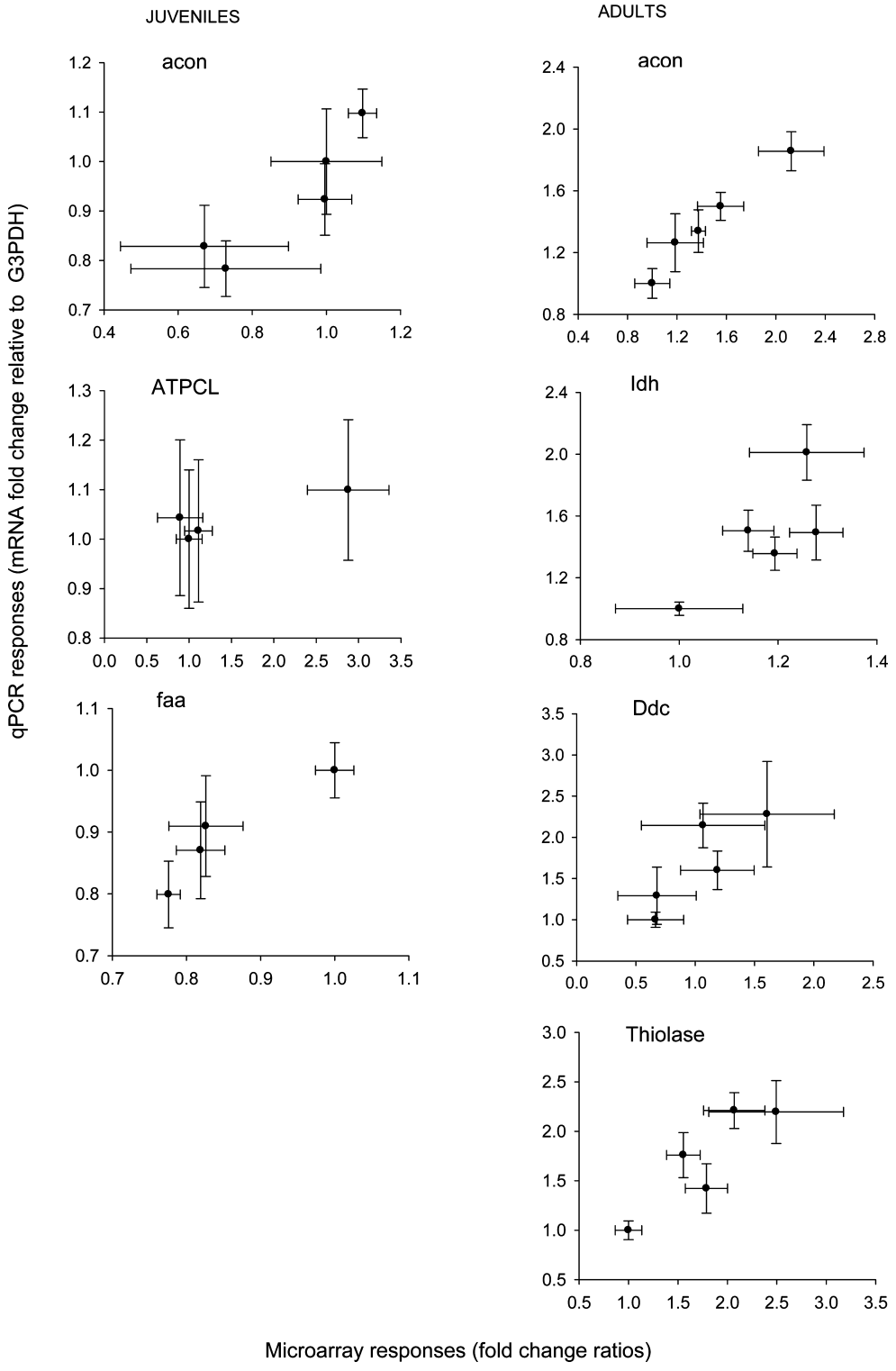

Figure 2. Confirmation of the array results with qPCR. Array and qPCR results are represented as gene fold change ratios. Errors bars are standard errors of the mean (SE, $N = 4$).

