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ORIGINAL ARTICLE

Strong resistance to β -cyfluthrin in a strain of the beetle *Alphitobius diaperinus*: a *de novo* transcriptome analysis

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Abstract The lesser mealworm, *Alphitobius diaperinus*, is an invasive tenebrionid beetle and a vector of pathogens. Due to the emergence of insecticide resistance and consequent outbreaks that generate significant phytosanitary and energy costs for poultry farmers, it has become a major insect pest worldwide. To better understand the molecular mechanisms behind this resistance, we studied a strain of *A. diaperinus* from a poultry house in Brittany that was found to be highly resistant to the β -cyfluthrin. The strain survived β -cyfluthrin exposures corresponding to more than 100 times the recommended dose. We used a comparative *de novo* RNA-Seq approach to explore genes expression in resistant versus sensitive strains. Our *de novo* transcriptomic analyses showed that responses to β -cyfluthrin likely involved a whole set of resistance mechanisms. Genes related to detoxification, metabolic resistance, cuticular hydrocarbon biosynthesis and proteolysis were found to be constitutively overexpressed in the resistant compared to the sensitive strain. Follow-up enzymatic assays confirmed that the resistant strain exhibited high basal activities for detoxification enzymes such as cytochrome P450 monooxygenase and glutathione-S-transferase. The in-depth analysis of differentially expressed genes suggests the involvement of complex regulation of signaling pathways. Detailed knowledge of these resistance mechanisms is essential for the establishment of effective pest control.

Key words *Alphitobius diaperinus*; *de novo* transcriptome; insecticide resistance; P450; xenobiotic metabolism

Introduction

In recent decades, pesticides have been increasingly used, especially in agriculture for crop protection (Walker *et al.*, 2012). Prolonged and cross-generational exposure to insecticides gradually eliminates nonresistant insects and reduces the genetic diversity of targeted populations (Medina *et al.*, 2007). This has contributed to the selection of pesticide-resistant strains (Rezende-Teixeira *et al.*, 2022), as observed in disease vectors such as *Anopheles gambiae* and *Aedes aegypti* (Martinez-Torres *et al.*, 1998;

Rodriguez *et al.*, 2001 and see Freeman *et al.*, 2021 for a review), or in agricultural pests such as in *Alphitobius diaperinus* (Hamm *et al.*, 2006; Renault & Colinet, 2021; Colinet *et al.*, 2023).

Insecticide resistance can result from (i) behavioral resistance (Zalucki & Furlong, 2017), (ii) penetration resistance (Lilly *et al.*, 2016), (iii) metabolic resistance (Ahmad *et al.*, 2006), and/or (iv) target site insensitivity (Soderlund & Knipple, 2003). Often, insecticide resistance has evolved through the two latter mechanisms (Soderlund & Knipple, 2003; Hemingway *et al.*, 2004; Li *et al.*, 2007; Panini *et al.*, 2016). Three main enzyme groups generally contribute to metabolic resistance: (i) glutathione-S-transferases (GST) (Pavliidi *et al.*, 2018), (ii) cytochrome P450 monooxygenases (P450) (Feyereisen, 2012; Liu *et al.*, 2015), and (iii) esterases (Est)

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(Hemingway & Ranson, 2000; Li *et al.*, 2007). In insects, P450s are involved in the metabolism and detoxification of almost all insecticides, as reported in *Drosophila melanogaster*, *Drosophila simulans* and *Musca domestica* (Li *et al.*, 2007; Nauen *et al.*, 2022). Increased activity of these detoxification enzymes is often triggered by gene upregulation, modulating enzyme and detoxification pathway expression, coding sequence mutations or a combination of these mechanisms (Li *et al.*, 2007; Nauen *et al.*, 2022). Complex signaling pathways are involved in the transcriptional regulation of detoxification genes and include regulators as aryl hydrocarbon receptor (AhR)/AHR nuclear translocator (ARNT), G protein-coupled receptors (GPCRs), Hormone Receptor-like in 96 (HR96), Cap “n” collar isoform C/Kelch-like ECH associated protein 1 (CncC/Keap1) and mitogen-activated protein kinase (MAPK)/cAMP-response element binding protein (CREB) (Amezian *et al.*, 2021; Black IV *et al.*, 2021; Lu *et al.*, 2021). For target site insensitivity, the modification of the insecticide target site results from random mutations or selection of variants of target sites (Hawkins *et al.*, 2018). The development of a single resistance type can provide cross-resistance against other chemical compounds, and a combination of these mechanisms can provide a multilayered protection against insecticides that reaches extremely high levels (Ahmad *et al.*, 2006).

Organophosphate or pyrethroid insecticides have been intensively sprayed for decades to control insect pests and vectors (Dunford & Kaufman, 2006; Zhu *et al.*, 2020). Pyrethroids are classified as type 1 (e.g., permethrin) or type 2 (e.g., cyfluthrin or its isomeric enriched form, β -cyfluthrin) (Baskar & Murthy, 2018), the latter being generally more effective (Wadleigh *et al.*, 1991; Palmquist *et al.*, 2012; Casida & Durkin, 2013). In insect, resistance to pyrethroids is linked to two main mechanisms (Silva *et al.*, 2014). A first mechanism involves mutation of the pyrethroid target site, the voltage-gated sodium channel (VGSC) (Soderlund & Knipple, 2003) conferring a knockdown resistance (*kdr*). The second mechanism involves the overexpression of genes leading to metabolic detoxification through the activities of P450, GST, and Est enzymes (Wang *et al.*, 2022). Both resistance mechanisms can coexist, but in some highly pyrethroid-resistant populations no *kdr* mutation has been found showing the importance of metabolic detoxification mechanisms in resistance phenomena (Zhong *et al.*, 2013).

The tenebrionid beetle *A. diaperinus* is a serious pest in broiler facilities (Dunford & Kaufman, 2006), as the warm and humid conditions of intensive poultry farming are highly suitable for its development. Insects thrive

in the mixture of spilled chicken feed, litter, excrement, and other avian detritus which provides a permanent source of food (Runge *et al.*, 2007). The intensive use of pyrethroids in poultry facilities caused the emergence of multiple resistant strains of *A. diaperinus* (Hamm *et al.*, 2006; Chernaki-Leffer *et al.*, 2011; Tomberlin *et al.*, 2014; Singh & Johnson, 2015; Renault & Colinet 2021; Colinet *et al.*, 2023). For instance, Lambkin (2005) revealed that the majority of 27 investigated poultry farms in Australia contained resistant *A. diaperinus* strains. Some *A. diaperinus* strains were described as resistant to cyfluthrin (Renault & Colinet, 2021). Recently, Colinet *et al.* (2023) isolated a strain highly resistant to β -cyfluthrin among populations collected in farms from Northern Brittany (France). A dose–response approach assessed that this strain tolerated more than 500 fold the recommended dose (RD) of β -cyfluthrin (i.e., label rate). No significant mortality had been observed at 500-RD in this strain.

Surprisingly, despite its importance as a pest species and evidence of insecticide resistance, the mechanisms by which resistance is attained in *A. diaperinus* have not been characterized. Kozaki *et al.* (2008) explored two cDNAs encoding acetylcholinesterase (AChE) genes (*AdAce1* and *AdAce2*) and found no evidence of mutations in tetrachlorvinphos-resistant strains. By using piperonyl butoxide (PBO) in their bioassays, a microsomal monooxygenase and esterase inhibitor, Lambkin and Furlong (2011) reported indirect evidence of metabolic resistance in an *A. diaperinus* strain resistant to cyfluthrin. However, this work was not developed further, so that the hypothesis of metabolic resistance has, to our knowledge, never been explored by genomic or biochemical studies.

Here, we explored molecular and biochemical mechanisms that could potentially be responsible for the high pyrethroid resistance of the *A. diaperinus* strain described in Colinet *et al.* (2023). These molecular mechanisms of resistance were examined by performing the first *de novo* transcriptomic analysis in *A. diaperinus*. Specifically, we focused on metabolic detoxification mechanisms and we aimed to test the hypothesis that genes involved in metabolic resistance are highly constitutively expressed in the resistant strain compared to a sensitive strain. To further test our hypothesis, we performed biochemical assays to assess the activity of candidate detoxification enzymes: GSTs, P450s, and Ests. Our study is the first mechanistic analysis in *A. diaperinus* that suggests the existence of metabolic resistance mediated by altered gene expression, as well as changes in detoxification enzyme activities.

