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Effects of Pentachlorophenol on *Drosophila melanogaster* transcriptome

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INDEX

ABSTRACT

Chlorinated molecules, among synthetic products, have been extensively applied as pesticides and Pentachlorophenol (PCP) has been one of the most extensively used biocides in the United States and in Europe. In the last decades its use has been restricted in several countries worldwide, due to its toxicity to wildlife, long persistence in the environment and bioaccumulation in fat tissues. Public increasing interest in an ecologically safe environment remediation, together with the awareness that biotechnology has high potential to satisfy this need, is leading towards different approaches for bioremediation. PCP-degrading bacteria and fungi are known, but looking for diverse and not yet characterized efficient degradation pathways can result in improving the available tools. Insects represent a potential and relatively unexplored source of metabolic mechanisms for detoxification of a wide variety of both natural and synthetic compounds due to their genetic plasticity.

Drosophila melanogaster was used as a model insect, and a set of differentially expressed genes associated with PCP response in the fruitfly was identified on the basis of genome-wide microarray analysis. Two PCP doses were tested, 20 and 2000 ppm, the latter causing a higher level of response, in terms of significantly differentially expressed genes. Overexpression of the main detoxifying gene families involved in the PCP response (five CYPs and one GST) was confirmed by qRT-PCR analysis, as well as two ABC transporter genes. Furthermore the enrichment analysis of the overexpressed 2000 ppm PCP treatment highlighted a strong response in the biogenic amine metabolic pathways, with the induction of genes potentially involved in degradation pathways, such as the tyrosine monooxygenase.

Two 2000 ppm PCP resistant *Drosophila* strains were selected, in order to obtain a deeper knowledge of the effects of PCP on *Drosophila*, and to elucidate PCP metabolic pathways. For this purpose HPLC analyses were carried out on PCP supplied diet on which resistant larvae fed on, evidencing a reduction of PCP as a consequence of feeding activity.

INTRODUCTION

Human activities, such as urbanization, agriculture, and industrialization, have produced increasing impact on the environment, compromising limited natural resources, through both waste production (sewage, wastewater, kitchen waste, industrial waste, effluents, agricultural waste, food waste) and dispersal of various chemicals (pesticides, chemical fertilizers, toxic products and by-products from chemical industries). An actual dramatic problem concerns the continuously increasing levels, in air, water or soil, of stable toxic chemicals such as halogen aliphatics, aromatics, polychlorinated biphenyls and other organic and inorganic pollutants. These compounds may adversely affect the environment, compromising the self-regulating capacity of the biosphere (Sen and Chakrabarti 2009; Beltrame *et al.* 2010; Prasad *et al.* 2010). Some toxics may reach high levels at the points of discharge, but even those which have low levels can be highly toxic for the organisms or can increase in concentration as they pass through the food chain due to biomagnification or bioaccumulation (Davies *et al.* 2006; Kelly *et al.* 2007; Fatemi and Baher 2009).
For public protection against toxic effects of pesticides, several countries have established standards specifying the acceptable residual levels of each pesticide in diverse food products (Tawara *et al*. 2006). The World Health Organization (WHO) has sets of basic acceptable minimum standards which are evaluated and reviewed periodically.

Chlorophenols

European Union (EU) categorized 132 dangerous substances that should be monitored in waters based on toxicity, stability, and bioaccumulation, among which are included the chlorophenols such as 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,5-trichlorophenol, and pentachlorophenol (European Environment Agency 2007). The EU has also set concentration limits for single and total pesticides (and their degradation products) in the environment (EU 2003). For the USA the Environmental Protection Agency (EPA) establishes the maximum level for each pesticide and related

transformation product (U.S. EPA 2004). Both European Union and the United States have restricted the use of chlorophenols.

Environmental and occupational exposures to pesticides as a risk factor for human health have been widely studied mainly among workers involved in agricultural and many industrial activities (e.g. textiles, leather, petrochemical, chlorinated pesticides or fungicides, incinerators). Occupational exposures have been observed to occur through inhalation and dermal contact and have been associated with increased mortality due to cancer, lymphoma, and myocardial ischaemia (Hoovield *et al.* 1998; Buckley *et al.* 2000; Mundt *et al.* 2000; Van Maele-Fabry and Willems 2003; Van Maele-Fabry *et al.* 2007). Primary environment concerns regard ground and surface water ecosystems and consequent risk for organisms associated with the food chain (Hoovield *et al.* 1998). The fact that chlorophenols are recalcitrant increases enormously this risk (Igbinosa *et al.* 2007).

Among chlorophenols, pentachlorophenol (PCP) has been widely used as wood preservative and in textile industries, but it is now considered as a priority pollutant both in the USA and in EU, even if it is still commonly utilized with the same purpose in China and in less developed countries (Catallo and Shupe 2008). Chlorophenol derivatives as tetrachloro-hydroquinone, chlorocatechols and chloroguaiacols, exhibit toxic properties including cytotoxic, mutagenic, and cancerogenic activity (Michałowicz and Majsterek 2010). Moreover, the number of chlorine atoms may be directly associated to toxicity increase and prolongation of the period of bioaccumulation (Michałowicz 2005).

Bioremediation

Nature and magnitude of pollution are continuously changing, bringing new challenges and creating a constant need for developing newer and more appropriate technologies.

An increasing awareness of effects of pollution on both human and environment health has being acquired in industrialized countries, and consequently governmental agencies across the globe have set environmental policy to limit the risks of pollutants. Recovery of polluted compartments has been dealt with different approaches,

including chemical treatment, volatilization, incineration, leaching etc. This so called "traditional remediation" have drawbacks, do not always offer the perfect solution, being economically restrictive and not ecologically safe. In this context, biotechnology has tremendous potential to cater for the needs and holds hope for environmental protection, sustainability and management (Hatti-Kaul *et al.* 2007; Azadi and Ho 2010).

Currently techniques based on the ability of organisms to transform complex organic compounds, including pollutants, into simpler ones (biodegradation) appear to be a valid alternative. Indeed organisms, above all microorganisms, have been able to extend their metabolic capabilities adapting to the presence of pollutants which are transformed into less toxic compounds or even completely degraded, thus being integrated into the natural biogeochemical cycles (Diaz 2004).

The combination of techniques aiming to enhance biodegradation where natural systems have limited capacity to reduce spontaneously and rapidly the contaminants is called Bioremediation. Potentially, bioremediation can be used to cope with a variety of xenobiotics, such as hydrocarbons (aliphatic, aromatic, BTEX, PAHs, etc.), chlorinated hydrocarbons (PCB, TCE, PCE, pesticides, herbicides, etc.), nitro aromatic compounds (TNT), organ phosphorus compounds, cyanides, etc. (Alexander 2001). Since the early 1980s, genes encoding for enzymes able to degrade xenobiotics started to be characterized and cloned. These efforts led to patent a modified strain of *Pseudomonas* able to degrade camphor, octane, salicylate and naphthalene (US Patent #4259444). Later, a genetically modified *Pseudomonas* strain with improved degradation pathway for alkyl-chlorobenzoate was obtained (Ramos *et al.* 1987; Rojo *et al.* 1987). Genetic transformation might be a tool for solving the problem of pollution, but to be really efficient a comprehension of biodegradation processes, with the analysis of the complex web of metabolic and regulatory interactions, is required (Cases and de Lorenzo 2005). The recent emergence of 'omics' technologies (genomics, transcriptomics, proteomics and metabolomics) and the development of methodology for network analysis have offered new perspectives for the development of biodegradation processes. In particular, this approach made easier to discover interconnected enzyme clusters working together (Ravasz *et al.* 2002).

Biodegradation and PCP

Degradation of recalcitrant molecules is critically based on the assembly of new pathways by a novel association of preexisting enzymes (Jensen 1976), a strategy that became more likely as life became more complex and the number of enzymes for organisms increased. PCP-degrading bacteria are present in soils worldwide (Saber and Crawford 1985; Tiirola *et al.* 2002; Kao *et al*. 2005; Mahmood *et al*. 2005; Yang *et al*. 2006). Since natural sources of PCP are not known, the degradation pathway employed by bacteria to degrade PCP likely evolved during the approximately 60 years since the human introduction of PCP into the environment (Copley 2000). The white rot fungi, which can decompose lignin, can effectively transform PCP and were widely studied for the treatment of PCP-contaminated soil (Rubilar *et al*. 2011; Yu *et al.* 2011) and wastewater (Pedroza *et al*. 2007). Dechlorination and methylation are the two major pathways of PCP biotransformation and both led to detoxification of PCP. Aerobic dechlorination of PCP is known to be catalyzed by hydroxylase (a flavoprotein in *Flavobacterium* sp.) (Xun and Orser 1991) and various peroxidases, such as laccase (Peng *et al*. 2008), lignin peroxidase (LiP), manganese peroxidase (MnP) (Reddy and Gold 2000) in the white rot fungi, and horseradish peroxidase (Zhang *et al.* 2007). PCP methylation was found in some bacteria (Neilson *et al.* 1988), and much more commonly among fungi, including the white rot fungi (Machado *et al*. 2005; Szewczyk and Długoński 2009).

Why *Drosophila*?

In general microorganisms are quite efficient agents for bioremediation, since during this process they degrade xenobiotics utilizing them as carbon and energy sources. Many microorganisms are known to transform xenobiotic compounds (Hussain *et al*. 2007), the use of which may be subject to various limitations, such as the need to verify optimal conditions for their growth, or for the enzymes involved (temperature, pH, presence/absence of oxygen, light, nutrients, inhibition factors). One approach for increasing the range of optimal conditions for detoxification would be to look for catabolic pathways in alternative organisms. Under this perspective, a rather neglected

group is represented by insects. Instead, this group might be an important source of useful molecules due to its surprising genetic plasticity and adaptability. Indeed, during their evolutionary history, insects have confronted with a variety of toxic substrates (mainly phytotoxins), developing, both at cellular and organismal level, defensive strategies (above all detoxification) to deal with them. Insects supported with such an efficient defense mechanism against natural compounds, are ready to react to the many classes of synthetic insecticides recently been introduced into their environments (Schuler 2012). During the last fifty years the massive employment of toxic chemicals to control noxious pests has favoured numerous resistance instances, with more than 500 species reported as resistant to all the major insecticide classes (http://www.pesticideresistance.com). Resistance emergence, even if problematic from the practical standpoint, since it resulting in toxin with a reduced or null effect, is a biologically interesting phenomenon of rapid and contemporary evolution. Its comprehension has two practical applications: the management of resistance and the search of biomolecules useful for decontamination of polluted environments.

Non-pest organisms have been frequently used to study the phenomenon of resistance. Among these, the fruit fly, *Drosophila melanogaster*, has received considerable interest from both evolutionary (Russell 1990) and practical (Daborn *et al.* 2012) standpoint. Even though *D. melanogaster* is not generally considered to be a pest species, field resistance to the main insecticide classes has been repeatedly discovered (Wilson 2001, 2005; Daborn *et al.* 2002; Bogwitz *et al.* 2005). Moreover genetic plasticity found in this genus is highlighted by the adaptations of several *Drosophila* species to phytotoxins (such as isoquinoline alkaloids) of their host plants (Frank 1992). Furthermore, usefulness of *D. melanogaster* to elucidate mechanisms of resistance is due to its biology and the ever increasing genetic resources. Indeed, rearing of this fly is facilitated by its rapid life cycle and ease of husbandry. From the genetic standpoint, this organism is valuable for the ease of manipulation of its small set of chromosomes and genetic transformation (Rubin and Spradling 1982). Moreover, the complete decoding of its genome (Adams *et al*. 2000) and transcriptome (Rubin *et al.* 2000) and the easy access to genomic resources by on-line bioinformatic platforms allow the usage of a wide range of technologies for the monitoring of gene expression in response to specific conditions and stimuli (St. Pierre

et al. 2014). *D. melanogaster* is an established model to study insecticide resistance (ffrench-Constant *et al.* 1992; Morton 1993; Perry *et al.* 2007). To date several cytochrome P450 genes and one GST have been identified as important factors contributing to insecticide resistance in several *D. melanogaster* populations (Daborn *et al.* 2002; Bogwitz *et al.* 2005; Low *et al.* 2010). Among these genes the most studied is the cytochrome P450 *Cyp6g1*, whose overexpression determines resistance to several insecticide classes (neonicotinoids, DDT and IGR) (Daborn *et al.* 2001, 2002). The role of this gene has been validated by the approach of transgenic overexpression (Chung *et al.* 2007; Daborn *et al.* 2002) which was later used to clarify the function of some other *Drosophila* cytochrome P450 genes (Bogwitz *et al.* 2005; Daborn *et al.* 2007).

Why Microarrays?

In the past decade, the completion of sequencing of higher organisms has led to the development of whole transcriptome analysis techniques. Among the most important innovations in this field is the microarray technology that allows to quantify the expression for thousands of genes simultaneously by measuring the hybridization from a tissue or cell of interest to probes immobilized on a solid surface. This powerful technology has been applied throughout the life sciences (Lockhart and Winzeler 2000; Young and Peppard 2000), addressing many biological questions at genomic scale that were not approachable previously. mRNA-expression profiling is one of the most frequent application. Indeed, parallel quantification of large numbers of messenger RNA transcripts using microarray technology promises to provide detailed insight into cellular processes involved in the regulation of gene expression. This should allow new understanding of signaling networks that operate in the cell, and of the molecular basis underlying cellular processes.

With the advent of complete, annotated, genome sequences for different insects such as *Drosophila* (Misra *et al*. 2002) and *Anopheles* (Holt *et al.* 2002), the study of insecticide genetic response is entering a genomic era (Oakeshott *et al*. 2003). In recent years, several papers have been published studying the expression of various genes under the action of chemical stressors by using microarray analysis. In

Drosophila this technique has more often been used to detect genes that are constitutively overexpressed in insecticide-resistant strains of *Drosophila* and mosquitoes (Daborn *et al.* 2002; Le Goff *et al.* 2003; Pedra *et al.* 2004; David *et al.* 2005; Vontas *et al.* 2005), and less frequently to identify responses to chemical stressors in susceptible strains (Zou *et al.* 2000; Girardot *et al.* 2004; Silva *et al.* 2012). One approach is to use microarray selectively representing only genes belonging to well defined categories, as the so called "detoxification chip", including Cytochrome P450 and Glutathione-S Transferases, genes usually involved in xenobiotic responses (David *et al.* 2005). This approach, even if permitting a simpler analysis, does not allow a full, broad comprehension of the mechanisms of the induced response, and the identification of genes not previously described as involved in detoxification mechanisms. For this reason it has been chosen for this project to carry out a genome wide transcription analysis to evaluate the transcriptomic response of *D. melanogaster* challenged with PCP.

Aim of the work

In the present study we evaluated the transcriptomic response of *D. melanogaster* challenged with PCP, used as a model for chlorophenols, with two different concentrations.

Our main aim was to elucidate the molecular response to PCP in *D. melanogaster* with the use of transcriptomic analysis. This technique, one of the most powerful and versatile, allows to compare simultaneously the expression profile of thousands of genes. The focus of our research was the identification of genes potentially involved in PCP degradation pathway comparing expression patterns of treated and untreated *Drosophila* larvae. On a subset of overexpressed genes with a putative detoxifying function results were validated by quantitative reverse transcription PCR (qRT- PCR). To our knowledge this is the first transcriptomic study of PCP effects in a non-target organism.

