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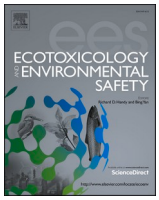

Differential metabolome responses to deltamethrin between resistant and susceptible *Anopheles sinensis*

Yueyue Li [a,1], Yashu Li [a,1], Guanxi Wang [a], Julin Li [a], Meihua Zhang [a], Jingyao Wu [a], Cheng Liang [a], Huayun Zhou [a], Jianxia Tang [a,b,*], Guoding Zhu [a,b,*]

[a] National Health Commission Key Laboratory of Parasitic Disease Control and Prevention, Jiangsu Provincial Key Laboratory on Parasite and Vector Control Technology, Jiangsu Institute of Parasitic Diseases, Wuxi 214064, China
[b] Center for Global Health, School of Public Health, Nanjing Medical University, Nanjing 211166, China

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ABSTRACT

Insecticide-based vector control measures play an important role in the prevention and control of insect-borne infectious diseases such as malaria; however, insecticide resistance has become a severe global problem for vector control. To date, the metabolic mechanism by which *Anopheles sinensis*, the most widely distributed malaria vector in China and Asia, detoxifies insecticides is not clear. In this study, the molecular metabolite changes in both the larval and adult stages of deltamethrin susceptible (DS) and deltamethrin-resistant (DR) *An. sinensis* mosquitoes were analysed by using liquid chromatography tandem mass spectrometry (LC-MS/MS) after exposure to deltamethrin. There were 127 differential metabolites in larval DR *An. sinensis* and 168 in adults. Five metabolites (glycerophosphocholine, deoxyguanosine, DL-methionine sulfoxide, D-myo-inositol-3-phosphate and N-acetyl-alpha-D-glucosamine1-phosphate) were downregulated in both DR larvae and adults, and one metabolite (aspartyl-glutamine) was upregulated, and the ratio of down- and up-regulation of these metabolites was 5:1. The differential metabolites between the DS and DR mosquitos were mainly classified into organic oxygen compounds, carboxylic acids and their derivatives, glycerophospholipids and purine nucleotides, and the common pathway enriched in both the larval and adult DR *An. sinensis* was glycerophospholipid metabolism. The findings of this study provide further mechanistic understanding of insecticide resistance in *An. sinensis*.

1. Introduction

Anopheles sinensis is a widely distributed vector in China and other Asian countries that can transmit malaria, filariasis and other vector-borne diseases. Insecticide-based vector control interventions have played a significant role in the prevention and control of malaria and other diseases (Engels and Zhou, 2020; Shaw and Catteruccia, 2019). Due to their safety, efficacy, and cost, pyrethroids have become the most popular insecticides and are widely used for vector and pest control in both the public health and agricultural fields (Weedall et al., 2019).

Unfortunately, the long-term and repetitive use of pyrethroids has led to increasing insecticide resistance in the majority of mosquito vectors. Currently, there are two major mechanisms involved in the insecticide resistance of mosquitoes: metabolic resistance and target insensitivity (Hemingway and Ranson, 2000). Metabolic resistance refers to the enhancement of detoxifying enzyme activities, such as the activity of the cytochrome P450 enzyme system, glutathione reductase system and carboxylesterase (Li et al., 2021, 2007; Wang et al., 2018), and the acceleration of the metabolic process when insects ingest exogenous poisons, including insecticides.

The metabolic process of insecticides involves complex detoxification pathways and multiple enzymatic metabolic reactions in mosquitoes, and the specific metabolic process and insecticide resistance mechanism are still unclear (Wang et al., 2020). In this study, liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to screen for differential metabolites and analyse the classified compounds and the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways that are enriched after exposure to deltamethrin, one of the

* Corresponding authors at: National Health Commission Key Laboratory of Parasitic Disease Control and Prevention, Jiangsu Provincial Key Laboratory on Parasite and Vector Control Technology, Jiangsu Institute of Parasitic Diseases, Wuxi 214064, China
E-mail addresses: tangjianxia78@163.com (J. Tang), jipdzhu@hotmail.com (G. Zhu).
[1] Contributed to this work equally

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most widely used pyrethroids in vector control, in deltamethrin-susceptible (DS) and deltamethrin-resistant (DR) *An. sinensis* mosquitos in both the larval and adult stages; these results contribute to a deeper understanding of the mechanism involved in the increasing insecticide resistance, and provide a more theoretical reference for resistance management and development of the novel insecticides in this malaria vector.

2. Materials and methods

2.1. Mosquito breeding and collection

A deltamethrin-sensitive strain of *An. sinensis* was collected in Jiangsu, China, in the late 1970 s and has been colonized in the vector laboratory of the Key Laboratory of Parasitic Disease Prevention and Control Technology, National Health Commission for more than 40 years; this strain has not been exposed to any insecticides during the past several decades. In 2020, blood-engorged female anopheles mosquitoes were collected from cattle sheds in Huai'An city, Jiangsu Province, China, where previous vector surveillance results showed that *A. sinensis* is the major distributed vector and exhibits deltamethrin resistance. Each captured engorged sample was transferred to a single tube that already had moist filter paper inside. Then, the samples were brought back to the laboratory, and their laid eggs were identified as *A. sinensis* using key morphology characteristics (Rueda et al., 2017). After hatching, the third- or fourth-instar larvae and the larvae three to five d post-emergence from the pupae stage of the same batch were selected for further experiments. The culture conditions were as follows: temperature (25 ± 1) °C, relative humidity (75 ± 5) %, and illumination 10 h/d. Larvae were fed tropical fish food, and adults were fed 5% glucose.

