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Metabolomics Reveals Target and Off-Target Toxicities of a Model Organophosphate Pesticide to Roach (*Rutilus rutilus*): Implications for Biomonitoring

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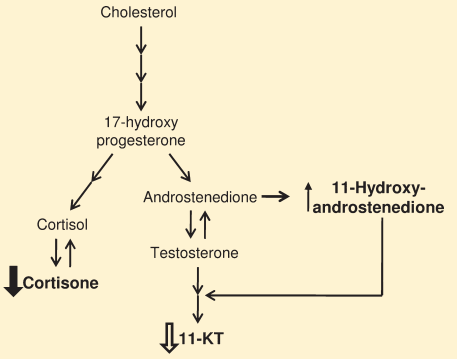
Supporting Information

ABSTRACT: The ability of targeted and nontargeted metabolomics to discover chronic ecotoxicological effects is largely unexplored. Fenitrothion, an organophosphate pesticide, is categorized as a "red list" pollutant, being particularly hazardous to aquatic life. It acts primarily as a cholinesterase inhibitor, but evidence suggests it can also act as an androgen receptor antagonist. Whole-organism fenitrothion-induced toxicity is well-established, but information regarding target and off-target molecular toxicities is limited. Here we study the molecular responses of male roach (*Rutilus rutilus*) exposed to fenitrothion, including environmentally realistic concentrations, for 28 days. Acetylcholine was assessed in brain; steroid metabolism was measured in testes and plasma; and NMR and mass spectrometry-based metabolomics were conducted on testes and liver to discover off-target toxicity. O-demethylation was confirmed as a major route of pesticide degradation. Fenitrothion significantly depleted acetylcholine, confirming its primary mode of action, and 11-ketotestosterone in plasma and cortisone in testes, showing disruption of steroid metabolism. Metabolomics revealed significant perturbations to the hepatic phosphagen system and previously undocumented effects on phenylalanine metabolism in liver and testes. On the basis of several unexpected molecular responses that were opposite to the anticipated acute toxicity, we propose that chronic pesticide exposure induces an adapting phenotype in roach, which may have considerable implications for interpreting molecular biomarker responses in field-sampled fish.



INTRODUCTION

Fenitrothion [*O*,*O*-dimethyl *O*-(4-nitro-*m*-tolyl)phosphorothioate] is an organophosphate pesticide widely used as a broad-spectrum contact insecticide for crop protection in the United Kingdom, Europe, Australia, and Japan.[1−3] In the United States, fenitrothion is licensed for use in household and industrial pest control products.[4] It is classed as a "red list" pollutant by the U.K. Environment Agency, that is, one of the most dangerous substances to the aquatic environment whose discharge into water is to be minimized as far as possible,[5] and consequently its presence in the environment is of particular concern.[1] This pesticide can contaminate surface water from soil runoff,[1] with up to 8 μg/L reported in streams receiving input from Japanese paddy fields,[3] up to 150 μg/L detected in surface waters in Spain,[6] and levels in excess of 100 μg/L in runoff from an Italian vineyard.[2] Although fenitrothion has been reported to biodegrade relatively rapidly, for example., ca. 4 days in natural waters,[7] regular input into the aquatic environment can result in sublethal exposure of nontarget organisms.

As for other organophosphates, fenitrothion acts by inhibiting the activity of acetylcholinesterases (AChE). While observations of whole-organism toxicity have been documented, including increased mortality rates in bees,[8] altered foraging habits in birds,[9] and fatigue in mammals,[10] no extensive investigations have been reported into the underlying target and off-target molecular toxicities of fenitrothion. Previous molecular studies have reported that fenitrothion bioaccumulates in the brain, liver, muscle, and blood of exposed fish.[11] Furthermore, at water concentrations of 40 μg/L it can significantly deplete hepatic glycogen and total lipid levels and significantly increase plasma glucose and lactate in fish, with alterations in these biomarkers indicating a shift toward glycolysis-based energy production.[12] Fenitrothion also reduces 3-β-hydroxysteroid dehydrogenase

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activity in carp gonads at exposure levels of 1.5 μg/L, with likely impacts on sex steroid metabolism.[13] Further studies show that fenitrothion acts as an androgen receptor antagonist in vitro, which is associated with reduced prostate and seminal vesicle tissue masses in rats in vivo.[14] Exposure of stickleback to 10 μg/L fenitrothion significantly reduced spiggin (an androgen-dependent protein used for nest construction) production,[15] while levels of only 1 μg/L disrupted their androgen-dependent reproductive behaviors.[16]

The occurrence of fenitrothion in the environment at concentrations believed to cause sublethal toxicity was a major driver for our investigations into the molecular mechanisms of this pesticide's effects in a fish species. We chose to study the roach (*Rutilus rutilus*) as it is a sentinel species for assessing endocrine disrupting chemicals,[17] and is widespread and ecologically important in lowland rivers throughout Eurasia. Adopting a chronic, environmentally relevant exposure, we aimed primarily to provide a more comprehensive understanding of the molecular toxicity of fenitrothion. A secondary aim was to identify a potential suite of molecular biomarkers of chronic effect and to discuss their applicability to environmental biomonitoring. Both targeted and nontargeted metabolomic analyses were conducted on selected tissues from male roach following a 28-day exposure to 2, 20, and 200 μg/L fenitrothion. Metabolomics can be used to investigate metabolic changes within a biological system in response to toxicant exposure or disease, and has considerable potential as a tool in the fields of environmental risk assessment and monitoring.[18] For example, nontargeted metabolomic approaches can provide an unbiased determination of metabolic pathway perturbations in response to toxicant exposure and can lead to determination of toxicant mode of action.[19] Key metabolic changes indentified from such studies, once understood and validated, can then in principle be used to monitor for environmental toxicant exposure.