Materials and methods

Insects

The β -cyfluthrin-resistant strain (RES) of *A. diaperinus* was field-collected from a poultry house in Northern Brittany (France) (Miniac sur Bécherel: 48°16'44.2"N, 1°55'46.8"W) in 2018. A sensitive strain (SENS) was used as control. This SENS strain was previously described as sensitive to insecticides exposure in two consecutive studies carried out in 2014 (Renault & Colinet, 2021) and 2018 (Colinet *et al.*, 2023). Individuals from this SENS strain have since been continuously maintained under insecticide-free rearing cultures under controlled conditions. Both strains were kept separately in large plastic boxes. The insects were supplied with dry dog food *ad libitum*, pieces of carrots, and StyrofoamTM to stimulate pupation as described in Renault and Colinet (2021). Water was supplied with moist cotton wool. Both strains were maintained at 25 ± 1 °C in continuous dark.

Bioassays

Bioassays were conducted in circular glass Petri dishes (90 mm \times 20 mm) on impregnated filter papers (63.615 cm²). β -cyfluthrin PESTANAL[®], analytical standard, was used (UPAC chemical name: (R)- α -cyano-4-fluoro-3-phenoxybenzyl (1S,3S)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate and (S)- α -cyano-4-fluoro-3-phenoxybenzyl (1R,3R)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarbox; purity (HPLC AREA %) \geq 98.0%; source: Sigma-Aldrich International GmbH). The recommended dose (RD) of β -cyfluthrin (i.e., label rate) for surface application is 0.2 g β -cyfluthrin/m². Filter papers were treated with acetone alone (i.e., control: ORD) or β -cyfluthrin dissolved in acetone at two concentrations: (1) 4 fold the recommended dose (4RD: 8.010 μ g β -cyfluthrin/cm²) or (2) 100 fold the recommended dose (100RD: 200.249 μ g β -cyfluthrin/cm²). Acetone was completely evaporated under a fume hood for at least 15 min before introducing the insects in the Petri dishes. For each treatment \times strain combination, three Petri dishes were set up each with 20 (range 17–21) adults to score the mortality until nine days of continuous exposure. Both SENS and RES insects were exposed to 4RD, but exposure to 100RD was only applied to RES beetles because pretests showed that this high concentration kills all SENS insects. At each sampling time (from 1 to 9 days), beetles were scored as alive and active (“Active”) or alive but knocked

down (“KD”) and the total number of living insects, called “Alive,” included the total of “Active” and “KD.” This resulted in five experimental treatments: RES strain control (RES-ORD) or exposed to 4RD (RES-4RD) or 100RD (RES-100RD) and SENS strain control (SENS-ORD) or exposed to 4RD (SENS-4RD). The data were analyzed using generalized linear models with logit link function for binary outcome (dead vs. alive). To account for complete separation, a bias reduction method was implemented by using “brglmFit” as fitting method. The differences among treatments were analyzed by comparing least-squares (Holm-adjusted) means in the “emmeans” R package.

Biochemical assays

Enzyme extraction Twelve samples per treatment (12 replicates), each consisting of a pool of three adults were collected after 24 h exposure to acetone control or to β -cyfluthrin at the concentrations indicated above. Samples were snap frozen in liquid nitrogen and stored at -80 °C until use. Beetles were homogenized in 0.1 mol/L KPO₄ buffer (pH 7.3) with tungsten beads (3 mm) and centrifuged for 5 min at $4000 \times g$. Collected supernatants were used for enzyme activity and protein assays.

Enzymatic assays Cytochrome P450 monooxygenase (Cyp), Glutathione S-transferase (GST), and esterases (Est) activities were determined spectrophotometrically in a 96-well plate reader (Molecular Devices, San Jose, CA, USA) using methods described in Brogdon *et al.* (1997) and Brogdon (2015) with minor modifications. Substrates used for the determination of Cyp, GST, and Est activities were 3,3',5,5'-tetramethylbenzidine (TMBZ), 1-chloro-2,4-dinitrobenzene (CDNB), and β -naphthyl acetate, respectively. The protein amount was determined with Bradford assay (Bradford, 1976). Mean activities were compared among the five treatments by ANOVA (“lm” function in R) followed by multiple comparisons (Holm-adjusted) in “emmeans” R package.

RNA extraction and purification

Given that 4RD β -cyfluthrin treatment induced significant deaths to the SENS strain from 24 h to 9 days, we chose to study beetles at 24 h of exposure when primary effects of insecticide could be analyzed. After 24 h of exposure to either the acetone control, 4RD β -cyfluthrin or 100RD β -cyfluthrin, three independent biological replicates, each consisting of a pool of three alive insects,

were collected from both strains (SENS or RES). As for the enzyme assays, 100RD exposure was only applied to RES strain. Each sample was snap frozen in liquid nitrogen and stored at -80°C for downstream analysis. Total RNA was extracted from whole bodies after homogenization using a NucleoSpin[®] RNA Kit (Macherey-Nagel), following the manufacturer's protocol. The RNA quality and quantity were assessed with NanoDrop and Bioanalyzer RNA nano 6000. Bioanalyzer electropherograms revealed excellent RNA integrity in all samples.

cDNA library generation and Illumina sequencing and quality trimming

Fifteen *A. diaperinus* libraries were prepared (i.e., three replicates and five treatments): RES strain control (RES-0RD) or exposed to 4RD (RES-4RD) or 100RD (RES-100RD) and SENS strain control (SENS-0RD) or exposed to 4RD (SENS-4RD). cDNA libraries were prepared using the TruSeq[®] Stranded mRNA kit (Illumina) and sequenced by Genoscreen (Genoscreen, Lille, France) using an Illumina HiSeq 4000 platform (Illumina, San Diego, California, USA) with sequence runs of 2×150 -bp paired-ends. All bioinformatic analyses were conducted via the Galaxy instance of GenOuest at <https://galaxy.genouest.org/>. RNA reads obtained from sequencing were quality assessed using FastQC (Andrews, 2010; Galaxy Version 0.72). Illumina adapter sequences were removed using Cutadapt (Martin, 2011; Galaxy Version 1.16.6). rRNA were also discarded using SortMeRNA (Kopylova *et al.*, 2012; Galaxy Version 2.1b.6). Resulting sequencing reads were quality-checked using FastQC (Galaxy Version 0.11.8).

De novo transcriptome assembly

The clean-reads were *de novo* assembled using Trinity software (Grabherr *et al.*, 2011; Haas *et al.*, 2013; Galaxy Version 2.9.1) using a *in silico* normalization of reads and enabling the “jaccard clip” flag used for compact genomes (351 012 sequences). The quality of RNA-Seq samples and biological replicates was assessed by principal component analysis (PCA) in the Trinity suite using raw counts. Then, *A. diaperinus* assembled contigs were filtered according to their length allowing sequences greater than 200 bases. CD-HIT-EST (Fu *et al.*, 2012; Galaxy Version 1.3) was used to cluster the contigs into nonredundant representative sequences. BUSCO tool (Simão *et al.*, 2015; Galaxy Version 4.1.4) was used to assess transcriptome assembly and complete-

ness against a database of Arthropoda universal single copy orthologs. Transdecoder (Haas *et al.*, 2013; Galaxy Version 5.5.0) was used to find coding regions within contigs. Functional annotation was carried out using Trinotate (Grabherr *et al.*, 2011; Galaxy Version 3.2.1) with representative sequences and Transdecoder generated peptide results and BLASTP, BLASTX (Galaxy Version 2.10.1), Hmmscan (Galaxy Version 3.3), TMHMM 2.0 (Galaxy Version 0.0.16), and SignalP 4.1 (Galaxy Version 0.0.19) tools. BLAST searches were conducted with a cutoff E-value of 10^{-5} against UniProt/Swiss-Prot and the close relative *Tribolium castaneum* (tenebrionid beetle) (Tcas5.2) databases. Trinotate results were compiled to reduce unannotated sequences. Differentially expressed genes (DEGs) with first high similarities in UniProt/Swiss-Prot database with a non-*T. castaneum* sequence (15%) were converted to red flour beetle ortholog.