2.2. LC_{50} Determination of Susceptible An. sinensis

2.2.1. Gradient concentration method for larvae

According to the previously tested LC_{50} (5.1×10^{-3} μg/mL) of deltamethrin to *An. sinensis* in the laboratory, upper and lower sides of three concentration gradients were calculated with 1.5 times coefficient, a total of seven exposure concentration gradients of deltamethrin (deltamethrin powder with 99.95% effective ingredients was obtained from the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention) were set up. For each concentration, twenty third-instar DS larvae were treated with each corresponding deltamethrin concentration up to a final volume of 300 μL of solution, and a control group was also set without insecticide exposure. After 24 h of exposure, the dead mosquitoes were recorded, and the mortality rate was calculated. If the mortality fitting model of each concentration gradient has not reached the linear standard, the appropriate gradient will be further selected for the experiment. The experiment was repeated at least three times until the fitted mortality model of the seven concentration gradient groups was nearly linear and showed statistical significance.

2.2.2. CDC-biological bottle detection for adult

Seven exposure concentration gradients of deltamethrin were set up. For each concentration, twenty female adults three-five d post emergence from DS pupae were treated with each concentration in an 250 μL CDC bottle for 30 mins, and a control group was also set up without insecticide exposure. Then, the tested samples were transferred to a mosquito cage and fed 5% glucose, the number of dead mosquitoes was recorded, and the mortality rate was calculated after 24 h recovery. Similarly, the experiment was repeated at least three times until the fitted mortality model was nearly linear and showed statistical significance.

2.2.3. Determination of LC_{50} and the confidence interval (CI)

The average larval and adult mortality data was subjected to probit analysis using IBM SPSS Statistics 23.0 for calculating lethal concentration 50% (LC_{50}) and 95% confidence interval (CI) of larvae and adults and for getting dose and time mortality regression lines. The linear model and the virulence regression equation Y=b+aX (dependent variable was mortality, independent variable was insecticide concentration) were established, and the dose and time mortality regression curves were obtained. LC_{50} and 95%CI were calculated by probability response regression line.

2.3. Collection of field An. sinensis

After the morphological characteristic identification and confirmation of the first generation (F1) of captured *An. sinensis* mosquitoes described in Section 2.1, the larvae and adults hatched from the eggs were collected to test their susceptibility to deltamethrin. Twenty third-instar F1 larvae were treated with ten times the LC_{50} ($10 \times LC_{50}$) of deltamethrin identified in colonized susceptible larvae mosquitoes in the laboratory; similarly, twenty F1 female adults 3–5 d after eclogenation were treated with ten times the LC_{50} ($10 \times LC_{50}$) of deltamethrin measured in colonized sensitive adult mosquitoes in the laboratory by using the abovementioned larval and adult testing method, respectively, and both experiments were repeated in triplicate. After 24 h, the mortality rates of both larvae and adults were calculated and used for the phenotype classification of the field-collected *An. sinensis*. The resistant samples were called DR.

2.4. Sample preparation for LC-MS/MS analysis

Fifty third instars from the DS and DR *An. sinensis* mosquito groups were treated with deltamethrin LC_{50} isolated from the DS larvae in the laboratory, and a control group was set up by introducing only the anhydrous ethanol ingredient. Both the DS and DR experiments were repeated six times. After 24 h of exposure, all surviving larvae were collected and immediately treated with liquid nitrogen for 20 mins within 5 min and transferred to and saved in a − 80 °C refrigerator for subsequent metabolomics detection.

For adults, twenty female mosquitoes (3–5 d after eclosion, without blood feeding) from both the DS and DR *An. sinensis* groups were treated with deltamethrin LC_{50} isolated from the DS adults in the laboratory by a CDC bottle assay; similar to the process described above, only anhydrous ethanol was introduced to the control group, both the DS and DR experiments were repeated six times, and after 24 h of exposure, all the surviving adults were collected and immediately treated with liquid nitrogen and preserved in a − 80 °C refrigerator, as mentioned above.

Samples of 20 mg of larvae and adult mosquito from the DS and DR groups were transferred to a 1.5-mL Eppendorf tube, followed by the addition of two small steel balls. Twenty μL of internal standard (2-chloro-L-phenylalanine in methanol, 0.3 mg/mL) and 400 μL of extraction solvent with methanol/water (4/1, v/v) were added to each sample. The samples were stored at − 20 °C for 2 min, ground at 60 Hz for 2 min, ultrasonicated at 0 °C for 10 min, and incubated for 20 min at − 20 °C. The extract was centrifuged at 13000 rpm and 4 °C for 15 min, and 300 mL of supernatant in a brown glass vial was dried in a freeze concentration centrifugal dryer. A 400-μL mixture of methanol and water (1/4, vol/vol) was added to each sample, vortexed for 30 s, and centrifuged at 13000 rpm and 4 °C for 10 min. Then, 150-μL supernatant was collected from each tube using a crystal syringe, filtered through 0.22-μm microfilters and transferred to LC-MS vials. The vials were stored at − 80 °C until the LC -MS analysis was performed.

2.5. Data preprocessing and statistical analysis

In the process of metabolome data collection, UNIFI 1.8.1 software is used for the collection of original data. The Progenesis QI V2.3 software (Nonlinear Dynamics, Newcastle, UK) is used for data pre-processing (baseline filtering, peak recognition, integration, retention time

correction, peak alignment and normalization), and its main parameters are:Aprecursortolerance: 5 PPM, product tolerance: 10 PPM, product ion threshold: 5%; Compounds are identified based on accurate mass number, secondary fragments, and isotope distribution, using The Human Metabolome Database (HMDB), Lipidmaps (V2.3), and METLIN Database for qualitative analysis.After data collection, the ion peaks with all missing values (0 value) > 50% in the extracted and identified data were deleted, and the 0 value was replaced with half of the minimum value. The qualitative compounds were screened according to the compound qualitative Score (Score), with a screening standard of 30 points (full Score of 60 points). Below 30 points shall be deemed as inaccurate qualitative results and deleted.Finally, the positive and negative ion data are combined into a data matrix table, which contains all the information extracted from the original data that can be used for analysis, and the subsequent analysis is based on this.

