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Molecular impact of propiconazole on *Daphnia magna* using a reproduction-related cDNA array

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Abstract

We have developed a first version cDNA microarray of the cladoceran *Daphnia magna*. Through Suppression Subtractive Hybridisation PCR (SSH–PCR) 855 life stage-specific cDNAs were collected and used to document the toxicological mode of action of the pesticide propiconazole. DNA sequencing analysis revealed gene fragments related to important functional classes such as embryo development, energy metabolism, molting and cell cycle. Major changes in transcription were observed in organisms exposed for 4 and 8 days to 1 μg/mL. After 4 days a 3-fold down-regulation of the gene encoding the yolk protein, vitellogenin, was observed indicating impaired oocyte maturation. Moreover, genes such as a larval-specific gene and chaperonin were repressed, whereas the heat shock 90 protein and ATP synthase were induced. Organismal effects clearly confirmed the major molecular findings: at the highest concentration (1 μg/mL) adult growth was significantly ($p < 0.05$) impaired and increased developmental effects in the offspring could be noted. We have demonstrated the potential of microarray analysis in toxicity screening with *D. magna*. The use of vitellogenin mRNA as a rapid biomarker of reproductive effects in chronic toxicity studies with cladocerans is suggested.

Keywords: Crustacean; *Daphnia magna*; Embryo toxicity; Gene expression; Microarray; Propiconazole; Suppression subtractive hybridization; Toxicogenomics

1. Introduction

Thanks to the rapid progress in the development of molecular biological techniques, tools are provided that may assist our understanding of how chemicals can affect human and ecosystem health. Through gene expression analysis after toxicant exposure, molecular mechanisms underlying toxic responses to environmental contaminants can be unraveled. For this differential gene expression analysis various strategies can be distinguished, such as DNA arrays and Subtractive Hybridization methods (Moens et al., 2003; Larkin et al., 2003). DNA arrays consist of nitrocellulose membranes (macroarrays) or microscopic glass slides (microarrays) containing small quantities of oligonucleotides or cDNAs and are used increasingly to screen for differential expression of gene transcripts. The main advantage of DNA arrays is the high number of genes that can be studied in a single analysis. Subtractive Hybridization methods such as Suppression Subtractive Hybridization (SSH) and Differential Display, on the other hand, can be used for isolation and identification of unknown genes. These genes can then be spotted on an array for high-throughput identification and quantification of differentially expressed genes (Schena et al., 1995; Moens et al., 2003; Snell et al., 2003; Snape et al., 2004). The use of subtracted hybridization methods and DNA arrays is widely regarded as a revolutionary technology in cancer research, developmental biology and endocrinology (Bertucci et al., 1999; Clarke et al., 2001; Livesey, 2002; Inoue et al., 2002; Linney et al., 2004). While these technologies are relatively new for aquatic toxicology, several research groups have recently published articles using these methods to study environmental contaminants in fish (Larkin et al., 2002; Williams et al., 2003; Koskinen et al., 2004; Van der Ven et al., 2005).

The combination of these methodologies enables environmental toxicologists to monitor gene expression changes, resulting from toxicant exposure in animal species whose

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genomes are still poorly characterized for example for ecotoxicological standard test organisms like *Daphnia magna*. Moreover, the standardized methods used to assess the potential impact of chemicals on *D. magna* rely solely on measuring whole-organism responses (e.g. mortality, growth and reproduction) while no insight in the mechanisms of toxicity is provided. Custom-made arrays may prove to be valuable tools in identifying mode of toxic action (Gracey and Cossins, 2003; Snape et al., 2004).

Daphnids are very suitable to perform aquatic toxicity tests because of their high sensitivity to a broad range of chemicals, short generation time and ease of manipulation. Moreover, the widely distributed daphnids play a central role in the aquatic food chain. They are often the predominant primary consumers, responsible for the transfer of energy from plants to animals within ecosystems (Dodson and Hanazato, 1995). Chemical stress reduces the amount of energy that is allocated to growth and reproduction (De Coen and Janssen, 2003). This reduction of their cyclic parthenogenetic reproduction not only affects their population density but can also adversely impact energy allocation within an ecosystem with possible effects on the whole (eco)system. On the other hand, reduced offspring for example, can also be caused by direct chemical interaction, inducing abnormalities in the embryo. This makes it necessary to study developmental effects in daphnids in toxicology experiments.

The daphnid embryonic development can be divided into six different stages (Kast-Hutcheson et al., 2001). Aberrations in this developmental pattern arise upon exposure to concentrations lower than the lethal thresholds for neonatal organisms (Shurin and Dodson, 1997; Kast-Hutcheson et al., 2001). The main characterized abnormalities include dead embryos at various stages of development, embryos with little sign of embryonic development and neonates with poorly developed second antennae, poorly extended shell spine or carapace deformities. For example, the alkylphenol degradation product 4-nonylphenol caused the arrest of the embryonic development resulting in dead embryos and showed deformities of neonates shell spine and second antennae (LeBlanc et al., 2000; Zhang et al., 2003). A second example is ethylenethiourea, a teratogenic compound for mammals. This carbamate insecticide degradation product caused embryo lethality in the final developmental stages and carapace malformations (Otha et al., 1998).

One of the best characterized chemicals which interferes with the embryonic development of *D. magna* is the azole fungicide propiconazole. Azole pesticides are used in a variety of agricultural applications. They inhibit the ergosterol biosynthesis, which is critical for the formation of cell walls of fungi, leading to growth arrest of the fungus (Zarn et al., 2003). Furthermore this fungicide interferes with the embryonic development of *D. magna* resulting in development abnormalities and embryonic death. More specifically, it causes underdeveloped second antennae and no extensions of shell spines in neonates (Kast-Hutcheson et al., 2001).

The first objective of this study was to isolate adult- and juvenile-related genes of the crustacean *D. magna* using Suppression Subtractive Hybridization–PCR. This method enables us to identify genes that are differentially expressed between two (mRNA) populations. cDNA from adults containing eggs in the brood pouch (reproductively active) was subtracted from juveniles (not reproductively active), which resulted in differentially expressed, and thus life stage-specific and reproduction-related genes. These genes were spotted on a microarray for further evaluation and characterization of environmental toxicants. For this purpose, exposure experiments were conducted with propiconazole to study the effect of this fungicide on the expression of the isolated genes, leading to a better understanding on the mode of action of this chemical and to evaluate the usefulness of the developed microarray.