Relating molecular responses to phenotypic effects is crucial in environmental risk assessment. A promising approach is the adverse outcome pathway (AOP) framework, which is a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome at a biological level of organization relevant to risk assessment.[29] The application of AOP has been restricted to model organisms (i.e., few fish species).[30−32] In a previous study[5] we showed that 4-nonylphenol increased offspring production but decreased at the same time offspring size. According to several studies being smaller at birth bears a fitness disadvantage since smaller neonates are more sensitive to environmental stress and have lower fitness than bigger ones.[14,33] SSRIs also increased offspring production with apparently no effects on offspring size, but such effects were related with increasing oxygen demand and being less tolerant

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Table 1. GO Terms for the Obtained Six SOM and Two PLS Gene Clusters for SSRI's and 4-Nonylphenol (N) Treatments[a]

	SOM-SSRI's				SOM-N		PLS	
	JUV1↑	JUV2↓	JUV3↓	ADT 4↓	ADT5↓	ADT6↑	SSRI↓↑	N↓↑
total genes in group	27	42	82	7	6	4	33	28
Biological Processes								
Developmental Process								
GO:0048856 anatomical structure development			12**	4**				
GO:0048513 organ development			39***				12***	
GO:0009888 tissue development		17***	32***				7*	
Nervous System Development								
GO:0007399 nervous system development		11*	22***				10*	
GO:0048666 neuron development			12**				8**	
GO:0007411 axon guidance		10*	21***				5*	
Sensory Organ Development								
GO:0001654 eye development							6*	
GO:0001751 compound eye photoreceptor cell differentiation			6*					
Morphogenesis								
GO:0009653 anatomical structure morphogenesis			22***				11*	
GO:0046664 dorsal closure, amnioserosa morphology change								2*
Synaptic Vesicle Transport								
GO:0048488 synaptic vesicle endocytosis		2*					3*	
GO:0031629 synaptic vesicle fusion to presynaptic membrane			10***					
Structural Constituents								
GO:0003735 structural constituent of ribosome	17**	29***	49***			4***		
GO:0005214 structural constituent of chitin-based cuticle						2*		
Localization								
GO:0051179 localization			23***				15***	
Response to Stimulus								
GO:0040011 locomotion							8**	
GO:0006935 chemotaxis							6***	
Molecular Functions								
GO:0003824 catalytic activity		21***	47***					16***
GO:0005488 binding		15***	20***				21***	17**
GO:0000166 nucleotide binding		24***	56***		6*			
GO:0001882 nucleoside binding		12***	20***					
GO:0008289 lipid binding								6***

[a]SOM clusters of juveniles (JUV 1, 2,3) and of adults (ADT 4, 5, 6) and PLS ones for SSRIs and 4-nonylphenol (N) are depicted in Figures 1 and S4. * $0.05 < P < 0.01$; ** $0.01 < P < 0.001$; *** $P < 0.001$. Further information of GO terns and annotated genes belonging to each group are in Table S5. Arrows indicate the direction of deregulation.

to low oxygen levels.[12] Thus in the two studied chemical groups (4-nonylphenol and SSRIs), adverse effects were directly or indirectly associated with increasing reproduction at the selected exposure levels. In the present study the combination of further annotation establishing gene homologies with known genomes like *D. melanogaster* improved the functional enrichment of deregulated *D. magna* gene sequences, and hence it was possible to obtain reliable gene pathways using GO and KEGG. Furthermore, the use of multivariate methods like PLS and SR to identify deregulated genes related with observed phenotypic effects in chronic exposures performed with adult females identified 111 annotated genes related to effects observed in reproduction. More standard clustering methods like SOM only allowed to identify 17 deregulated genes in adult females. PLS methods are already being used to relate gene transcription patterns with toxicological effects.[34] The combination of PLS with SR, however, offers the possibility to select deregulated genes highly correlated with phenotypic responses (see Table S4, Supporting Information).

Juvenile SOM clusters included several differentially expressed genes involved in developmental processes after SSRI exposure, which can be linked to the reported increased juvenile developmental rates.[5] The SSRI exposures also deregulated ribosomal structural constituents, which could be linked to protein biosynthesis or cell proliferation. Dom et al.[34] reported the up-regulation of ribosomal constituents related genes in *D. magna* exposed to polar narcotics as an intent to overcome toxicant stress and/or to restore the damage elicited by exposure to those narcotics. Neuronal pathways, including synaptic vesicle transport, establishment and maintenance of localization, and response to stimuli were GO functional terms down-regulated in both juvenile and adults. The down regulation of neuronal pathways may be associated to an adaptive gene response to the presence of neuronal active compounds, as a similar response has been observed for the neurotoxic insecticide methomyl.[35]

Three genes involved in the ecdysone regulatory pathway that controls morphogenesis and integrin expression during *Drosophila* metamorphosis[36] appeared specifically down-regulated by 4-nonylphenol treatment: papss (3′-phosphoadenosine 5′-phosphosulfate synthetase), got1 (glutamate oxaloacetate transaminase), and btk29A (Bruton's tyrosine kinase). This is consistent with the hypothesis that 4-nonylphenol can affect *D. magna* by disrupting ecdysone metabolism.[13] However, the