The positive and negative data were combined to get a combine data which was imported into R ropls package. Principle component analysis (PCA) and orthogonal partial least-squares-discriminant analysis OPLS-DA were carried out to visualize the metabolic alterations among experimental groups, after mean centering (Ctr) and Pareto variance (Par) scaling, respectively. The Hotelling's T2 region, shown as an ellipse in score plots of the models, defines the 95% confidence interval of the modeled variation. Variable importance in the projection (VIP) ranks the overall contribution of each variable to the OPLS-DA model, and those variables with VIP > 1 are considered relevant for group discrimination. In this study, the default 7-round cross-validation was applied with 1/seventh of the samples being excluded from the mathematical model in each round, in order to guard against overfitting.

The differential metabolites were selected on the basis of the combination of a statistically significant threshold of variable influence on projection (VIP) values obtained from the OPLS-DA model and p values from a two-tailed Student's t test on the normalized peak areas, where metabolites with VIP values larger than 1.0 and p values less than 0.05 were considered as differential metabolites, and the pathway enrichment analyses of different metabolites were performed using the KEGG database. The Metabolomics data have been deposited to the EMBL-EBI MetaboLights database. (https://www.ebi.ac.uk/metabolights/MTBLS3773).

3. Results

3.1. Determination of LC_{50} in DS larvae and adults

The 7 concentration gradients used for LC_{50} determination in the DS larvae were as follows: 1.29×10^{-3} μg/mL, 1.94×10^{-3} μg/mL, 2.91×10^{-3} μg/mL, 4.36×10^{-3} μg/mL, 6.54×10^{-3} μg/mL, 9.81×10^{-3} μg/mL, 14.72×10^{-3} μg/mL. The LC_{50} of deltamethrin obtained by fitting the equation with the experimental results repeated 3 times at these 7 concentrations was 4.4×10^{-3} μg/mL, with a 95% CI of 3.6×10^{-3} - 5.2×10^{-3} μg/mL), with the following fitted regression equation of virulence: $Y = 0.6 + 1.7 \times X$ ($F=101.491$, $P < 0.001$, $R^2 = 0.830$, $P < 0.001$). The LC_{50} of DS adults was determined by a biological bottle assay, and the LC_{50} of deltamethrin was 0.96 μg/mL, with a 95% CI of 0.49–1.4. The regression equation of virulence was fitted as follows: $Y = 0.4 + 0.15 \times X$ ($F=97.329$, $P < 0.001$); the nonparametric test coefficient R^2 of the regression equation was 0.847 ($P < 0.001$).

3.2. Phenotypes of field-collected An. sinensis

The average mortality rates of the field-collected *An. sinensis* larvae at the LC_{50} and 10 $\times LC_{50}$ concentrations were 0.69% and 17.14%, respectively, indicating 72.46 and 5.83 times lower mortality rates than those identified in the DS samples. For the adult field-collected *An. sinensis*, the average mortality rates at the LC_{50} and 10 $\times LC_{50}$ concentrations were 3.5% and 10.00%, respectively, 14 and 10 times lower than those found in the DS samples; both the larvae and adults were classified as DR.

3.3. Principal component analysis

The PCA results showed that all four groups, DS larvae (A), DS adults (B), DR larvae (C) and DR adults (D), can be clustered together and clearly separated between the experimental group and the control group, as shown in Fig. 1(A-D), suggesting that the samples in one group have similar characteristics; thus, these results could be used for further data processing and analysis.

3.4. OPLS-DA and response permutation testing

In this experiment, the orthogonal projection analysis (OPLS-DA) of potential structure discriminant analysis was used to establish the relationship model between metabolite expression and sample concentration groups of sensitive and resistant strains. OPLS-DA is a supervised discriminant analysis statistical method. The Abscissa PC1 and ordinate PC2 of the score map represent the first and second principal component scores respectively, and the scatters of different colors and shapes represent the experimental grouping of the samples. In order to prevent over-fitting after the construction of the model, the quality of the model was investigated by means of seven-cycle interactive verification and 200th response permutation testing (RPT). RPT is a random ranking method used to evaluate the accuracy of OPLS model, and it is not accidental to avoid classification by supervised learning method. We give the response ranking test result diagram of OPLS-DA model in Fig. 1 (I-L). Through the fitting test of the model, the slope of the straight line is very large, indicating that the model does not exceed the fitting, the Q^2Y value was greater than 0.5, and the differences between the R^2Y scores were no more than ± 0.5, indicating that the model could effectively explain and predict the differences between two sample groups (Qi et al., 2019). which means that the OPLS-DA model has a good ability to explain and predict, and can reflect the differences in the actual response of different sample groups. As shown in Fig. 1(E-L), the variances in each pair of groups were compared using OPLS-DA methods, obvious group separation was obtained after supervised clustering by OPLS-DA in groups of DS and DR *An. sinensis*, demonstrating that the sample classes were clearly separated between groups, indicating a reliable difference between different deltamethrin exposures in the DS and DR mosquitoes.

3.5. Differential metabolites in DS and DR

The metabolites produced in different groups were analysed, and the results are shown in Fig. 2. There were 183 differential metabolites in DS larvae vs. EOH larvae, 129 in DR larvae vs. EOH larvae, 234 in DS adults vs. EOH adults, and 178 in DR adults vs. EOH adults.

