

Flavin-containing monooxygenase acts as a metabolic signaling hub linking AhR-CYP transcriptional reprogramming to insecticide resistance

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Abstract

Insecticide resistance is a complex adaptive trait that cannot be fully explained by individual detoxification enzymes. Flavin-containing monooxygenases (FMOs) are conserved but limited in gene number in insects, raising the question of how they contribute to broad-spectrum resistance. Whether FMOs act beyond direct xenobiotic metabolism remains unclear. We investigated FMO function in metaflumizone resistance using *Spodoptera exigua* and *Drosophila*, combined with transcriptomics, pharmacological inhibition, enzymatic assays, and molecular docking analyses. Field populations showed strong metaflumizone resistance associated with FMO upregulation, but recombinant FMOs did not directly metabolize the insecticide. In *Drosophila*, FMO overexpression reduced metaflumizone sensitivity, whereas knockdown increased susceptibility. Mechanistically, FMO activates the tryptophan–kynurenine metabolic pathway, thereby promoting AhR signaling and subsequently upregulating CYP detoxification genes. Pharmacological modulation of FMO or AhR consistently altered CYP expression and insecticide sensitivity in both model and field systems. These results suggest FMO contributes to insecticide resistance indirectly by functioning as a metabolic signaling regulator that links endogenous tryptophan metabolism to AhR-mediated CYP transcriptional reprogramming. These findings uncover a previously unrecognized mechanism by which an evolutionarily ancient detoxification enzyme regulates the CYP detoxification network through metabolic signaling.

Introduction

Insecticide resistance in agricultural pests represents one of the most pressing challenges to global crop protection^{1, 2}. Although numerous enzymatic detoxification systems, including cytochrome P450 monooxygenases (CYPs), glutathione S-transferases, and carboxylesterases, have been implicated in resistance development, increasing evidence suggests that resistance is not merely the result of individual enzyme upregulation, but rather a coordinated, systems-level metabolic adaptation involving transcriptional and signaling reprogramming^{3, 4, 5, 6}. In this context, understanding how endogenous physiological networks are reorganized to support xenobiotic detoxification has become a central question in insect toxicology.

Among lepidopteran pests, *Spodoptera exigua* has emerged as a representative species exhibiting severe and rapidly evolving resistance to multiple classes of insecticides. Field populations have developed high-level resistance not only to classical neurotoxic compounds but also to structurally diverse newer chemistries, suggesting the existence of convergent and possibly upstream regulatory mechanisms governing detoxification capacity^{7, 8, 9, 10, 11}.

Flavin-containing monooxygenases (FMOs) are conserved phase I oxidative enzymes and are considered sister enzymes of CYPs. FMOs catalyze the oxygenation of soft nucleophilic heteroatoms such as nitrogen and sulfur, and are traditionally viewed as auxiliary detoxification enzymes involved in xenobiotic metabolism^{12, 13, 14, 15, 16}. However, a fundamental paradox exists in insects: unlike the highly expanded CYP superfamily¹⁷, FMOs are represented by only a few genes per genome¹⁸, yet they have

repeatedly been associated with resistance to structurally diverse insecticides^{19, 20, 21}. For example, in *S. exigua*, only three FMO genes have been identified²², whereas dozens of CYP genes are present in the same genome²³. Despite this limited gene repertoire, FMO expression has been linked to resistance phenotypes across multiple insect species^{19, 20, 21, 22}. This discrepancy raises an unresolved question: how can such a small enzyme family contribute to broad-spectrum insecticide resistance? Importantly, although FMOs can directly metabolize certain xenobiotics¹², accumulating evidence indicates that their substrate range does not fully explain the diversity and magnitude of resistance phenotypes observed in field populations. This suggests that FMOs may possess additional, non-canonical functions beyond direct detoxification^{24, 25, 26, 27, 28}.

