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Gene transcription in *Daphnia magna*: Effects of acute exposure to a carbamate insecticide and an acetanilide herbicide

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ABSTRACT

Daphnia magna is a key invertebrate in the freshwater environment and is used widely as a model in ecotoxicological measurements and risk assessment. Understanding the genomic responses of *D. magna* to chemical challenges will be of value to regulatory authorities worldwide. Here we exposed *D. magna* to the insecticide methomyl and the herbicide propanil to compare phenotypic effects with changes in mRNA expression levels. Both pesticides are found in drainage ditches and surface water bodies standing adjacent to crops. Methomyl, a carbamate insecticide widely used in agriculture, inhibits acetylcholinesterase, a key enzyme in nerve transmission. Propanil, an acetanilide herbicide, is used to control grass and broad-leaf weeds. The phenotypic effects of single doses of each chemical were evaluated using a standard immobilisation assay. Immobilisation was linked to global mRNA expression levels using the previously estimated 48 h-EC_1s, followed by hybridization to a cDNA microarray with more than 13,000 redundant cDNA clones representing >5000 unique genes. Following exposure to methomyl and propanil, differential expression was found for 624 and 551 cDNAs, respectively (one-way ANOVA with Bonferroni correction, $P \leq 0.05$, more than 2-fold change) and up-regulation was prevalent for both test chemicals. Both pesticides promoted transcriptional changes in energy metabolism (e.g., mitochondrial proteins, ATP synthesis-related proteins), moulting (e.g., chitin-binding proteins, cuticular proteins) and protein biosynthesis (e.g., ribosomal proteins, transcription factors). Methomyl induced the transcription of genes involved in specific processes such as ion homeostasis and xenobiotic metabolism. Propanil highly promoted haemoglobin synthesis and up-regulated genes specifically related to defence mechanisms (e.g., innate immunity response systems) and neuronal pathways. Pesticide-specific toxic responses were found but there is little evidence for transcriptional responses purely restricted to genes associated with the pesticide target site or mechanism of toxicity.

1. Introduction

The freshwater habitat is often contaminated with agrochemicals applied to control insect pests, weeds or pathogens. Pesticide contamination can result from spray drift during application, surface runoff and/or leaching (Brown et al., 1995; Carter, 2000; Reichenberger et al., 2007). Contemporary pesticides were developed in the mid-1970s as a less hazardous alternative to e.g., persistent organochlorines (Barr and Needham, 2002). Despite their relatively rapid degradation in the field these pesticides have been detected in water at concentrations frequently exceeding reference safety levels (e.g., Barr and Needham, 2002; Cerejeira et al., 2003; García de Llasera and Bernal-González, 2001; Guest et al., 2006; Wilson and Foos, 2006). The insecticide tested here, methomyl [S-methyl N-(methylcarbamoyloxy)thioacetimidate] and the herbicide propanil (3,4-dichloropropioanilide) are examples of these agrochemicals.

Methomyl is a monomethyl carbamate widely used to control a large range of insects and spider mites through direct contact and ingestion (Tomlin, 2001). Carbamates reversibly inhibit cholinesterase enzymes, such as acetylcholinesterase (AChE), which hydrolyses the cationic neurotransmitter acetylcholine at very high rates; these pesticides inactivate the enzyme through carbamylation of its active serine, hence compromising the normal neurotransmission function (Quinn, 1987). The potential

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of AChE inhibition as a biomarker of exposure to carbamates in *Daphnia* has been studied (Barata et al., 2004; Printes and Callaghan, 2004). However, these chemicals are able to significantly inhibit other esterases (Barata et al., 2004) and the relationship between the biomarker and the observed response at the individual level has already been shown to be dependent on the acting chemical (Printes and Callaghan, 2004). Such experimental evidence provides clues to the actual mechanism of carbamate toxicity to non-target organisms. Genomic investigation may provide further insight into the mechanism of carbamate toxicity.

Propanil is an anilide herbicide that is commonly applied in the post-emergence of rice and acts through direct surface contact to control grass and broad-leaf weeds (Tomlin, 2001). Its specific mechanism of toxicity in target species involves an enzyme-mediated process of disruption of the electron flow in the Photosystem II, therefore inhibiting the light reaction of photosynthesis (e.g., Mitsou et al., 2006). Propanil is known to elicit deleterious effects in *Daphnia* related to survival, life-history and feeding (e.g., Pereira et al., 2007; Villarroel et al., 2003). Information on cellular and sub-cellular toxicological pathways of propanil in non-target systems is limited, but a few focussed studies are available in the vertebrate literature (Blyler et al., 1994; Cuff et al., 1996; Guilhermino et al., 1998; Li et al., 2003; Malerba et al., 2002).

Daphnia have been widely used to study the effects of pesticides in freshwater ecosystems (e.g., Hanazato, 2001; Poynton and Vulpe, 2009; Sarma and Nandini, 2006) because they occupy a central position in the food web (e.g., Lampert, 2006) and are readily tested in the laboratory. Recent progresses in sequencing and annotating the *Daphnia pulex* genome and, to a lesser extent, *Daphnia magna* (Shaw et al., 2008; http://daphnia.cgb.indiana.edu; http://www.jgi.doe.gov) now allow to study their genomic responses.