There were 8 identical differential metabolites between the 183 and 129 differential metabolites in DS larvae and DR larvae after exposure to deltamethrin, 6 of which showed opposite change trends and 2 of which showed the same change trends (Table 1); therefore, there were a total of 127 specific differential metabolites in DR larvae. Similarly, there were 24 identical differential metabolites between the 234 and 178 differential metabolites in DS adults and DR adults, among which 14 exhibited opposite change trends and 10 showed the same change trends (Table 2); therefore, there were a total of 168 specific differential metabolites in DR adults (Fig. 2).

Between the 127 specific differential metabolites in DR larvae and 168 specific differential metabolites in DR adults, there were 7 identical differential metabolites, 6 of which showed the same change trends (Table 3), among which glycerophosphocholine, deoxyguanosine, DL-methionine sulfoxide, D-myo-inositol-3-phosphate and N-acetyl-alpha-D-glucosamine-1-phosphate were downregulated in both DR larvae and adults; however, aspartyl-glutamine was upregulated in both DR larvae and adults.

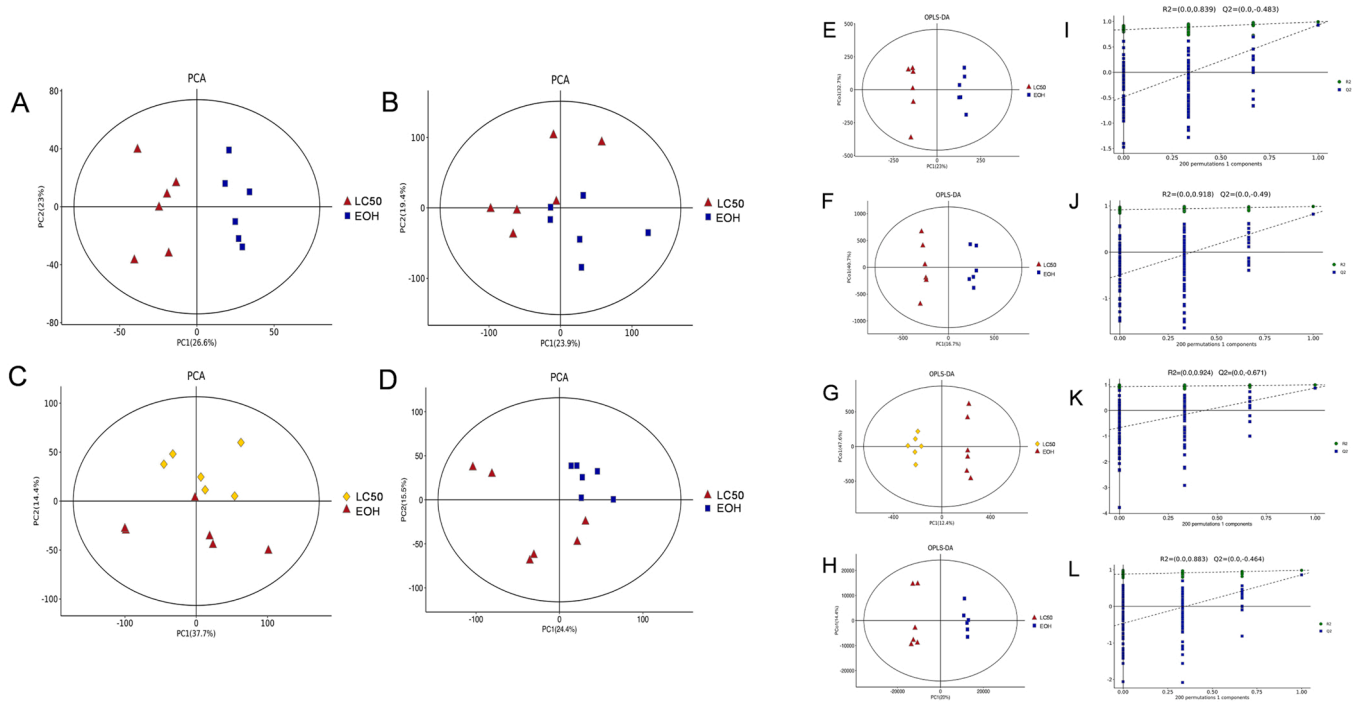

Fig. 1. Principal component analysis of the samples in the metabolomics analysis. Significant intergroup isolation was observed between mosquitoes from DS larvae (A), DS adults (B), DR larvae (C) and DR adults (D); OPLS-DA scores plots of DS larvae (E), DS adults (F), DR larvae (G), and DR adults (H) and response permutation testing of OPLS-DA models for DS larvae (I), DS adults (J), DR larvae (K) and DR adults (L).