A second aim was to obtain a broader information on resistance mechanisms and PCP insects metabolic pathways. For this purpose *D. melanogaster* strains have been selected in laboratory at 2000 ppm PCP under different conditions to evaluate their

potential to degrade PCP. This analysis was performed by HPLC at Departamento de Ciencias Quimicas y Recursos Naturales, Laboratorio de Quimica Ecologica (Universidad de La Frontera) under the supervision of Prof. Andres Quiroz Cortez.

MATERIALS AND METHODS

Drosophila strains and rearing

Flies and larvae of Canton - S strain of *D. melanogaster* were cultured on *Drosophila* agar-gelled food, containing agar 3 g/l, sucrose 100 g/l, commercial cornmeal 100 g/l, heat inactivated brewer's yeast 50 g/l and Nipagin (methyl 4-hydroxybenzoate) 2 g/l, at 25 ± 1°C, with 16 hours light /8 hour dark cycle. Additional suspension of yeast was provided to maximize oviposition. Adult flies were transferred to fresh diet twice a week to maintain the strain.

For PCP treatments and selection of resistant strains in order to achieve a more homogeneous distribution of the toxicant and an easier recovery of larvae, a different medium was prepared with agar 10 g/l, sucrose 50 g/l, heat inactivated brewer's yeast 100 g/l and Nipagin 1.5 g/l (without cornmeal) (Bass *et al*. 2007, slightly modified). The third-instar larvae due to their active crawling, boring, and vigorous feeding habit were chosen for the study. Synchronization of third-instar larvae was achieved by allowing oviposition for a short time (6 h).

Two 2000 ppm PCP resistant strains were selected under different conditions: the first strain (PCPR1) was obtained rearing the wild strain with progressively increasing concentrations of PCP (1000 and 1500 ppm) for the first 15 generations; the successive 10 generations were alternately reared on 2000 ppm PCP treated and untreated diet. From the 25th generation the strain was constantly maintained on 2000 ppm PCP supplied diet. The second strain (PCPR2) was selected, starting directly at the 2000 ppm dose, for 10 generations alternately reared on PCP treated and untreated diet, and then constantly maintained on 2000 ppm PCP supplied diet. This selection procedure was designed to exert low (PCPR1) and high (PCPR2) selection pressures favoring polygenic or monogenic forms of pesticide resistance, respectively (McKenzie 2000).

Determination of LC_{50} (48 h) of PCP

The 50% lethal concentration (LC_{50}) of third-instar larvae to PCP at 48h was determined using the sodium salt of PCP, technical-grade (nominally 86%; Aldrich Chemicals) soluble in water. The treatments tested were 500, 1000, 2000, 4000, 6000 and 12000 ppm of PCP. To record the natural mortality a control was also run by side. The doses were prepared using a PCP stock solution (30 mg/ml) dissolved in water, added to the food prior to gelification in petri dishes. The experiments consisted of 5 replicas each dose, with 20 third-instar larvae each. The bioassays were repeated 4 times. Controls were performed after 48h, larvae unable to move were counted as dead. The statistical analysis of the data was made following the probit analysis outlined by Finney (1952), with the STATGRAPHICS Plus Version 5 software.

PCP treatments

To prove that the larvae fed in presence of PCP, an ingestion assay was set (Coulson *et al.* 2005): twenty third-instar larvae were placed directly on artificial diet mixed with 0, 20 and 2000 and 4000 ppm PCP in presence of 0.05% of bromophenol blue, an indigestible dye. The presence of bromophenol blue in the medium makes food in the larvae gut visible: if the larvae eat, they uptake the dye. After 3 h of feeding larvae were removed from food and scored for dye uptake: those positive for ingestion had dark blue midgut, those negative had no or very light blue staining.

For microarray experiment two PCP concentrations were chosen: 20 and 2000 ppm. The 20 ppm concentration is the same order of magnitude of the upper Italian concentration threshold for PCP in soils (D.Lgs 152/2006), ranging from 0.01 mg/kg to 5 mg/kg, depending on the use of the soils.

The high dose treatment, 2000 ppm, of the same order of magnitude of the determined LC_{50}, was chosen on the basis of preliminary experiments of ingestion, since with treatment with 4000 ppm PCP, after 3 h, larvae showed only lightly blue stained guts.

No significant effect on vitality and feeding behavior of the larvae was observed at the lower dose, whereas a sluggish movement, and a lower attraction for food was exhibited by the larvae in the treatment with the higher dose.

For PCP treatment three independent biological replicates were performed. Each biological replica consisted of three petri dishes with 50 larvae each, for control and 20 ppm dose, and of 6 petri dishes for 2000 ppm treatment, in order to be able to choose only larvae deep in the diet. Larvae were allowed to feed for 3 h. Following feeding, the larvae were taken out, washed thoroughly with Phosphate Buffered Saline (PBS) to remove the adhering food and pesticide, transferred to petri dishes containing fresh food only, allowed to feed for 3 h, washed again with PBS and thereafter the larvae were frozen in liquid nitrogen. Since the detoxification process reaches a plateau in 3 h, the time schedule chosen was the optimum required for monitoring the response elicited by the toxicant (Chowdhuri *et al.* 1999). Totally, about 150 larvae /treatment were collected for each biological replica.

Expression profiling

a) Total RNA extraction

Samples were homogenized in liquid nitrogen, and total RNA extraction was carried out in a nuclease-free environment, using 3 ml of TRI-Reagent (Applied Biosystem)/sample, according to the manufacturer's instructions. After lyses of samples (5 min at room temperature), 0.2 ml of chloroform (per 1ml TRI-Reagent reagent) was added, and the tubes were shaken vigorously by hand for 15 seconds, followed by incubation at room temperature for 15 min. Tubes were centrifuged at 12,000 x g for 15 min at 4°C, and the top aqueous phase containing RNA was transferred to a new tube; 0.5 ml isopropyl alcohol (per 1ml TRI-Reagent used) was added to the tubes, followed by an incubation of 10 min at room temperature. Tubes were centrifuged at 12,000 x g for 10 min at 4°C, the supernatant was removed, and the pellet was washed with 1 ml of 75% ethanol. After removing ethanol the pellet was air dried for 5 min and dissolved in 100 ml DEPC H2O, by vortexing it and incubating at 65°C for 10 min. RNA samples were then treated with DNAse I and further purified on silica columns with RNeasy Plant Mini Kit (RNeasy Quiagen) to remove potential contaminants, such as polysaccharides and residues of genomic DNA, following the manufacturer’s protocol. RNA purity was spectrofotometrically assayed with the NanoPhotometer P300 (Implen), a UV-Vis spectrophotometer which allows analysis

of samples as little as 2 µl. The concentration of an RNA solution can be determined by measuring its absorbance at 260 nm and by multiplying the A260 by the dilution factor and the extinction coefficient (1 A260 = 40 µg RNA/ml). The obtained value expresses the sample concentration in µg/ml.

A first quality assessment is achieved by evaluating the ratio A260/280 (higher than 1.9) and A260/230 (higher than 2), where A280 is the absorbance of proteins, phenol or other contaminants, and A230 of phenols and carbohydrates.

A more complete evaluation of RNA integrity was assayed by capillary electrophoresis on a 2100 Bioanalyzer (Agilent) with the Eukaryote Total RNA Nano assay. This is the miniaturized version of a gel electrophoresis, where samples are separated in micro-channels by capillary electrophoresis, detected by their fluorescence and translated into gel-like images (bands) and electropherograms (peaks), the bioanalyzer provides a fast and accurate size distribution profile of RNA samples, thus allowing evaluation of its integrity.

b) RNA Retrotranscription, Amplification and Labeling

For each sample, starting from 2 mg of total RNA from control and treated larvae, unmodified amplified RNA was generated and ULS-Cy5 labeled, with the "RNA amplification and labelling Kit for Combimatrix arrays" kit (Kreatech Biotechnology), based on Eberwine's RNA amplification protocols (Van Gelder *et al.* 1990), following the manufacturer's instructions. In Vitro Transcription of the ds-cDNA, with a linear amplification of RNA, is based on the use of a DNA dependent RNA polymerase and maintains the proportionality of each RNA species present in the original sample. cDNA, aRNA (antisense RNA) synthesis and labeling reactions were performed independently for each replicate. An overview of the procedure is the following:

- First Strand cDNA Synthesis with oligo(dT) primers:

For each sample, in one 0.2 ml RNase-free tube were added: 2 µg of total RNA, 1 µl of T7-Oligo(dT) Primer and Water-DEPC to 12 µl. Mixes were incubated at 70°C for 10 min, kept on ice for 1 min, and briefly centrifuged. At room temperature a Reverse Transcription Master Mix was prepared in a nuclease-free tube with 2 µl of 10X First-Strand buffer, 4 µl of dNTPs mix, 1 µl of RNase Inhibitor and 1 µl of Array –Script

and added to each sample. Samples were briefly centrifuged and incubated at 42°C for 2 h. The tubes were then placed on ice.

- Second strand cDNA synthesis:

In a nuclease free tube a Second Strand Master Mix was prepared by adding 63 µl Water-DEPC, 10 µl 10X Second-Strand buffer, 4 µl of dNTPs mix, 2 µl DNA Polymerase I, 1 µl RNase H and transferred to each sample. Samples were incubated at 16°C for 2h and then put on ice.

- Double stranded cDNA cleanup, to prevent carryover of non-incorporated dNTP, primers and inactivated enzymes:

250µl of cDNA Binding Buffer was added to each sample and the mix was loaded onto the center of a cDNA Filter Cartridge; samples were centrifuged at 10,000 x g at room temperature for 1 min, the flow-through was discarded, and the cDNA Filter Cartridge replaced in the wash tube; cDNA were washed with 500 µl of Wash Buffer and centrifuged twice for 1 min at 10,000 x g to remove all of the Wash Buffer. cDNA Filter Cartridges were transferred to cDNA Elution Tubes and 10 µl of nuclease-free water (preheated to 50-55°C) was added to the center of the filter in the cDNA Filter Cartridge; after 2 min at room temperature, samples were centrifuged for 2 min at 10,000 x g to collect purified cDNAs. A second elution with 10 µl of preheated nuclease-free water was performed to maximize the yields.

- In Vitro Transcription (IVT) and synthesis of aRNA:

At room temperature, to 16 µl of each Double-stranded cDNA in nuclease-free water, a IVT mix was added, prepared with 16 µl T7 rNTP mix (75 mM), 4 µl T7 10X Reaction Buffer, 4 µl T7 Enzyme Mix; samples were incubated at 37°C for 14h. To stop the reaction, nuclease-free water was added up to 100 µl to each sample.

- aRNA purification, performed to remove enzymes, salts, and unincorporated nucleotides:

350 µl of aRNA Binding Buffer and 250 µl of 100% ethanol were added to each sample, mixtures were pipetted onto the center of the filter in an aRNA Filter Cartridge and centrifuged for 1 min at 10,000 x g; the flow-through was discarded and the aRNA Filter Cartridges replaced back into the aRNA Collection Tubes; 650 µl Wash Buffer was applied to each aRNA Filter Cartridge and centrifuged for 1 min at 10,000 x g; the flow-through were again discarded and the aRNA Filter Cartridges

replaced back into the Collection Tubes; 650 µl 80% ethanol was applied to each aRNA Filter Cartridge and centrifuged twice for 1 min at 10,000 x g to remove trace amounts of ethanol and Wash Buffer; Filter Cartridges were transferred to fresh Collection Tubes and 100 µl nuclease-free water (preheated to 50-60°C) were added to the center of the filter; samples were incubated at room temperature for 2 min and then centrifuged at 10,000 x g for 2 min.

The concentration of the amount and the quality of aRNA was assessed by UV absorbance and by evaluation of the OD260/280 and OD260/230 ratios. For all RNAs OD260/280 has to be >1.9 and OD260/230 has to be >2.1.

- Non enzymatic labeling of aRNA with ULS-Cy5, followed by a dye removal step:

to each aRNA sample (6 µg) were added 6 µl of Cy5-ULS, 2 µl volume of 10x Labeling solution and RNase-free water to final volume of 20 µl, and mixed; samples were incubated for 45 min at 80ºC, then placed on ice and spun down.

Dye removal was performed by using KREApure columns following the manufacturer's protocol.

The Degree of Labeling (DoL) was determined by measuring the absorbance at 260 and 650 nm in order to have a good indication of the efficiency of the labeling reaction.

The DOL is calculated from the measured values according to the following equation:

$$\text{Conc. of nucleic acid (ng/µl)} = \frac{(\text{OD260} - (\text{ODdye} * \text{corr. factor})) * \text{dilution factor} * 40}{\text{cuvette length (cm)}}$$

$$\text{DoL} = \frac{340 * \text{Conc. of dye (pmol/µl)} * 100\%}{\text{Conc. of nucleic acid (ng/µl)} * 1000}$$

A DoL higher than 2 was accepted. DoL values lower than 1.0 might not produce enough signal, whereas DoL values higher than 3.6 might cause high background levels.

All the processed samples had a DoL higher than 2, included in the optimal DoL range (1.0-3.6 for aRNA) and were homogeneous.

c) Chip design

Microarray experiment was designed and conducted on 90K microarray chip, synthesized on the CombiMatrix platform at the Plant Functional Genomics Center of the University of Verona (http://ddlab.sci.univr.it/FunctionalGenomics/), containing multiple specific 35–40mer probes for 30,000 out of the 36,335 Tentative Consensus (TC) retrieved from DFCI Drosophila Gene Index database release 12 of DFCI Drosophila Gene Index (http://compbio.dfci.harvard.edu/tgi/). Every target gene was represented on the chip with three replicates, casually spread in the array, to avoid bias due to position effects, and where possible with different oligonucleotide probes.

Together with specific probes for *Drosophila*, the CombiMatrix chips presented, as internal controls, three clusters of negative controls (from plants, bacteria and phage) and a set of empty features (not synthesized).

Microarray experimental design included 3 replicates for both PCP treatments and the control (no PCP) for a total of 9 hybridizations. For this purpose two microarray chips were synthetized, and used 4 or 5 times each.

d) Microarray hybridization and Imaging

Prehybridization, RNA fragmentation, hybridization and successive post-hybridization washings were performed according to CombiMatrix protocols PTL020_00_90K_Hyb_Imaging.pdf.

Microarray slides were incubated with a prehybridization solution, in order to saturate unspecific sites, then the hybridation mix, containing labeled RNA target, was incubated overnight, and finally unhybridized labeled RNA was removed through washing steps.

The main steps are briefly described:

Target RNA was chemically fragmented, as 50-200 base pairs RNA fragments are more easily removed during stripping procedure. Reaction mix was composed by labeled RNA (5 µg) and 5X RNA Fragmentation solution. The mix was incubated at 95°C for 20 min, then placed on ice to stop the reaction.

130 µl of pre-hybridization solution, containing salmon sperm DNA and proteins to saturate unspecific sites was used for the prehybridization step, a small air bubble was

introduced to improve the mixing process, and the microarray was incubated for 30 min at 45°C, with gentle rotation.