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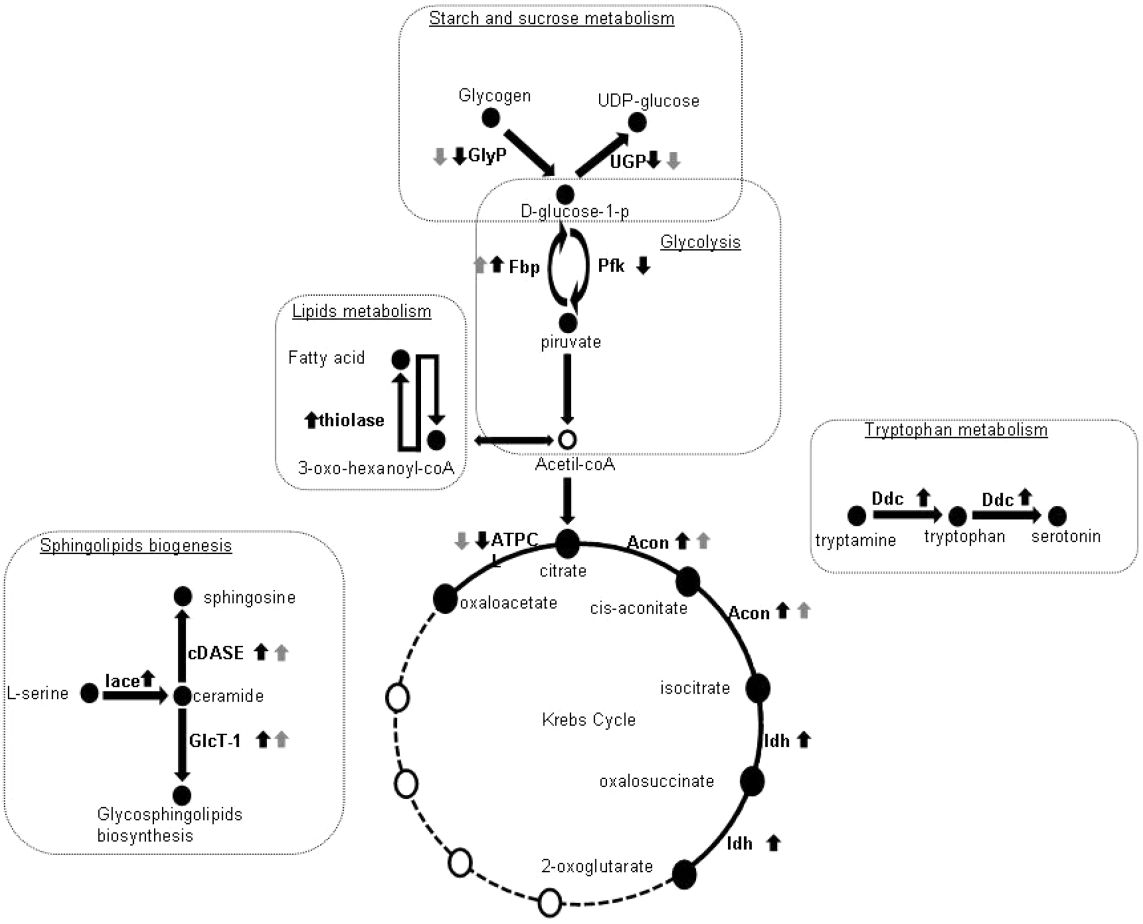

Figure 3. KEGG diagram of metabolic pathways with the genes up and down regulated across SSRIs (in bold) and 4-nonylphenol (in gray) treatments in adult females. Arrows indicated deregulated direction relative to control treatments. Further details of genes are in Table S7, Supporting Information.

number of affected genes related to this pathway was too low to be considered significantly enriched by the AmiGO! algorithm. Further research on ecdysone metabolism-related genes in *Daphnia* is thus required to prove or to refute this hypothesis.

Common pathways deregulated by both SSRIs and 4-nonylphenol involved enzymes that regulate morphogenesis and tissue differentiation probably involved in molting and reproduction, which are essential processes for growth and energy supply (e.g., cuticle formation, ribosomes, catabolism, transport and binding of essential macromolecules). Similar deregulated pathways were found to be altered in *D. magna* individuals exposed to narcotic chemicals, pesticides, ammunitions constituents, silver nanoparticles, metals, and temperature changes.[18,19,21,34,35,37−42] The simplest explanation to this finding of similar responses to disparate substances is that these pathways are related to general stress responses and not to the observed specific hormetic responses on juvenile developmental rates and female reproduction.