Effects of environmental stressors, such as pesticides, on non-target organisms have generally been assayed using whole-organism or population responses. Despite providing valuable insight and useful information for regulatory purposes, such assessments rarely explain the mechanisms of toxicity underlying the observed response. The integration of genomic-based tools and ecotoxicology is a promising approach that may provide a broad view of how living systems respond to a given stressor (Neumann and Galvez, 2002; Robbens et al., 2007; Snape et al., 2004). Transcription profiling using microarrays (first described by Schena et al., 1995) is one of the most prominent genome-wide technologies within ecotoxicogenomics since it provides an overview of changes in gene expression linked to chemical exposure. With such an approach, we can try to establish a relationship between exposure and response effects. Very recently, cDNA microarray-related techniques have been successfully used to address transcriptional responses of *D. magna* to different environmental toxicants, including pharmaceuticals, heavy-metals, pesticides and PAHs (Connon et al., 2008; Heckmann et al., 2008; Soetaert et al., 2006, 2007a; Watanabe et al., 2007).

Here we investigate phenotypic and molecular responses of *D. magna* to the pesticides methomyl and propanil and highlight the complex nature of molecular-level stress response resulting in immobility in this non-target organism. Our approach was to compare the response to equitoxic concentrations of each pesticide, using a previously estimated effect concentration (EC) EC_1. This allowed the use of strictly comparable exposure concentrations and hence responses. The EC1 concentration was chosen in order to detect sub-lethal transcriptional responses that could be linked to phenotypic responses.

2. Materials and methods

2.1. Test organisms

D. magna were obtained from the Water Research Centre (WRc), Medmenham, UK and cultured as a single clonal lineage at the University of Reading, UK for at least 2 years before testing. For full details of culturing conditions see Hooper et al. (2006). Third to fifth brood juveniles <24 h old and differing in age by <3 h were used for testing.

2.2. Chemicals and range-finding assays

Methomyl (Pestanal®, 99.5% purity) and propanil (Pestanal®, 99.7% purity) were supplied by Sigma Aldrich (Seelze, Germany). Stock solutions were freshly prepared prior to experiments by directly dissolving methomyl or propanil in culture medium. The acute toxicity of each pesticide to *D. magna* was assessed following OECD guideline 202 (OECD, 2004). In brief, 48 h exposures were carried out under a static design using twenty juveniles (<24 h old) per treatment. Incubation conditions were as described for culturing (see Section 2.1). The tests were conducted in glass beakers, each containing 50 mL test solution. Dissolved oxygen and pH were monitored at the beginning and the end of the tests for validation purposes. Immobilised individuals were counted at the end of the test. Effect concentrations were estimated via Probit analysis (Finney, 1971).

2.3. Experimental treatments, RNA extraction and target labelling

Neonate *D. magna* (<24 h old, 3 h age-range), were obtained from 40 bulk cultures (see Section 2.1) and were exposed to each treatment for 48 h (1-L test solution). A randomised block design with three treatments was followed: negative control, methomyl EC_1 (10.5 $\mu g\,L^{-1}$ with a 95% confidence interval of 8.82–11.7 $\mu g\,L^{-1}$) and propanil EC_1 (363 $\mu g\,L^{-1}$ with a 95% confidence interval of 302–401 $\mu g\,L^{-1}$). Five replicates were used per block and thirty juveniles were randomly assigned to each replicate. After the 48 h static exposure, the organisms were collected into sterile 1.5 mL micro-centrifuge tubes with 150 μL RNA*later*® (Ambion, UK), using a previously described approach (Heckmann et al., 2007) and stored at −80°C. Total RNA was extracted using the RNeasy Mini kit with on-column DNase treatment (Qiagen, UK), following the manufacturer's instructions. RNA concentrations were determined on a GeneQuant Pro spectrophotometer (Biochrom, UK) and RNA integrity was verified using the BioAnalyser 2100 and RNA 6000 Nano Kit (Agilent Technologies, UK). For each sample, total RNA was amplified and labelled with Aminoallyl Message Amp aRNA Amplification Kit (Ambion, UK) from 400 ng of starting material. Reference material was created by pooling 10 μg of aRNA from each sample followed by labelling with Alexa Fluor dye 555. Individual samples were labelled with Alexa Fluor 647.

2.4. Microarray experiments

The *D. magna* microarray used in this study was produced at the Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, UK. Good agreement between QPCR data and microarray data using this chip has already been confirmed in previous studies (e.g., Heckmann et al., 2008). This indicates good chip quality and validates its use in further ecotoxicological assessments. The chip cDNA content and manufacturing protocols, pre-hybridization and hybridization buffers and protocols are described in Connon et al. (2008). In brief, a mix of 5 μg labelled sample and 5 μg labelled reference material, together with blocking reagents, were hybridized in 50% formamide, 5× SSC and 0.1%SDS to individual microarray