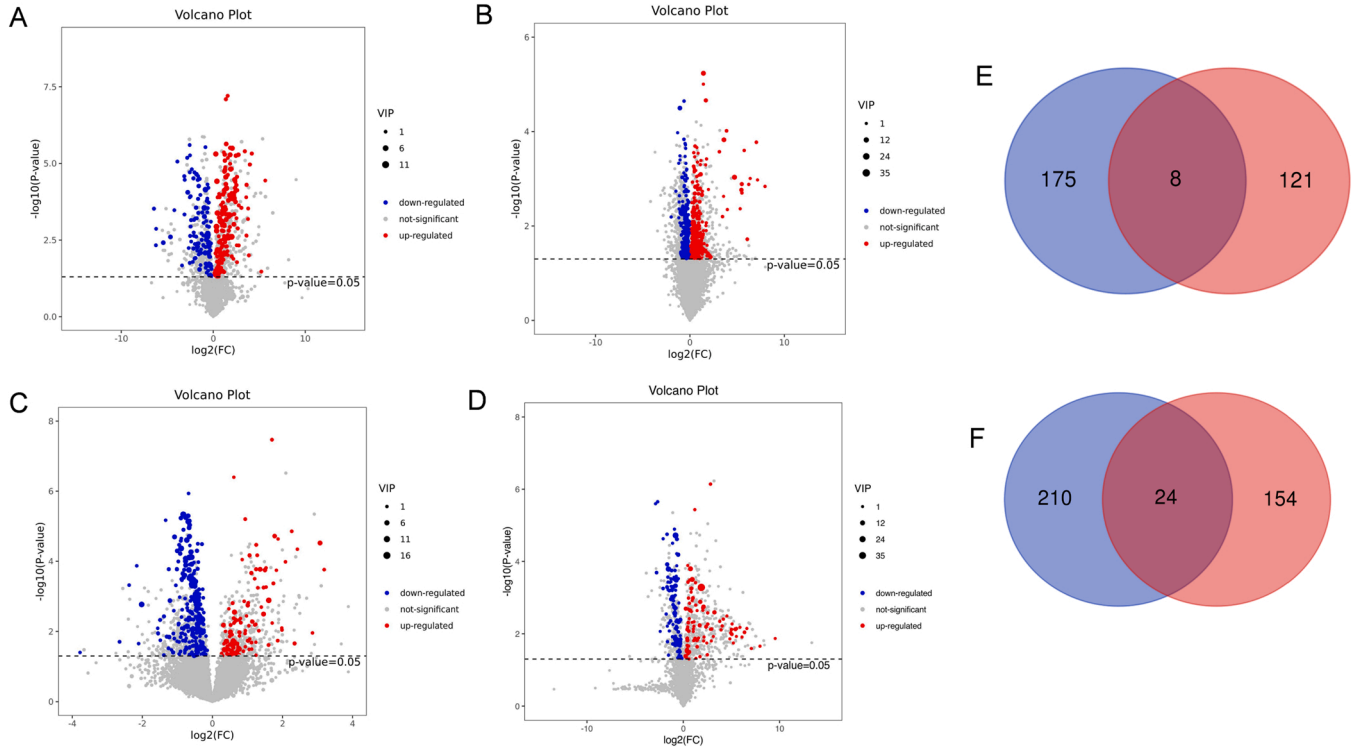

Fig. 2. Volcano plot comparing the LC_{50} and EOH groups of DS larvae (A), DS adults (B), DR larvae (C) and DR adults (D) after exposure to deltamethrin for 24 h. Venn diagram of differential metabolites in DS vs. DR larvae (E) and DS vs. DR adults (F). Each point in the volcano diagram represents a metabolite. The horizontal axis represents the logarithm of FC (fold change) values based on 2, the vertical axis represents the p-value obtained by Student's t test, and the scatter size represents the VIP value.

3.6. Biochemical functions of differential metabolites

Most of the biochemical functions of the 183, 234, 129 and 178 differential metabolites found in DS and DR *An. sinensis* mosquitoes are concentrated in the carboxylic acid and its derivatives, organic oxygen compounds, glycerophospholipids, fatty acyls groups, enol lipids, purine

Table 1
Common different metabolites of DS and DR larvae.

Metabolite	Formula	Class	DS	DR
Hydroxypropionylcarnitine*	$C_{10}H_{19}NO_5$	Fatty Acyls	↑	↓
Succinoadenosine	$C_{14}H_{17}N_5O_8$	Purine nucleosides	↑	↑
1-(sn-Glycero-3-phospho)− 1D-myo-inositol*	$C_9H_{19}O_{11}P$	Glycerophospholipids	↑	↓
Uridine diphosphate-N-acetylglucosamine	$C_{17}H_{27}N_3O_{17}P_2$	Pyrimidine nucleotides	↑	↑
Glycerol 3-phosphate*	$C_3H_9O_6P$	Glycerophospholipids	↑	↓
Guanosine monophosphate*	$C_{10}H_{14}N_5O_8P$	Purine nucleotides	↑	↓
Mumiamicin*	C15H22O3	Unclassified	↑	↓
11-deoxy-PGF2a*	$C_{20}H_{34}O_4$	Fatty Acyls	↓	↑

The * symbol indicates the metabolites that showed opposite change trends between DS and DR mosquitoes.

Table 2
Commonly different metabolites of resistance and sensitive adult.

Metabolites	Formula	class	DS	DR
PC(16:1(9Z)/0:0)[U]*	$C_{24}H_{48}NO_7P$	Unclassified	↑	↓
Adenosine 3′-monophosphate	$C_{10}H_{14}N_5O_7P$	Ribonucleoside 3′-phosphates	↑	↑
PKOHA-PG*	$C_{29}H_{51}O_{12}P$	Glycerophospholipids	↑	↓
Biflorin	$C_{16}H_{18}O_9$	Organooxygen compounds	↑	↑
Cefadroxil*	$C_{16}H_{17}N_3O_5S$	Lactams	↑	↓
D-Glucuronic acid	$C_6H_{10}O_7$	Organooxygen compounds	↑	↑
Orcein	$C_{28}H_{24}N_2O_7$	Benzofurans	↑	↑
PKOHA-PG*	$C_{25}H_{47}O_{12}P$	Glycerophospholipids	↑	↓
PS-PC*	$C_{28}H_{54}NO_{10}P$	Glycerophospholipids	↑	↓
Galactose 1-phosphate	$C_6H_{13}O_9P$	Organooxygen compounds	↓	↓
Galactonic acid	$C_6H_{12}O_7$	Hydroxy acids and derivatives	↑	↑
2-Formaminobenzoylacetate*	$C_{10}H_9NO_4$	Unclassified	↓	↑
LysoPC(14:1(9Z))*	$C_{22}H_{44}NO_7P$	Glycerophospholipids	↑	↓
LysoPC(17:0)*	$C_{25}H_{52}NO_7P$	Glycerophospholipids	↑	↓
Leucyl-Leucine*	$C_{12}H_{24}N_2O_3$	Carboxylic acids and derivatives	↓	↑
Phosphoribosyl-AMP*	$C_{15}H_{23}N_5O_{14}P_2$	Purine nucleotides	↑	↓
Isoleucyl-Glutamate*	$C_{11}H_{20}N_2O_5$	Carboxylic acids and derivatives	↓	↑
GlcCer(d18:1/16:0)	$C_{40}H_{77}NO_8$	Sphingolipids	↑	↑
(+)-Gallocatechin*	$C_{15}H_{14}O_7$	Flavonoids	↑	↓
Caryoptosidic acid	$C_{16}H_{24}O_{11}$	Prenol lipids	↑	↑
Phenylglucuronide	$C_{12}H_{14}O_7$	Organooxygen compounds	↑	↑
N-Acetyl-leu-leu-tyr*	$C_{23}H_{35}N_3O_6$	Unclassified	↑	↓
3-Deoxyguanosine*	$C_{10}H_{13}N_5O_4$	Unclassified	↑	↓
D-N-(Carboxyacetyl)alanine	$C_6H_9NO_5$	Carboxylic acids and derivatives	↓	↓