Here, targeted metabolite analyses of testes, brain, and plasma were focused on preselected metabolites and/or metabolic pathways that we hypothesized were involved in the toxic response, specifically the disruption of acetylcholine (ACh) metabolism and steroid metabolism, utilizing liquid chromatography−mass spectrometry (LC-MS) and immunoassays with high analytical sensitivity and specificity.[20] In addition, nontargeted metabolomics of testes and liver was used to simultaneously measure as large a proportion of the metabolomes as possible to investigate for unanticipated metabolic toxicity as well as metabolic degradation products of the pesticide. This utilized both ^{1}H nuclear magnetic resonance (NMR) spectroscopy and direct infusion mass spectrometry (DIMS) -based metabolomics. To further investigate some of the perturbed metabolic pathways identified by the metabolomics approaches, expression levels of selected genes were measured by real-time quantitative polymerase chain reaction (RT-QPCR).

MATERIALS AND METHODS

Fish Maintenance, Fenitrothion Exposure, and Whole-Organism Toxicity. Sexually mature roach of a mixed-sex population (laboratory-maintained first-generation fish derived from wild parental stock; Calverton Fish Farm, U.K. Environment Agency) were randomly allocated into groups of 12 fish in 150 L tanks. Each tank was exposed to nominal concentrations of 2, 20, and 200 μg/L fenitrothion (Sigma Aldrich) suspended in ethanol (each exposure was done in duplicate; see Supporting Information for further details). Water control (WC) and solvent control (SC, ethanol added) tanks were run under the same conditions without fenitrothion. Fish underwent a 28-day (chronic) exposure under continuous flow-through conditions (tank water fenitrothion concentrations were confirmed by gas chromatography (GC)−MS; Supporting Information) and then sacrificed by terminal anesthesia with benzocaine. Fork length, wet body mass, condition factor (fork length^3/body mass), and gonadosomatic index (GSI; testes mass/body mass) were recorded. The liver, gonads, and brain were dissected rapidly and, along with plasma (from caudal sinus), were frozen in liquid nitrogen and stored at −80 °C until analysis. A section of gonad underwent histology to confirm each fish was male (see Supporting Information). Only male fish were used in this study.

Metabolite Extraction from Tissues. Metabolites were extracted from brain, testes and liver using a two-step methanol/chloroform/water method with a final solvent ratio of 2:2:1.8.[21] Extract blanks were produced by the same protocol but in the absence of tissue. The extracted polar phase was split into 2 × 50 μL aliquots for DIMS or 1 × 600 μL aliquot for NMR and then dried. The DIMS aliquots were resuspended in 80:20 methanol/water containing either 20 mM ammonium acetate (for negative-ion DIMS) or 0.25% formic acid (positive-ion DIMS). The NMR aliquot was resuspended in a sodium phosphate-based buffer (see Supporting Information).

Nontargeted Metabolomics. All treatment groups (WC, SC, and 2, 20, and 200 μg/L fenitrothion exposures; see Table S1 in Supporting Information for group sizes) were analyzed by nontargeted metabolomics.

^{1}H NMR Analysis and Data Preprocessing. Liver and testes extracts were analyzed by one-dimensional (1D) ^{1}H and two-dimensional (2D) ^{1}H *J*-resolved NMR spectroscopy on a 500 MHz spectrometer equipped with a cryoprobe (Bruker BioSpin, UK). Preprocessing included taking skyline projections of the 2D *J*-resolved spectra (termed pJRES).[22] Residual water and chloroform peaks were removed, and spectra were normalized to a total area of unity and then binned prior to statistical analysis, described below[22] (further details in Supporting Information).

Mass Spectrometry Analysis and Data Preprocessing. Liver and testes extracts were analyzed by DIMS in negative-ion mode by the selected ion monitoring (SIM) stitching method[23] on a hybrid linear ion trap/Fourier transform ion cyclotron resonance mass spectrometer (LTQ FT-ICR, Thermo Fisher Scientific, Germany). Each sample was analyzed in triplicate and filtered into a single peak list (see Supporting Information; internal calibrants in Table S2). Final matrices containing peak intensity data were normalized by the probabilistic quotient method[24] prior to statistical analysis, described below. Selected peaks were subsequently identified by collision induced dissociation-based MS/MS in the linear ion trap (energy applied 35 eV), and fragments were recorded in the ion cyclotron resonance detector.

Targeted Metabolite Analyses. *Acetylcholine Analysis in Brain.* Polar brain extracts (from SC and 2 and 200 μg/L fenitrothion exposures, each $n = 6$) resuspended in methanol/water were analyzed by DIMS and processed as for nontargeted analysis. SIM windows at 90−120 and 130−160 m/z were recorded (positive ion) to measure choline and ACh, respectively.