After prehybridization solution removal, the hybridization solution (denatured at 95°C for 3 min) was pipetted into the hybridization chamber, again leaving a small air bubble and microarray was then incubated for about 16 h (overnight) at 45°C, with gentle rotation.

Unhybridized target removal was performed with 6 washing steps, consisting in rinsing the chamber with the washing solution: the first washing step was performed with 6X SSPET (preheated to 45°C) 5 min at 45°C, then at room temperature for 1 min each, with 3X SSPET, 0.5X SSPET, PBST and two final washes with PBS. Microarray slides were dipped in imaging solution, covered with LifterSlip™, and then scanned with Axon 4300B Microarray Scanner and GenePix® Pro 7 Software.

e) Microarray stripping

The CombiMatrix microarray can be stripped of hybridized targets and re-used several times. Hybridized RNA is removed by using strong chemicals and high temperature. Stripping was performed after imaging as described in the protocol Stripping and Preparation of CombiMatrix 90K Microarrays for Re-hybridization (PTL025) and stripping quality was checked by scanning the chip with same scan settings as those used for imaging the array.

f) Data processing and Statistical analysis

Raw data were median centered, log transformed and quantile normalized by using the SPSS (Statistical Package for Social Sciences) statistical package release 18.0. Probe signals with a variability coefficient higher than 0.5 as well as spikes and factory probes were filtered out. Also, probes with signal intensities in the upper most and lower most 10% of fluorescence intensity distributions were deleted.

The signals differentially expressed were identified using Linear Models for Microarray Data (LIMMA) analysis (Smyth 2004), implemented in the TMEV (TIGR Multiple Experiment Viewer) package version 4.9.0 (http://www.tigr.org/software/tm4/; Saeed *et al*. 2003). This is a flexible frequently used method, based on the fitting of each gene to a linear model, and an empirical

Bayes for error smoothing. LIMMA uses a moderated t-statistic to test the average difference in log expression levels between the two groups for each gene. The moderated t-statistic is the average log ratio divided by a standard error which is calculated using information from the replicates of the given gene and information from across all genes. Once all possible tests have been done, a variety of multiple comparison procedures are available to control for the false discovery rate of the experiment (Smyth 2004).

A functional interpretation of the set of differentially expressed genes was obtained by GO categorization and enrichment analysis using BLAST2GO (http://www.blast2go.com/; Conesa *et al*. 2005) integrated by categorizations performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID v 6.7) software (Huang *et al*. 2009).

Experimental validation by quantitative real-time PCR (qRT-PCR)

TCs expression profiles of genes considered to be key control points for potential PCP degradation in *D. melanogaster* were validated by qRT-PCR.

Same aRNAs used for microarray assays were used as starting material, cDNA synthesis was carried out with the SuperScript III reverse kit adding for each reaction 1 µg aRNA, 250 ng Random Primers, 1 µl dNTPs Mix (10mM), nuclease free water up to 13 µl. The mixture was heated to 65°C for 5 min, cooled on ice 1 min, and then 4 µl of 5x First Strand Buffer, 1 µl of 0.1M DTT, 1 µl of SuperScript III RT (200 units/µl) were added. The 20 µl reaction was incubated at 25°C for 5 min, followed by an incubation at 55°C for 1 h. The reaction was inactivated by heating at 70°C for 15 min.

Gene specific primer pairs were designed with Universal ProbeLibrary (UPL) ProbeFinder, a web-based software tool. Based on the user-defined target information the software designs real-time PCR specific primer pairs. ProbeFinder assay design software is based on Primer3 software using optimized settings as default, to give best results. All primer pairs designed by ProbeFinder are checked with an in silico PCR algorithm. The algorithm searches the selected transcriptome for possible mispriming sites both primers.

The selected primer pair sequences are listed in table 1.

Primer pairs were validated using a standard curve over a template dilution range, 10^{-1}-10^{-3} (R2 > 0.98; slope close to -3.23). For each TC three biological replicates for treatments were considered and for each experiment three technical replicates were performed, and two negative controls were included.

Normalization of gene expression data is used to correct sample-to-sample variation. Starting material obtained from different individuals usually varies in tissue mass or cell number, RNA integrity or quantity, or experimental treatment. Therefore, real-time PCR results are usually normalized against a control gene that may also serve as a positive control for the reaction. The ideal control gene should be equally expressed regardless of experimental conditions, including different tissue or cell types, developmental stage, or sample treatment. Because there is no one gene that meets this criterion for every experimental condition, the expression stability of a control gene for the specific requirements of an experiment should be validated prior to its use for normalization (Schmittgen and Zakrajsek 2000). For this project as endogenous control the gene encoding for the β tubulin 56D (reported in DFCI as TC236999 and in FlyBase as FBtr0086537) was chosen on the basis of the microarray results. This gene is among the most commonly employed standards used to normalize gene expression in *Drosophila* stressed by xenobiotic treatments (Moskalev *et al*. 2014).

All amplifications were performed in 25 μl reaction volumes using a Power SYBR® Green PCR Master Mix (Applied Biosystems) in a 7900HT Fast Real-Time PCR System (Applied Biosystems), according to the manufacturer's instructions. Each primer final concentration was 300 nM, and cDNA was diluted 10^{-1}.

PCR conditions commonly used included an initial step at 95°C for 10 min, denaturation at 95°C for 15 seconds, annealing/extension for 1 min at 60°C. The total number of PCR cycles used was 40. Finally, a melting curve step was used to determine if the primers amplified one specific product. The right PCR product was identified using the melting curve. If the melting curve showed one single peak at the right melting temperature predicted for the PCR product, then the expression data were further analyzed.

Table 1. Primer pairs used to perform qRT-PCR analysis.

Annotation	Flybase ID	DFCI code	Sequence
Cyp6a8	FBgn0013772	NP920106_Fw	5'-TTGGTGTGAAAGACGGAATCT-3'
		NP920106_Rev	5'-TGGCTTTTAGTTGAGAGTTTTCAG-3'
Cyp6d2	FBgn0034756	TC227621_Fw	5'-CCATCGCTTCGATTCAAATAA-3'
		TC227621_Rev	5'-GACCCTCTCCAAAAGGCATA-3'
Cyp6a2	FBgn0000473	TC227973_Fw	5'-TTTATGTGGCCGGATTTGAG-3'
		TC227973_Rev	5'-GAGCCAACTCATACAGGCAGT-3'
Cyp6a17	FBgn0015714	TC228776_Fw	5'-CATCATTCCAAACCGCAGA-3'
		TC228776_Rev	5'-CGAGACCCTCGTGCACTT-3'
Cyp28d2	FBgn0031688	TC228845_Fw	5'-GATGGACCTCGTCACTGTCC-3'
		TC228845_Rev	5'-CCTTGATTTCAAAGTTCCTCAAG-3'
Cyp12e1	FBgn0037817	TC228865_Fw	5'-CTGACAAGCAGGCTCGACT-3'
		TC228865_Rev	5'-ATGGTCAGCGATGAGTCCTT-3'
Cyp4d2 non coding region	FBgn0011576	TC236293_Fw	5'-GCTCTGAGTTTTCCATCTAACTTTG-3'
		TC236293_Rv	5'-GCACAATATGCACAGCATTAAAC-3'
Cyp4d2	FBgn0011576	TC228895_Fw	5'-GCTGTGGGATTTCCTCTGG-3'
		TC228895_Rv	5'-AGACCGCGGTACATGAGC-3'
Cyp12a5	FBgn0038680	TC254277_Fw	5'-CTGTGCCTCGCCAAGAAT-3'
		TC254277_Rev	5'-GTTGGGCAGCACCTTCATA-3'
Cyp4d14	FBgn0023541	TC256061_Fw	5'-CGCAATGCTCGAGATGAAG-3'
		TC256061_Rv	5'-GGCAGCAACTCAAAGTGTCTC-3'
GstD4	FBgn0010040	TC233952_Fw	5'-ACAATGGATTCGCCATTTG-3'
		TC233952_Rev	5'-GGAGTCGTCCTTGCCGTA-3'
GstD9	FBgn0038020	TC234547_Fw	5'-CCGGAAGTGGTTAGGTGGTA-3'
		TC234547_Rev	5'-GCCCTCCCAGTTCTCCTC-3'
GstS1	FBgn0010226	TC239235_Fw	5'-CAGCTGAGGGAGCACCAC-3'
		TC239235_Rev	5'-CGTGTAGCTGTGCTTGATGG-3'
Fmo-I	FBgn0034943	TC229242_Fw	5'-GCAGTGCATCAACATCAGGA-3'
		TC229242_Rev	5'-CAAACGTAAAACGGCAGTCC-3'
Mdr50	FBgn0010241	NP029605_Fw	5'-GCGCAATGTTTCTATGACCA-3'
		NP029605_Rev	5'-ATCGAAAAGAATGGGCTCCT-3'
CG4562	FBgn0038740	TC222449_Fw	5'-GCCTCCAAGCAGAAGTTGTT-3'
		TC222449_Rev	5'-TCAGTGGGGATGTCTGCTG-3'
β-Tubulin at 56D	FBgn0003887	Tub56D_F	5'-GCAGATGCTGAACATCCAGA-3'
		Tub56D_Rev	5'-TTGTTGGGGATCCATTCG-3'

The data obtained was then expressed as fold difference on the basis of CT values using the ΔΔCT method (Livak *et al.* 2001). Relative expression data was converted in fold change variations compared to the average relative expression in the control treatment. Significance of variation in the expression of mRNAs was assayed by

Student's t test coupled with 1000 re-sampling by a bootstrap procedure using SPSS statistical package release 18.0.

HPLC analysis

HPLC analyses were performed to determine the uptake of PCP in *Drosophila* resistant larvae after feeding on PCP supplied diet.

a) Treatments

Resistant flies (about 10 males and 10 females) of two different strains were allowed to oviposit for 24 h on diet supplied with 2000 ppm of PCP, after 8 days third instar larvae were taken out from the diet, rinsed with PBS, and frozen. Diets were collected and frozen too. Three replicates for each strains and control (diet without larvae) were carried out.

b) Sample preparation

Samples of diet and larvae were freeze dried and PCP was extracted with 80 ml/g or 40 ml/g of EtOH 50% for larval tissues and diets respectively. Samples were sonicated at room temperature for about 2 h; the samples were evaporated at 60°C using a rotavapor (Laborota 4000, Heidolph) under reduced pressure, and re-suspended in methanol HPLC grade. Samples were filtered through an ultra membrane filter (pore size 0.45 µm) prior to HPLC analysis.

c) HPLC run

The HPLC system (Shimadzu Corporation, Kyoto, Japan) was equipped with a Shimadzu LC-20 AT pump, a diode array detector Shimadzu SPD M 20 AVP and a Rheodyne Model 7725 injector with a loop volume of 20 µl. The chromatographic analysis was carried out in isocratic conditions using a C-18 column at 25°C. Running conditions included methanol: water (80:20 v/v) as mobile phase, a flow rate of 1 ml/min and analytical wave length of 220 mm. PCP sodium salt was used for the calibration curve (from 1000 ppm to 0.5 ppm). The peak area was calculated with the Winchrom integrator. Statistical analysis of PCP content in diet after larval feeding was performed by Anova and multiple range test.

RESULTS

LC_{50} determination

LC_{50} values were based on the cumulative mortality observed at the end of a desired exposure period (48h). LC_{50} values and 95% confidence intervals (CI) were calculated by probit analysis transformation method and by plotting graph of percent mortality (probit value) against PCP concentrations (ppm). LC_{50} calculated through probit method at 95% confidence limit was found to be 5771 ppm (5.8 g/l). The estimated mortality percentages and confidence intervals are listed in table 2. Graphic representation of the probit transformation for PCP is presented in Fig. 1.

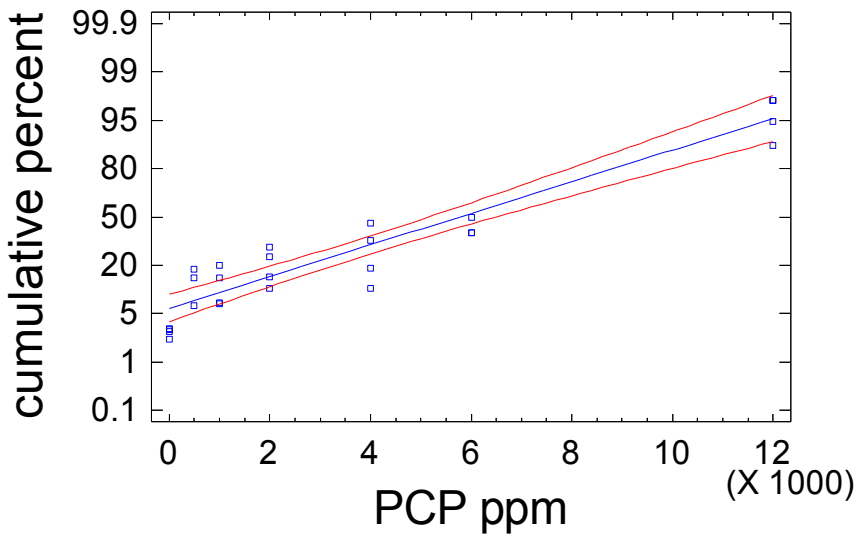

Fig. 1. Probability plot for the fitted probit regression model. The percentage of deviance in mortality explained by the model equals 86.2%.

Table 2. 48-h percent mortalities corresponding to PCP concentrations and their confidence limits for *D. melanogaster* according to Finney's Probit Analysis.

		95% Confidence limit	
% mortality	PCP ppm	Lower	Upper
10	699	-	1400
20	2440	1773	3029
25	3101	2486	3675
30	3695	3109	4273
40	4768	4192	5395
50	**5771**	5158	6490
60	6773	6092	7617
70	7846	7066	8848
75	8440	7598	9537
80	9101	8186	10309
90	10842	9719	12355
95	12280	10974	14055
99	14977	13316	17257
99.5	15964	14171	18432
99.9	18000	15930	20857

RNA extraction

From the bioanalyzer analysis the *D. melanogaster* rRNA profiles consistently showed two very close rRNA peaks instead of two clearly separated peaks expected for the two large rRNA species, 18S and 28S (Fig. 2). This result is consistent with those reported by other insect studies (Applebaum *et al.* 1966; Greenberg 1969; Gillespie *et al.* 2006) and does not represent RNA degradation. In fact, the 28S rRNA of most insects consists of two separate fragments, that are hydrogen-bonded together, and during the denaturation step pretreatment disruption of these hydrogen bonds occurs: the two fragments co-migrate with the 18S rRNA. The 5.8S rRNA is also base-paired to this 28S complex and is likewise released in denaturing conditions. Therefore, the

typically observed insect rRNA profile reflects endogenously present components of the insect rRNA rather than degradation during the extraction process (Winnebeck *et al.* 2010).