Recent advances in metabolic biology have revealed that xenobiotic resistance is often regulated by endogenous metabolic state^{4, 5, 29, 30}. In particular, tryptophan metabolism and its kynurenine pathway derivatives, including kynurenine (Kyn) and kynurenic acid (KynA), have been identified as endogenous ligands capable of activating the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor traditionally known for mediating adaptive responses to environmental toxins^{31, 32, 33}. Upon activation, AhR translocates into the nucleus and forms a transcriptional complex with ARNT, thereby regulating xenobiotic response elements (XREs) and inducing detoxification-associated genes, including multiple CYP family members^{34, 35, 36}. Although this AhR-centered regulatory system has been extensively studied in mammals³⁷, its functional role in insect xenobiotic adaptation remains incompletely understood^{38, 39}. Notably, AhR homologs in insects, such as *Drosophila* Spineless and its orthologs in lepidopteran species, share conserved PAS domain architecture, suggesting potential conservation of ligand responsiveness and transcriptional function^{40, 41, 42}. However, whether endogenous metabolic enzymes upstream of AhR can actively reshape CYP-mediated detoxification programs in insects remains largely unexplored. These observations collectively suggest a conceptual gap: the possibility that metabolic enzymes may not only act as detoxification catalysts but also function as regulators of signaling networks controlling xenobiotic responses.

Based on these considerations, we hypothesized that FMOs may function beyond classical oxidative detoxification and instead act as metabolic signaling regulators that connect endogenous metabolic pathways with xenobiotic detoxification networks. Specifically, we propose that FMO-mediated modulation of tryptophan-kynurenine metabolism generates endogenous ligands capable of activating AhR signaling, thereby inducing a transcriptional CYP detoxification program. Under this framework, insecticide resistance is not solely determined by enzyme-substrate interactions, but emerges from a hierarchical regulatory architecture in which metabolic state reprogramming drives transcriptional remodeling of detoxification capacity. To test this hypothesis, we combined field population analyses of *S. exigua*, genetic manipulation in *D. melanogaster*, transcriptomic profiling, pharmacological perturbation, and biochemical validation. This cross-species strategy allows us to dissect whether FMO-mediated metabolic signaling represents a conserved mechanism underlying insecticide resistance.

Results

FMO contributes to metaflumizone resistance through a mechanism independent of direct insecticide metabolism

To characterize the current resistance status of field populations, we first compared the susceptibility of a laboratory susceptible strain (Sus) and two field-collected populations (HZ21 and HZ24) to five insecticides representing different modes of action. The HZ24 population exhibited varying levels of resistance to all tested insecticides, but the magnitude and temporal trends differed among compounds. Resistance to lambda-cyhalothrin remained relatively stable between HZ21 and HZ24 (RR = 64.1 and 64.6, respectively), whereas resistance to chlorantraniliprole and emamectin benzoate increased substantially. In contrast, resistance to chlorpyrifos declined from 239.4-fold in HZ21 to 29.4-fold in HZ24. Notably, resistance to metaflumizone continued to increase and reached the highest level among all insecticides tested, with an LC_{50} of 1892.2 mg/L and a resistance ratio of 567.9-fold in HZ24, representing an approximately 2.7-fold increase relative to HZ21 (Fig. 1A). These results indicate that metaflumizone resistance remains under strong selection and is a prominent component of the multidrug-resistant phenotype in field populations of *S. exigua*.

To examine whether FMO is associated with this resistance phenotype, we quantified the expression of the three known FMO genes in *S. exigua*. Compared with Sus strain, both *SeFMO1* and *SeFMO3* were markedly upregulated in resistant larvae. In the third-instar larvae of HZ24, *SeFMO1* and *SeFMO3* transcript levels increased by 6.0-fold and 11.6-fold, respectively, while in fifth-instar larvae the increases reached 6.1-fold and 307.3-fold (Fig. 1B,C). In contrast, *SeFMO2* expression showed no significant difference between susceptible and resistant populations.

To evaluate the functional contribution of FMO activity, resistant larvae were treated with the FMO inhibitor methimazole (MMI). MMI markedly increased the susceptibility of HZ24 larvae to metaflumizone, reducing the LC_{50} from 1892.2 mg/L to 383.1 mg/L (synergism ratio, SR = 4.9). However, MMI did not significantly alter susceptibility to chlorpyrifos, beta-cypermethrin, chlorantraniliprole or emamectin benzoate (Fig. 1D). These results suggest a specific association between FMO activity and metaflumizone resistance.