2. Materials and methods

2.1. D. magna culturing

D. magna cultures were held in 1-L glass beakers containing aerated and bio-filtered tap water. The temperature was maintained at 20±1 °C and a 14-h light/10-h dark photoperiod was used. Three times weekly, the culture medium was renewed and the daphnids were fed a mixture of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 ratio (4×10^5 cells/mL). These conditions maintained the daphnids in the parthenogenetic reproductive stage.

2.2. Development of a life stage-specific cDNA library

Fifteen adults carrying eggs and 75 juveniles were collected and pooled. Total RNA was extracted using the Totally RNA™ Kit (Ambion, USA) according to the manufacturer's protocol. After DNase treatment (Sambrook et al., 1989), RNA concentration was determined by spectrophotometry (~50 μg) and the quality was checked by a denaturing formaldehyde–agarose gel. For the construction of the subtracted library, by means of Suppression Subtractive Hybridization, the PCR Select cDNA Subtraction Kit (Clontech, USA) in combination with the Smart PCR cDNA Synthesis Kit (Clontech) was used (Moens et al., 2003). The subtraction was performed using pooled RNA from adults carrying eggs and juveniles.

2.3. Cloning and sequencing

The adult and juvenile gene pools, enriched for differentially expressed genes, were ligated in a TA-vector system using the pGEM-T easy vector (Promega, USA). *Escherichia coli* competent cells (JM109, Promega) were transformed through heat shock. Adult-specific and juvenile-specific recombinant clones were grown in 96-well plates. Lysates, replicas and glycerols were made. Clones were amplified with vector-specific primers (M13 forward and M13 reverse) and purified by an exosap reaction (Werle et al., 1994) based on exonuclease I and shrimp alkaline phosphatase (Fermentas, USA). The PCR products were sequenced using the Ceq™ Dye terminator cycle sequencing kit (Beckman Coulter, USA) and a CEQ™8000 Genetic analysis system (Beckman Coulter).

After sequencing, the cDNA fragments were identified based on their similarity to sequences in the National Center for Biotechnology Information (NCBI) database as determined by the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The sequences were compared with DNA and protein databases using BLASTN and BLASTX analysis software respectively. All unique sequences were published on the NCBI website (accession numbers: DV075760 to DV07850).

2.4. Microarray

In preparation for spotting, cDNA-inserts of the clones were amplified using adaptor-specific primers (nested primers, Clontech), purified by Montage PCR Plate (Millipore, USA) and loaded into 384-well plates (Genetix, UK) in 50% dimethylsulfoxide at ~75 ng/μL. A Qarray Mini Robot (Genetix) was used to spot the cDNA library in triplicate onto aminosilane-coated glass slides (Generoma microarray slides; Asper Biotech, Estonia). In addition, a set of artificial control genes (Lucidea Universal Scorecard, Amersham Biosciences, UK), containing calibration controls, ratio controls and negative control genes, was spotted over the arrays in 20 replicates. These spiked-in labeled controls at known concentrations provide information on cDNA labeling efficiency, sensitivity and intra-array variability of replicates. Negative controls–cDNAs with no provided mRNA spike–were included to assess non-specific hybridization and quality of blocking at the prehybridization step. After rehydration and drying, the slides were cross-linked by UV-radiation at 300 mJ (UV Stratalinker 2400; Stratagene, USA).

2.5. Exposure experiment

In an initial experiment, the acute toxicity of propiconazole (CAS 60207901; Dr. Ehrenstorfer, USA) to *D. magna* was assessed to ensure proper concentration ranges were selected in the 8 days of exposure. Organisms were exposed during 48 h to 5 concentrations (0.32, 1, 3.2, 10, and 32 μg/mL). This test was performed according to the OECD-guidelines (no. 202, Part I). Propiconazole was dissolved in acetone and added to OECD-water to reach the appropriate concentration. The concentration of acetone (0.001%) was the same in all treatment and control solutions. Groups of 10 neonates were exposed in a total volume of 20 mL water. The experiment was conducted in quadruplicate and a temperature of 20±1 °C and a 14-h light/10-h dark cycle was maintained. The 48-h LC_{50} value, calculated using the Trimmed Spearman–Karber software, was 8.5 μg/mL (95% CI: 7.03 μg/mL and 10.19 μg/mL). This value is comparable with the 48-h LC_{50} found in the literature (Kast-Hutcheson et al., 2001) (48 h LC_{50}=5 μg/mL).

In a second experiment, the exposure conditions were chosen based on the OECD-guidelines (no. 202, Part II) to determine the effect of propiconazole on gene expression, length and embryo abnormalities. During the experiment, the temperature was maintained at 20±1 °C and a 14-h light/10-h dark photoperiod was used. Thrice a week, the toxicant concentrations were renewed and the organisms were fed a mixture of *P. subcapitata* and *C. reinhardtii* in a 3:1 ratio (4×10^5 cells/mL). All test concentrations were confirmed by HPLC analysis. Aliquots of 100 μL of sample were loaded on an Omnispher C18 column at a flow rate of 0.5 mL/min. The mobile phase was acetonitrile/H_2O, starting at 67% acetonitrile. All measurements were done according to the instructions of Dr. Ehrenstorfer (USA). For each of the 3 toxicant concentrations (0.25, 0.5 and 1 μg/mL; 0.001% acetone) and the control, a total of 75 neonates were divided over three 250-mL replicate flasks. After 4 days of exposure, all daphnids were collected. For each flask, 7 daphnids were put on sucrose–formol for length measurements and 18 daphnids were put in RNALater (Ambion) and shock-frozen in liquid nitrogen for gene expression analysis. Using the same setup, daphnids were also collected after 8 days. Moreover, embryos were collected and further exposed in multiwell plates. After 2 days, neonates were collected and put on sucrose–formol for detection of embryo abnormalities.