Steroid Analysis in Testes and Plasma. Prior to testes extraction, 2 ng of deuterated testosterone-d_3 and 4 ng of deuterated cortisol-d_4 were added to each sample (sample group sizes, Table S3 in Supporting Information). Testes were extracted in

methanol followed by two solid-phase extractions.[20] Steroid standards [cortisone, cortisol, 17-hydroxyprogesterone, androstenedione, 11-ketotestosterone (11-KT), testosterone, and 11-hydroxyandrostenedione; Steraloids] were made both with and without a pooled testes extract (equivalent of 100 mg of testes/sample) and 2 ng of testosterone-d_3 and 4 ng of cortisol-d_4 per LC-MS injection. Roach samples and steroid standards (in 20 μL of 50:50 methanol/water) were analyzed by ultraperformance liquid chromatography time-of-flight MS (Waters, U.K.).[20] The mobile phases were (A) 95% water, 5% acetonitrile (ACN), and 0.25% formic acid and (B) 100% ACN. The gradient used was 0−14.9 min, from 20% to 80% B; 14.9−15.0 min, from 80% to 100% B; 15.0−25.0 min, 100% B. For the analysis of plasma 11-KT, 20 μL of plasma was extracted[25] and the androgen was quantified by radioimmunoassay.[26]

Gene Expression Analysis. Total RNA was extracted from 10 mg of liver (from the methanol/water homogenate prepared for the nontargeted metabolomics, n = 8) or 10 mg of testes (n = 7−8), by use of the RNeasy system with DNase treatment (Qiagen, Crawley, U.K.) according to the manufacturer's protocol (Supporting Information). cDNA was synthesized from 1 μg of total RNA by use of random hexamers and Moloney murine leukemia virus reverse transcriptase (Promega, Southampton, U.K.) following manufacturer's instructions. RT-QPCR (gene-specific primer sequences, Table S4 in Supporting Information) was performed on relevant target genes including creatine kinase, glutathione S-transferase, phenylalanine hydroxylase, androgen receptor, and gonadal aromatase using the iCycler iQ Real-time Detection System (Bio-Rad Laboratories Inc.), and expression levels were normalized to expression of the ribosomal protein L8 gene (further details in Supporting Information).

Data Analysis. Analysis of variance (ANOVA; one-way, with Tukey's post hoc testing) was conducted on whole-organism responses, targeted metabolite levels, and gene expression levels, while the Kruskal−Wallis method (with Dunn's all-pairwise multiple comparison) was used on data shown to be nonparametric (11-KT radioimmunoassay data);[27] both methods were conducted in Minitab.

Removal of Fenitrothion Metabolites from Metabolomics Data Sets. To avoid biasing the statistical analyses, peaks in the NMR and mass spectra from fenitrothion metabolism were removed. Specifically, DIMS peaks corresponding to desmethylfenitrothion $[M-H]^-$ or *S*-methylglutathione $[M-H]^-$ were removed (identities confirmed by MS/MS; Figure S1A,B in Supporting Information). In the NMR spectra of liver extracts, bins from 2.83 to 2.86 ppm that were highly suspected to correspond to desmethylfenitrothion (Figure S2 in Supporting Information) were removed.

Multivariate Statistical Analyses of Metabolomics Data. A generalized log transformation was applied to the normalized pJRES NMR and DIMS data sets, and then principal component analysis (PCA) was conducted using PLS_Toolbox (Eigenvector Research, Wenatchee, WA) in MatLab (version 7, The MathWorks, Natick, MA). ANOVA was conducted on the scores for the first five PCs from each model [with a false discovery rate (FDR) of <10% to correct for multiple hypothesis testing][28] to establish significance of treatments.

Univariate Statistical Analyses of Metabolomics Data and Metabolite Identification. ANOVA was conducted on each variable (pJRES NMR bin or DIMS peak) in the normalized data sets across SC and 2, 20, and 200 μg/L fenitrothion treatment groups (at FDR < 10%). Significantly changing NMR bins were screened against an in-house NMR library of 210 metabolite standards, and then the definitively identified metabolites were quantified in the 1D biological NMR spectra, normalized to the total spectral area, and tested by ANOVA (Supporting Information and Table S5). Significant peaks in the DIMS data were annotated by use of MI-Pack software,[29] described in Supporting Information. Metabolite x-fold changes were calculated as the average intensity in a treated group relative to the average of the SC group.

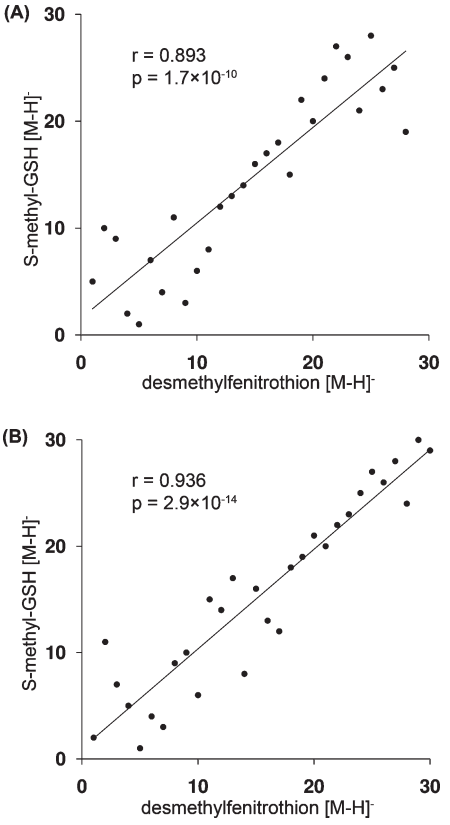

Figure 1. Correlations between the ranked intensities of *S*-methylglutathione $[M-H]^-$ and desmethylfenitrothion $[M-H]^-$ measured in (A) liver and (B) testes of roach exposed to fenitrothion, confirming that O-demethylation is a major route of pesticide degradation.

RESULTS

Water Chemistry and Whole Organism Toxicity. Mean (±SEM) measured fenitrothion exposure concentrations were 2.9 ± 0.9, 30 ± 9.7, and 193 ± 61 μg/L for the nominal 2, 20, and 200 μg/L exposures, respectively. No mortalities or significant changes to fork length (p = 0.543), body mass (p = 0.439), GSI (p = 0.542), or condition factor (p = 0.940) occurred during the 28-day experiment (data not shown), confirming that the selected exposure concentrations did not induce overt toxicity.