Given that FMOs are generally regarded as xenobiotic-metabolizing enzymes, we next examined whether *SeFMOs* directly metabolize metaflumizone. Recombinant *SeFMO1*, *SeFMO2* and *SeFMO3* proteins were expressed and incubated with metaflumizone *in vitro*. HPLC analyses detected no measurable depletion of the parent compound by any of the three recombinant enzymes (Fig. 1E), indicating that the contribution of FMO to metaflumizone resistance cannot be explained by direct metabolic detoxification.

To further investigate this unexpected observation, we employed *Drosophila* as a genetically tractable model system. Constitutive overexpression of either *FMO1* or *FMO2* significantly reduced fly mortality under metaflumizone exposure, whereas RNAi-mediated knockdown of either gene markedly increased susceptibility. No significant differences in metaflumizone mortality were detected between GAL4/+

progeny and the corresponding UAS/+ progeny (Fig. 1F,G). Similar bidirectional effects were observed when FMO expression was manipulated in metabolically important tissues, including the malpighian tubule and fat body (Fig. 1H, I), demonstrating that FMO expression levels are sufficient to modulate metaflumizone tolerance.

We next tested whether this phenotype depended on FMO catalytic activity. Treatment with MMI largely abolished the enhanced tolerance of FMO-overexpressing flies without significantly affecting control flies (Fig. 1J, K), confirming that FMO enzymatic activity is required for the observed phenotype.

The inability of recombinant FMOs to directly metabolize metaflumizone prompted us to investigate whether downstream detoxification systems might be involved. Because cytochrome P450 enzymes are major determinants of metabolic resistance, we examined the effect of the P450 inhibitor piperonyl butoxide (PBO). PBO substantially suppressed the metaflumizone-tolerant phenotype conferred by FMO overexpression (Fig. 1L). In contrast, PBO had no effect on FMO-mediated tolerance to cartap, an insecticide that can be directly oxidized by FMO (Fig. 1M; Fig. S1A). These results indicate that FMO contributes to metaflumizone tolerance through a P450-dependent mechanism distinct from its conventional role in direct xenobiotic metabolism.

Collectively, these findings reveal a mechanistic paradox: although FMO activity is clearly required for metaflumizone resistance, the resistance phenotype cannot be explained by direct insecticide metabolism. Instead, FMO appears to promote metaflumizone tolerance through an indirect detoxification pathway involving P450-dependent metabolic processes.

FMO activates a selective CYP detoxification program associated with insecticide tolerance

The observation that PBO markedly suppressed FMO-mediated metaflumizone tolerance suggested that cytochrome P450-dependent detoxification processes may function downstream of FMO activity. To identify the molecular pathways underlying this response, we performed transcriptome sequencing on flies overexpressing FMO1 or FMO2 and compared their transcriptional profiles with those of corresponding control flies.

Principal component analysis revealed a clear separation between FMO-overexpressing and control samples, indicating substantial transcriptional reprogramming associated with elevated FMO expression (Fig. 2A). Differential expression analysis identified 465 upregulated and 310 downregulated genes in FMO1-overexpressing flies, and 453 upregulated and 360 downregulated genes in FMO2-overexpressing flies (Fig. 2B). Among the commonly induced genes, *Cyp4p2* was consistently upregulated in both FMO-overexpression backgrounds, suggesting a potential link between FMO activity and CYP-dependent detoxification pathways.

To obtain a broader view of the biological processes affected by FMO activation, we performed KEGG enrichment analyses. In both FMO1- and FMO2-overexpressing flies, the most significantly enriched pathway was cytochrome P450-mediated drug metabolism (Fig. 2C, D). Additional detoxification-related

pathways, including drug metabolism by other enzymes and glutathione metabolism, were also enriched. These findings indicate that FMO activation is accompanied by coordinated remodeling of xenobiotic detoxification networks, with CYP-mediated metabolism representing the most prominent transcriptional response.

Given the strong enrichment of CYP-associated pathways, we next examined the expression patterns of CYP genes in greater detail. Among the 179 annotated CYP-like transcripts detected in the transcriptome, approximately twenty exhibited significant upregulation in at least one FMO-overexpression background. Following further filtering based on expression abundance and statistical significance, thirteen candidate CYP genes were identified as putative FMO-responsive targets (Fig. 2E). Notably, several of these genes showed highly consistent induction in both FMO1- and FMO2-overexpressing flies, suggesting that FMO activation triggers a coordinated CYP transcriptional program rather than isolated gene-specific responses.