2.6. Hybridizations

Fluorescently labeled cDNA, from daphnids exposed during 4 and 8 days to 0, 0.25, 0.5 and 1 μg/mL propiconazole, was synthesized for microarray analysis. Total RNA was isolated, using the Totally RNA™ Kit (Ambion) and a DNase treatment was performed. As we are interested to detect the major molecular effects of propiconazole on *D. magna*, we used RNA from pooled organisms. The assessment of the intra-individual variation among the daphnids is beyond the scope of the present study. Seven micrograms total RNA, spiked with the mRNA mix from the Lucidea Universal Scorecard (Amersham Biosciences), was converted into single-stranded cDNA with incorporated aminoallyl-dUTPs (Sigma, USA) through a random primer (Invitrogen SA, Belgium) extension with superscript II reverse transcriptase (Invitrogen). After purification with the QIAquick PCR purification kit (Qiagen Inc., USA), the aminoallyl-labeled cDNA was covalently coupled with Cy Dye esters (Amersham Biosciences). Aminoallyl cDNA from exposed and control daphnids were coupled, respectively, with Cy5 and Cy3. The uncoupled Cy3/Cy5 was removed by the QIAquick PCR purification kit (Qiagen) and labeling efficiency was determined by spectrophotometry. Microarrays were incubated in a coplin jar with prehybridization solution (50% formamide, 5× SSC, 0.1% SDS, 0.1 mg/mL BSA) at 42 °C for 60 min. After washing with deionized water and isopropanol, the arrays were immediately dried with compressed N_2. Vacuum-dried probes were resolved in hybridization solution (50% formamide, 5× SSC, 0.1% SDS, 0.1 mg/mL BSA, 0.1 mg/mL salmon sperm) and incubated at 95 °C for 5 min. Then, cDNA containing 150 pmol of each incorporated dye was mixed and applied on the array slides. The cover-slipped slides (Menzel Glaser, Germany) were placed in a hybridization chamber (Genetix) and hybridized at 42 °C overnight. Thereafter, slides were washed with the following wash buffers: 2× SSC and 0.1% SDS (at 42 °C for 1 and 5 min), 0.1× SSC and 0.1% SDS (room

temperature two times for 10 min), 0.1× SSC (room temperature for 15 s, twice for 2 min and 1 min) and 0.01× SSC (room temperature for 15 s). Finally slides were rinsed with deionized water and isopropanol and dried with compressed N_2.

2.7. Scanning and data analysis

Microarrays were scanned at 532 and 635 nm using the Genepix Personal 4100A Scanner (Axon Instruments, USA). For each glass slide the photomultiplier tube voltage (PMT) was adjusted to obtain a ratio (Cy5/Cy3) close to one and no oversaturation in the set of artificial control spots (Lucidea Universal ScoreCard, Amersham Biosciences). The images were analyzed by means of the Genepix pro Software (Axon Instruments) for spot identification and for quantification of the fluorescent signal intensities. Subsequently data were put into a MIAME-compliant database. The fluorescent signal intensity for each DNA spot (average of intensity of each pixel present within the spot) was calculated using local background subtraction. Low intensity spots (mean foreground < mean background + 2× S.D.) and saturated signals were filtered out. The ratio (Cy5/Cy3) was calculated for each spot, transformed into a logarithmic value ($\log_2$), and normalized using Locally Weighed Scatterplot Smoothing (Lowess) (Yang et al., 2002). By this method, intensity-dependent normalization was applied where the ratio was reduced to the residual of the Lowess fit of the intensity vs. ratio curve. Using expression data of multiple self–self-hybridization experiments (split control experiments), where RNA from a single pool was labeled with both dyes and hybridized to the same arrays, the average standard deviation for all genes on the array was calculated. Based on the average standard deviation we determined the expected variance of the genes on the array leading to a predicted confidence interval for testing the significance of the ratios obtained. Ratios outside the 99.7% confidence interval were determined to be significantly different following the treatment. All differentially expressed genes were sequenced and identified.

2.8. Real-time PCR

Real-time PCR was carried out on a Roche Molecular Biochemicals LightCycler 3.5 with the LightCycler FastStart DNA Master^{PLUS}SYBR Green I Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Reverse transcription was performed using the Superscript First-Strand Synthesis System for RT–PCR (Invitrogen). Primers were designed using Roche Molecular Biochemicals Light-Cycler Probe Design Software 1.0. Relative quantification was calculated using glyceraldehyde-3-phophate dehydrogenase for normalization. This gene was chosen because of its apparent lack of differential expression as demonstrated by microarray analysis (1.06-fold induction). The primer sequences used for amplification of the target transcripts are: vitellogenin: forward primer 5′–AGCGAATCCTACACCG–3′, reverse primer 5′–CGACGAAGCTCAGCAA–3′; glyceraldehyde-3-phophate dehydrogenase: forward primer 5′–GGATTCGGTCGTATTGGC–3′, reverse primer 5′–TCAGCTCCAGCAGTTC–3′. The relative expression of the target transcript sequences was calculated as reported by Pfaffl et al. (2002) including efficiency corrections for each transcript. The formation of a specific PCR product was checked by melting curve analysis.

2.9. Organismal effects

Embryos, removed from the brood chamber from 8 days exposed gravid daphnids, were further exposed for 2 days in 96-well plates and examined microscopically for embryo abnormalities during the various stages of their development. Exposure experiments on embryo development were evaluated statistically using a Chi-square test (Excel software, Microsoft). The carapace length of the adult organisms (after 4 and 8 days of exposure), from the top of the daphnids head to the base of its spine, was determined using a microprojector (Projectina). Analyses were done with the software package Statistica (Stat Soft Inc.). Data were checked for normality and homogeneity of variance and the effect of treatment was tested for significance using two-way ANOVA (Newman–Keuls test).

3. Results

3.1. Development of a life stage-specific cDNA library

By performing the Suppression Subtractive Hybridization using pooled RNA from adults carrying eggs and juveniles, genes that are differentially expressed in both populations were enriched in the final cDNA library. A total of 609 and 246 specific clones, respectively, were picked for the adults (i.e. carrying eggs) and for the juveniles. From these clones, a custom microarray was developed. Preliminarily, 90 inserts of randomly selected clones were sequenced and subjected to blast homology search (BLASTX as well as BLASTN) resulting in 56 sequences with similarity to sequences in the public databases. This group could be reduced to 34 unique sequences, as some of the clones were found several times i.e.