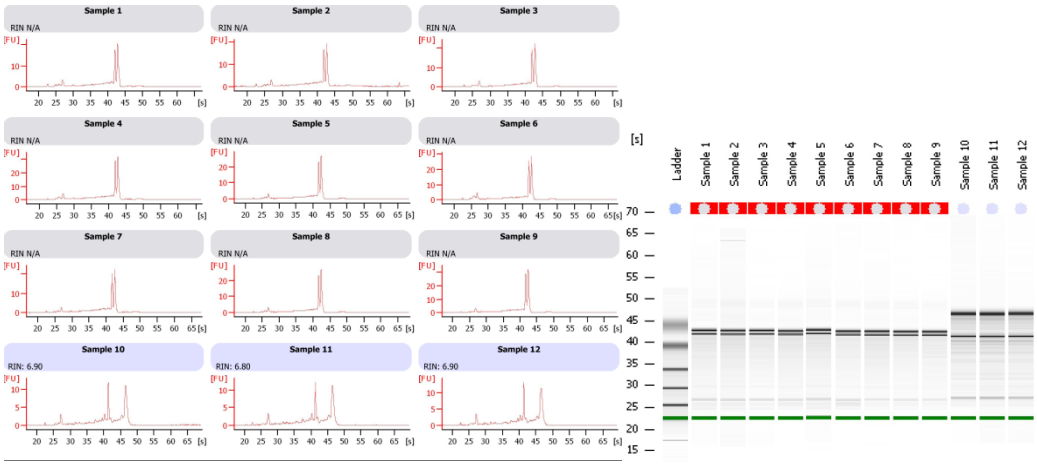

Fig. 2. Bioanalyzer analysis of rRNA. Samples from 1 to 9 are from *D. melanogaster*, and from 10 to 12 are from tomato plant.

Expression profiling

A 90K Combimatrix Chip was used to analyze the *D. melanogaster* trancriptome. It includes 92,500 probes, corresponding to 34,500 TCs; annotation by FlyBase (flybase.org; St. Pierre *et al.* 2014) was performed allowing to match TCs with 12,564 unique genes. Differentially expressed genes (DEs) were identified by LIMMA procedure. In particular, 811 mRNAs out of 25,000 analyzed were recovered as differentially expressed ($p \leq 0.05$) in PCP treated larvae. 81.7% (663) of DE genes were functionally annotated, while for 15.3% no annotation was recovered, because no correspondence was found in FlyBase database.

The t-statistic used in LIMMA assures that the final list of genes includes genes that are consistently different between groups. LIMMA will choose a gene that is moderately different and consistent, before it will choose a gene that is extremely different in each sample, but whose expression is highly variable. The fold change expression of the differentially expressed genes ranged between values -1.2 and 2.71.

Statistically significant downregulated genes ($p<0.05$) were 86 and 241 in 20 and 2000 ppm treatments, respectively, with all 20 ppm dose transcripts except three, shared with those of the higher dose treatment. The statistically significant upregulated genes ($p<0.05$) were 62 and 327 in 20 and 2000 ppm treatments, respectively, with all 20 ppm dose transcripts except two, shared with those of the higher dose treatment.

The enrichment analysis allowed to highlight the over-represented GO terms compared to the background gene list from the combimatrix chip design. The analysis revealed that Biological Processes (BP) (Fig. 3) and Molecular Functions (MF) (Fig. 4) were significantly over-represented among the DE gene set in the PCP challenged larvae respect to control. Within BP the GO categories of Anatomical Structure Development and Single Organism Developmental and Cellular Process were significantly enriched, while within the MF GO categories the monooxygenase activity group was particularly interesting.

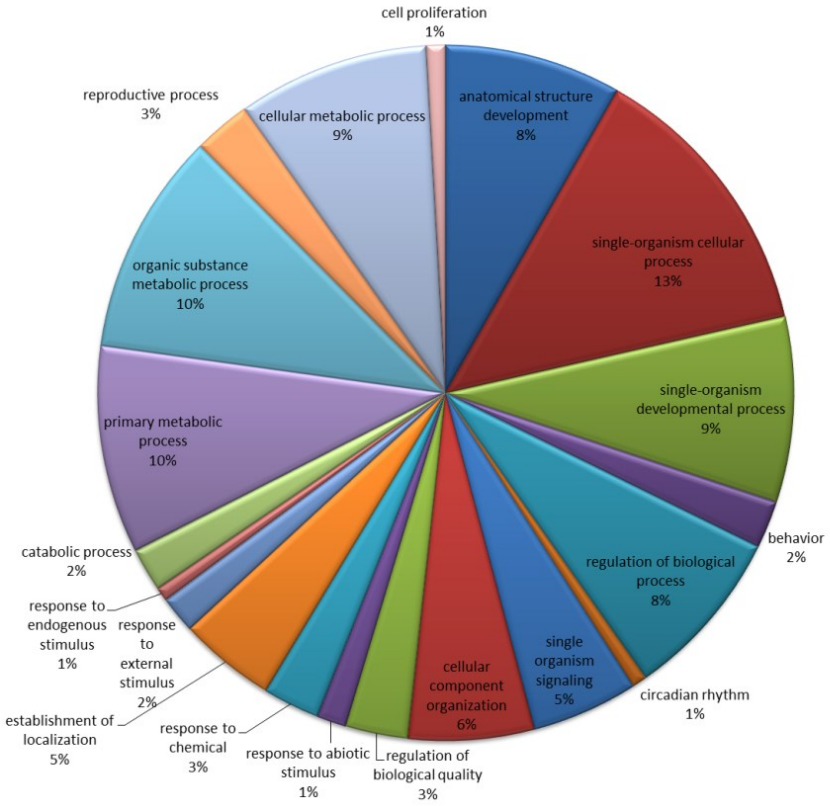

Fig. 3. Distribution of GO IDs at the 3rd level based on the participation in Biological Process of DE genes.

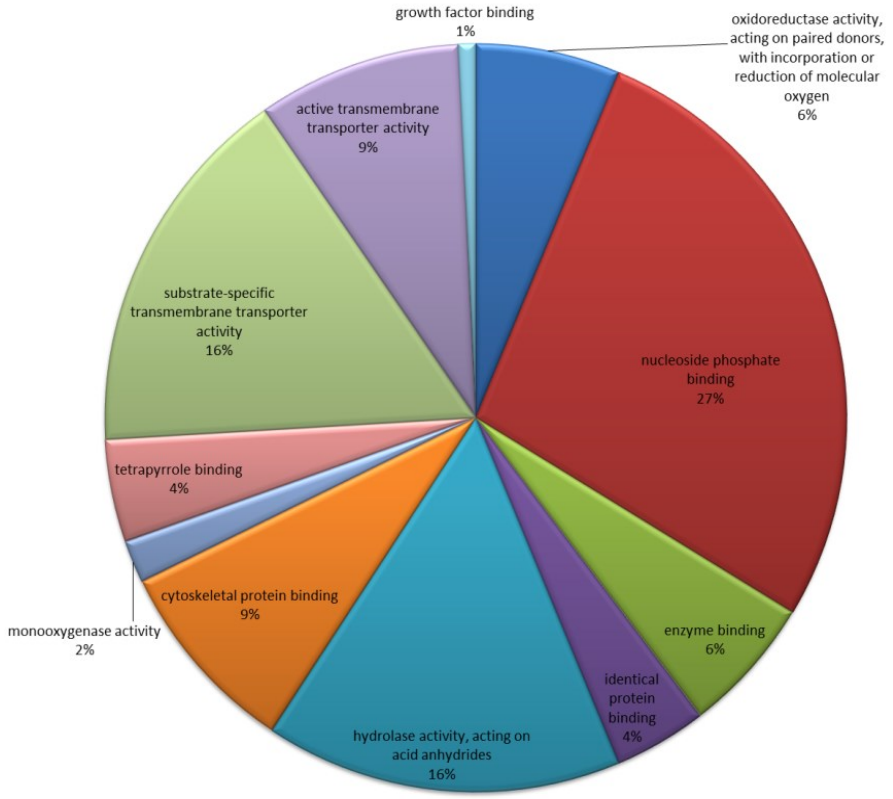

Fig. 4. Distribution of GO IDs at the 4th level based on the participation in Molecular Functions of DE genes.

In tables 3 and 4 the most enriched GO terms for Biological Processes for upregulated genes in 20 and 2000 ppm treatments are presented. The analysis for BP categories revealed activities involving protein and lipid metabolism in 20 ppm PCP treatments, probably to meet an increased energy demand in response to the PCP stress. With the 2000 ppm treatment, other than the previous ones, more specific categories appeared to be enriched, such as biogenic amine metabolic process, transport, and response to chemical stimulus.

Table 3. Enriched Biological Process GO terms and functional annotation clustering of upregulated genes after 20 ppm PCP treatment, with $p<0.05$.

GO ID		Number of genes	%	Fold Change
Cluster 1 (Enrichment Score: 1.7)				
GO:0044267	cellular protein metabolic process	13	21.7	2.30
GO:0006464	protein modification process	9	15.0	2.80
GO:0043412	biopolymer modification	9	15.0	2.70
GO:0043687	post-translational protein modification	7	11.7	2.70
GO:0044260	cellular macromolecule metabolic process	16	26.7	1.60
GO:0019538	protein metabolic process	14	23.3	1.70
Cluster 2 (Enrichment Score: 1.64)				
GO:0009966	regulation of signal transduction	5	8.3	3.70
GO:0010646	regulation of cell communication	5	8.3	3.50
Cluster 3 (Enrichment Score: 1.32)				
GO:0007304	chorion-containing eggshell formation	3	5.0	8.20
GO:0030703	eggshell formation	3	5.0	8.20
Not Clustered				
GO:0060191	regulation of lipase activity	2	3.3	46.00
GO:0010517	regulation of phospholipase activity	2	3.3	46.00

Table 4. Enriched GO Biological Process terms and functional annotation clustering of upregulated genes after 2000 ppm PCP treatment, with $p<0.05$.

	GO ID	Number of genes	%	Fold Change
	Cluster 1 (Enrichment Score: 2.07)			
GO:0006576	biogenic amine metabolic process	7	2.3	8.80
GO:0006575	cellular amino acid derivative metabolic process	7	2.3	5.00
GO:0042439	ethanolamine and derivative metabolic process	3	1	15.00
GO:0006584	catecholamine metabolic process	3	1	11.00
GO:0018958	phenol metabolic process	3	1	11.00
	Cluster 2 (Enrichment Score: 1.99)			
GO:0044106	cellular amine metabolic process	13	4.2	3.30
GO:0006519	cellular aa and derivative metabolic process	13	4.2	2.90
GO:0009309	amine biosynthetic process	6	1.9	5.60
GO:0009308	amine metabolic process	15	4.8	2.00
GO:0006520	cellular amino acid metabolic process	9	2.9	2.60
GO:0006082	organic acid metabolic process	12	3.9	2.00
GO:0019752	carboxylic acid metabolic process	12	3.9	2.00
GO:0043436	oxoacid metabolic process	12	3.9	2.00
	Cluster 3 (Enrichment Score: 1.8)			
GO:0044271	nitrogen compound biosynthetic process	16	5.1	3.00
GO:0006163	purine nucleotide metabolic process	9	2.9	3.00
GO:0006164	purine nucleotide biosynthetic process	8	2.6	2.80
GO:0046483	heterocycle metabolic process	12	3.9	2.10
GO:0015985	energy coupled proton transport	5	1.6	3.90
GO:0015986	ATP synthesis coupled proton transport	5	1.6	3.90
GO:0055085	transmembrane transport	7	2.3	2.70
GO:0034220	ion transmembrane transport	5	1.6	3.80
	Cluster 4 (Enrichment Score: 1.61)			
GO:0016042	lipid catabolic process	5	1.6	4.90
GO:0044255	cellular lipid metabolic process	10	3.2	2.40
GO:0006629	lipid metabolic process	13	4.2	1.90
	Not clustered			
GO:0008152	metabolic process	122	39.2	1.2
GO:0007611	learning or memory	6	1.9	3.4
GO:0051234	establishment of localization	44	14.1	1.3
GO:0007612	learning	5	1.6	4
GO:0007163	establishment or maintenance of cell polarity	7	2.3	2.8
GO:0045165	cell fate commitment	11	3.5	2.1
GO:0055114	oxidation reduction	22	7.1	1.6
GO:0042221	response to chemical stimulus	15	4.8	1.8
GO:0048699	generation of neurons	18	5.8	1.7
GO:0007399	nervous system development	24	7.7	1.5
GO:0006457	protein folding	7	2.3	2.7
GO:0006810	transport	42	13.5	1.3

The MF enrichment analysis was carried out with a lower stringency compared to the enrichment analysis for BP, in order to minimize the exclusion of interesting GO terms. The MF enrichment for the 20 ppm treatment resulted in very few clusters, involving signaling and transport activities (table 5), while the most enriched GO terms for MF in the 2000 ppm PCP treatment included response to wound healing and neurogenesis (tubulin-tyrosine ligase activity), monooxygenase (many cytochrome P450s), transcriptional repressor, transporter (ABC and vesicular transporters), transferase (GSTs and UGTs), and catalytic activities (including transcripts involved in biogenic amine - acting as neurotransmitters) (table 6).

Table 5. Enriched GO Molecular Function terms and functional annotation clustering of upregulated genes after 20 ppm PCP treatment, with $p<0.5$.

GO ID		Number of genes	%	Fold Change
Cluster 1 (Enrichment Score: 0.57)				
GO:0005525	GTP binding	3	5.0	4.00
GO:0032555	purine ribonucleotide binding	6	10.0	1.50
GO:0017076	purine nucleotide binding	6	10.0	1.40
Cluster 2 (Enrichment Score: 0.53)				
GO:0022857	transmembrane transporter activity	6	10.0	1.90
GO:0008324	cation transmembrane transporter activity	3	5.0	1.80
Cluster 3 (Enrichment Score: 0.33)				
GO:0046872	metal ion binding	9	15.0	1.20
GO:0043169	cation binding	9	15.0	1.20
Cluster 4 (Enrichment Score: 0.3)				
GO:0060089	molecular transducer activity	4	6.7	1.50
GO:0004871	signal transducer activity	4	6.7	1.50
Not Clustered				
GO:0009055	electron carrier activity	3	5.0	3.4
GO:0005509	calcium ion binding	3	5.0	2.8
GO:0016773	phosphotransferase activity	3	5.0	2.0
GO:0005488	binding	34	56.7	1.0
GO:0003824	catalytic activity	19	31.7	1.1

Table 6. Enriched GO Molecular Function terms and functional annotation clustering of upregulated genes after 2000 ppm PCP treatment, with p<0.5.