Fenitrothion Degradation Pathway. Two peaks were identified by MS/MS as desmethylfenitrothion (only detected in exposed fish, including within the liver, testes, and brain) and *S*-methylglutathione (≥10-fold increase in liver, testes, and brain of exposed fish versus controls, $p \leq 3.14 \times 10^{-9}$) (Figures S1A,B and S3 and Table S6 in Supporting Information). The normalized intensities of these two peaks in the liver and testes data sets were significantly correlated ($p = 1.7 \times 10^{-10}$ and 2.9×10^{-14},

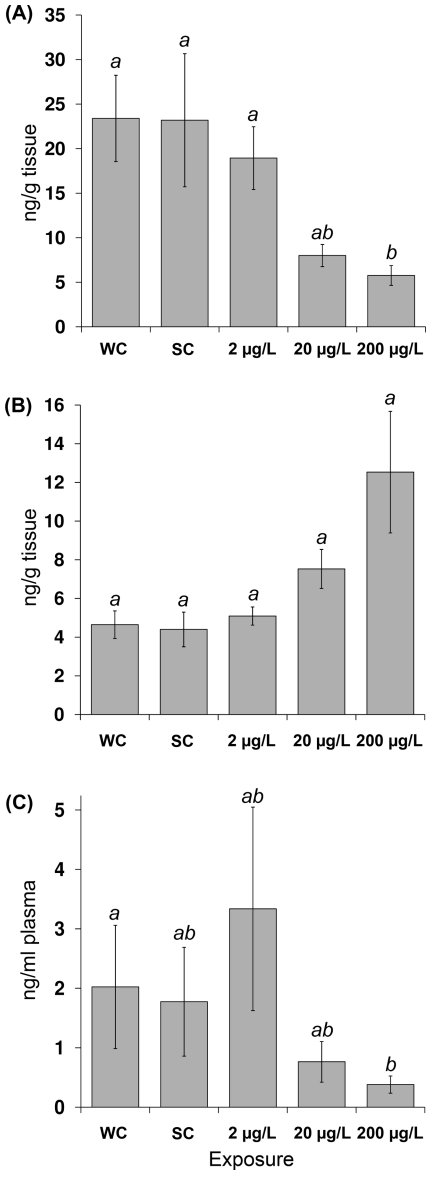

Figure 2. Mean concentrations (±SEM) of (A) cortisone and (B) 11-hydroxyandrostenedione in roach testes and (C) 11-ketotestosterone in plasma. Key: water control (WC), solvent control (SC), and 2, 20, and 200 μg/L fenitrothion exposures. Lowercase letters indicate significance, such that bars with the same letter are not significantly different.

respectively; Figure 1), strongly suggesting that they result from the metabolic breakdown of fenitrothion (see Supporting Information for further details).

Acetylcholine Metabolism. A peak at 146.117 56 m/z in the DIMS of brain extract was confirmed by MS/MS as $[\text{ACh-e}]^+$ (0.035 ppm mass error; Figure S1C in Supporting Information). $[\text{ACh-e}]^+$ was depleted following exposure to 2 μg/L (1.80-fold, significant by Tukey's post hoc) and 200 μg/L fenitrothion (1.36-fold) compared to the SC group, confirming disruption at the target site (ANOVA, $p = 0.009$). The intensity of a peak putatively identified as $[\text{choline-e}]^+$ (104.107 00 m/z) was not significantly changed ($p = 0.291$) across the treatment groups.

Steroid Metabolism. Cortisone showed a significant 4.0-fold depletion, from SC to 200 μg/L exposure, in response to fenitrothion ($p = 0.004$, post hoc: 200 μg/L group significantly different from WC, SC, and 2 μg/L exposure), and 11-hydroxyandrostenedione showed a near-significant 2.9-fold increase from SC to 200 μg/L exposure ($p = 0.057$, Figure 2A,B). Standard curves for these steroids showed similar gradients with or without the presence of tissue extract, indicating that the biological matrix exerts minimal ion suppression on them (Figure S4, Supporting Information). Cortisol ($p = 0.34$), androstenedione ($p = 0.87$), and 11-KT ($p = 0.93$) levels in testes extracts did not change significantly on exposure (Figure S5, Supporting Information), while 17-hydroxyprogesterone and testosterone were below the LC-MS detection limit. Plasma 11-KT levels were significantly ($p = 0.038$) reduced in response to fenitrothion (5.3- and 4.7-fold reductions for the 200 μg/L exposure compared with WC and SC, respectively; Figure 2C).

Nontargeted Metabolomic Analysis. PCA was conducted on the DIMS and NMR data sets (representative spectra, Figure 3) of testes and liver (PCA scores plots, Figure 4), and the resulting scores were evaluated by ANOVA (Table S7, Supporting Information). Significant differences existed between the five treatments groups (FDR < 10%) along two of the top five PC axes for the NMR liver and testes models and for the DIMS liver model. No significant differences between treatments were revealed in the PCA of the DIMS testes data set. PC1 from the NMR liver model and PC5 from the DIMS liver model showed the highest significance ($p = 8.3 \times 10^{-11}$ and 9.4×10^{-6}, respectively; post hoc: 200 μg/L exposure significantly different from all other groups along both axes), indicating a greater overall metabolic response in the liver metabolome compared to the testes metabolome (post hoc summary, Table S8, Supporting Information). The WC and SC groups were not significantly different (t test, $p > 0.1$ in all cases) along the first five PCs in all models, confirming that the solvent had no significant affect on the observed liver or testes metabolomes.