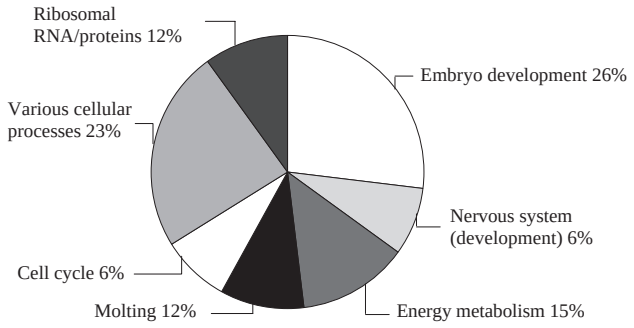

Fig. 1. The distribution of the SSH isolated transcripts based on known functions. A number of isolated gene fragments were randomly selected for sequencing. Only the unique gene fragments with 'known' functions were classified into various functional groups. The genes with unknown functions or the sequences that did not have any homology to known sequences in public databases are not shown.

duplicates, overlapping regions or different fragments of the same gene. The remaining 34 sequences showed no homology with sequences in the database; 22 of these sequences were non-redundant. The 34 different sequences could be classified in functional categories such as embryo development, nervous system (development), energy metabolism, molting, cell cycle, various cellular processes and ribosomal RNA/proteins (Fig. 1). All different sequences are listed in Table 1. Fifty-six percent of the similarities were found with the group of invertebrate species.

3.2. Gene expression profiles

Fluorescently labeled cDNA originating from daphnids exposed to propiconazole were hybridized on microarrays containing all SSH gene fragments and compared to the control population. The ratios of 855 clones were acquired from triplicate experiments and the median of all values at each concentration was defined as the expression levels (fold change vs. control) at a given time interval. Our numerical assessment of the quality of the microarrays focused on the spiked-in

Table 1
Blast homology search results of gene sequences isolated by SSH

Accession clone	Gene most similar to	Species	Accession	Size	*E*	(%)
Embryo development						
DV075792	Larval-specific gene	*Balanus amphitrite*[a]	BAA99543	300	5e−4	63
DV075833	Filamin	*Drosophila melanogaster*[a]	AAF04108	388	1e−9	65
AB114859	Vitellogenin	*Daphnia magna*[a]	AB114859	360	0e0[b]	98[c]
DV075771	Cyclin B	*Asterina pectinifera*[a]	AAL05452	479	4e−34	75
DV075814	Cyclin B3	*Xenopus laevis*	CAC24491	623	4e−64	75
DV075816	Chromobox 1	*Homo sapiens*	AAH02609	593	2e−34	58
DV075778	Histone deacetylase-2	*Gallus gallus*	NP_990162	517	5e−75	88
DV075800	Homeobox gene (Xvent-1)	*Xenopus laevis*	CAA63437	303	2e−2	54
DV075803	BicaudalD protein	*Drosophila melanogaster*[a]	CAA63437	804	2e−44	67
Nervous system (development)						
DV075767	Nucleosome assembly protein 1	*Danio rerio*	NP_958475	570	2e−36	63
DV075794	Neuronal survival protein	*Gallus gallus*	XP_419467	641	9e−4	64
Energy metabolism						
DV075780	Fatty acyl-CoA-reductase 1	*Homo sapiens*	NP_115604	589	3e−15	76
DV075850	ADP/ATP translocase	*Anopheles gambiae*[a]	AAO32818	369	3e−50	90
DV075765	Amino transferase 2	*Danio rerio*	NP_001002676	608	4e−52	75
DV075810	GAPDH	*Daphnia pulex*[a]	CAB94909	535	1e−35	97
DV075770	NADH ubiquinone oxidoreductase	*Bos taurus*	CAA32523	250	1e−23	72
Molting						
DV075764	Cuticle protein	*Bombyx mori*[a]	BAC16225	216	1e−6	60
DV075837	Cuticle protein	*Drosophila melanogaster*[a]	AAB88066	225	3e−5	78
DV075835	Cyp 306a1 like	*Bombyx mori*[a]	AB162964	437	2e−15[b]	96[c]
DV075783	Cuticle glycoprotein (peroxidase)	*Brugia pahangi*[a]	CAA48882	320	3e−3	56
Cell cycle						
DV075776	DNA helicase (xnp/atr-x)	*Drosophila melanogaster*[a]	AAG40586	446	1e−11	76
DV075782	Pre-mRNA processing factor 39	*Danio rerio*	NP_001004520	407	5e−5	70
Various cellular processes						
DV075795	β tubulin	*Homo sapiens*	AAN87335	594	1e−108	98
DV075790	Death associated protein 1	*Apis mellifera*[a]	XP_392446	334	3e−10	56
DV075832	Protein kinase	*Homo sapiens*	NP_057235	575	1e−42	87
DV075768	Syndecan binding protein	*Danio rerio*	AAQ97846	446	3e−10	52
DV075769	Peroxinectin	*Penaeus monodon*[a]	AAL05973	311	8e−19	60
DV075796	Golgi snare protein	*Xenopus laevis*	AAH45246	419	4e−34	71
DV075791	Lysosomal associated protein	*Drosophila melanogaster*[a]	AAK69597	603	4e−16	48
DV075811	Proteasome subunit α type 7	*Carassius auratus*	Q9PTW9	176	1e−17	81
Ribosomal RNA/proteins						
DV075834	Ribosomal RNA 16S	*Daphnia lumholtzi*[a]	AF064189	577	1e−172[b]	91[c]
DV075777	Ribosomal protein L17A	*Drosophila melanogaster*[a]	JC1253	331	3e−49	99
DV075820	Ribosomal protein L35A	*Apis mellifera*[a]	XP_393102	476	1e−27	82
DV075785	Ribosomal protein S8	*Apis mellifera*[a]	NP_001011604	482	8e−63	83

From all clones that were obtained, only a fraction was sequenced. These genes are listed above.
[a] Invertebrates.
[b] *E*−values were obtained by BLASTN search. The others by BLASTX search.
[c] Percent identities. The others are percent positives with fraction of residues that are identical or similar.