	GO ID	Number of genes	%	Fold Change
Annotation Cluster 1 (Enrichment Score: 1.88)				
GO:0004497	monooxygenase activity	11	3.5	4.3
GO:0009055	electron carrier activity	12	3.9	2.6
GO:0046906	tetrapyrrole binding	9	2.9	2.8
GO:0020037	heme binding	9	2.9	2.8
GO:0005506	iron ion binding	11	3.5	1.9
GO:0016491	oxidoreductase activity	19	6.1	1.3
Annotation Cluster 2 (Enrichment Score: 1.07)				
GO:0004835	tubulin-tyrosine ligase activity	3	1.0	11.0
GO:0016881	acid-amino acid ligase activity	6	1.9	2.4
GO:0016874	ligase activity	9	2.9	1.7
Annotation Cluster 3 (Enrichment Score: 1.05)				
GO:0016853	isomerase activity	7	2.3	2.9
GO:0003755	peptidyl-prolyl cis-trans isomerase activity	3	1.0	4.5
Annotation Cluster 4 (Enrichment Score: 0.84)				
GO:0016564	transcription repressor activity	7	2.3	2.8
GO:0016563	transcription activator activity	4	1.3	1.9
Annotation Cluster 5 (Enrichment Score: 0.57)				
GO:0004091	carboxylesterase activity	5	1.6	2.1
GO:0016298	lipase activity	4	1.3	2.0
Annotation Cluster 6 (Enrichment Score: 0.55)				
GO:0005215	transporter activity	27	8.7	1.4
GO:0008324	cation transmembrane transporter activity	13	4.2	1.5
GO:0022857	transmembrane transporter activity	21	6.8	1.3
GO:0015075	ion transmembrane transporter activity	14	4.5	1.2
GO:0046873	metal ion transmembrane transporter activity	5	1.6	1.5
GO:0022804	active transmembrane transporter activity	10	3.2	1.2
GO:0005261	cation channel activity	4	1.3	1.6
Annotation Cluster 7 (Enrichment Score: 0.54)				
GO:0042626	ATPase activity, coupled to transmembrane movement of substances	6	1.9	2.0
GO:0015399	primary active transmembrane transporter activity	6	1.9	1.7
GO:0008553	hydrogen-exporting ATPase activity, phosphorylative mechanism	3	1.0	2.8

GO:0022890	inorganic cation transmembrane transporter activity	5	1.6	1.7
GO:0015078	hydrogen ion transmembrane transporter activity	4	1.3	1.9
GO:0015077	monovalent inorganic cation transmembrane transporter activity	4	1.3	1.9
GO:0022804	active transmembrane transporter activity	10	3.2	1.2
Annotation Cluster 8 (Enrichment Score: 0.53)				
GO:0015020	glucuronosyltransferase activity	3	1.0	3.6
GO:0016757	transferase activity, transferring glycosyl groups	6	1.9	1.6
Annotation Cluster 9 (Enrichment Score: 0.48)				
GO:0043167	ion binding	45	14.5	1.1
GO:0046872	metal ion binding	43	13.8	1.1
GO:0046914	transition metal ion binding	34	10.9	1.1
Annotation Cluster 10 (Enrichment Score: 0.42)				
GO:0008237	metallopeptidase activity	6	1.9	1.6
GO:0008238	exopeptidase activity	4	1.3	1.8
GO:0008233	peptidase activity	17	5.5	1.1
Annotation Cluster 11 (Enrichment Score: 0.42)				
GO:0016773	phosphotransferase activity, alcohol group as acceptor	10	3.2	1.3
GO:0004674	protein serine/threonine kinase activity	6	1.9	1.3
Annotation Cluster 12 (Enrichment Score: 0.37)				
GO:0032183	SUMO binding	3	1.0	2.1
GO:0032182	small conjugating protein binding	3	1.0	2.0
Annotation Cluster 13 (Enrichment Score: 0.37)				
GO:0050662	coenzyme binding	5	1.6	1.5
GO:0050660	FAD binding	3	1.0	2.0
GO:0048037	cofactor binding	6	1.9	1.4
Not clustered				
GO:0003824	catalytic activity	111	35.7	1.2
GO:0016829	lyase activity	6	1.9	1.9
GO:0051082	unfolded protein binding	4	1.3	2.4
GO:0005509	calcium ion binding	8	2.6	1.4
GO:0016787	hydrolase activity	48	15.4	1.1
GO:0016765	transferase activity, transferring alkyl or aryl (other than methyl) groups	3	1.0	2.2
GO:0042802	identical protein binding	5	1.6	1.5
GO:0030234	enzyme regulator activity	9	2.9	1.2
GO:0015370	solute:sodium symporter activity	3	1.0	1.9

Enrichment analysis for KEGG pathways for 20 ppm PCP treatment did not recover any pathway, while for 2000 ppm pathways such as those involved in signaling, drug, aromatic compounds and glutathione metabolism, ABC transporters resulted enriched (table 7).

Table 7. KEGG pathways influenced by 2000 ppm PCP treatment, with p<0.25.

	KEGG pathway	Number of genes	%	Fold Change
dme00982	Drug metabolism	6	1.9	2.4
dme00903	Limonene and pinene degradation	7	2.3	2.2
dme04310	Wnt signaling pathway	6	1.9	2.2
dme00564	Glycerophospholipid metabolism	5	1.6	2.1
dme02010	ABC transporters	2	0.6	8.9
dme00980	Metabolism of xenobiotics by P450	5	1.6	2.1
dme00565	Ether lipid metabolism	3	1	3.1
dme04320	Dorso-ventral axis formation	3	1	3.1

From the Gene enrichment and the Functional annotation analyses of the upregulated sequences, several genes were selected as potentially associated with PCP response and degradation according to the published literature (Li *et al.* 2007, Chahine and O'Donnell 2011). In order to validate the expression data of microarray experiments, a set of 16 upregulated transcripts was tested by qRT-PCR. In particular all the nine CYPs and the Flavin-monooxygenase-I (Monooxygenase activity), all the three GSTs (Catalytic activity), and two out of the seven ABC transporters (Transmembrane transporter activity). The complete set of upregulated ABC transporters is presented in table 8.

Table 8. ABC transporters in the upregulated gene set (GO:0042626).

Gene symbol	FlyBase id	FC20 mean	FC20 SE	FC2000 mean	FC2000 SE	deltaFC 2000 vs 20
Multi drug resistance 50	FBgn0010241	0.178	0.179	0.678**	0.133	0.501
Dmel_CG4562	FBgn0038740	0.074	0.099	0.532**	0.054	0.458
Dmel_CG5421	FBgn0032434	0.098	0.097	0.460**	0.084	0.362
Vacuolar H[+] ATPase 16kD subunit	FBgn0004145	0.037	0.007	0.311**	0.091	0.274
ATP synthase subunit a	FBgn0013672	0.022	0.045	0.293*	0.058	0.271
Dmel_CG12602	FBgn0032373	0.012	-	0.605**	0.089	0.593
Dmel_CG32089	FBgn0028668	-0.047	0.032	0.165*	0.084	0.212

Fold changes (FC) for microarray expressions and qRT-PCR of chosen genes are reported in tables 9 and 10 respectively.

Table 9. Microarrays expression fold changes (FC) of candidate genes involved in PCP detoxification. Statistically significant difference at $p<0.01$ (**), $p<0.05$ (*).

Annotation	FlyBase id	FC20 mean	FC20 SE	FC2000 mean	FC2000 SE	deltaFC 2000 vs 20
Cyp6d2	FBgn0034756	0.067	0.0125	0.317*	0.130	0.250
Cyp6a2	FBgn0000473	0.242	0.0164	0.887**	0.258	0.645
Cyp6a17	FBgn0015714	0.122	0.1515	0.444*	0.129	0.322
Cyp28d2	FBgn0031688	0.247	0.096	0.853**	0.248	0.606
Cyp12e1	FBgn0037817	-0.002	0.136	0.577**	0.133	0.579
Cyp12a5	FBgn0038680	0.354*	0.184	0.360*	0.119	0.006
Cyp4d2 non coding region	FBgn0011576	-0.129	0.029	-0.360**	0.088	-0.227
Cyp4d14	FBgn0010226	0.285*	0.119	0.648**	0.086	0.363
Cyp6a8	FBgn0013772	0.138	0.100	1.595**	0.461	1.457
Fmo-1	FBgn0034943	-0.043	0.059	0.308*	0.085	0.351
GstD9	FBgn0010040	-0.132	0.041	0.313**	0.210	0.445
GstS1	FBgn0010226	0.201	0.012	0.386*	0.008	0.185
Mdr50	FBgn0010241	0.178	0.179	0.678**	0.133	0.501
CG4562	FBgn0038740	0.074	0.099	0.532**	0.054	0.458

Table 10. Change in the expression of candidate genes according to PCP treatments as assessed by qRT-PCR. Statistically significant difference at one-sample Student's t test coupled with 10,000 bootstrap re-sampling (*)

Target	Treatment	Fold change out of the control		95% CI (10000 bootstrap re-sampling)	
		Mean	SE	lower bond	upper bond
Cyp6d2	T20	0.023	0.340	-0.365	0.412
	T2000	0.509*	0.479	0.107	0.936
Cyp6a2	T20	-0.031	0.303	-0.351	0.333
	T2000	1.218*	0.527	0.671	1.765
Cyp6a17	T20	0.359	0.305	-0.032	0.759
	T2000	1.073	0.300	0.741	1.462
Cyp28d2	T20	0.498*	0.099	0.398	0.622
	T2000	1.567*	0.081	1.474	1.660
Cyp12e1	T20	-0.750*	0.493	-1.360	-0.099
	T2000	0.360*	0.178	0.157	0.564
Cyp4d2 (non coding region)	T20	-0.124	0.206	-0.297	0.059
	T2000	-0.223	0.317	-0.587	0.141
Cyp4d2	T20	-0.184	0.211	-0.428	0.059
	T2000	0.126	0.307	-0.247	0.531
Cyp4d14	T20	0.428*	0.233	0.171	0.691
	T2000	1.391*	0.075	1.303	1.471
Cyp6a8	T20	0.157	0.419	-0.289	0.603
	T2000	3.674*	0.418	3.247	4.189
GstD4	T20	-0.132	0.381	-0.591	0.267
	T2000	0.521*	0.511	0.000	1.151
GstD9	T20	-0.234	0.651	-0.961	0.494
	T2000	-0.981*	0.504	-1.501	-0.365
GstS1	T20	-1.740*	1.431	-3.513	-0.291
	T2000	-0.120	0.146	-0.288	0.039
Fmo-I	T20	-0.023	0.130	-0.170	0.123
	T2000	0.249	0.264	-0.048	0.546
Mdr50	T20	-0.766	1.004	-1.883	0.352
	T2000	1.445*	0.290	1.155	1.806
CG4562	T20	0.517*	0.217	0.261	0.748
	T2000	1.017*	0.249	0.734	1.300

Most of the obtained qRT-PCR results were concordant with the microarray data. A correlation analysis between microarray and qRT-PCR expression data (Fold Change average of the expression value) yielded a Pearson's value of 0.80 ($p \leq 0.05$). The qRT-PCR approach confirmed that exposure to 20 and 2000 ppm PCP is associated with a significant increase in relative mRNA expression for six P450 genes (Fig. 5). In particular, the *Cyp6a8* and *Cyp6a2* genes increased their mRNA expression levels when larvae were challenged with PCP, with the highest expression reached at 2000 ppm PCP. On the whole, the 20 ppm treatment showed a minor effect on the expression of those CYP genes compared with the 2000 ppm. For *Cyp4d2* a non-coding region was analyzed with qRT-PCR, since from the microarray it resulted differentially expressed, and to achieve a more complete information on the behavior of this gene the analysis of a second region (in the coding portion) was included. Real time PCR did not confirm significant change in the expression of any of the two *Cyp4d2* analyzed regions. Similarly, the qRT-PCR approach did not confirm upregulation of the flavin-monooxygenase gene (*fmo-1*).

The Glutathione S-Tranferase (GST) genes showed an expression profile by qRT-PCR analysis that did not completely confirm microarray data. In fact *GSTD4* was the only one showing the same upregulated trend in both analyses. For *GSTD9* and *GSTS1* qRT-PCR results were not in accordance with microarray data, showing a significant downregulation in 2000 and 20 ppm treatments respectively.

Among all the transporters within differentially expressed genes, two were tested by qRT-PCR, *mdr*50 (multi drug resistance) and the transcript corresponding to the FlyBase code CG4562. For both genes qRT-PCR analysis confirmed a significant upregulation in response to 2000 ppm treatment, and moreover showed an increased expression for CG4562 even at the low dose treatment.

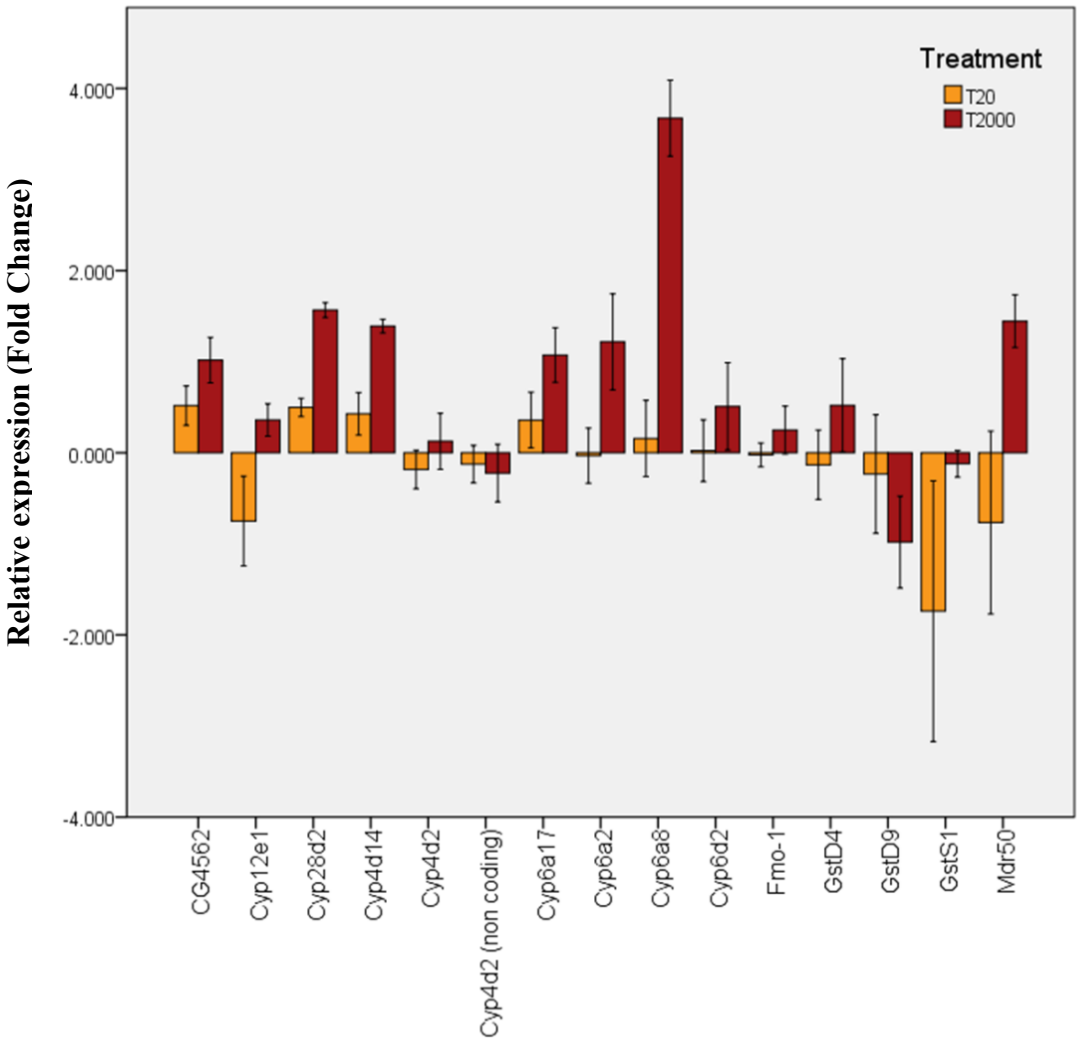

Fig. 5. Quantification of relative expression in PCP treated larvae respect to the control. Data are shown as means ± SE.