Multiple univariate statistical testing across the SC and 2, 20, and 200 μg/L fenitrothion treatment groups revealed 9.34% and 5.84% of the variables in the NMR and DIMS liver data sets were significantly different (FDR < 10%), respectively, while 0% and 1.56% of the variables differed significantly in the NMR and DIMS testes data sets, respectively (Table S7, Supporting Information). This indicated that fenitrothion induced a larger metabolic response in the liver versus testes metabolome, in agreement with the findings from the PCA. The NMR liver data set contained 161 significant bins (of a total of 1724, FDR < 10%) and of these, four metabolites (creatine, phosphocreatine, isoleucine, and valine) were positively identified (Table S9, Supporting Information). In the DIMS liver data set, 243 peaks (of a total of 4162) were significant (FDR < 10%) and of these, 20 were putatively identified from the KEGG database (Table S10, Supporting Information). In the DIMS testes data set there were 50 significant peaks (of a total of 3709), of which 10 were putatively identified from KEGG (Table S10, Supporting Information). Tables S11 and S12 (Supporting Information) provide extensive annotation of the entire 4162-peak liver and 3709-peak testes data sets, comprising m/z data, associated summary statistics (FDR q-value, metabolite x-fold changes), and putative identifications [empirical formula(s) and metabolite names from KEGG database]. Table 1 shows a selection of data from Tables S9, S11, and S12 (Supporting Information), focusing upon key metabolic changes in the DIMS and NMR data sets of liver and testes that are discussed below. In the DIMS liver data set the identities of creatine, phosphocreatine, and tyrosine were confirmed by observation of highly significant correlations between

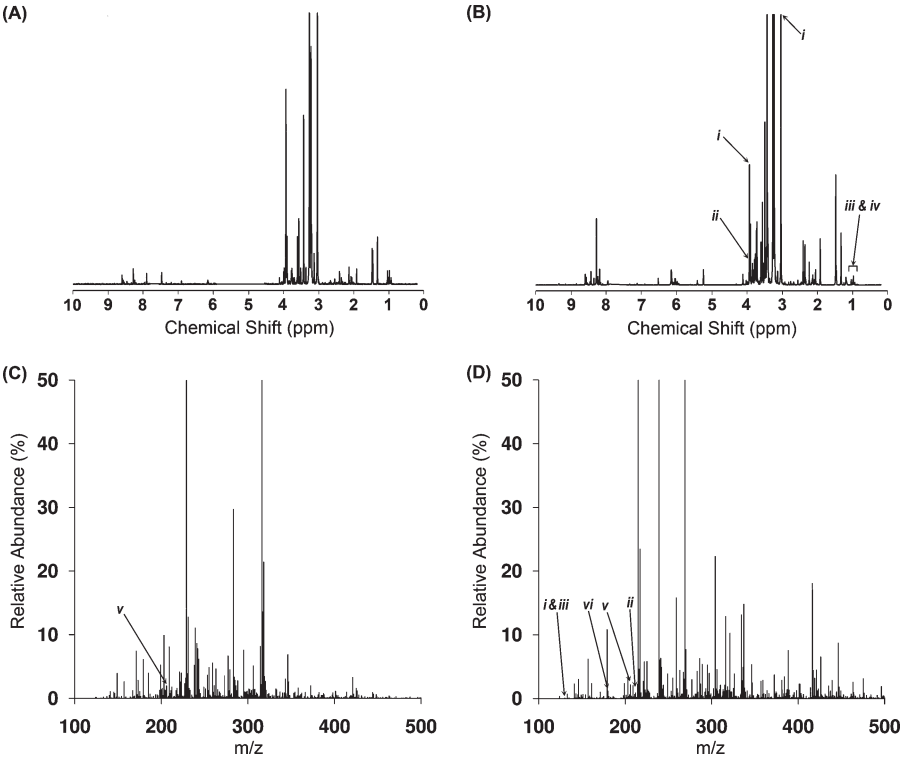

Figure 3. Representative spectra of tissue extracts from an untreated roach: 1D projections of *J*-resolved ^{1}H NMR spectra of (A) testes and (B) liver, and negative-ion direct infusion mass spectra of (C) testes and (D) liver. Selected metabolites that changed significantly in response to fenitrothion exposure are indicated: (i) creatine, (ii) phosphocreatine, (iii) isoleucine, (iv) valine, (v) *N*-acetylphenylalanine, and (vi) tyrosine.

the NMR and DIMS peak intensities (Table S10, Supporting Information), which also illustrated the high consistency between the analytical methods for these metabolites (further highlighted in Table S13 in Supporting Information).

Significant changes to creatine and phosphocreatine levels in liver indicated a perturbation to energy metabolism. In the testes, phosphocreatine levels were substantially increased in the highest exposure compared to SC, though not significantly at FDR < 10% (Table 1). Consistent with these findings and strengthening the case for perturbed energy metabolism, a peak putatively identified as the doubly charged ion of ATP (252.4906 m/z) showed near-significant changes similar to those of phosphocreatine in testes and liver tissue (Table 1). Significant increases in *N*-acetylphenylalanine levels (confirmed by MS/MS; Figure S1D in Supporting Information) in both tissues, together with altered tyrosine and phenylalanine levels in the testes and liver DIMS and liver NMR data sets (Table 1), indicated a fenitrothion-induced effect on phenylalanine metabolism.