The * symbol indicates the metabolites that showed opposite change trends between DS and DR mosquitoes.

Table 3
Common differential metabolites of DR larvae and adults.

Metabolites	Formula	class	larvae	adult
Choline	$C_5H_{13}NO$	Organonitrogen compounds	↑	↓
Deoxyguanosine*	$C_{10}H_{13}N_5O_4$	Purine nucleosides	↓	↓
Glycerophosphocholine*	$C_8H_{20}NO_6P$	Glycerophospholipids	↓	↓
D-myo-Inositol-3-phosphate*	$C_6H_{13}O_9P$	Unclassified	↓	↓
DL-Methionine sulfoxide*	$C_5H_{11}NO_3S$	Carboxylic acids and derivatives	↓	↓
N-Acetyl-alpha-D-glucosamine1-phosphate*	$C_8H_{16}NO_9P$	Organooxygen compounds	↓	↓
Aspartyl-Glutamine*	$C_9H_{15}N_3O_6$	Carboxylic acids and derivatives	↑	↑

The * symbolled indicates the metabolites that showed the same change trends between DR larvae and DR adults.

nucleosides, and pyrimidine nucleosides categories. However, the distribution differed between different deltamethrin-related phenotype groups; for example, in DS larvae, polyketones had the highest abundance, accounting for 17.5%, while this metabolite was tenth most abundant in DR larvae, accounting for only 2.8%. Furthermore, in DS adults, the most-accumulated compound was carboxylic acid and its derivatives, which accounted for 17%; however, the most accumulated compound in DR adults was organic oxygen compounds, which accounted for 31.6%, followed by carboxylic acid and its derivatives, which accounted for 10.5% (Fig. 3).

3.7. Significant pathways enriched by differential metabolites

According to the pathway enrichment analysis in the KEGG database of differential metabolites in DS and DR *A. sinensis* mosquitoes, the pathways specifically enriched in DR larvae were as follows: tryptophan metabolism; glycine, serine and threonine metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; and glycerophospholipid metabolism (Fig. 4A). The pathways specifically enriched in DR adults were ABC transporters, starch and sucrose metabolism, pentose and glucuronate interconversions, glycerophospholipid metabolism and ether lipid metabolism (Fig. 4B). The above results show that glycerophospholipid metabolism is the common pathway enriched in both larval and adult DR *An. sinensis*.