Lucidea genes. The spiked-in labeled Lucidea controls at known concentrations provide information on cDNA labeling efficiency, sensitivity and intra-array variability of replicates. Fig. 2 shows data from a representative array with excellent spikes. No significant overlap between the intensities of the different calibration genes is observed, except at the low copy numbers. This implies that the arrays can be used reliably to measure expression levels across a dynamic range spanning several orders of magnitude and that the coefficient of variation is sufficiently small. No significant location effects were observed. The background fluorescence in both channels was low and homogeneously spread across the microarrays as assessed by using signal to noise ratio plots. Overall, we conclude that the microarray measurements took place in good conditions. Negative controls were included to assess quality of blocking at the prehybridization step and nonspecific hybridization. No detectable hybridization signal was observed for any of the negative controls. Based on self–self-hybridizations, run at several occasions, the average standard deviation and a 99.7% confidence interval could be calculated. We chose three standard deviations from the mean as a conservative threshold, therefore ratios outside the 99.7% confidence interval were deemed to be significantly different following treatment. Based on these values, the cutoff thresholds of the median $\log_2(R/G)$

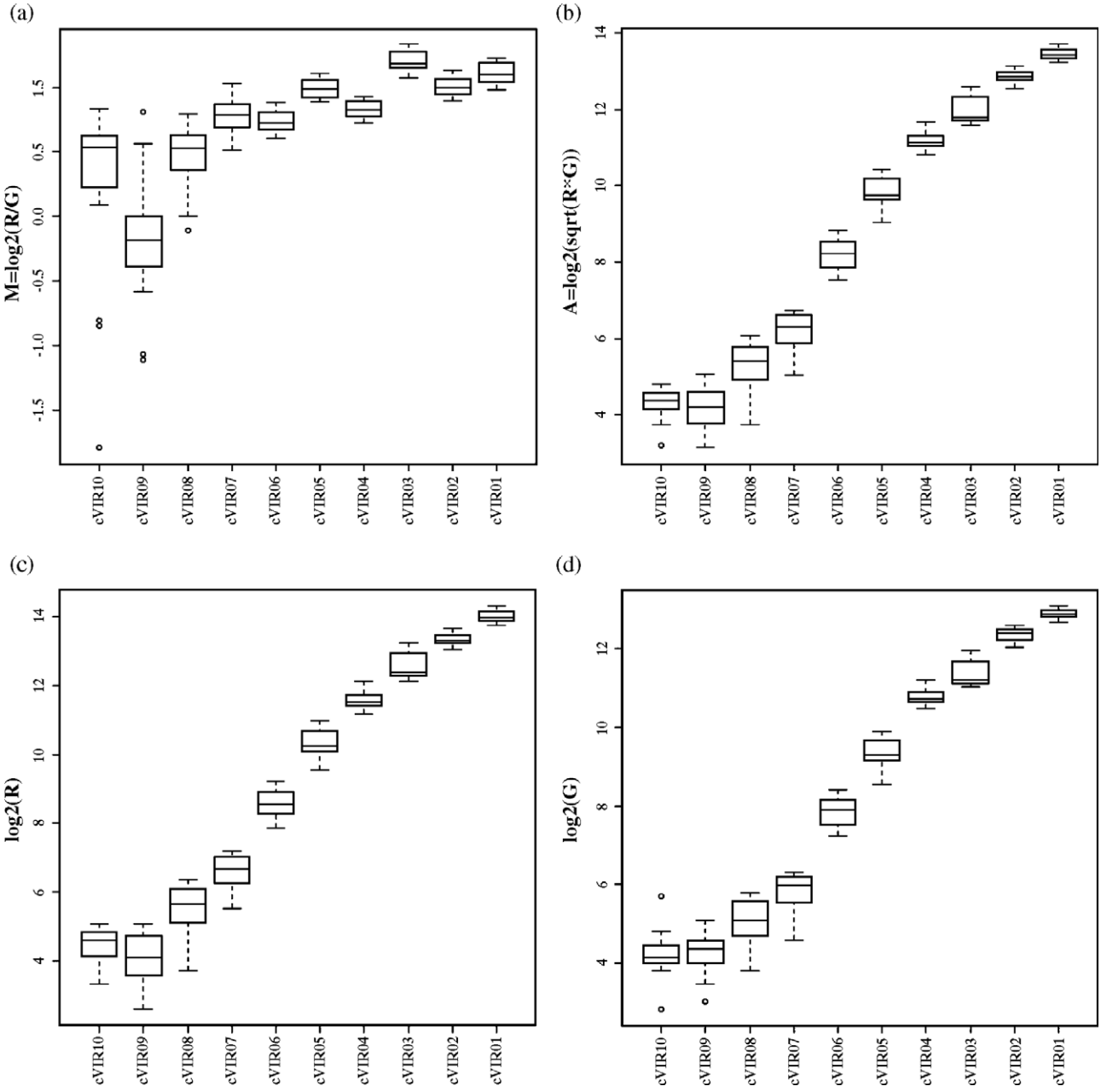

Fig. 2. Statistics of the (a) M value ($\log_2$(red/green)), (b) A value ($\log_2$(square-root(red * green))), (c) intensities in the red channel, and (d) intensities in the green channel of the 10 calibration genes, each measured in 20 replicas for one representative array. Note the satisfying separation between the groups in the range (cYIR7–cYIR2, or 30 to 10,000 copies). The boxplots represent the median, the lower (25th percentile) and the upper hinges (75th percentile) and the lower and upper whiskers.

for up-regulation and down-regulation were determined to be 0.8 and −0.8, respectively.

In total, for both experiments (4 days and 8 days), 104 of the 855 gene fragments were differentially expressed after propiconazole exposure. Identity information of some of these gene fragments was already known while for the others sequencing and blast homology search was performed. In total, from the 104 differentially expressed gene fragments, 43 unique gene fragments were found including 19 sequences with similarity to sequences in the databases and 24 unknown sequences. The newly identified genes are listed in Table 2.

Gene expression profiles of the three concentrations (1, 0.5 and 0.25 μg/mL) and the two points in time (4 and 8 days) are shown in Fig. 3. The differential expressed gene fragments found were related to embryo development, molting, stress response/protein folding, energy metabolism, various cellular processes and transcription/translation. A numerical overview of all the distinct differentially expressed genes is listed in Table 3. In general, the expression profiles show more down-regulated than up-regulated genes (37 down- vs. 26 up-regulated genes). Comparison between the gene profiles of the 4-day and 8-day exposure shows that more genes are differentially expressed after 4 days (37 vs. 26 genes). After 4 days of exposure at the highest concentration, most differentially expressed genes occurred: 14 of the 19 differentially expressed genes appeared to be down-regulated.