Targeted Gene Expression Analysis. Relevant target genes were selected partly from prior knowledge of fenitrothion toxicity, for example, androgen receptor (*ar*) and aromatase (*cyp19a1a*), and partly from the discoveries of perturbed metabolic pathways, for example, glutathione S-transferase (*gst*), creatine kinase (*ck*), and phenylalanine hydroxylase (*pah*). In regard to pesticide degradation, testes *gst* was not significantly changed (p = 1.0), while hepatic *gst* expression showed a significant increase in response to fenitrothion (p = 0.007); however, WC was not significantly different from any exposed group (Figures S6 and S7, Supporting Information). Concerning energy metabolism, although *ck* showed no significant change in expression in either liver (p = 0.20) or testes (p = 0.07), a trend toward decreasing *ck* expression in the liver with increasing fenitrothion concentration was observed (Figure S6, Supporting Information; 2.0-fold decrease in 200 μg/L exposure relative to SC). Fenitrothion did not significantly alter *pah* expression in either testes or liver (p = 0.05 and 0.64, respectively; Figures S6 and S7 in Supporting Information). The measurement of endocrine-related genes in the testes revealed no significant changes in *ar* (p = 0.58), while *cyp19a1a* showed a significant decrease in response to fenitrothion (p = 0.03) (Figure S7, Supporting Information); however, SC was not significantly different from, any exposed groups suggesting that the solvent caused this effect.

DISCUSSION

Fenitrothion Degradation Pathway. The metabolic degradation product of fenitrothion, desmethylfenitrothion, was detected in brain, testes, and liver, confirming uptake of the pesticide by roach. The observed significant increase in *S*-methyl-GSH in fenitrothion-treated fish, and its significant correlation with desmethylfenitrothion (Figure 1), strongly suggests that both compounds result from metabolic decay of fenitrothion. Organophosphate (OP) compounds can undergo O-demethylation catalyzed by glutathione S-transferase in the presence of reduced glutathione, producing demethylated OP and *S*-methyl-GSH.[30] Here we confirm this as a major route of fenitrothion degradation in roach.

Alterations to Steroid Metabolism. The significant depletion of cortisone and suggested increase in 11-hydroxyandrostenedione in the testes, together with the significant depletion of 11-KT in the plasma, confirms that fenitrothion disrupts steroid metabolism in roach. Plasma 11-KT in fish is a much more

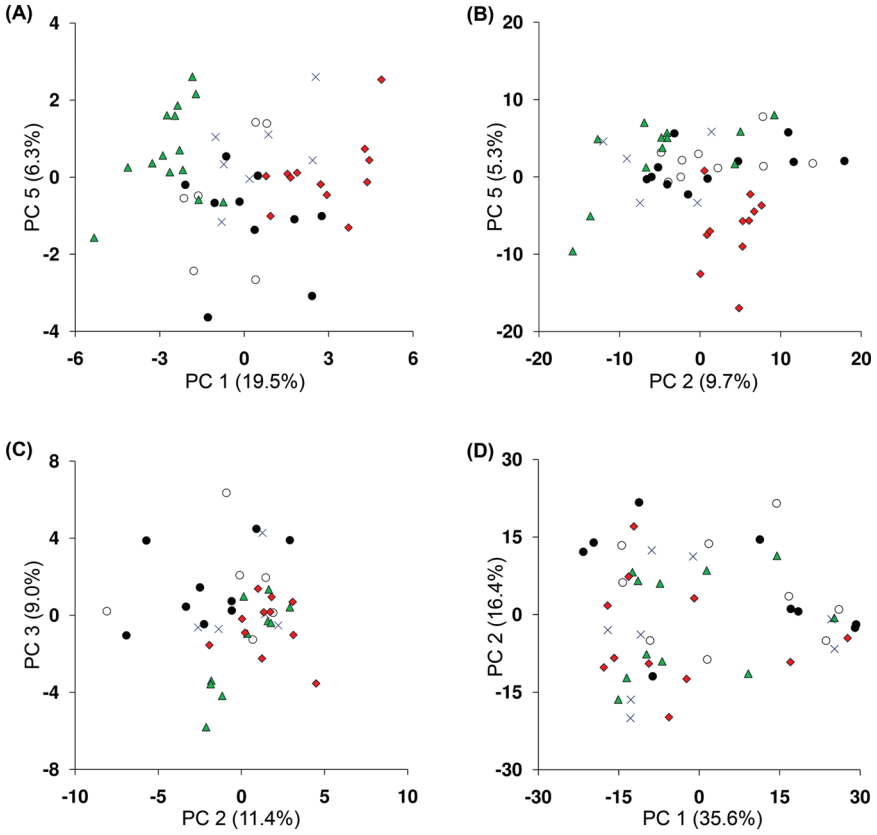

Figure 4. PCA score plots for (A) NMR liver data, (B) DIMS liver data, (C) NMR testes data, and (D) DIMS testes data. Key: (●) water control, (○) solvent control, and (▲, green) 2 μg/L, (×, blue) 20 μg/L, and (◆, red) 200 μg/L fenitrothion exposures. The PC axes were selected (up to PC5) based upon which axes showed a significant difference between groups (FDR < 10%; see Table S7 in Supporting Information), except for panel D, in which no PCs were significant so the axes capturing the highest variance were plotted.