Differential gene expression analysis and functional enrichment

The differential gene expression analyses were performed in Galaxy using the “Align reads and estimate abundance” tool (Grabherr *et al.*, 2011; Galaxy Version 2.9.1) with Bowtie tool to align clean-reads and final transcriptome assembly version and with RSEM tool to estimate the transcript abundance. The *Trimmed Mean of M-values (TMM)* method implemented in the *edgeR* package was used for normalization. The false-discovery rate (FDR) < 0.05 from corrected *P*-value and the absolute value of $\log_2(\text{fold-change (FC)}) \geq 1.58$ (i.e., $\text{FC} \geq 3$) were used as thresholds to select differentially expressed genes (DEGs) between two compared conditions. In the RES strain, the transcriptomes of 4RD and 100RD treatments were compared to that of untreated ORD, yielding to the comparisons RES-4RD/RES-0RD and RES-100RD/RES-0RD, respectively. In the SENS strain, the transcriptome of 4RD treatment was also compared to that of untreated, giving the SENS-4RD/SENS-0RD comparison. Finally, the transcriptomes of ORD and 4RD were compared between RES and SENS strains, yielding to the comparisons RES-0RD/SENS-0RD and RES-4RD/SENS-4RD, respectively. The ClueGO (v.2.5.7) Cytoscape (v.3.8.2) plug-in was used to calculate and visualize Gene Ontology (GO) and KEGG pathway enrichments for each comparison (Bindea *et al.*, 2009). The enrichment significance in terms and groups was calculated with a threshold of $P < 0.001$. Complete transcriptome data was deposited in the Transcriptome Shotgun Assembly (TSA) database under BioProject ID: PRJNA928577.

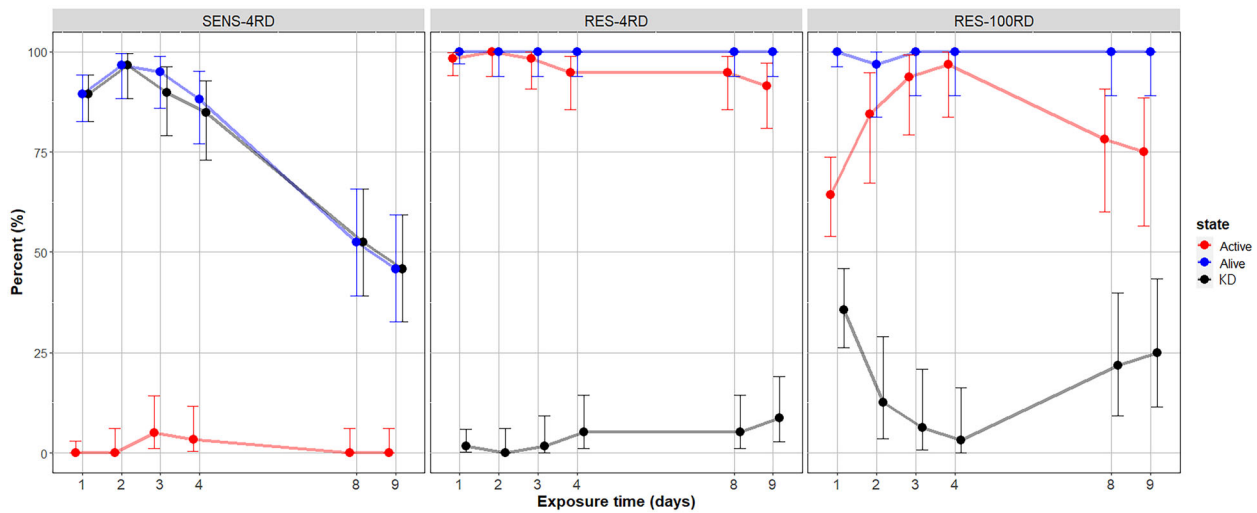


Fig. 1 Susceptibility of SENS and RES strains to β -cyfluthrin. SENS and RES insects were exposed to 4RD β -cyfluthrin (SENS-4RD, RES-4RD). Exposure to 100RD β -cyfluthrin was only applied to resistant beetles (RES-100RD). At each sampling time (from 1 to 9 days), beetles were scored as alive and active (“Active”) or alive but knocked down (“KD”) and the total number of living insects, called “Alive,” included the total of “Active” and “KD.” Each dot represents a mean of three replicates with 95% CI.

Validation by RT-qPCR

RT-qPCR was used to assess and validate expression of 20 different DEGs, which were either strongly up- or downregulated (i.e., with absolute $\text{Log}_2\text{FC} > 2$ and $\text{FDR} < 0.05$) among at least one of the five comparisons. Specific primers were designed according to sequencing using Primer3 software (Untergasser *et al.*, 2012; Table S1). Total RNA from three biological replicates was reverse transcribed to cDNA (SuperScriptTM III First-Strand Synthesis SuperMix, Invitrogen). qPCRs were then conducted using LightCycler[®] 480 (Roche Diagnostics, Meylan, France). Conditions were: 95 °C, 3 min, and 40 cycles (95 °C, 15 s; 60 °C, 30 s; 72 °C, 30 s). Gene expression was expressed relative to the most stable housekeeping gene among four tested genes. Selection of the best housekeeping gene was done using the Refinder pipeline (which integrates the major computational programs geNorm, Normfinder, BestKeeper). For each primer pair, raw amplification data were used to calculate the efficiency using the Miner pipeline (Zhao & Fernald, 2005; Tellinghuisen & Spiess, 2014). Relative expression ratios (i.e., Log_2FC) were then calculated using the efficiency calibrated model of Pfaffl (Pfaffl, 2001). Log_2 mean expression of the RNA-Seq data was regressed against that of the RT-qPCR data with “lm” function in R, assuming a slope equal to 1 in the case of a good match between both datasets (“linearHypothesis” test in R).

Results and discussion

Lesser mealworms collected from a conventional broiler farm are highly resistant to β -cyfluthrin

Almost no insect from the SENS strain was active when exposed to 4RD β -cyfluthrin at any exposure duration (Fig. 1). SENS insects were mostly knocked down during the first 4 days of exposure, and instead of recovering with time like the RES strain, they progressively died with only 46% insects being alive after 9 days of exposure (Fig. 1). In contrast, in the presence of 4RD β -cyfluthrin, most RES insects remained fully active (~90%) throughout the test and no mortality was observed (Fig. 1). This RES strain was able to tolerate a dose of β -cyfluthrin 100 times the recommended dose (i.e., 100RD). At 100RD, a portion of RES insects was initially KD, but they gradually recovered to become fully active again (Fig. 1). The GLM model showed a significant effect of time on survival probability ($\chi^2 = 74.88$, P -value < 0.001), as well as a treatment effect ($\chi^2 = 171.49$, P -value < 0.001) in which the SENS-4RD was clearly different from both RES-4RD and RES-100RD. Resistance to β -cyfluthrin has already been reported in several insect species. For instance, Holderman *et al.* (2018) observed resistance to β -cyfluthrin in horn fly population. Likewise, Adelman *et al.* (2011) reported strong resistance to β -cyfluthrin in *Cimex lectularius*. In *A. diaperinus*, resistance to cyfluthrin was also previously reported (Lambkin & Furlong, 2011; Renault & Colinet, 2021).

Characterization of β -cyfluthrin resistance mechanisms

Whole transcriptome sequencing and data assembly

Despite its major economic impact on the poultry industry, so far, no genomic information on the lesser mealworm was available. To decipher the transcriptomic basis of resistance, we thus carried out the first *de novo* transcriptome analysis in *A. diaperinus* by comparing basal and β -cyfluthrin-exposed transcriptomes in SENS versus RES strains.

Illumina sequencing of the fifteen libraries yielded a total of 21 157 854 to 32 923 608 paired-end reads per library for a total of 818 380 906 reads reduced to a total of 765 083 122 clean-reads after quality trimming 93.48% of total reads (Table S2). The final *A. diaperinus de novo* transcriptome assembly version included 249 946 distinct contigs with an N50 sequence length of 1467 bp, a mean sequence length of 793 bp and a longest sequence of 35 405 bp. Overall statistics of the final Trinity-assembled *A. diaperinus* transcriptome are listed in Table S2. PCA showed that RNA-Seq samples were mainly grouped by strains (Fig. S1). Indeed, the first axe of the PCA (PC1) could explain 94% of the variance between SENS and RES strains suggesting profound differences in expression profile. BUSCO analysis showed high completeness of the transcriptome with 99.1% of conserved Arthropoda genes (Table S3), with only 0.8% and 0.1% of genes fragmented or missing, respectively. A total of 47 514 contigs were mapped to Swiss-prot and 63 991 contigs, representing 94.6% of the matches to databases of its closer relative *T. castaneum* (Tcas5.2), leading overall to a total of 67 620 unique annotated contigs. RNA-Seq results were validated with RT-qPCRs based on 20 target genes spread across 36 comparisons (Fig. S2). Differential expression patterns were highly similar between RNA-Seq and qPCR datasets (Pearson's correlation: P -value < 0.001; linear regression: P -value < 0.001; $R^2 = 0.90$). The slope of the relation between expression of RNA-Seq versus qPCR was not significantly different from 1 ($F = 0.067$; P -value = 0.797), indicating a strong similarity between both methods (Fig. S2).