3.3. Organismal effects

For each concentration, on average, 56 embryos were examined for developmental abnormalities. The observed abnormalities included neonates with underdeveloped second antennae, curved or unextended shell spines and dead embryos due to an arrest in egg development. The incidence of embryo abnormalities was significantly elevated at 1 μg/mL (Fig. 4).

Length measurements of the adult organisms revealed a significant impact at the highest propiconazole concentration (1 μg/mL) after 4 as well as 8 days of exposure (Fig. 5).

4. Discussion

4.1. Development of a life stage cDNA library

Using Suppression Subtractive Hybridization PCR, a cDNA library was developed for the crustacean *D. magna*. This technique was used to create, in a relatively fast way, a cDNA library for *D. magna* which was enriched with target genes. Differentially expressed genes from adults carrying eggs in the brood pouch (reproductively active) and juveniles (not reproductively active or in preparation for reproduction) were isolated in order to obtain life stage-specific genes. The obtained cDNA library contained multiple genes with a clear function related to reproduction. Previous studies already demonstrated the usefulness of the SSH method in combination with the microarray technology in the field of aquatic toxicology (Williams et al., 2003; Koskinen et al., 2004; Van der Ven et al., 2005). In the present study, this method has been applied for the first time to isolate genes of the crustacean *D. magna*. Microarray technology was used for the evaluation and characterization of the effects of environmental toxicants on these life stage-specific genes. For this purpose, the embryotoxicant propiconazole was used as a model compound. As the ultimate goal of ecotoxicology is to predict effects at higher levels of biological organization (population, community, ecosystem), we have generated a strategy to collect those genes that are relevant for reproductive processes. Knowledge of reproduction-related mechanisms of toxicity can provide us

Table 2
Blast homology search results of gene sequences that are differentially expressed upon propiconazole exposure (the ones that are not yet present in Table 1)

Accession clone	Gene most similar to	Species	Accession	Size	*E*	(%)
Stress response/protein folding						
DV075812	Chaperonin (hsp60 domain)	*Danio rerio*	AAM34670	623	4e−82	80
DV075823	Heat shock protein 90	*Chiromantes haematocheir*[a]	AAS19788	546	4e−47	60
Energy metabolism						
DV075825	ATP synthase	*Drosophila melanogaster*[a]	P23380	485	6e−34	92
Various cellular processes						
DV075804	Stromal cell derived fact.	*Branchiostoma belcheri*	AAT34988	431	7e−6	92
DV075819	Sorting nexin	*Rattus norvegicus*	NP_037478	404	4e−38	92
Transcription/translation						
DV075799	Ribonucleoprotein 87F	*Drosophila melanogaster*[a]	P48810	430	5e−3	38
DV075844	Sm like protein	*Homo sapiens*	NP_055277	411	2e−32	90
DV075798	Ribosomal protein L37	*Homo sapiens*	AAR38839	593	1e−12	48
DV075846	Translation elongation factor	*Drosophila melanogaster*[a]	T13689	594	1e−43	81
DV075839	Ribosomal RNA 5.8S	*Eubosmina longicornis*[a]	AY264773	519	3e−75[b]	97[c]
DV075845	Ribosomal protein L3	*Spodoptera frugiperda*[a]	AAL62468	661	2e−90	80
DV075849	Ribosomal RNA 18S	*Daphnia pulex*[a]	AF014011	208	3e−79[b]	97[c]

[a] Invertebrates.
[b] *E*-values were obtained by BLASTN search. The others by BLASTX search.
[c] Percent identities. The others are percent positives with fraction of residues that are identical or similar.

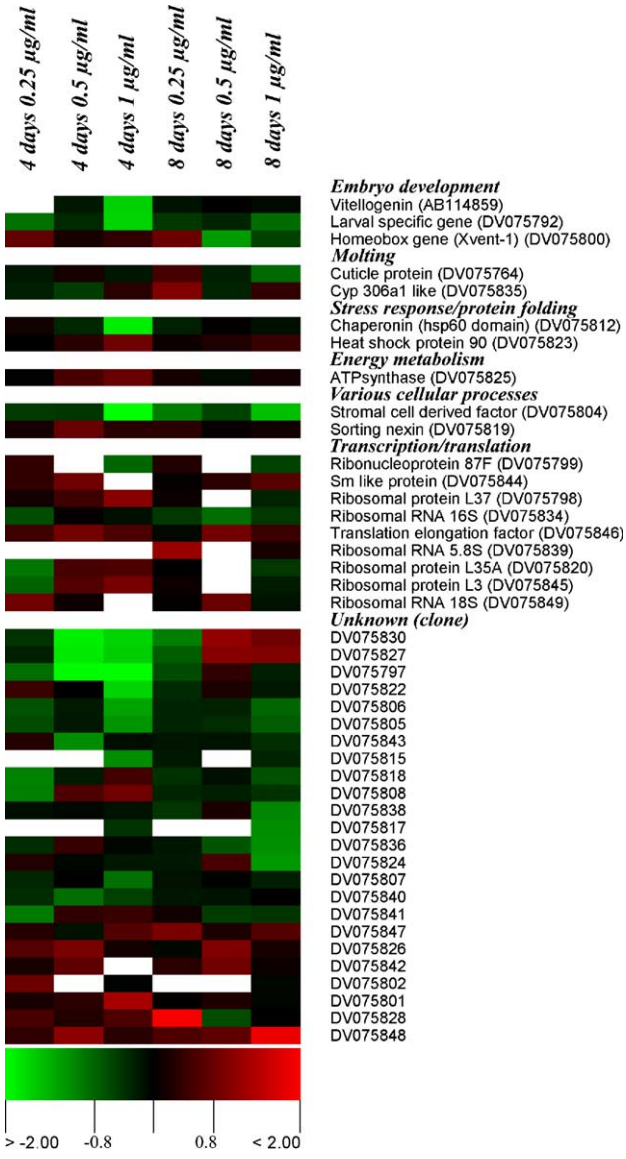

Fig. 3. Gene expression profiles after propiconazole exposure. Median $\log_2$ ratios were used to construct a heat map, presenting all the unique gene fragments with differential expression during at least one time point. The expression pattern of each gene fragment is displayed as a horizontal strip. The intensities of red and green colors are proportional to the relative gene induction and repression, respectively. Colorless boxes indicate missing data. Accession numbers of the *Daphnia magna* clones are present.

with early warning biomarkers predictive for effects at higher levels of organization. Through the development of this cDNA microarray, we have set a first step into ecotoxicogenomics applications for this species.