Table 1. Key Putatively Identified Metabolic Perturbations from the DIMS and NMR Metabolomic Analyses of Liver and Testes from Fenitrothion-Exposed Roach[a]

		liver				testes			
			avg *x*-fold change[b]				avg *x*-fold change[b]		
putative metabolite identification	analytical method	*p*-value	2 μg/L	20 μg/L	200 μg/L	*p*-value	2 μg/L	20 μg/L	200 μg/L
creatine	DIMS	***1.9 × 10^{-5}**	0.51	1.09	1.22	0.656	1.19	1.07	1.13
creatine	NMR	***4.5 × 10^{-7}**	0.71	1.21	1.32	0.340	1.32	1.25	1.40
phosphocreatine	DIMS	***1.3 × 10^{-3}**	0.42	1.11	1.47	0.265	1.90	1.75	2.51
phosphocreatine	NMR	***4.3 × 10^{-4}**	0.69	1.32	1.45	0.204	2.10	1.76	2.35
ATP	DIMS	0.060	1.00	1.40	1.47	0.088	1.27	2.00	1.68
N-acetylphenylalanine	DIMS	***1.3 × 10^{-3}**	1.83	2.29	2.90	***3.9 × 10^{-4}**	1.05	1.27	2.05
N-acetylphenylalanine	NMR		nd	nd	nd		nd	nd	nd
phenylalanine	DIMS	0.045	1.16	1.28	0.92	***1.5 × 10^{-3}**	0.55	0.45	0.53
phenylalanine	NMR	0.068	1.24	1.30	0.90		nd	nd	nd
tyrosine	DIMS	***4.0 × 10^{-3}**	1.12	1.44	0.74	0.086	0.64	0.61	0.57
tyrosine	NMR	0.014	1.28	1.18	0.81		nd	nd	nd

[b] Relative to solvent control. [a] *Asterisk and boldface type indicates significant *p*-value, FDR < 10%. Complete lists of annotated peaks in the liver and testes data sets are shown in Tables S9, S11, and S12 in Supporting Information.

effective stimulator of secondary sexual characteristics and spermatogenesis than testosterone[31] and appears to play a key role in driving male sexual behaviors.[25] Thus, the effects seen on 11-KT could have a major bearing on sexual function in males. Previously it has been suggested that fenitrothion acts as an androgen receptor antagonist.[14] The consequences of such inhibition are 2-fold: first, depending on the extent of inhibition, receptor-mediated androgen signaling will be decreased. Second,

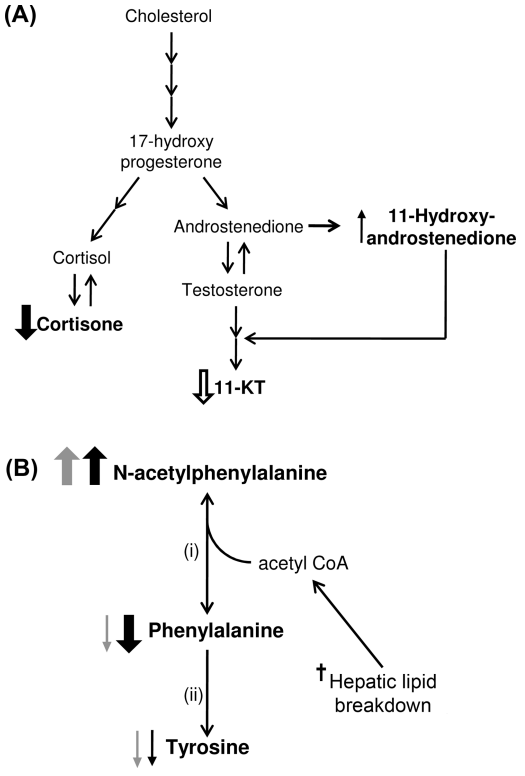

Figure 5. Overview of metabolic changes discovered in (A) steroidogenesis and (B) phenylalanine metabolism in roach. Observed metabolites are in boldface type, with arrows (broad = significant, narrow = near-significant) indicating the intensity change from solvent control to 200 μg/L fenitrothion exposure. Biological compartments of the observations: black arrow = testes; gray arrow = liver; open arrow = plasma. †Data from Sancho et al.[12] (i) Phenylalanine N-acetyltransferase; (ii) phenylalanine hydroxylase.

over time, androgen synthesis may increase to compensate for this reduction in receptor-mediated signaling.[14] Androgen synthesis, which occurs in the testes, requires cholesterol as its precursor, as is also the case for glucocorticoid synthesis (Figure 5A). The observed decrease in cortisone and increase in 11-hydroxyandrostenedione in the testes may indicate that steroid synthesis is directed away from glucocorticoids and toward androgens. Since the exposure of roach to fenitrothion was chronic, this may represent an adaptive cellular response to the continued inhibition of the androgen receptor, ultimately enabling androgen receptor-mediated activity even in the presence of the antiandrogenic effects of fenitrothion. The suggested increase in androgen synthesis in the testes was not reflected in the plasma, where 11-KT was significantly depleted. This may indicate a fenitrothion-induced inhibition of 11-KT secretion from the testes into the plasma, highlighting the importance of studying more than one tissue or biofluid compartment.

Metabolic Changes Revealed by Nontargeted Metabolomics. The liver is the major site of OP metabolism in mammals, including fenitrothion O-demethylation,[30] and fenitrothion has been demonstrated to bioconcentrate in European eel liver.[11] The considerable metabolic changes observed in roach liver, which were much greater than in testes, suggest this organ also plays the major responsive role in this species. Energy metabolism was affected in liver and testes, with perturbations to both creatine and the phosphagen phosphocreatine (Table 1); the latter is known to decrease during high energy demand to buffer ATP levels.[32] The *x*-fold change responses of these metabolites differed between tissues, with the liver showing a bimodal dose dependency (i.e., metabolites were depleted in the 2 μg/L treatment and increased in the 20 and 200 μg/L exposures, discussed below) and testes showing an increase across all fenitrothion doses. Interestingly, it is thought that such a bimodal dose dependency effect is actually more common than the threshold model.[33] In mammals, creatine synthesis is initiated in the kidney, completed by the liver,[34] and then is distributed to tissues (including testes) that have high energy demand. Hence differing responses of liver and testes are not unexpected. Overall our observations are consistent with the reported disruption to hepatic energy metabolism in eels acutely exposed to 40 μg/L fenitrothion.[12]