Differentially expressed genes set differs between RES and SENS strains

We first compared transcript expression of the treated strains to those of the untreated strains (i.e., SENS-4RD/SENS-0RD, RES-4RD/RES-0RD, and RES-100RD/RES-0RD). Only contigs with a fold-change (FC) variation in expression superior to three between two conditions (i.e., $|\text{Log}_2\text{FC}| > 1.58$ and $\text{FDR} < 0.05$) were considered as differentially expressed genes (DEGs) (Table S4). In the SENS strain, 205 DEGs (105 induced and 100 repressed) were found in the comparison

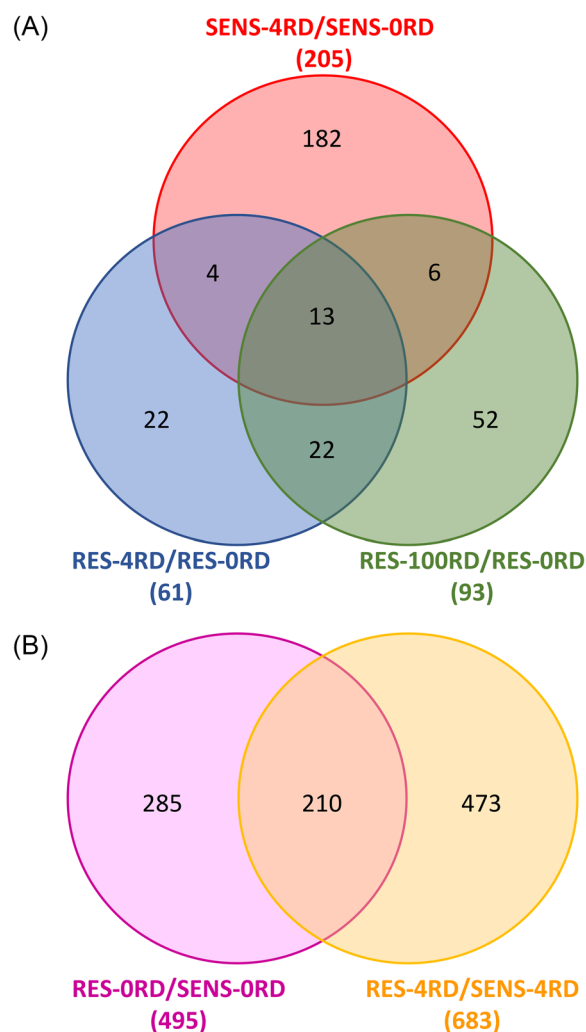


Fig. 2 Venn diagram representing the common and specific DEGs according to the different comparisons. (A) Number of detected DEGs between the SENS strain in response to the 4RD β -cyfluthrin (SENS-4RD/SENS-0RD) and the RES strain in response to the 4RD (RES-4RD/RES-0RD) and 100RD (RES-100RD/RES-0RD) β -cyfluthrin. (B) Number of detected DEGs between the untreated RES strain and the untreated SENS strain (RES-0RD/SENS-0RD) and the 4RD treated RES strain compared to the 4RD treated SENS strain (RES-4RD/SENS-4RD).

son SENS-4RD/SENS-0RD. For the RES strain, 61 (38 induced and 23 repressed) and 93 (45 induced and 48 repressed) DEGs were found in RES-4RD/RES-0RD and RES-100RD/RES-0RD, respectively (Table S4, Fig. 2A). Hence, in the RES strain, significantly fewer genes responded to β -cyfluthrin compared to the SENS strain, suggesting a lower plasticity or the existence of a constitutive resistance mechanism. The low number of common DEGs between comparisons (Fig. 2A) suggested distinct

molecular responses between the two strains in response to β -cyfluthrin and could partly explain the distinct behavioral and physiological responses between the strains. Indeed, DEGs in SENS-4RD/SENS-0RD reflected β -cyfluthrin-induced coma and progressive death in the SENS strain (Fig. 1), while DEGs in RES-4RD/RES-0RD and RES-100RD/RES-0RD were associated with maintenance of activity and survival in the RES strain, even in presence of high doses of β -cyfluthrin (Fig. 1). The comparison RES-0RD/SENS-0RD provided insight into the basal gene expression profile between both strains (Table S4). In the absence of the insecticide, basal expression of RES differed considerably from that of SENS with 495 DEGs (301 induced and 194 repressed) (Table S4, Fig. 2B). Comparing gene expression between RES and SENS in response to 4RD β -cyfluthrin (i.e., the RES-4RD/SENS-4RD comparison), 683 DEGs were found in total of which 467 were induced and 216 repressed (Table S4, Fig. 2B). Hence, the RES strain differed from the SENS strain first by specific basal expression (285 DEGs) in absence of insecticide but also by specific changes in gene expression (473 DEGs) in the presence of β -cyfluthrin (Fig. 2B). These expression profiles suggested that β -cyfluthrin resistance of the RES strain could in part be due to protective activities already constitutively expressed.

GO terms and KEGG terms enrichment according to strains To characterize the transcriptomic responses, functional enrichment analysis was performed for each comparison (Table 1, see Figs. S3–S6 for detailed enrichments in GO and KEGG terms).

Among upregulated DEGs in the SENS-4RD/SENS-0RD comparison (Table 1, Fig. S3A), only two GO terms linked to catabolic processes in biological process category were overrepresented, while downregulated DEGs were characterized by enriched terms linked to biosynthesis, metabolism pathways, proteolysis, oxidoreductase activities or xenobiotics biodegradation (Table 1, Fig. S3B). These GO and KEGG terms are closely related since, in an efficient system, xenobiotic exposure generates oxidative stress attenuated by oxidoreductase activities, detoxification processes including P450 (Wang *et al.*, 2016; Black IV *et al.*, 2021) and by maintenance of a functional system through “Pentose and glucuronate interconversions” pathway (Fig. S3B, Agledal *et al.*, 2010). Indeed, this last pathway generates NADPH and pentoses, to allow, *inter alia*, homeostasis through the maintenance or regeneration of cellular detoxifying and antioxidative defense systems (Agledal *et al.*, 2010). Downregulations of these pathways showed that the response mechanisms of the SENS strain were not efficient enough to overcome

the chemical stress bringing insects into coma when exposed to β -cyfluthrin. Insecticide treatment also gave rise to the activation of genes involved in catabolism (modified amino acid catabolism, cofactor catabolism) and to the repression of genes involved in protein synthesis, metabolic processes and the proteolytic process. These expression variations then caused energy deficit and dysfunctions. The detoxification tools were not sufficiently effective in SENS strain to overcome the adverse effect mediated by the insecticide, leading insects to exhibit altered functions (“KD”) and finally death.

The exposure to 4RD β -cyfluthrin did not prevent activity in the RES strain (Fig. 1). Intriguingly, the functional enrichment for RES-4RD/RES-0RD showed only one cluster of overrepresented GO and KEGG terms for upregulated DEGs, related to fatty acid synthesis, whereas for downregulated DEGs no enrichment was observed (Table 1, Fig. S4A). Such overrepresentation of terms linked to lipid biosynthesis and degradation has also been described for a pyrethroid-resistant *Anopheles coluzzii* population in response to deltamethrin (Ingham *et al.*, 2021). Moreover, Hu *et al.* (2020) clearly demonstrated that *lipase A*, encoding an enzyme that catalyzes the hydrolysis of cholesteryl esters and triglycerides, was involved in deltamethrin resistance in *Culex pipiens pallens*. The fatty acid composition is known to differ between resistant and sensible populations, as demonstrated for neonicotinoid-resistant *Leptinotarsa decemlineata* (Clements *et al.*, 2020), suggesting that RES strain responded to 4RD β -cyfluthrin with specific lipid synthesis. In contrast, with exposure to 100RD in RES, we found downregulated DEGs enriched in GO and KEGG terms related to fatty acid biosynthesis. So, although not lethal, the higher concentration of insecticide affected lipid composition. The analysis of downregulated DEGs after the 100RD treatment showed the overrepresentation of oxidoreductase activity and some pathways of molecular regulation (Table 1, Fig. S4C). Likewise, the higher β -cyfluthrin treatment (Fig. S4B) led, for upregulated DEGs in RES strain, to the enrichment in GO terms related to the regulation of transcription, suggesting some induction of molecular regulation. Analysis of the overrepresentation of GO and KEGG terms in RES strain in response to insecticide treatments provided a scattered overview of its resistance mechanisms, suggesting that constitutive expression of key proteins allowed *de facto* a strong protection. The 100RD treatment showed some altered functions that reflected a few toxic side effects (Table 1).