Table 3
Number of differentially expressed genes after propiconazole exposure

Concentration (µg/mL)	4 days				8 days				Total
	0.25	0.5	1	Subtotal	0.25	0.5	1	Subtotal	
Down-regulated	6	5	14	25	2	2	8	12	37
Up-regulated	2	5	5	12	5	6	3	14	26
Total	8	10	19	37	7	8	11	26	

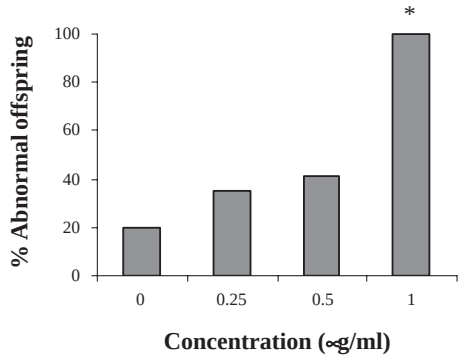

Fig. 4. Embryo toxicity of propiconazole. An asterisk denotes a significant difference from the control ($p < 0.05$, Chi-square).

Gene fragments with a putative role in embryo development occur at a high frequency in the library (26% of the genes showed similarity with the NCBI database). The yolk protein vitellogenin, essential as a feeding reserve for the developing embryo, was present in multiple copies in the library. Other genes could also be linked to embryo development. Histone deacetylase, for example, is a chromatin remodeling factor through deacetylation of histones resulting in condensed (inactive) chromatine (which is difficult to enter into the transcriptional machinery). In the sea urchin histone deacetylase is involved in the gastrulation process (Nemer, 1998). Also a chromobox protein with its typically chromodomain, a highly conserved sequence element, was isolated. This gene seems to be involved in transcriptional silencing by recognizing and binding to histones which is important in progression of

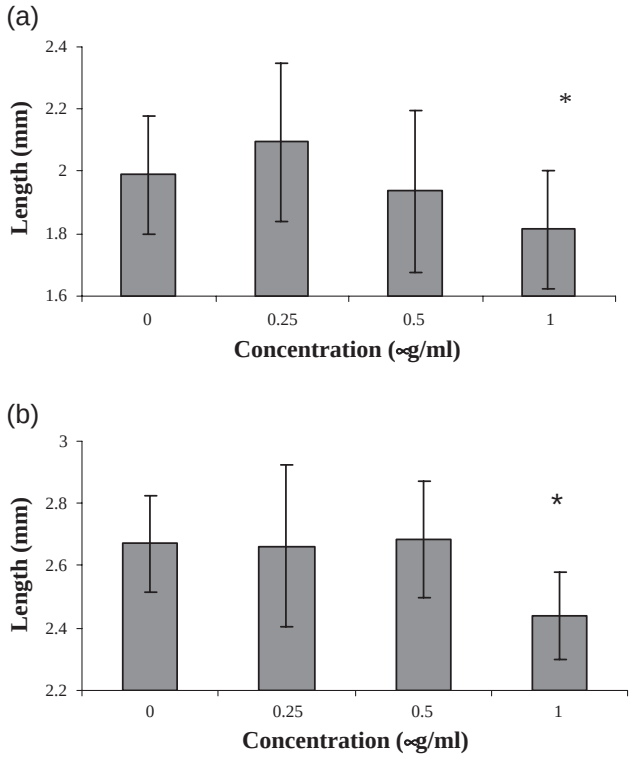

Fig. 5. Length measurements of daphnids after (a) 4 days and (b) 8 days exposure to propiconazole (mean ± S.D.). An asterisk denotes a significant difference from the control ($p < 0.05$, ANOVA).

developmental programmes (Yamaguchi et al., 1998; Lachner et al., 2001). Other genes in the library have a general role in cell cycle events but can also be linked to organismal development. For example cyclin B, with a crucial role in the regulation of the cell cycle, is required for final oocyte maturation (Hochegger et al., 2001; Gutierrez et al., 2003). The actin-binding protein filamin, with a pivotal role in reorganizing the actin cytoskelet (essential during cell division), is also involved during oogenesis in *Drosophila*, building ring canal structures for establishing cytoplasmic bridges between cells of the germline (Li et al., 1999). Further genes related to dorsoventral patterning of the mesoderm (homeobox gene Xvent-1), development asymmetry (bicaudalD protein) and a larval-specific gene were also isolated through SSH.

Another interesting category of genes consists of possible nervous system (development) related genes including a neuronal survival protein and a nucleosome assembly protein. The latter facilitates the packaging of DNA into nucleosomal structures leading to the inhibition of processes that require access to DNA, including transcription. This nucleosome assembly protein has, beside this general function also a specific role in the control of neurulation (Rogner et al., 2000) and seems to be necessary during early development of an embryo (Quivy et al., 2001).

Energy metabolism related genes belonging to different pathways such as glycolysis (glyceraldehydes-3-phophate dehydrogenase (GAPDH)), electron transport chain (NADH ubiquinone oxidoreductase), lipid metabolism (fatty acyl-CoA-reductase) and amino acid breakdown (amino transferase) were isolated. The ADP/ATP translocase, required for the exchange of ADP and ATP across the inner mitochondrial membrane, has also a link to development of an organism: its expression is high during oogenesis and the formation of the anteroposterior dorsal axis and is dynamically regulated during neurulation (Crawford et al., 2001).

Other sequences (53%) showed similarities to molting, cell cycle, various cellular processes and ribosomal RNA/protein-related genes.