Another notable and previously undocumented effect of fenitrothion on metabolism, which was consistent across liver and testes, was the increase in *N*-acetylphenylalanine. Related metabolic changes were observed in phenylalanine and tyrosine (Table 1). The novelty of this observation is highlighted by the lack of literature on altered *N*-acetylphenylalanine levels in response to toxicant exposure. *N*-Acetylphenylalanine is generated from the reaction of phenylalanine and acetyl-CoA, catalyzed by phenylalanine N-acetyltransferase. Acetyl-CoA can be generated by the breakdown of stored lipids.[35] Fenitrothion has previously been shown to induce large-scale hepatic lipid depletion,[12] which, via acetyl-CoA production, could explain the observed significant increase in the acetylation of phenylalanine as well as the observed decreases in both phenylalanine and tyrosine (Figure 5B).

Chronic Exposure Suggests an Adapting Phenotype. The molecular changes detected could potentially act as biomarkers to assess fenitrothion toxicity in field-sampled chronically exposed roach. Our results, however, highlight the considerable challenge of interpreting molecular responses to *chronic* exposure. For example, OPs primarily exert toxicity via AChE inhibition that under acute exposure leads to ACh accumulation at nerve synapses. Although the significant effect on brain ACh observed here confirms that fenitrothion acts upon this target in roach, the *decrease* of ACh was unexpected and suggests that chronic OP exposure results in a degree of adaptation. This is consistent with a recent hypothesis that AChE recovery following chronic OP exposure can be overcompensated, leading to increased AChE activity and a cholinergic deficit.[36] Such compensatory shifts in molecular pathways—leading to an adapted phenotype—could also explain the large *x*-fold *increases* of phosphocreatine, in liver and testes, in response to the higher fenitrothion doses, again opposite to the anticipated acute response to high energy demand. Metabolic adaptation during the exposure period has been documented previously in a metabolomics study of fathead minnow dosed with 17α-ethynylestradiol.[37] Even if these underlying metabolic changes can be understood, the implications for interpreting molecular biomarker responses in field-sampled organisms are considerable. Specifically, does an adapted phenotype remain constant over time? This would be the ideal scenario, providing definitive criteria for field assessment (but requiring a multi-time-point toxicity study to address this). Second, can this phenotype provide sufficient evidence of mode of action(s) of pollutants and simultaneously reveal downstream (off-target) molecular effects that relate to whole-organism health? Our results from this single-toxicant study suggest that the answer is yes but that

unanticipated molecular responses should be expected, as adaptation is a complex phenomenon that will be challenging to unravel.

Discovery Role for Metabolomics in Environmental Biomonitoring. Several off-target perturbations were discovered by use of metabolomics, including disruption of phenylalanine metabolism. While this observation is robust, significant, and occurs in both liver and testes—therefore hinting at being a useful biomarker of chronic effect—it is unproven on two points: first, what are the functional implications of this perturbation? And second, how specific is this response to one pesticide, pesticide class, or even to toxicant exposure in general? While the considerable value of metabolomics for the initial discovery of toxicant-induced perturbations to molecular pathways is proven,[18,38,39] verifying the value of these discoveries prior to application in biomonitoring is essential and has rarely been achieved to date. This was addressed recently by Hines et al.,[39] who confirmed that selected metabolic biomarker signatures (discovered via metabolomics) were predictive of physiological toxicity in field-sampled marine mussels.

There are further reasons why shotgun metabolomics approaches will have limited direct application in biomonitoring. By definition, metabolomics measurements comprise vast numbers of analyses. Hence the threshold for statistical significance for any one response is penalized by the correction for multiple hypothesis testing (e.g., using the widely applied false discovery correction method).[28] Put simply, by such an approach, the primary mode of action of fenitrothion may not have been detected (significantly) in the brain if ACh was measured as part of a 4000-peak fingerprint. Furthermore, although we have demonstrated the value of metabolomics for detecting changes in both the endometabolome (roach biochemistry) and exometabolome (pesticide metabolism), separating these metabolomes in field-sampled fish that have been exposed to chemical mixtures would be a considerable challenge (at least until automated protocols for large-scale, definitive metabolite identification are developed). Overall we regard metabolomics as having an important role in the *discovery* of molecular pathways that are predictive of both mode of action and whole-organism chronic toxicity, but implementation of this knowledge into environmental biomonitoring should utilize *targeted* technologies, for example, multiplexed LC-MS.[40] Arguably a biomarker profile comprising ACh, 11-KT, cortisone, creatine, phosphocreatine, and *N*-acetylphenylalanine could convey considerable diagnostic information on target and off-target chronic toxicity of fenitrothion.

In conclusion, focusing on the specific actions of fenitrothion, we have confirmed that it significantly disrupts acetylcholine metabolism in roach brain and induces changes in key steroids in testes and plasma. This confirms that fenitrothion acts both via its primary mode of action and as an endocrine disruptor at environmentally realistic levels. Using discovery-driven approaches, we have provided the first evidence that fenitrothion can disrupt phosphocreatine regulation, providing a molecular rationale for previous reports of fenitrothion-mediated perturbations to whole-organism energetics. Furthermore, among several off-target effects, we have discovered a perturbation to phenylalanine metabolism that results in a significant elevation of *N*-acetylphenylalanine in both liver and testes. Collectively these findings have enhanced our understanding of the molecular toxicities of fenitrothion.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Further details on the methodology and results obtained, with seven figures and 13 tables, as indicated in the main text. This material is available free of charge via the Internet at http://pubs.acs.org.

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