HPLC analysis

Two resistant *D. melanogaster* strains have been selected in laboratory to obtain a deeper knowledge of the effects of PCP on *D. melanogaster*, and to understand the genetic bases of the resistance mechanisms and PCP insect metabolic pathways. For this purpose, HPLC analyses were performed to compare the amount of PCP in *Drosophila* diet, with and without larval feeding and for determining the uptake of PCP in resistant larvae after feeding on PCP supplied diet. Typical chromatograms

obtained from the HPLC run of the extracts from the diet and from the larval tissue are shown in Figg 6 and 7 respectively. HPLC traces show only one single peak, identified as PCP on the basis of the retention time of the standard (about 9.2 minutes).

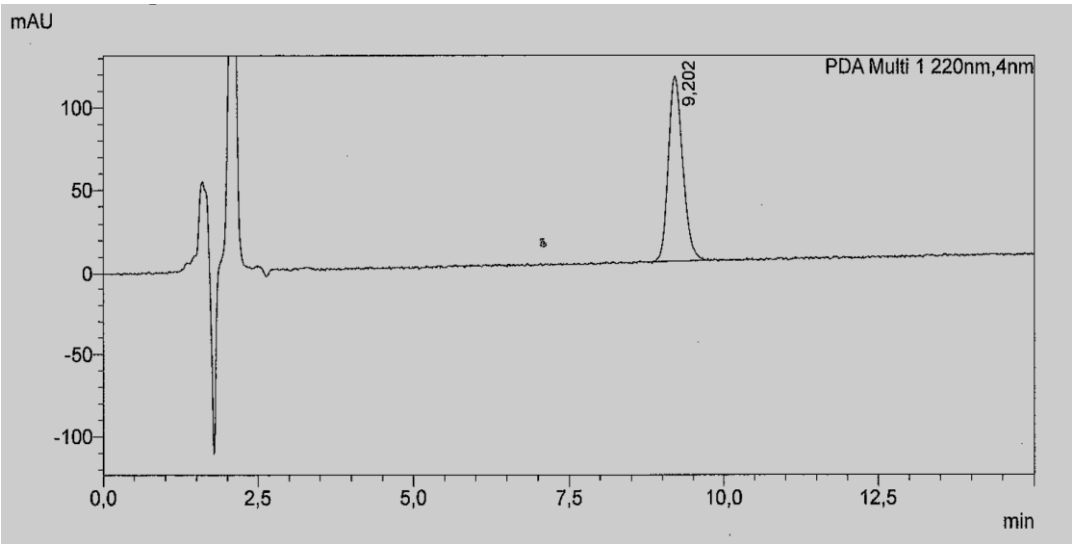

Fig. 6. Chromatogram obtained from the HPLC run of the extracts from the *Drosophila* diet supplied with 2000 ppm PCP, after larval feeding.

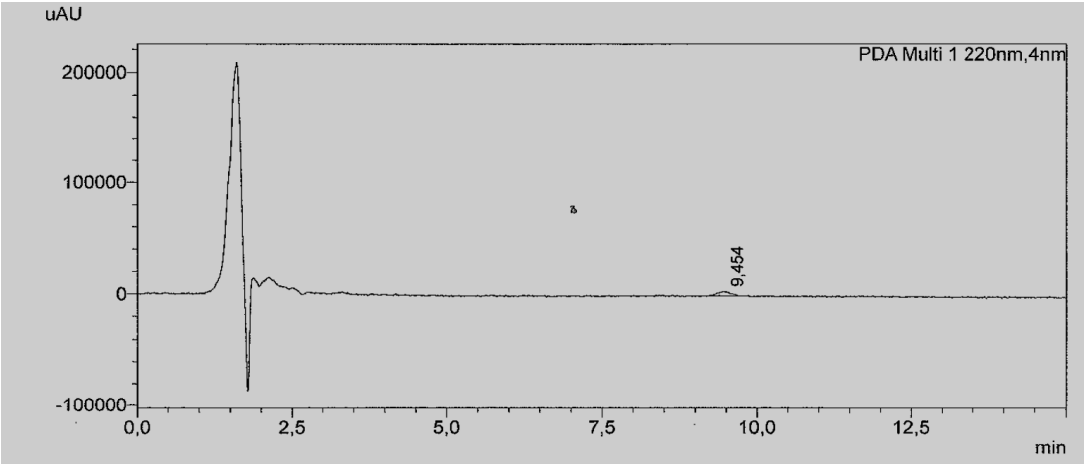

Fig. 7. Chromatogram obtained from the HPLC run of the extracts from resistant *Drosophila* larvae fed upon 2000 ppm PCP diet.

Results show a reduction of PCP content in diets for both the resistant strains compared to the control (Fig. 8). For larval tissues the amount of PCP was too close to the detection limit of the instruments.

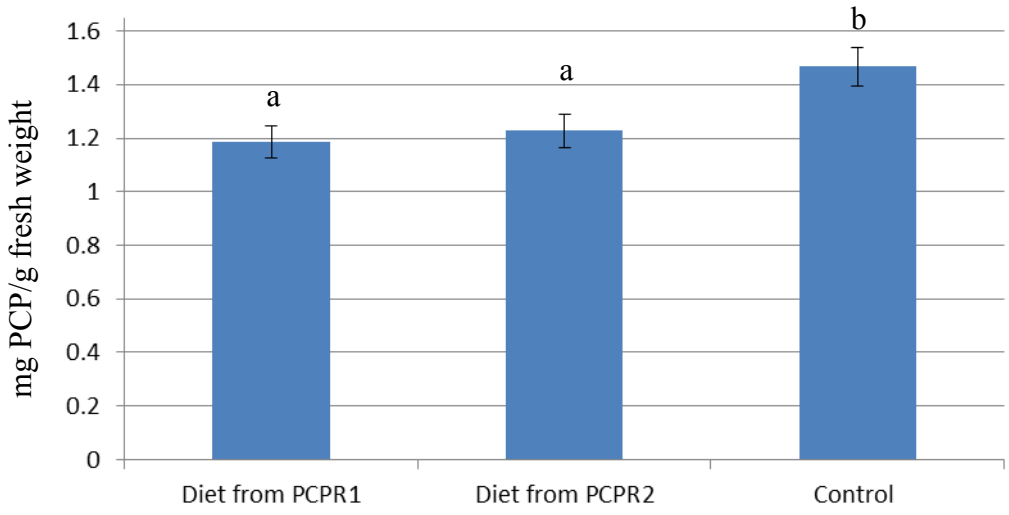

Fig. 8. PCP content by HPLC analysis of diet with and without feeding activity of *Drosophila* resistant larvae (Error bars = DS). Significant differences indicated by different letters ($p \leq 0.05$).

DISCUSSION

Insects, including *Drosophila*, readily respond to toxins such as phytotoxins, metal ions, and insecticides present in their environment by evolving resistance. Although *Drosophila* are seldom target for insecticides, nevertheless populations worldwide have evolved resistance in the field to a variety of insecticides such as DDT (Kikkawa 1961), cyclodienes (ffrench-Constant *et al.* 1990), organophosphorus chemicals (Windelspecht *et al.* 1995; Miyo 2001), carbamates (Wilson and Cain 1997), insect growth regulators (Wilson and Cryan, 1996) and neonicotinoids (Daborn *et al*. 2002). Furthermore, *D. melanogaster* has been suggested as, and is proving to be, a model organism to study the evolution of resistance (ffrench-Constant *et al.* 1990, 2000; Daborn *et al.* 2000, 2002; Wilson 2001).

In many cases, *Drosophila* uses the same genetic and biochemical mechanisms that underlie resistance in pest insects, including single-site changes in target molecules and upregulation of degradative enzymes, particularly cytochrome P450 enzymes and Glutathione S-Transferases (Wilson 2001).

D. melanogaster is a genetically well-known and accessible animal model, widely used in studying various biological processes. In this study the attention has been focused on the identification of PCP inducible genes mainly in order to identify those potentially involved in its detoxification. The exploitation of these genes is expected to step forward research on biotechnological solutions for bioremediation approaches.

The effect of many different xenobiotics has been examined on *D. melanogaster.* There are many examples on how insects in general, and *Drosophila* in particular, can develop resistance to chemicals, even if they are not direct target, demonstrating that also non-target organisms are affected by allochemicals released in the environment (Wilson 2001). This is the first attempt to analyze the effects of PCP using the whole transcriptome in *D. melanogaster*.

General response to PCP treatment

Among the DE genes found in *D. melanogaster* after PCP treatment a large group of detoxification enzymes was overexpressed (see below), but the total transcriptional profile showed a more complex reaction, involving a number of different ongoing processes.

The transcriptomic response at 20 ppm treatment was limited compared to the 2000 ppm in terms of number of transcripts differentially expressed. Indeed for the low dose treatment results suggest above all the activation of genes involved not only in response to a generic stress (protein and lipid metabolism and signal transduction), but also in response to oxidative stress (GTPase mediated signal, apoptosis, DNA metabolism and neuronal function) (Weber *et al*. 2012). For the high dose the transcription profile suggested a similar trend, but with the induction of a wider range of responses.

The large amount of genes involved in Anatomical Structure Development and Single Organism Developmental and Cellular Process (with 106 genes categorized in Neurogenesis GO term, 22 in Synapse Organization GO term, and 7 genes in DNA Damage Checkpoint GO term), reflect the known effects of cell exposure to PCP. Previous studies indicate that PCP toxicity is related to the uncoupling of oxidative phosphorylation in mitochondria and the generation of reactive oxygen species (ROS) such as its metabolite tetrachlorohydroquinone. Among the main consequences of PCP exposure there are DNA damages (as double or/and single strand breaks or DNA base oxidation) (Valic *et al.* 2004), and neurotoxic effects (Folch *et al.* 2009). Furthermore some transcripts encoding chitin binding proteins (CG8756, CG9369), and chitinases (CG9307), together with CG876 (Peritrophin-A chitin binding protein), were also upregulated in the high dose treatment. These gene products could be involved in the molting process or the formation of the peritrophic envelope. The peritrophic envelope is excreted by the gut epithelial cells in most arthropods, and is a thin membrane which has protective functions against abrasive food particles, invading pathogens, plant toxins, and oxidative damage due to PCP generated ROS (Barbehenn *et al*. 2004).

Among Cellular Metabolic Process enriched GO term, one interesting category is the Biogenic Amine Metabolic Process, which involves any of a group of naturally occurring, biologically active amines, such as dopamine (belonging to catecholamine, important for insect development and known to be involved in insect stress responses), norepinephrine, histamine, and serotonin, many of which act as neurotransmitters. Higher organisms respond to many stressors by modulating the production of catecholamines (Neckameyer and Weinstein 2005). In *Drosophila*, some of the key controlling factors for the dopaminergic pathway are mainly GTP cyclohydrolase I (GTPCH, encoded by *punch*), the rate-limiting enzyme in the biosynthesis of tetrahydrobiopterin (BH4), which is the regulatory cofactor of the tyrosine monooxygenase (TH, encoded by *pale*) (Chaudhuri *et al*. 2007). Both enzymes are upregulated during exposure to a wide range of stressors. Some of the genes belonging to DE gene set take part in the pathway of the Tyrosine Metabolism and Catecholamine Biosynthesis; among these, *punch* and *pale* are both upregulated, probably due to an increased request of neurotransmitter molecules, but *pale* might also have a role in PCP detoxification. Other monooxygenases, such as mushroom tyrosinases, have been shown to be active in removing phenolic compounds from wastewaters (Ikehata and Nicell 2000), and a cluster of genes involved in tyrosine metabolism in the filamentous fungus *Aspergillus fumigatus* was associated with phenolic compound degradation (Greene *et al.* 2014).

Five transcripts encoding ribosomal proteins (*RbL28*, *RbL8*, *RbS3A*, *RbL21*, *RbS31*) were all downregulated, indicating a suppression of protein translation. The suppression of these ribosomal proteins is a clear difference between the low and high PCP concentration exposure, being absent in the low dose. Protein synthesis is an energy costly process, and therefore it is often suppressed in stressful situations, like in detoxification of xenobiotics, in order to reallocate the energy budget.

Surprisingly three heat shock genes (*hsp83* and *hsp70Ba*, and CG4461), present in DE gene set, and belonging to the GO category of Response to Abiotic Stimulus, are downregulated, following PCP treatments. Induction of heat shock proteins (Hsps), as a response to any stressor that threatens macromolecule homeostasis, is among the most common features of the non-specific cellular stress response. The Hsps are indeed thought to assist the cells during the adverse conditions by transiently

preprogramming cellular metabolic activity which protects cells from further oxidative and heat damage (Nover 1991). Chowdhuri *et al*. (2001) found a high expression of a heat shock gene at the initial stages (2-12 h) of PCP exposure of *D. melanogaster* larvae, but this result has not been confirmed by our data. In the GO term Response to Chemicals are included most of the known enzymes involved in xenobiotic detoxification such as CYPs (more evidently grouped under the Molecular Function GO term Monooxygenase activity), GSTs and many transporters (grouped under the Molecular Function GO term Active transmembrane transporter activity).

Transcripts coding for Detoxification genes

Xenobiotics are usually non-polar compounds, therefore they can easily enter the cell through the membrane barriers; organisms had to develop biological processes for selectively inactivate and detoxify non-polar compounds. To achieve this aim they often evolve enzymes with broad substrate specificities so that they can metabolize almost any non-polar toxic compound. Mechanisms of response to xenobiotics are known to involve an elaborate three-phase system, leading xenobiotics to be converted into less harmful substances and facilitating their excretion (Xu *et al.* 2005) (Fig. 9). Interestingly, all living beings share the main features of this pathway.

The first step (phase I) mainly consists of oxidation, hydrolysis, and reduction reactions, with a prominent role played by enzymes belonging to the cytochrome P450 family (CYP) which exhibit a prevalent monooxygenasic activity. The phase I detoxification enzymes represent the most abundant class of xenobiotic-metabolizing enzymes, playing a determinant role decreasing the biological activity of a broad range of substrates (even if it can happen that they increase their toxicity).

During the second step of detoxification (phase II) the by-products of the phase I are modified by enzymes such as Glutathione S-Transferases (GSTs), UDP-glycosyltransferases (UGTs), and carboxylesterases (Misra *et al*. 2011). GSTs and UGTs add bulky side groups onto toxic compounds increasing their hydrophilicity, with production of more soluble compounds which are easily excreted from the organism.