4.2. Gene expression profiles

A custom array for the crustacean *D. magna* was constructed and its usefulness for ecotoxicological screening was investigated. The fungicide propiconazole was used here as a model compound to evaluate the impact on the expression of the life stage-specific genes. Differential gene expression analysis revealed a number of genes showing up- and down-regulation. More specifically, a clear time- and concentration-dependent alteration in gene expression profiles could be observed. Clearly, the expression profiles showed more down- than up-regulated genes and more genes were differentially expressed after 4 days of exposure (compared to 8 days of exposure). Especially after 4 days of exposure at the highest concentration, most repressed genes were found (Table 3).

Gene expression profiles after 4 days of exposure to 1 μg/mL show differentially expressed genes related to embryo development, stress response/protein folding, energy metabolism, various cellular processes and transcription/translation. Some of them like vitellogenin, larval-specific gene, stromal cell-derived factor and chaperonin were more than 3 times down-regulated (Fig. 3). The yolk protein vitellogenin serves as a source of nutrition to the developing embryo. Its down-regulation was confirmed by real-time PCR analysis showing a 7.9-fold inhibition (variation = 0.06; $p = 0.001$). It was only recently that this lipoprotein of *D. magna* was fully sequenced (Kato et al., 2004). Accumulation of vitellogenin in the oocytes is one of the key events in ovarian maturation. In the 1 μg/mL propiconazole treatment we found significant increases in embryo deformities. Down-regulation of this lipoprotein clearly indicates impaired reproduction functioning of the maturing daphnids. In fish, a similar inhibition of vitellogenin after exposure to the azole fungicide fadrozole was reported (Ankley et al., 2002). However, until now, no reports were made on chemical-induced vitellogenin reductions in daphnids. Looking at its physiological importance for the developing embryo, this gene certainly seems an interesting marker to predict reproductive effects in daphnids.

Other strongly repressed genes after 4 days of exposure to 1 μg/mL include a ribonucleoprotein (87F) and some of the sequences with no known similarity (labeled as “unknown”) (Fig. 3). Clearly some of these genes were affected in a concentration-related manner. However, their physiological function remains to be elucidated through future research. Moreover, since sequence information databases are continuous growing, more identity information for these sequences could become available in the future. Especially sequencing information of the *Daphnia* Genomics Consortium will be of great benefit (Thomas and Klaper, 2004).

Up-regulated genes after 4 days of exposure 1 μg/mL are for instance heat shock protein 90, and ATP synthase (Fig. 3). In general, heat shock proteins function as molecular chaperones – assisting the folding and maturation of proteins – and are induced by many different environmental stressors including extremes of temperature and various toxic substances. More specifically, HSP 90 also appears to have a possible role in embryonic development providing protection to all cells of the embryo following stress (Sass and Krone, 1997; Feder and Hofmann, 1999; Lewis et al., 1999). The induction of ATP synthase is pointing towards a higher need of energy (ATP) in response to propiconazole exposure. The effect of toxic stress on the energy budget of *D. magna* was already intensively examined, demonstrating that toxic stress causes an increase in the basic energy metabolism resulting in reduced energy budgets (De Coen and Janssen, 2003). The induction of ATP synthase after propiconazole exposure points towards the same direction.

In parallel with differential gene expression analysis, effects at the organismal level were studied. At the highest concentration, significant differences in embryo abnormalities were found. This corroborates the results of Kast-Hutcheson et al., 2001 who also observed abnormalities in embryos of *D. magna* exposed to 1 μg/mL propiconazole. Next to the deformities, we also observed a reduced growth in the population exposed to

the highest concentration of propiconazole (both after 4 and 8 days). Other organismal endpoints (survival, duration of the molt cycle, days to reproductive maturity and offspring per female) were not affected according to literature (Kast-Hutcheson et al., 2001).

Comparing the results at the gene expression level with those measured at the organismal level shows that both levels of biological organization respond differently to propiconazole stress. While at the organismal level a growth- and developmental-based LOEC of 1 μg/mL could be derived, at the molecular level lower LOEC-threshold values were obtained. However, it is clear from our microarray results that deriving LOEC values based on gene expression analysis is not straightforward: at the three different concentrations and exposure periods different genes were significantly affected. Moreover, these genes were not always affected in a concentration-dependent manner. For example, vitellogenin showed no clear dose–response, although its significant inhibition after 4 days of exposure at 1 μg/mL clearly suggested an adverse effect on embryonic development. Among the genes with an unknown function, some showed a concentration-dependent inhibition (e.g. DV075797), which indicates that our cDNA collection contains potential early warning biomarkers for chronic (reproductive) effects. Future research will be conducted to validate the gene fragments for toxicity screening purposes.

Table 3 demonstrates that most of the differentially expressed genes were measured in organisms exposed to 1 μg/mL. A shorter exposure period affected a higher number of genes. Increasing the stressor dose clearly caused a higher number of genes to be affected. A similar effect was seen at the enzyme level (De Coen et al., 2001). Using a set of 5 different enzymes of the energy metabolism of *D. magna* exposed to lindane and mercury, more enzymes were significantly induced or inhibited at higher toxicant concentrations after 2 and 4 days of exposure. Also in this study different concentration response relationships were observed for the different enzymes. In general, it can be stated that, with increasing toxic stress, an increasing number of pathways are affected. The effects at the transcriptional level demonstrate that significant changes do not necessarily imply adverse acute effects at the organismal level. However, the effects of prolonged exposure periods need to be investigated in order to validate the full ecological relevance of these molecular biomarkers. Only after assessing the long-term impact of propiconazole on the growth, survival and reproductive capacity of these exposed populations, it will become clear how toxicogenomics-based measurements can be used in a quantitative and predictive manner.

In conclusion, this study demonstrates the usefulness of SSH–PCR to produce specific cDNA libraries of ecotoxicological standard organisms, like *D. magna*. Moreover, the obtained adult and juvenile related genes were applied on a cDNA microarray to monitor gene expression changes resulting from toxicant exposure. After exposure to the embryotoxicant propiconazole, several differentially expressed genes were identified. One of the major down-regulated genes was vitellogenin, which plays a crucial role in the embryonic development. In future experiments this custom array will be extended with genes related to other physiological processes. Testing with other chemicals will help us to further evaluate this tool for toxicant characterization and to investigate the relationship between gene expression profiles and organismal and population parameters.

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