Even without treatment, constitutive expression of genes differed considerably between the strains (i.e., RES-0RD/SENS-0RD; see Fig. 2B, Table S4). Enriched

Table 1 Functional enrichment analysis.

Transcriptome comparison	Enriched GO term or KEGG pathway in upregulated DEGs (<i>P</i> -value)	Enriched GO term or KEGG pathway in downregulated DEGs (<i>P</i> -value)
SENS-4RD/SENS-0RD	<ul style="list-style-type: none"> Cellular modified amino acid catabolic process (2.7×10^{-4}) 	<ul style="list-style-type: none"> Monosaccharide biosynthetic process (3.9×10^{-6}) Serine-type endopeptidase activity (1.5×10^{-5}) Pentose and glucuronate interconversions (9.3×10^{-5}) Monoxygenase activity (1.1×10^{-4}) Insect hormone biosynthesis (2.6×10^{-3}) Cysteine-type endopeptidase activity (4.9×10^{-3}) Alpha-amino acid metabolic process (1.1×10^{-2}) Chitin metabolic process (1.2×10^{-2})
RES-4RD/RES-0RD	<ul style="list-style-type: none"> Fatty acid synthase activity (2.2×10^{-4}) 	NE
RES-100RD/RES-0RD	<ul style="list-style-type: none"> Core promoter binding (9.6×10^{-4}) 	<ul style="list-style-type: none"> 2-alkenal reductase [NAD(P)] activity (1.1×10^{-5}) Second-messenger-mediated signaling (6.9×10^{-3}) Fatty acid biosynthesis (1.4×10^{-3}) Regulation of protein serine/threonine kinase activity (6.6×10^{-3})
RES-0RD/SENS-0RD	<ul style="list-style-type: none"> Monoxygenase activity (2.8×10^{-7}) Metabolism of xenobiotics by cytochrome P450 (1.9×10^{-6}) Serine-type endopeptidase activity (1.5×10^{-5}) RNA polymerase II core promoter sequence-specific DNA binding (2.9×10^{-4}) 	<ul style="list-style-type: none"> Serine-type endopeptidase activity (2.0×10^{-4}) Monoxygenase activity (2.7×10^{-3})
RES-4RD/SENS-4RD	<ul style="list-style-type: none"> Monoxygenase activity (3.0×10^{-15}) Endopeptidase activity (2.8×10^{-7}) Metabolism of xenobiotics by cytochrome P450 (8.43×10^{-6}) Carboxylic acid metabolic process (1.4×10^{-4}) Carboxylic ester hydrolase activity (2.5×10^{-4}) Valine, leucine, and isoleucine degradation (5.9×10^{-4}) 	<ul style="list-style-type: none"> Drug metabolic process (2.3×10^{-5}) Protein folding chaperone (2.3×10^{-4}) Monosaccharide binding (3.9×10^{-4}) Serine-type endopeptidase activity (7.5×10^{-4}) Hexose metabolic process (1.2×10^{-3}) RNA polymerase II core promoter sequence-specific DNA binding (1.7×10^{-3})

The enrichment significance in terms and groups was calculated with a threshold of *P*-value < 0.001. NE: no enrichment.

GO terms and KEGG terms emerging from the up-regulated DEGs in the RES-0RD/SENS-0RD comparison (Table 1, Fig. S5A) showed first two large clusters

that confirmed the implication of constitutive expressions in RES strain. A first cluster involved KEGG terms related to “Metabolism of xenobiotics by cytochrome

P450,” suggesting that RES strain exhibited at least some metabolic resistance mechanisms of Phase I linked to P450s. A second cluster grouped GO terms linked to the first one and related to “monooxygenase activity” (Table 1, Fig. S5A). This was in line with the fact that constitutive overexpression and/or induction in pesticide-resistant strains are a feature of effective pesticide detoxification mechanisms, especially concerning the detoxification mechanisms linked to P450s (Zhu *et al.*, 2010, 2016; Dang *et al.*, 2017). Moreover, pyrethroids, such as permethrin, are known to cause oxidative stress (Wang *et al.*, 2016), so the constitutive expression of efficient antioxidant defense systems may alleviate toxic effects of β -cyfluthrin in the RES. A third cluster of upregulated DEGs was related to proteolysis through enrichment GO terms like “serine-type endopeptidase activity” (Table 1, Fig. S5A). Such an enriched cluster was also found downregulated in the SENS-4RD/SENS-0RD comparison (Table 1, Fig. S3B). Hou *et al.* (2014) demonstrated that detoxification mechanisms require energy that can be provided by the action of proteinases. Serine peptidases have been particularly and indirectly implicated in these processes through their capability to supply necessary energy and amino acids for homeostasis. Basal expression of DEGs related to the regulation of transcription was higher in the RES strain than in the SENS strain, showing the constitutive expression of regulation pathways (Table 1, Fig. S5A). Among the 194 repressed DEGs in the RES-0RD/SENS-0RD comparison (Table 1, Fig. S5B), enrichments of GO terms related to “serine-type endopeptidase activity” and to “monooxygenase activity” were detected, suggesting that related genes that were expressed in SENS strain in control condition were different from those expressed in RES strain.

In the presence of β -cyfluthrin at 4RD, functional enrichment patterns of induced DEGs in the RES strain compared to the SENS strain (RES-4RD/SENS-4RD) changed little compared to those observed without any treatment (Table 1, Fig. S5A and S6A). In addition to the activities related to “Metabolism of xenobiotics by cytochrome P450,” to “proteolysis” and to “monooxygenase,” the overrepresentation of terms linked to “carboxylic ester hydrolase activity,” “carboxylic acid metabolic process” and “valine, leucine and isoleucine degradation” were found. The last two clusters are, like proteolysis, related to the energy requirements of cells during treatment application and insecticide detoxification. Carboxylic ester hydrolases or carboxylesterases, known as detoxification enzymes of Phase I involved in pyrethroid metabolism (Zhu *et al.*, 2010, 2013a, 2013b; Xi *et al.*, 2015; Black IV *et al.*, 2021; Li *et al.*, 2021),

were more expressed during exposure to β -cyfluthrin in the RES strain.

Regarding the overrepresentation of terms related to downregulated DEGs in the RES-4RD/SENS-4RD comparison (Table 1, Fig. S6B), a very large cluster was related to the biological process “protein folding chaperone.” Hence, in the presence of β -cyfluthrin, chaperone activities were repressed in the RES strain compared to the SENS strain. The SENS strain must therefore have activated a chaperone pathway to eliminate unfolded proteins, probably because the cells were not already protected against the insecticidal effects. Another cluster related to “drug metabolic processes” was repressed in the RES-4RD/SENS-4RD comparison, suggesting that it was overexpressed in SENS strain. These detoxification processes that took place in the SENS strain appeared distinct from those upregulated in the RES strain; they mainly involved several metabolic processes and chitin binding that were not efficient against β -cyfluthrin effects (Fig. S5). Overall, the contrasted responses of *A. diaperinus* strains revealed several processes mainly involved in detoxification, proteolysis and fatty acid metabolism.

Detoxification responses involved in β -cyfluthrin resistance To test whether the constitutive overexpression of genes related to metabolic resistance and antioxidant defense in RES strain resulted in higher enzymes activities, we assessed three enzymes: GST, P450 and Est. These enzymes are known to underlie metabolic resistance against pyrethroids (Dang *et al.*, 2017; Zhu *et al.*, 2020) (Fig. 3).