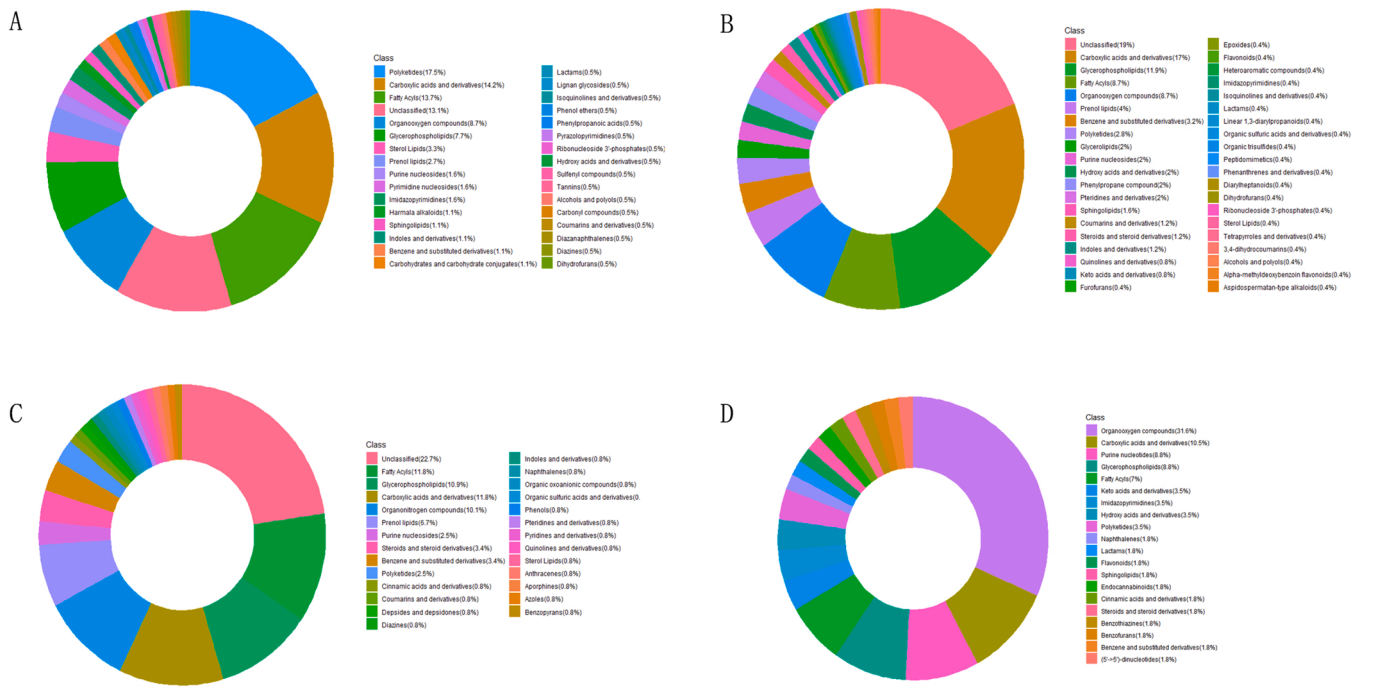

Fig. 3. Circular diagram of the biochemical classification of the differential metabolites of DS larvae (A), DS adults (B), DR larvae (C) and DR adults (D).

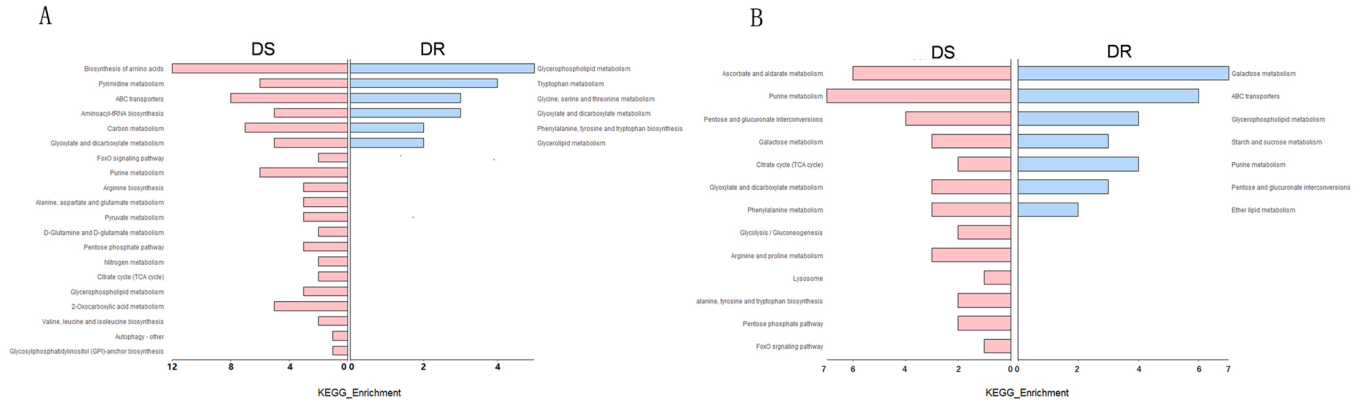

Fig. 4. TOP20 of the KEGG enrichment pathway of differential metabolites in DS vs. DR *A. sinensis* larvae (A) and adults (B) treated with deltamethrin.

4. Discussion

In this study, DS and DR *An. sinensis* mosquitoes at their two major developmental stages, larvae and adults, were collected from the laboratory and the field and examined using the LC-MS/MS method for differential metabolite analysis after deltamethrin treatment. Seven identical differential metabolites, including glycerophosphocholine, deoxyguanosine, DL-methionine sulfoxide, D-myo-inositol-3-phosphate, N-acetyl-alpha-D-glucosamine1-phosphate and aspartyl-glutamine, were identified in DR *An. sinensis* mosquitoes, and organooxygen compounds, glycerophospholipids and purine nucleic acids might play an important role in the metabolic detoxification of deltamethrin. Furthermore, one commonly enriched glycerophospholipid metabolism pathway was identified in both larval and adult DR *An. sinensis* mosquitoes.

There were a total of twenty (six from DS vs. DR larvae and fourteen from DS vs. DR adults) common differential metabolites in DS vs. DR *An. sinensis*, among which four of them, 2-formaminobenzoylacetate, leucyl-leucine, isoleucyl-glutamate and 11-deoxy-PGF2a, showed upregulation in DR and downregulation in DS, while the others showed opposite trends, suggesting that amino acids and other compounds could be candidate metabolites involved in the insecticide resistance of mosquitoes. Our results showed that one of the most enriched metabolic pathways in DR involves amino acid metabolism and amino acid biosynthesis. It has been reported that the concentration of most amino acids in *Tribolium castaneum* larvae exposed to organophosphorus pesticides increased (Saleem and Shakoori, 1993), and the same has been observed to occur in *Drosophila* after exposure to pyrethroids (Brinzer et al., 2015). A metabonomics analysis of *Drosophila melanogaster* treated with permethrin confirmed that tryptophan catabolism plays an unprecedented role in the defence against insecticides (Brinzer et al., 2015). It has been reported that amino acid supplementation has been used to change the metabolites of insects resistant to imidacloprid and restore the sensitivity of *brown planthoppers* to insecticides (Elzaki et al., 2020).

Amino acids such as glycine, serine and threonine are related to glucose, glycogen and pyruvate metabolism, and these types of metabolism are closely related to energy metabolism; phenylalanine, tyrosine and tryptophan are the end products of glycolysis and are aromatic amino acids consistent with the energy changes in other insects after

exposure to pesticides (Brinzer et al., 2015; Mansingh, 1965; Rand et al., 2015). The compound 11-deoxy-PGF2a is an aliphatic acyl compound that may result from the oxidative stress induced by exposure to deltamethrin, and high energy expenditures promote glycogen mobilization in the adipose bodies of insects (Grigoraki et al., 2020).