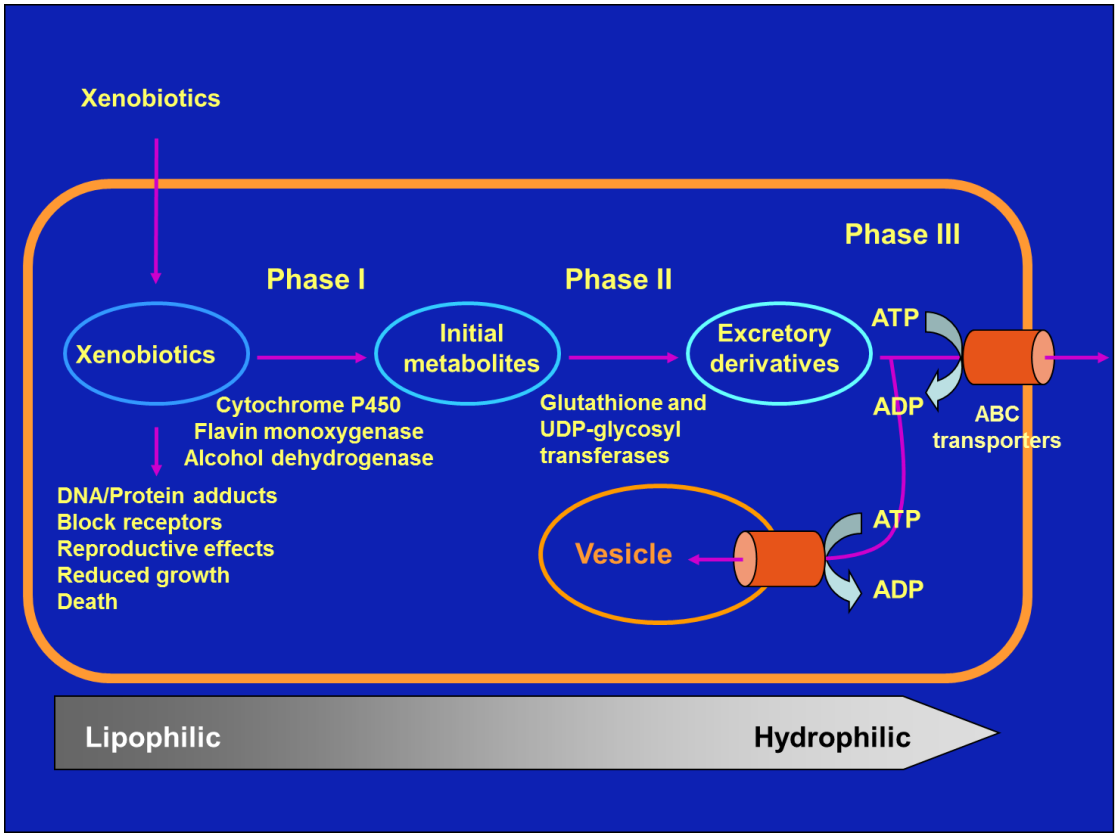

Fig. 9. Schematic pathway of xenobiotic degradation

Differently carboxylesterases catalyze the hydrolysis of ester-containing xenobiotics, leading to their detoxification. Hence, the first genes potentially involved in resistance are those coding for enzymes participating in these two phases. Finally, the phase III system consists of ATP binding cassette (ABC) and other transmembrane transporters that actively export the conjugated toxins out of the cell.

Within this study, analysis of differentially expressed genes, and in particular the upregulated transcripts, allowed the identification of a significantly enriched group with monooxygenase activity including 10 transcripts, eight of them encoding for cytochrome P450 genes.

The P450 category forms a diverse and important gene superfamily known to catalyze an extremely diverse range of chemical reactions, important both in developmental processes and in the detoxification of foreign compounds (Feyereisen 1999, 2012), with representatives in virtually all living organisms, from bacteria to protists, plants, fungi, and animals (Werck-Reichhart and Feyereisen 2000). In particular it is known

that insects have twice as many CYP genes as mammals, but only a third that of plants (Nelson 2009).

Insects exhibit a long co-evolution history with plants, during which they have been exposed to a variety of natural toxins, against which they have evolved very efficient metabolic pathways of detoxification. Insects equipped with such defense mechanisms against natural compounds are pre-adapted to deal with many classes of synthetic xenobiotics introduced into their environment by humans. Under this respect P450s predominate in the insect enzymatic system in the front-line catabolism of toxins (Li *et al*. 2007; Feyereisen 2011; Schuler 2011, 2012).

Cytochrome P450 enzymes are some of the most versatile redox proteins known. Collectively they use substrates ranging in size from ethylene to cyclosporin A. The so-called xenobiotic-metabolizing P450s are generally not considered to be individually critical for life, but collectively serve as a defense against the detrimental effects of natural products (e.g. phenolics, alkaloids, terpenes, etc), that would accumulate in the organism and be harmful. The general reaction can be described as the catalysis of the addition of oxygen to a substrate from P450 enzymes, via a heme cofactor (Coon *et al*. 1992; Hollenberg 1992). The additional oxygen atom may alter the stability of the substrate, leading to other molecular rearrangements (Bergé *et al.* 1998), or it may trigger conjugation by enzymes such as Glutathione S-Transferases (Tu and Akgül 2005). These processes lead to the detoxification and/or excretion of harmful compounds. The relatively low specificity of P450s provides a general defense system respect to drugs and other synthetic chemicals (Guengerich 2001).

Fig. 10 shows a genetic map indicating the distribution of *D. melanogaster* P450 genes throughout the chromosomes. More than half (47) of the P450s genes are distributed on chromosome 2; 15 genes are on the X chromosome and 26 on chromosome 3. No P450s were found on the mostly heterochromatic Y chromosome or on the small chromosome 4. The NADPH-cytochrome P450 reductase, adrenodoxin reductase, cytochrome b5 and nitric oxide synthase genes, coding for the redox partners of most P450 enzymes, are also localized on chromosome 2.

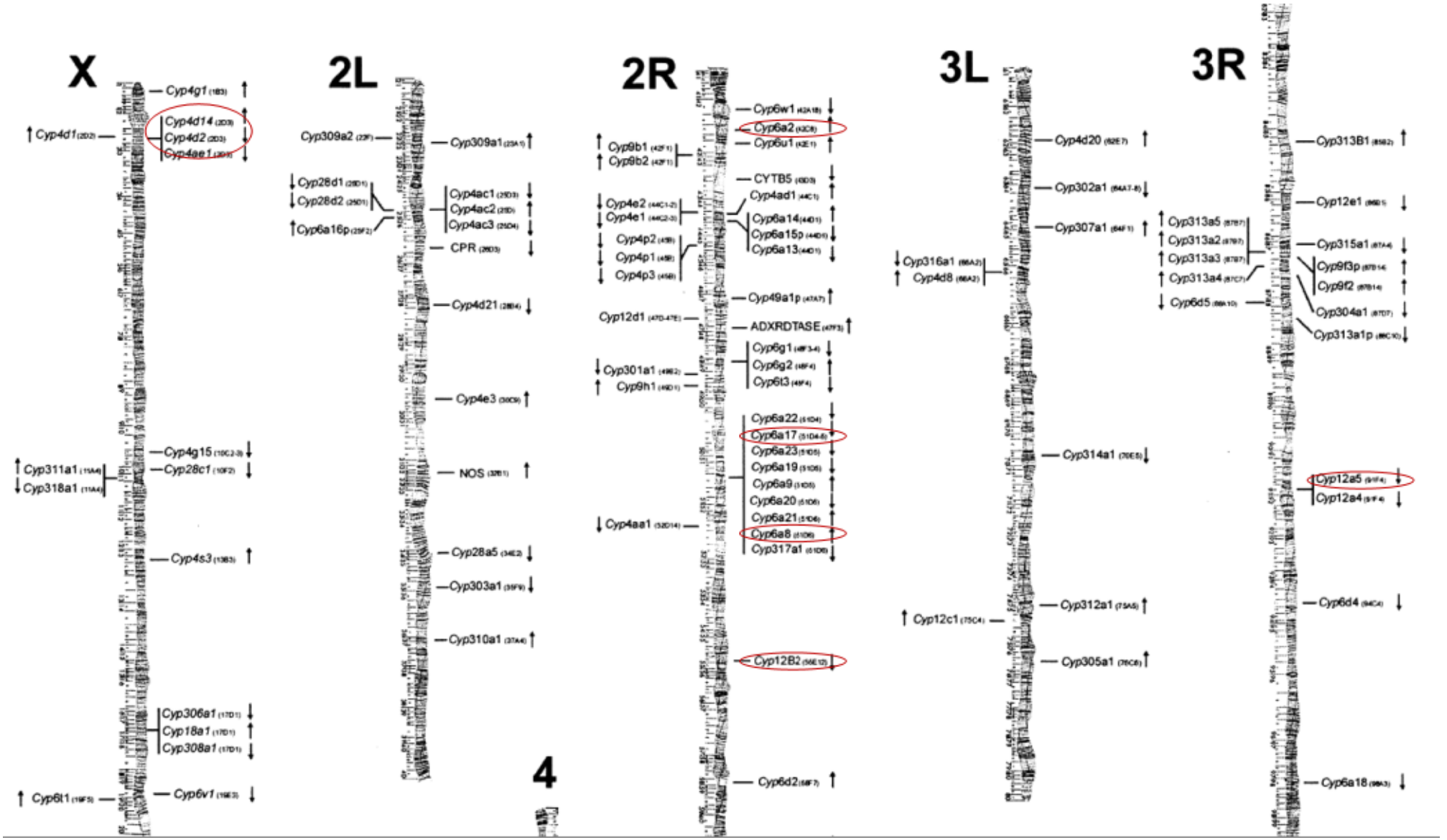

Fig. 10. Genetic map of the *D. melanogaster* P450 genes. The physical location of each P450 gene on the polytene chromosome map is marked; *Cyp*s discussed in the text are in red circles, and the orientation is shown by an arrow (up arrow is the reverse strand, down arrow is the forward strand). Adapted from Tijet *et al.* 2001.

It seems that P450s tend to be grouped in clusters. Eight clusters, defined arbitrarily as containing at least three genes, can be distinguished. On the right arm of chromosome 2, four clusters have been found. On these clusters, 78% of the genes are from the Cyp6 family. The presence of large P450 gene clusters, sometimes containing members of different P450 families, has also been observed in *Arabidopsis thaliana* (Paquette *et al.* 2000). The cluster of *Cyp6a* genes in *D. melanogaster* on the right arm of chromosome 2 recalls a similar cluster on chromosome 5 of the house fly (Cohen and Feyereisen 1995), showing synteny of these linkage groups (Foster *et al.* 1981). However it is difficult to assign orthologous relationships between the genes, because the ancestral cluster has evolved separately for over 100 million years in each species. On the X chromosome, *Cyp4d14*, *Cyp4d2* and *Cyp4ae1* form a cluster. It is generally believed that such gene clusters originate from ancient gene duplications and gene conversion events followed by divergence (Dunkov *et al*. 1997).

Several CYP genes showing overexpression in our experiment have been previously related to insecticide resistance but in most cases whether a specific CYP confers resistance or metabolizes the insecticide was not proved by a direct experimental approach such as gene disruption or transgenics.

In this experiment, a limited number of CYP genes evidenced changes in the expression of their mRNA, consistent with the challenging PCP supplementations and this is in agreement with previous reports. Indeed, microarray experiments carried out on different genera of mosquitoes (*Aedes* and *Culex*), to identify P450 genes involved in insecticide resistance, have identified a relatively restricted number of CYP genes overexpressed (compared to the total amount of CYP genes existing) and potentially involved in resistance. Interestingly in most cases several CYP genes are overexpressed altogether, albeit to different levels (Amenya *et al.* 2008; Hardstone *et al.* 2010).

Since it seems that increased CYP gene expression only occurs when exposed to a toxin, it could be concluded that transcriptional regulation is at work. This would be a clear advantage for insects as they would invest energy only when necessary (Schuler, 1996). Insects are indeed exposed to a variety of xenobiotics and require the presence of a number of different detoxification enzymes to combat these challenges. To constitutively express many different detoxification genes, at levels high enough to

detoxify compounds that the organism seldom encounters, would incur an unnecessarily high metabolic and fitness costs for the organism (Zeng *et al*. 2009), including increased levels of oxidative stress (Dostalek *et al.* 2007, 2008). Organisms have therefore evolved an induction system whereby the production of detoxification genes is increased only upon contact with the xenobiotic.

Insect P450 sequences are distributed on the basis of the sequence similarity in four major clades (Feyereisen 2006) that are strongly supported by bootstrap analysis. These correspond to four CYP "clans" which are named after the founding family in vertebrates (CYP3, CYP4, CYP2 clans) or their subcellular location (mitochondrial CYP clan) (table 11).

Table 11. Four clans of CYP genes in insects, with CYP family numbers (adapted from Feyereisen 2012).

CYP2 Clan	*CYP3 Clan*	*CYP4 Clan*	*Mitochondrial CYP Clan*
15	6	4	12
18	9	311-313	49
303-307	28	316	301-302
343	308-310	318	314-315
359	317	325	333-334
369	321	340-341	339
	324	349-352	353
	329	380	366
	332	367	
	336-338	405	
	345-348	411-412	
	354		
	357-358		
	365		
	395-400		
	408		
	413		

Among the *Drosophila* PCP induced CYP genes, representatives of microsomal CYP3, CYP4 and mitochondrial CYP clans have been found.

Genes from CYP3 group appear to share the characteristics of "environmental response genes" as defined by Berenbaum (2002), showing specifically very high diversity, proliferation by duplication events, rapid rates of evolution, occurrence in gene clusters, and tissue- or temporal-specific expression. Among genes of this group, the PCP induced *Cyp6a2* and *Cyp6a8* (with a FC in 2000 ppm treatment of 0.89 and 1.6 respectively), have been previously reported to be overexpressed as a consequence of exposure to different xenobiotics (Willoughby *et al.* 2006). *Cyp6a2* and *Cyp6a8* are among the best characterized CYP genes, in particular the first one is highly expressed in different insecticide resistant *Drosophila* strains (Kalajdzic *et al.* 2012) and its encoded enzyme has been proved to be able to metabolize organochlorine and organophosphorus insecticides (Saner *et al.* 1996; Dunkov *et al.* 1997; Giraudo *et al*. 2010). For both these genes also regulatory loci have been identified, both trans- and cis- acting (Li *et al*. 2007). Dombrowski *et al*. (1998) and Maitra *et al*. (2000) suggested that a repressor present on the third chromosome regulates the constitutive and induced expression of *Cyp6a2* and *Cyp6a8* genes present on the second chromosome.

The wild type function of these loci is to repress the expression of these two CYP genes and the overexpression of *Cyp6* genes in the resistant strain is due to a mutation in these regulatory loci (Maitra *et al*. 2000).

Overexpression of *Cyp6d2*, another overexpressed *Cyp* as a consequence of 2000 ppm treatment, has been reported in *Drosophila* larval fat body in response to camptothecin treatment, a type I topoisomerase inhibitor that creates DNA double-strand break (Thomas *et al*. 2013), with the same effects caused by the presence of Reactive Oxidative Species (ROS). ROS modify the purine and pyrimidine bases of DNA, thus inducing point mutations and breaks within DNA strands (Czekaj 2000). The exposure of cells to chlorinated compounds, such as PCP, usually results in DNA damage such as double or/and single strand breaks or DNA base oxidation (Valic *et al.* 2004). As suggested by Thomas *et al.* (2013), *Cyp6d2* might have one of the two roles, if not both: limiting DNA damages after ROS induction, or degrading xenobiotics after

having been expressed in fat body, where many detoxification reactions happen (consistently with known *Cyp6* activities).