P450 and GST baseline activities were significantly enhanced in the RES strain compared to SENS strain (GST: $F = 4.07$, P -value = 0.005; P450: $F = 5.92$, P -value < 0.001). These activities tended to increase slightly in the resistant strain after exposure to 4RD and 100RD β -cyfluthrin (Fig. 3). On the other hand, Est activities were not statistically different between strains nor in the presence of β -cyfluthrin ($F = 0.958$, P -value = 0.438). These results corroborated the gene expression profiles and suggested that increased basal GST and P450 activities may have played a role in resistance and detoxification of β -cyfluthrin in *A. diaperinus*. Adelman *et al.* (2011) demonstrated in the bed bug, *Cimex lectularius*, that general esterase and P450 activities were higher in a β -cyfluthrin-resistant strain than in a sensitive strain. Cyfluthrin resistance in *T. castaneum* was also associated with an increased expression of a constitutive form of GST (Reidy *et al.*, 1990).

The expression of DEGs involved in detoxification processes in the RES-0RD/SENS-0RD, RES-4RD/

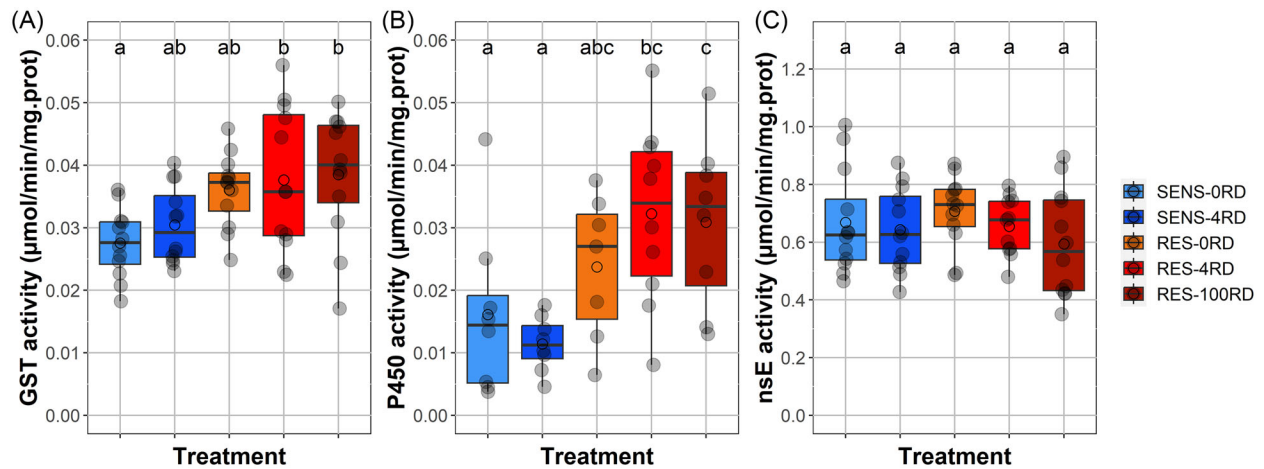


Fig. 3 Effects of β -cyfluthrin treatments on detoxification enzyme activities. (A) Glutathione S-transferase (GST) expressed in units corresponding to the production of μmol of conjugated CDNB per min per mg of protein, (B) Cytochrome P450 (Cyp) expressed in units corresponding to the production of μmol of P450 equivalent (oxidized TMBZ) per min per mg of protein, and (C) esterase (Est) activity expressed in units corresponding to the production of μmol of beta-naphthol per min per mg of protein in the sensitive (SENS, blue) and the resistant (RES, red) strains nonexposed or exposed to 4 time or 100 time the recommended dose (RD). Boxplot represents the lower quartile (Q1), the median, and the upper quartile (Q3). The different letters indicate significant differences resulting from Holm-adjusted pairwise comparisons at P -value < 0.05 .

SENS-4RD, and SENS-4RD/SENS-0RD comparisons were also represented in volcano plots and heat map (Fig. 4A, D). Looking at detoxification-related DEGs led to the identification of a great number of contigs involved in almost all the known steps of detoxification processes. Fig. 4A, D showed the expression of DEGs related to metabolic resistance from Phase I as Cytochrome P450 monooxygenase, Carboxylic ester hydrolase, and also from Phase II as GST, UDP-glycosyltransferase (UGT) and finally from Phase III as ABC-type xenobiotic transporter in the three comparisons. The basal expression of DEGs specifically involved in detoxification was drastically different in RES compared to SENS. Fifty-three and 19 DEGs were, respectively, induced and repressed in the RES-0RD/SENS-0RD comparison. In the presence of β -cyfluthrin at 4RD, the RES strain overexpressed 103 detoxification-related DEGs and under-expressed 14 others in RES-4RD/SENS-4RD (Fig. 4A, D). By contrast, in the comparison SENS-4RD/SENS-0RD, only 4 upregulations and 14 downregulations of detoxification-related DEGs were found.

Transcriptomic analysis showed that genes encoding the three components of the P450 monooxygenase system (Dang *et al.*, 2017; Black IV *et al.*, 2021), cytochrome P450, NADPH-cytochrome P450 reductase (CPR), and cytochrome b5 (Cyt) were found among expressed contigs in *A. diaperinus* (Fig. 4A–D, Table S4). In *T. castaneum*, a close relative of *A. diaperinus*, 143 cytochrome

P450 genes have been reported and annotated (Zhu *et al.*, 2013b). In *A. diaperinus*, our analysis revealed 88 contigs with very strong homologies with *T. castaneum* cytochrome P450 genes; these genes were annotated as their homolog followed by “-like” (Table S4). For instance, 34 P450-like contigs showed a higher basal and constitutive expression relative to SENS (Fig. 4A, D, Table S4). The exposure to β -cyfluthrin induced 39 other P450-like contigs (RES-4RD/SENS-4RD). Only 10 P450-like contigs were differentially expressed in the SENS strain (SENS-4RD/SENS-0RD), out of which six were repressed. The transcriptomic analysis highlighted Cyp-like genes that were exclusively detected or overexpressed in the RES strain. Among the four known P450 clans in *T. castaneum*, CYP2, the mitochondrial CYP clan, CYP3 and CYP4 (Zhu *et al.*, 2013b), genes belonging to the CYP4- and CYP3-like (*Cyp6* and *Cyp9*) clans were among the most highly expressed P450-like related contigs found in the RES strain. Among the CYP3 clan, genes belonging to the CYP6 family have been shown to be involved in pyrethroid insecticide resistance and detoxification through constitutive overexpression and/or induced expression (Yang *et al.*, 2006; Zhu *et al.*, 2008, 2013b; Feyerisen, 2012; Black IV *et al.*, 2021; Perini *et al.*, 2021; Nauen *et al.*, 2022). In *A. diaperinus*, 21 and 44 genes related to this CYP6 family were overexpressed in the RES strain in the absence or presence of β -cyfluthrin respectively compared to the SENS strain (Table S4). In