Deregulated metabolic pathways in *D. magna* adults chronically exposed to the studied chemicals were further analyzed using KEGG to identify altered metabolic pathways linked to reported effects on energy and carbohydrate metabolism. Both SSRIs and 4-nonylphenol deregulated similarly key genes such as glyp (glycogen phosphorylase) and ugp (UTP: glucose-1-phosphate), both involved in starch and sucrose metabolism, mainly in the transformation of glycogen to glucose.[43] Although both chemical families up-regulated fbp (fructose-1,6-bisphosphatase), only SSRIs down-regulated pfk (phospho-fructo kinase), which is involved in control of glucose homeostasis by allowing to switch from glycolysis to gluconeogenesis[44] and is a key gene in the regulation from aerobic to anaerobic metabolism. In a previous study we found that exposure to SSRIs decreases carbohydrate levels in adult *D. magna* females;[12] thus down-regulation of pfk may be an adaptive response to maintain minimum levels of glucose and may be linked to reported effects of SSRIs on oxygen consumption rates.[12] There are also evidence that serotonin, whose regulation is the pharmacological target of SSRIs, may be involved in the regulation of pfk. In human cells, serotonin increases pfk by binding to the 5-HT(2A) receptor, causing the tyrosine residue of pfk to be phosphorylated via phospholipase C. Because pfk regulates glycolytic flux, serotonin plays a regulatory role in glycolysis.[45]

SSRIs and 4-nonylphenol treatments deregulated acon (aconitase) and ATPCl (ATP citrate lyase), respectively. Additionally, SSRIs specifically up-regulated idh (isocitrate dehydrogenase). Thus SSRIs and 4-nonylphenol deregulated genes involved in 6 and 4 out of 10 reactions of the Krebs cycle, respectively. These effects could potentially affect oxygen consumption rates and the consumption of carbohydrates to produce energy. SSRIs indeed increased oxygen consumption rates and decreased levels of carbohydrates in *D. magna*.[12] 4-Nonylphenol is known to increase mRNA levels of *D. magna*

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hemoglobin,[46] which may indicate also increasing rates of oxygen consumption.

The beta-oxidation pathway was affected exclusively by SSRIs up-regulating thiolase, which is a key control gene of lipid metabolism and biosynthesis.[47] 4-Nonylphenol and SSRIs up-regulated two and three genes, respectively, from the sphingolipid biogenesis pathway (cDASE, Glct-1, lace) that are involved in the biogenesis of ceramide, glycosphingolipids, and sphingosines. Recently, sphingolipid metabolites, such as ceramide and sphingosine-1-phosphate, have been shown to be important mediators in the signaling cascades involved in apoptosis, proliferation, stress responses, necrosis, inflammation, autophagy, senescence, and differentiation.[48]

SSRIs up-regulated specifically dopa decarboxylase (Ddc) that catalyzes the conversion of 5-hydroxytryptophan to serotonin in response to several endogenous or exogenous signals and has already been shown to be involved in insect cuticle maturation, neuronal regulation, pigmentation patterning, and innate immunity.[49] Up-regulation of Ddc in rats exposed to SSRIs has been suggested as a response to the blocking of the reuptake mechanisms, which leads to low levels of serotonin inside the terminal neuron axon.[50]

The expression pattern of key genes from significantly enriched pathways such as lipids metabolism (thiolase), Krebs cycle (Acon, idh, and ATPCL), tryptophan metabolism (Ddc), and nucleotide metabolism (Faa) was validated by qPCR, confirming the microarray results.

In summary SSRIs and 4-nonylphenol deregulated different gene pathways in *D. magna* that in most cases could be related to reported phenotypic effects. SSRIs deregulated more genes than 4-nonylphenol specially in juvenile stages, which supports the hypothesis that these compounds act as neuronal disruptors in *Daphnia*.[12] SSRIs specifically deregulated one of their targeted pathways, serotonin metabolism, and key genes from the carbohydrate and Krebs cycle metabolism. 4-Nonylphenol specifically targeted genes related to ecdysone, carbohydrate, and Krebs cycle in adults. Taken together, the observed changes in the energy-related pathways are consistent with a decrease on reserve carbohydrates and an increase on respiratory metabolism, in line with our previous observations on the effects of SSRIs in *D. magna*.[12]

■ ASSOCIATED CONTENT

Supporting Information

Additional method details and supplementary tables and figures, including primer sequences for qPCR (Table S1), differentially expressed genes (Table S2), genes included in SOM clusters (Table S3), PLS-SR selected genes (Table S4), full GO terms table with gene symbol (Table S5), KEGG annotated geasnes with fold induction values (Table S6), total offspring production responses (Figure S1), heat map of differentially expressed genes of juveniles and adults with respective hierarchical clustering (Figure S2, S3), and SOM graphs results (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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