Cyp6a17 and *Cyp28d2* have been defined as belonging to CYP genes on the basis of sequence and structure similarity with other CYPs, but their functions are still unknown. *Cyp6a17* induction has been found in experiment of phenobarbital (a known P450 inducer) challenge of adult fruitflies (Le Goff *et al*. 2006) and hints of its detoxification role are the overexpression in Malpighian tubules as for most of detoxifying enzymes (FlyAtlas database, Chintapalli *et al.* 2007). Recently *Cyp6a17* has been associated with circadian cycles (Ranade *et al.* 2008) and temperature perception (Kang *et al.* 2011). Both those CYP genes show an increased expression corresponding to an increase of the dose treatment. It would be interesting to collect more information in order to corroborate their involvement in PCP degradation.

The CYP4 members are considered the least studied of the CYP clans in insects, and in mammals are known to be involved in fatty acid and xenobiotic metabolism (Simpson 1997). In *Drosophila mettleri* resistance to senita cactus alkaloids is linked to the action of multiple P450 enzymes (Cyp6, Cyp4, Cyp9, and a new Cyp28) induced by the alkaloid; again a cooperative manner to metabolize the toxins is hypothesized (Danielson *et al.* 1997). A further study identified a Cyp4d10 from *D. mettleri*, induced by isoquinoline alkaloids of saguaro cactus, providing the first evidence that a member of the CYP4 family can be involved in phytotoxin detoxification (Danielson *et al*. 1998). Moreover, overexpression of CYP4 family of genes was implicated in resistance to methyl parathion and carbaryl in the Nebraska western corn rootworm (Scharf *et al.* 2001). In PCP data set there are two members of CYP4 family, *Cyp4d14* and *Cyp4d2*. *Cyp4d14* is reported to be induced by phenobarbital and caffeine, both of them extensively used to study the induction mechanism of P450 genes. This gene, furthermore, is reported to participate in different KEGG degradation pathways, such as Bisphenol Degradation (KEGG#00363), Aminobenzoate Degradation (KEGG#00627), Limonene and Pinene Degradation (KEGG#00903), and Polycyclic Aromatic Hydrocarbon Degradation (KEGG#00624). Recently Kalajdzic *et al*. (2012) associated, for the first time, the overexpression of this gene with resistance to the neonicotinoid Imidacloprid. The C*yp4d2* was underexpressed in 20 ppm PCP treatment and reached a moderate

overexpression in the higher dose. Even though *Cyp4d2* has not been functionally characterized yet, it was found to result in lethality by RNAi at larval stages suggesting an essential role for viability (Chung *et al*. 2009).

P450s are dependent on redox partners for their supply of reducing equivalents from NADH or NADPH; for microsome P450 the redox partners can be either a NADPH cytochrome P450 reductase or cytochrome b5, while for mitochondrial P450s redox partners are either an adrenodoxin reductase (NADPH-dependent FAD flavoprotein), or an adrenodoxin (a [2Fe-2S] ferredoxin-type iron sulfur protein). In the overexpressed set of genes, both a NADPH cytochrome P450 reductase (FBgn0011676) and a ferredoxin-NAD(P) reductase (FBgn0032754) are present, showing a significant ($p<0.05$) overexpression in 2000 ppm PCP treatment.

The *D. melanogaster* Cyp12 family members (*Cyp12a4, 12a5, 12b2, 12c1, 12d1, 12e1*) have several structural features in common with mammalian mitochondrial P450s. The function of mitochondrial P450s of *D. melanogaster* is not well characterized. The vertebrate mitochondrial P450s are all involved in the metabolism of compounds related to hormonal steroids, bile acids and vitamin D. Biochemical data indicate that at least three steps in insect ecdysteroid metabolism are catalyzed by mitochondrial P450s (Feyereisen 1999), even though Guzov *et al*. (1998) reported for house fly CYP12A1 a xenobiotic metabolizing activity. Of the two PCP induced mitochondrial *CYP* genes, *Cyp12a5* either shares the same KEGG degradation pathways reported for *Cyp4d14*, and could therefore have a role in the metabolism of toxic compounds, or takes place into the ecdysteroid biosynthetic process with the other mitochondrial transcript, *Cyp12e1*, through the action of an intermediate gene, called *shade*. 20-hydroxyecdysone and the ecdysone receptor have been showed to participate in the regulation of genes involved in metabolism, stress, and immunity at the onset of metamorphosis (Beckstead *et al.* 2005).

Studies have shown that the induction of *CYP* genes has been found in various tissues of insects, including the nervous system, antennae, fat body, Malpighian (renal) tubules, and the midgut. Known insecticide metabolism genes in *Drosophila* larvae are highly enriched in Malpighian tubules and fat body (Yang *et al*. 2007) and midgut (Li *et al*. 2008) implicating them in xenobiotic metabolism.

One monooxygenase coding gene, a flavin-monooxygenase gene (*fmo-I*) was significantly overexpressed in 2000 ppm PCP treatment, and since it belongs to a group of known mammalian enzymes involved in xenobiotic detoxification, it has been included in the genes to be further analyzed by qRT-PCR. Its upregulation, though, was not confirmed by qRT-PCR.

FMO genes are found in all phyla (Hao *et al.* 2009), from vertebrates, where form a gene family of five similar genes that provide an efficient detoxification system for xenobiotics (Cashman 2002), to yeast (Suh *et al.* 1996), where only one gene is present; in *D. melanogaster* two FMOs have been detected (Scharf *et al.* 2004). It has long been suspected that FMOs in insects, on the basis of what happens in mammals, are involved in xenobiotic metabolism (Agosin 1985), even if it results quite difficult to separate the actions of the FMOs and cytochromes P450. For example, both enzymes catalyze oxidations, both share a requirement for NADPH as a co-factor, and both occur in the microsomal fraction (Cashman 1995). Recent studies have shown that FMOs might not play a central role in xenobiotic detoxification in insects, in contrast to mammals, sharing instead features in common with monoamine oxidases, which play important roles in the degradation of biogenic amine neurotransmitters (Gilbert *et al*. 2000). In PCP overexpressed gene set, therefore*, fmo-I* could have been involved more likely in a general response of *Drosophila* to PCP rather than in a detoxification mechanism.

The phase II of detoxification involves the conjugation of the by-products of the phase I with glutathione by Glutathione S-Transferases (GSTs), allowing the production of more soluble compounds which can be more easily excreted. Glutathione S-Transferases are enzymes ubiquitously widespread in organisms, having been reported in microbes, plants, insects and vertebrates (Hayes and Pulford 1995). Even though GSTs exhibit a variety of catalytic and non-catalytic functions, their genes are particularly investigated in detoxification mechanisms since they are involved in insecticide resistance (Ranson *et al*. 2001; Tu and Akgul 2005; Claudianos *et al*. 2006). GSTs catalyze the conjugation between the sulphur of the thiol group from the reduced glutathione (GSH) to compounds possessing an electrophilic center (Mannervik 1985). Xenobiotics are thus eliminated from the cell because more water soluble or targeting them to specific GSH multidrug transporters. In addition, some

GSTs dehydrochlorinate the substrate using reduced glutathione as a cofactor rather than a conjugate (Clark and Shamaan 1984). Several insect species show resistance to insecticides either by increased expression (Grant and Hammock 1992; Syvanen *et al*. 1994) or increased GST activity (Fournier *et al.* 1992). GSTs have been reported to modify organochlorine (Clark and Shamaan 1984; Tang and Tu 1994) and organophosphorous insecticides (Lewis and Sawicki 1971; Oppenoorth *et al.* 1979; Huang *et al.* 1998), and moreover they can confer resistance to pyrethroid, which damage the lipids of the nervous membrane, by reducing the oxidative injury (Vontas *et al*. 2001).

In insects, two separate superfamilies of GST show transferase activity: the cytosolic or soluble GSTs and the microsomal tranferases (membrane-associated proteins) (Hayes and Strange 2000); the latter ones, even if catalyzing similar reactions, are not involved in detoxification of insecticides (Gakuta and Toshiro, 2000).

Insect cytosolic GSTs comprehend a large number of enzymes, classified in six families (δ, ε, σ, θ, ω, ζ) on the basis of the mammalian system of GST nomenclature (Chelvanayagam *et al*. 2001), with δ and ε, insect specific, forming some of the largest gene clusters in insect genomes (Tang and Tu 1994; Ranson *et al*. 2001; Ding *et al.* 2003).

Surprisingly, among the differentially expressed genes following PCP treatment only the *GSTD4* showed an overexpression in 2000 ppm dose, confirmed by qRT-PCR assay, while the *GSTD9* and *GSTS1* were underexpressed. This is in contrast with the literature, not only regarding GSTs involvement in detoxification, but also regarding their role in cell protection resistance by reducing the oxidative damage caused by insecticides (Vontas *et al*. 2001; Enayati *et al*. 2005). Indeed, even though *GSTD4* has been shown to be overexpressed in response to oxidative stress (Sun *et al*. 2011), its GST activity has not been biochemically proved (Sawicki *et al.* 2003), while it has been confirmed for *GSTS1* (Singh *et al*. 2001) and *GSTD9* (Sawicki *et al.* 2003).

The UDP-glycosyltransferases (UGTs) are another superfamily of enzymes known to play a major role in the inactivation and excretion of a great variety of both endogenous and exogenous compounds (Huang *et al.* 2008). Members of this superfamily are present in animals, plants, bacteria, and viruses, and play an important role in the detoxification of both plant allelochemicals ingested by many herbivorous

insects with their food and xenobiotics. These enzymes catalyze the transfer of the glycosyl group from a nucleotide sugar (UDP-glucose for insects) to a variety of small hydrophobic molecules, including phenols, forming more hydrophilic compounds that are efficiently excreted. It is known that many plant phenolics can act as toxins or feeding deterrents to insects and thus play an important role in plant defense against herbivorous insects. The detoxification of ingested plant phenolics is believed to be one of the principle functions of insect UGT enzymes (Ahmad and Hopkins 1993). Three UGTs have been significantly induced in the 2000 ppm PCP treatment, *UGT35b* (CG6649), *UGT 86Da* (CG18578), *UGT86De* (CG6653). Enzymatic activities of the insect UGTs are detected in the fat body, midgut, Malpighian tubules, integument, and silk gland, consistently with functions in detoxification (Ahn *et al.* 2012), thus the overexpression of these genes in PCP high dose treatment might indicate their involvement in the inactivation and excretion of PCP, probably with a major role compared to that of GSTs.

Transcripts coding for Transporter genes

Following phase I and phase II detoxification, a variety of transporters are involved in phase III, including members of the ATP-binding cassette (ABC), responsible for the ATP-powered translocation of many substrates across membranes. These substrates include ions, sugars, amino acids, vitamins, peptides, polysaccharides, hormones, lipids and xenobiotics (Labbe *et al.* 2011).

In particular, *Mdr50* is one of the three known P-glycoproteins (P-gps) in the complete genome of *Drosophila* (*mdr49*, *mdr50*, and *mdr65*). It is a membrane-spanning protein of the ABCB subfamily. P-gps importance in removing xenobiotics from cells is increasingly being recognized and it is likely that they represent a first line of defense to the penetration of drugs and pesticides into the cell. A wide range of pesticides has been shown to interact with P-gps, including insecticides (cyclodienes, organophosphates, avermectins), fungicides (azoles) and herbicides (Buss and Callaghan 2008).

CG4562 belongs to the ABCC subfamily, including functionally diverse transporters, with broad-specificity, called multidrug resistance-associated proteins (MRPs). MRPs

are known for their involvement in translocation of a range of substrates, including drugs, endogenous compounds and their glutathione and glucuronyl conjugates, glutathione, and cyclic nucleotides (Sturm *et al.* 2009).

The role of ABC transporters has been previously described in the aphid *Myzus persicae*, where three encoding ATP-binding cassette transporters were found to be upregulated in a susceptible genotype after pirimicarb exposure (Silva *et al*. 2012). Differential transcription of ABC transporters has been also found in DDT resistant *Drosophila* strains (Pedra *et al.* 2004); in field populations of *Helicoverpa armigera* resistant to pyrethroids, organophosphates and cyclodienes, an elevated protein titer of P-gp has been reported (Srinivas *et al*. 2004). There is also evidence that the P-gp inhibitor verapamil significantly increases the toxicity of some insecticides (Buss and Callaghan 2008). Therefore, genes for ABC transporters appear to play an important role during insecticide elimination.

HPLC analyses

PCP resistant strains of *D. melanogaster* were selected in order to characterize an eventual PCP degradation pathway developed by this insect, and to further investigate the identification of PCP metabolites and their toxicity. Indeed, PCP transformations might be responsible for a variety of breakdown products, some of which may be as toxic, or even more toxic than the parent product. It has been estimated that there are over 30 PCP microbial transformation products and toxicity of biodegraded PCP samples towards fish embryos (*Menidia beryllina*) has been observed (Middaugh *et al*. 1993). For this purpose the two different selected strains were tested to detect potentially different degradation pathways. PCP was ethanol extracted from samples, and HPLC analyses were performed to compare the amount of PCP in *Drosophila* diet, with and without feeding by resistant larvae. Results showed a significant reduction of PCP content in diets where resistant larvae have fed, compared to control. The amount of PCP reduction (about 20%) was very similar for the two resistant strains.

The ethanol extracted samples after HPLC analysis showed for both diets and larval tissues only one single peak, corresponding to the PCP standard, as if the only

compound present was just unaltered PCP. It is realistic to suppose that *Drosophila* larvae, after up-taking PCP, metabolize it and excrete the by-products into the medium. A possible explanation for the lacking of other peaks could be the choice of extraction protocol and analysis method very focused on PCP, and thus not adequate to detect its metabolites. A chemical extraction method should therefore be developed in order to allow characterization of the poorly extractable fractions. Furthermore, analysis of diet and larval tissues by GC-MSMS will be useful for determining the presence of any PCP metabolite, gaining a broader comprehension of *D. melanogaster* genetic and enzymatic mechanisms involved in the response to PCP.

CONCLUSIONS

This first transcriptomic study on PCP effects on *Drosophila melanogaster*, a non-target organism, showed some remarkable results. With the low PCP concentration a smaller number of affected genes was identified, compared to the high dose, indicating a positive dose-response, with the significantly downregulated genes slightly less abundant for both treatments. Different transcriptomic profiles of the two treatments reflected quantitative and qualitative differences depending on the PCP dose. Most of the genes involved in the general and oxidative stress response were shared by both treatments, while genes involved in the detoxification processes (mainly CYPs, UGTs and ABC transporters) were induced mostly at the high dose suggesting the existence of a pathway of biotransformation of PCP.

This project could result in two different outcomes.

If the identified genes induced by PCP treatment are proved to be involved in its detoxification, then enzymes of an organism different from microorganisms will become available to be employed for decontamination of PCP polluted environments (Zhang and Qiao 2002). The contribute of organisms like insects, as a potential source of enzymes degrading contaminants and their unwanted by-products, will enrich the available arsenal of microorganisms, plants and derived molecules.

On the other hand, even if the differentially expressed genes were not involved in detoxification pathways, their different expression would make them suitable as biomarkers for a toxicity screening of potentially polluted sites (Nota *et al*. 2009).

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