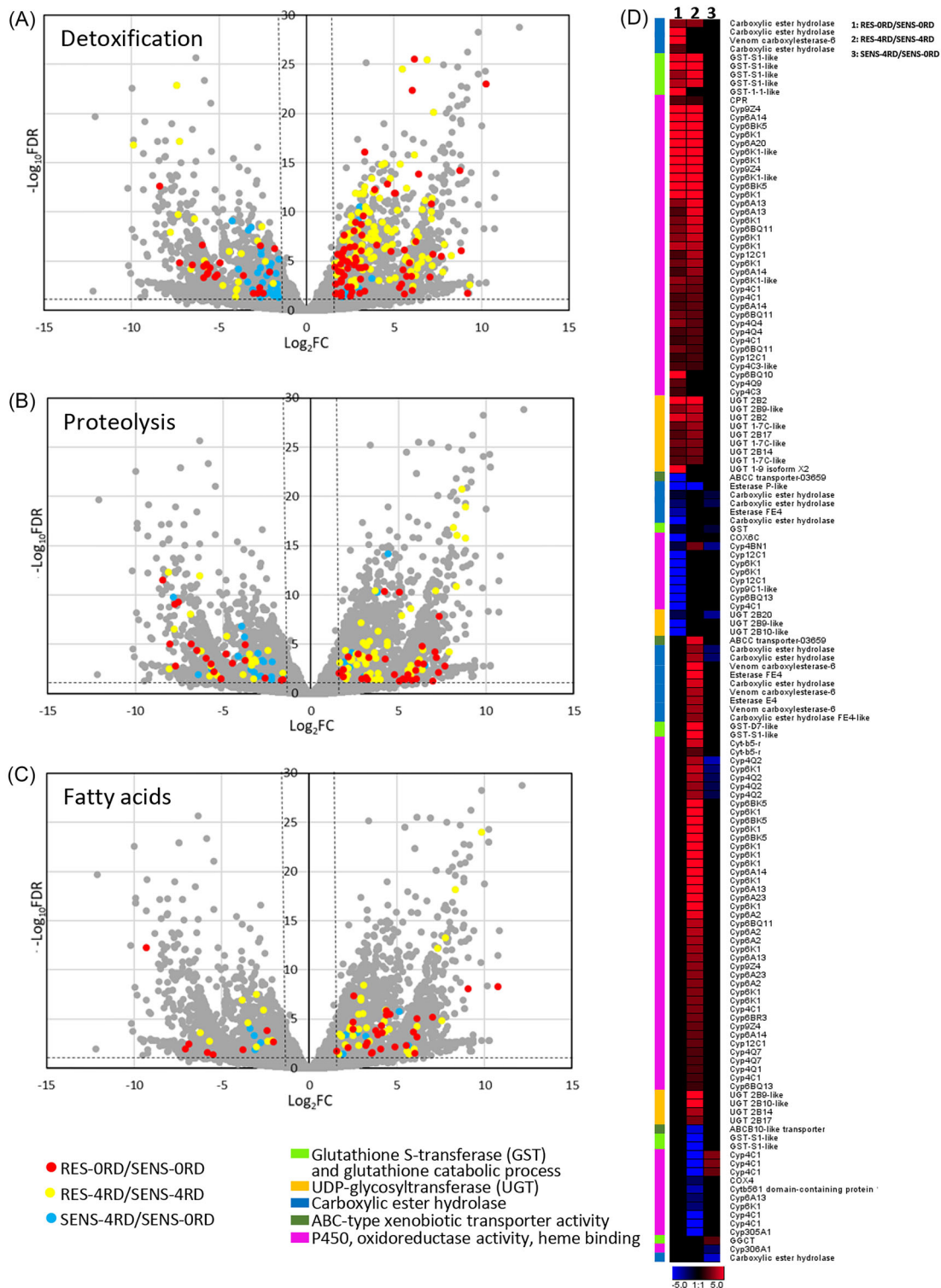


Fig. 4 Differential expression of DEGs involved in detoxification, proteolysis and related to fatty acids. Each dot in each volcano plot represents the values of expression of one contig. *Y*-axis shows values of $(-\text{Log}_{10}(\text{FDR}))$ and *X*-axis shows Log_2FC according to RES-ORD/SENS-ORD (red), RES-4RD/SENS-4RD (yellow) and SENS-4RD/SENS-ORD (blue) comparisons. The colored dots represent DEGs annotated as involved in detoxification (A), proteolysis (B), and related to fatty acids (C). Gray dots correspond to all other contigs according to their fold-change and FDR in the three comparisons. The dashed line shows the FDR cutoff with points above the

the CYP6BQ subfamily, *CYP6BQ11* is known to be involved in resistance to deltamethrin in *T. castaneum* and is overexpressed in a resistant strain (Zhu *et al.*, 2013b; Kalsi & Palli, 2015). Silencing of *Cyp6BQ11* also enhanced the susceptibility of *T. castaneum* to dichlorvos and carbofuran (Xiong *et al.*, 2019). Likewise, in the resistant strain of *A. diaperinus*, three Cytochrome P450 *Cyp6BQ11*-like coding genes were overexpressed compared to the SENS strain in the presence or absence of β -cyfluthrin (Table S4). Among the genes of the Cyp4 clan, *CYP4Q7* was induced in *T. castaneum* in the presence of eugenol, a toxic insecticidal molecule, and its inactivation by RNA interference increased the eugenol sensitivity of beetles (Zhang *et al.*, 2022). In *A. diaperinus*, the presence of β -cyfluthrin induced the expression of two *Cyp4Q7*-related genes in the RES compared to SENS strain (Table S4). All these overexpressions in the *A. diaperinus*-resistant strain could suggest a possible involvement of Cytochrome P450 in resistance, as described in many insects (Nauen *et al.*, 2022).

We also found some variation in the expression of genes encoding esterases, but the number of DEGs was much lower than for cytochromes. In the SENS strain, five DEGs related to carboxylic ester hydrolase activity were repressed when exposed to β -cyfluthrin. The RES strain overexpressed four carboxylic ester hydrolases without β -cyfluthrin and the presence of β -cyfluthrin induced nine DEGs related to that Phase I of detoxification (Fig. 4D, Table S4). Yet, the enzymatic assays on Est did not detect any difference between the RES and SENS strains (Fig. 3). Such discrepancy between gene expression and enzyme activity has already been observed in a malathion-resistant strain of *T. castaneum*. This strain had an induced malathion carboxylesterase activity, whereas esterase levels were lower than in the sensitive strain (Haubruge *et al.*, 2002). Several venom carboxylesterases-6-related contigs were found to be highly expressed in the RES strain, consistent with the capacity of carboxylesterase 6 (PxEst-6) in *Plutella xylostella* to metabolize cyfluthrin (Li *et al.*, 2021). Esterases E4 and FE4, whose genes were also overexpressed in the RES strain in response to β -cyfluthrin, are known to be implicated in insecticide resistance through metabolic detoxification (Field & Devonshire 1998; Lu *et al.*, 2021).

The RES strain in this study was also characterized by high basal expression of genes encoding Phase II enzymes: five GSTs (*GST-D7*-like, *GST-1-1*-like, *GST-SI*-like) and nine UGTs, nearly all of which were more expressed in response to β -cyfluthrin (Fig. 4A, D, Table S4). The β -cyfluthrin induced two other GSTs and four other UGTs in the RES insects, whereas DEGs related to these activities were repressed in the SENS strain, thus confirming the enzymatic activities (Fig. 3). Cyfluthrin and β -cyfluthrin resistances in, respectively, *Aedes aegypti* and *Cimex lectularius* have been linked to *GST-SI* overexpression (Adelman *et al.*, 2011; Seixas *et al.*, 2017). A γ -glutamylcyclotransferase encoding gene, responsible for glutathione catabolism, was found to be induced by the insecticide, but only in the SENS strain (Fig. 4D, Table S4), suggesting depletion of intracellular glutathione (Lilley *et al.*, 2018). Therefore, a reduced glutathione pool and a decrease of GST activity appeared here as a pattern for sensitivity, and the reverse would therefore reflect a biochemical response associated with resistance. Indeed, Reidy *et al.* (1990) showed that cyfluthrin resistance in *T. castaneum* was associated with increases in both glutathione pool and GST activity. Our enzymatic assays confirmed higher GST activity in the RES strain. Insecticide resistance has also been correlated to overexpression of the UGT2 genes family, including *UGT 2B2*, *UGT 2B17*, and *UGT 2B17* (Gao *et al.*, 2020; Perini *et al.*, 2021). These genes were all highly expressed in the RES strain and not detectable in the sensitive strain (Fig. 4D, Table S4).

Differentially expressed ABC transporters were poorly represented in our conditions (Fig. 4D, Table S4). Only one DEG encoding an ABCC Multidrug resistance-associated transporter was overexpressed in the RES strain compared to the SENS strain in the presence of β -cyfluthrin (RES-4RD/SENS-4RD). Such upregulation of ABCC genes was found to contribute to the diflubenzuron resistance phenotype in *T. castaneum* (Rösner & Merzendorfer, 2020). *ABCB10* was found to be repressed in *A. diaperinus* in the RES-4RD/SENS-4RD. The same gene was upregulated in ivermectin-resistant ticks (Pohl *et al.*, 2011). Contrary to observations in *Cimex lectularius* and *T. castaneum* (Zhu *et al.*, 2013b; Rösner *et al.*, 2021), β -cyfluthrin resistance in *A. diaperinus* does not seem to depend on multidrug resistance-associated

line having an FDR < 0.05. Vertical dashed lines represent the threshold level for 3 fold changes. Heat map (D) of contigs differentially expressed in RES-0RD/SENS-0RD (1), RES-4RD/SENS-4RD (2), and SENS-4RD/SENS-0RD (3) comparisons are represented in map as Log₂FC. Red, blue, and black colors indicate upregulated or downregulated, or unchanged expression, respectively. The colored sidebar legend corresponding to the description of the contigs is displayed at the bottom. CPR, NADPH-cytochrome P450 reductase; Cyp, Cytochrome P450; Cyt, Cytochrome b5; UGT, UDP-glycosyltransferase; GST, Glutathione S-transferase; COX, Cytochrome c oxidase; GGCT, Gamma-glutamylcyclotransferase.

proteins (e.g., ABC transporters) (Labbé *et al.*, 2011). Consistent with the enzymatic assays, the transcriptomic analysis showed that β -cyfluthrin-resistant *A. diaperinus* expressed a significant proportion of known detoxification genes encoding P450s, carboxylic ester hydrolases, GSTs and UGTs.