In *Cyprinus carpio*, chlorpyrifos exposure led to an increase in the cyclic concentration of glucose and other metabolites involved in energy metabolism (Kokushi et al., 2015). This enhancement is related to the need to provide energy through gluconeogenesis in response to higher metabolic needs after exposure to pesticides (Ramesh and Saravanan, 2008). Another metabonomic study based on nuclear magnetic resonance (NMR) analysis found significant differences in the contents of amino acids, organic acids and sugars between DR and DS *Aedes albopictus* strains (Huang et al., 2021).

In this study, most of the differential metabolites between DS and DR mosquitos were classified as organooxygen compounds, glycerophospholipids and purine nucleotides, all of which are essential for protein synthesis, DNA replication and energy production in *An. sinensis*. These results are consistent with the metabolic responses of *Aphis gossypii* treated with imidacloprid, which has a great influence on the carbohydrate metabolism of *An. gossypii* (Lv et al., 2021). A previous study showed that a blood component that initiates the production of phospholipids from scratch can reduce mosquito infection with dengue virus, (Vial et al., 2020) indicating that glycerol phospholipid family compounds may play an important role in vector biological control. In our study, purine metabolism was upregulated in DS mosquitoes, and hypoxanthine was converted to xanthine under the catalysis of xanthine oxidase, accompanied by the production of reactive oxygen species (ROS), which could lead to DNA damage and apoptosis in exposed individuals (Patetsini et al., 2013; Sigrist-Flores et al., 2021). High energy consumption and excitotoxicity may lead to ROS when *Aphis gossypii* is exposed to imidacloprid (Lv et al., 2021).

Other studies have provided evidence that exposure to oxidative stressors and metabolic inhibition of NADPH regeneration lead to redox transitions, which adversely affect mosquito fecundity and insecticide detoxification (Champion and Xu, 2018). In addition, NADPH-cytochrome P450 oxidoreductase (CPR) plays a key role in the chemical detoxification and insecticide resistance of other insects, such as *Anopheles gambiae* and *Culex quinquefasciatus*(Li et al., 2015; Lu-Yun et al., 2011).

Glyceropholipid metabolism was the common pathway enriched in both DR larvae and adults in this study. In 2015, Sieber and Spradling (Sieber and Spradling, 2015) showed that the steroid hormone ecdysone plays a role in controlling lipid metabolism and supporting oocyte formation in *Drosophila melanogaster*. Lipid metabolism is closely correlated with insect growth, development and reproduction, and a study of *Daphnia* showed that lipid metabolism is mainly involved in lipid distribution and the metabolism of fatty acids (Goulden and Place, 1990; Wacker and Martin-Creuzburg, 2007). The metabolic pathways of glycerol phospholipids and related genes in *Daphnia magna* exposed to TNT (2,4,6-trinitrotoluene) and different flame retardants have also changed (Scanlan et al., 2015; Stanley et al., 2013). The role of the signal transduction of lipid phosphatase has been proven in the development and metabolism of *Drosophila melanogaster* (Lehmann, 2021). Another study on the lipidomics of *Aphis gossypii* showed that the triacylglycerol content and acyl chain composition, glycerol phospholipid content and acyl chain subspecies composition changed significantly after *Aphis gossypii* was parasitized by Lysiphlebia japonica for 3 d (XueKe et al., 2017).

The ATP-binding cassette (ABC) transporter family is one of the largest membrane protein families found in all organisms. The ABC transporter is considered to be related to the resistance of some agricultural pests to pyrethroids ((Dermauw and Van Leeuwen, 2014). Some studies have provided the information framework of ABC transporter subfamily genes, thus laying an important foundation for better understanding and extensively researching the role of ABC transporters in insecticide poisoning (Epis et al., 2014; He et al., 2019). It has also been reported that mutations or downregulation of ABC transporter subfamily C genes ABCC2 and ABCC3 and a high level of resistance to the BtCry1 toxin were genetically associated with seven species of Lepidoptera (Guo et al., 2019). The above results indicate that ABC transporters may also play an important role in insecticide resistance in *An. sinensis* mosquitoes.

Overall, although the participation of target insensitivity mechanisms cannot be ruled out for formulating the insecticide resistance in this malaria vector, the metabolic resistance mechanism was mainly focused in this study. This study provided the candidate compounds, classifications and pathways that may be involved in insecticide resistance and enhanced our understanding of the mechanism of insecticide resistance in *An. sinensis*. Further research focusing on the discovery of molecular compound functions and the identification of the elements participating in the metabolic pathways is still needed to provide more direct molecular evidence and references for decelerating the spread of insecticide resistance management and the development of novel insecticides in *An. sinensis*.

5. Conclusion

In conclusion, organooxygen compounds, glycerophospholipids and purine nucleic acids might play an important role in the metabolic detoxification of deltamethrin to the major malaria vector *An. sinensis*. The glycerophospholipid metabolism-related pathway is proposed as a potential target for deltamethrin resistance management and vector control based on the further understanding of the resistance mechanism obtained in this study.

CRediT authorship contribution statement

Yueyue Li: Data collection and analysis, Writing – original draft. **Yashu Li:** Data collection and analysis. **Guanxi Wang:** Data analysis. **Julin Li:** Sample collection. **Meihua Zhang:** Data collection. **Jingyao Wu:** Sample collection and treatment. **Cheng Liang:** Sample collection and treatment. **Huayun Zhou:** Investigation. **Jianxia Tang:** Data analysis, Supervision. **Guoding Zhu:** Supervision, Writing – reviewing and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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