Involvement of proteolysis and lipid metabolic process in β -cyfluthrin resistance Proteolytic activities were downregulated in the SENS strain and expressed constitutively in the RES strain (Table 1, Figs. S3–S5), as can be seen in the volcano plot (Fig. 4B). β -cyfluthrin induced 10 genes coding proteases (trypsin-like serine proteases, cathepsin) or serine protease inhibitors in the SENS strain. These are known to regulate excessive activity of proteases (Krowarsch *et al.*, 2003). Downregulated DEGs (19) included mainly proteases and few protease inhibitors. The RES strain showed a constitutive overexpression of 30 DEGs coding for proteases (20) or protease inhibitors (10), while, for the SENS strain, DEGs coding for proteases (12) or protease inhibitors (4) were downregulated. So, resistance in the RES strain was linked to the activation and inactivation of specific proteases and protease inhibitors. Such parallel expression patterns between proteases and protease inhibitors have previously been described (Lin *et al.*, 2017), but their role in resistance mechanisms is poorly understood. The involvement of peptidases and proteolysis in response to insecticide-induced stress is possibly indirect. The resulting protein degradation could allow to fulfill the need for energy for proper functioning of resistance mechanisms (Silva *et al.*, 2012; Hou *et al.*, 2014; Perini *et al.*, 2021). Peptidases may also act on protein synthesis or on the activation of enzymes related to detoxification (Vontas *et al.*, 2005; Silva *et al.*, 2012).

The enrichment analysis suggested a role of lipid metabolic processes that were represented in a volcano plot (Fig. 4C). The number of DEGs in the RES strain compared to the SENS strain was very high. The RES-0RD/SENS-0RD and RES-4RD/SENS-4RD comparisons shared few common DEGs. Two of them were repressed DEGs concerning the lipid transport and 10 other upregulated genes were related to fatty acid biosynthetic processes or lipid homeostasis (Table S4). Apart from one induced DEG related to lipid transport, all the other common DEGs were not differentially expressed in the SENS strain in response to β -cyfluthrin. Furthermore, the few DEGs induced by β -cyfluthrin in the SENS strain and found repressed in the RES-4RD/SENS-4RD comparison concerned some processes of lipid storage, lipid catabolism, lipid transport and the negative regulation of fatty acid biosynthesis. This suggested a de-

crease in lipid biosynthesis in the SENS strain. On the contrary, the specific and induced DEGs from the RES-0RD/SENS-0RD comparison encoded mainly fatty acid synthases. Yang *et al.* (2020) demonstrated in *Locusta migratoria* that fatty acid synthases were responsible for cuticular hydrocarbons and inner hydrocarbons production and consequently for cuticle permeability. A defect of fatty acid synthases caused a significant increase in mortality in the presence of the pyrethroid deltamethrin. Among the specific and induced DEGs from the RES-4RD/SENS-4RD comparison, many were related as well to fatty acid biosynthesis processes but also linked to oxidoreductase activity through fatty acyl-CoA reductases (FARs), prostaglandin reductases, and hydroxyacyl-coenzyme A dehydrogenase (Table S4). The FARs gene family has been described to encode enzymes in insect cuticular hydrocarbons synthesis (Finet *et al.*, 2019). Thus, the RES strain expressed constitutive enzymes that may have contributed to xenobiotic resistance through the biosynthesis of an impermeable cuticle. This is in line with the variation in fatty acid composition, mainly arachidonic acid, observed in *Leptinotarsa decemlineata* populations exposed to neonicotinoids (Clements *et al.*, 2020). Prostaglandins are lipid compounds derived from arachidonic acid which act in a wide variety of physiological processes. Their degradation pathway involves prostaglandin reductases whose corresponding genes were induced in the RES strain.

Regulation of β -cyfluthrin resistance mechanisms

Our results showed that β -cyfluthrin resistance in our *A. diaperinus* line appeared to be linked to the constitutive overexpression of genes encoding many proteins and enzymes implicated in various detoxification mechanisms. Such processes would require a fine coordination of various signaling pathways, such as the GPCR signaling, the MAPKs-CREB, the AhR/ARNT, the HR96, and the CncC/Keap1 (Amezian *et al.*, 2021; Black IV *et al.*, 2021; Lu *et al.*, 2021). Our analysis actually found a few DEGs involved in transcriptional regulation of xenobiotic detoxification genes (Table S4). Among these, *Arrestin 2*, a locus belonging to the GPCR pathway, was highly upregulated in the RES-0RD/SENS-0RD and RES-4RD/SENS-4RD (Log_2FC of 6.2 and 4.0, respectively), but not differentially expressed in the SENS-4RD/SENS-0RD comparison (Table S4). Two genes encoding CREB-binding proteins were also highly induced in the RES-0RD/SENS-0RD and RES-4RD/SENS-4RD comparisons (Log_2FC of 6.9 and 7.3, respectively), whereas a gene encoding a mitogen-activated protein kinase 13, potentially involved in the MAPKs-CREB pathway, was highly repressed (Log_2FC of -7.3) in the RES-0RD/

SENS-ORD comparison. It is thus likely that β -cyfluthrin resistance in *A. diaperinus* involves coordinated regulation of signaling pathways such as GPCR and MAPKs-CREB. Of note, among DEGs, 25% had an unknown function and more than 10% had regulation and signaling functions not clearly defined, such as 50 DEGs coding for ankyrin repeat domain-containing proteins (Table S4). The role of all these loci and the regulation leading to the expression of detoxification genes remain to be elucidated.

Conclusions

To the best of our knowledge, this is the first *de novo* transcriptome of sensitive and insecticide-resistant strains in *A. diaperinus*. Obviously, we cannot completely rule out the possibility that parts of the differences we observed may result from a difference between strains. Yet, our combined biochemical and molecular approaches suggested that insecticide resistance in our RES strain may result from the constitutive overexpression of detoxification genes. The high resistance of the RES strain was associated to overexpression of genes encoding Cyp, GST, Carboxylic ester hydrolase, UGT, ABCC transporter loci, as well as an activated proteolysis and a potential cuticle strengthening by cuticular hydrocarbons synthesis. The possible regulation of these detoxification mechanisms by several signaling pathways was consistent with recent relevant studies (Amezian *et al.*, 2021; Black IV *et al.*, 2021; Lu *et al.*, 2021) and still need to be fully characterized. Such effective resistance mechanisms raise questions about the existence of cross-resistance. Knowledge of detoxification mechanisms and a better understanding of the full range of the resistance mechanisms are essential for the design of effective pest control methods.

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Disclosure

All authors declare no conflict of interest, financial or otherwise, that might potentially bias this work.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Description and primer sequences of the genes selected for qRT-PCR analysis.

Table S2 Summary statistics of Trinity-assembled *Alphitobius diaperinus* transcriptome.

Table S3 BUSCO on Assembled *Alphitobius* transcripts.

Table S4 Differentially expressed genes (Log₂FC) according to comparisons (excel format).

Fig. S1 Principal component analysis (PCA) of full transcriptomes of SENS and RES strains without and with β -cyfluthrin exposure.

Fig. S2 Validation of the transcriptome results by RT-qPCRs based on 20 different DEGs, which were either strongly up- or downregulated at least in one of the five different comparisons as listed in Table 1.

Fig. S3 Functional enrichment among DEGs in SENS strain after β -cyfluthrin exposure.

Fig. S4 Functional enrichment among DEGs in RES strain after β -cyfluthrin exposure.

Fig. S5 Functional enrichment of DEGs in RES strain compared to SENS strain.

Fig. S6 Functional enrichment of DEGs in RES pop strain compared to SENS strain after β -cyfluthrin exposure.