To validate these transcriptomic observations, seven representative CYP genes were selected for RT-qPCR analysis, while *Cyp4d21*, which showed no differential expression in the transcriptome, was included as a negative control (Fig. 2F). Consistent with RNA-seq results, *Cyp4p2*, *Cyp6d2*, *Cyp6a14*, *Cyp6a19*, *Cyp4e1*, *Cyp4g1* and *Cyp6a17* were all significantly upregulated in FMO-overexpressing flies. Importantly, several of these genes also exhibited reduced expression in FMO knockdown flies, demonstrating that CYP transcription responds bidirectionally to changes in FMO activity. In contrast, expression of the negative control gene *Cyp4d21* remained unchanged across all treatments. Together, these results establish a robust association between FMO activity and induction of specific CYP genes.

To determine whether these FMO-responsive CYPs contribute functionally to insecticide tolerance, we selected three representative candidates—*Cyp4p2*, *Cyp6d2* and *Cyp6a14*—for further analysis. Transgenic overexpression lines were generated, and RT-qPCR confirmed substantial increases in transcript abundance, with expression levels elevated by 65.7-, 56.2- and 185.9-fold, respectively (Fig. S2). Functional bioassays revealed that overexpression of either *Cyp4p2* or *Cyp6d2* significantly reduced mortality under metaflumizone exposure (Fig. 2G), indicating that these genes contribute directly to insecticide tolerance. In contrast, despite its strong transcriptional induction, *Cyp6a14* overexpression did not significantly alter metaflumizone susceptibility.

Interestingly, *Cyp6d2* overexpression also reduced susceptibility to indoxacarb (Fig. 2H), consistent with the altered indoxacarb sensitivity observed in FMO-manipulated flies (Fig. S1B). However, none of the three CYP genes reduced chlorpyrifos susceptibility (Fig. 2I), indicating that the detoxification function of FMO-responsive CYPs is insecticide-specific rather than broadly protective.

Collectively, these results demonstrate that FMO activation induces a selective CYP detoxification program, and that a subset of FMO-responsive CYP genes functions as downstream effectors contributing to insecticide tolerance. These findings provide a mechanistic explanation for the PBO-sensitive phenotype observed in FMO-overexpressing flies and suggest that FMO promotes

metaflumizone tolerance through transcriptional activation of specific CYP-dependent detoxification pathways.

AhR signaling provides the mechanistic link between FMO activity and CYP-dependent detoxification

The transcriptomic and functional analyses above demonstrated that FMO promotes metaflumizone tolerance through induction of specific CYP genes. However, the mechanism by which changes in FMO activity are translated into transcriptional activation of detoxification genes remained unclear.

FMOs have been reported to participate in tryptophan metabolism, and elevated FMO expression increases the abundance of several kynurenine-pathway metabolites, including Kyn, KynA, and XA. Because these metabolites have been reported as endogenous ligands of AhR, we hypothesized that AhR signaling may connect FMO-dependent metabolic remodeling to CYP induction.

To explore this possibility, we first examined the expression of the *Drosophila* AhR homolog *spineless* in FMO-overexpressing flies. Both *FMO1* and *FMO2* overexpression significantly increased *spineless* transcript abundance (Fig. 3A), suggesting activation of AhR-associated signaling. If FMO-induced kynurenine pathway metabolites function upstream of AhR, exogenous supplementation of these metabolites should recapitulate the transcriptional response observed in FMO-overexpressing flies. Consistent with this prediction, treatment with Kyn, KynA, or XA significantly increased *spineless* expression (Fig. 3B). Moreover, all three metabolites induced the expression of the FMO-responsive CYP gene *Cyp4p2*, while Kyn and KynA also elevated *Cyp6d2* expression. Similarly, strong induction of *Cyp6a14* was observed following treatment with each metabolite (Fig. 3C-E). These findings indicate that kynurenine pathway metabolites are sufficient to activate AhR-associated signaling and promote transcription of FMO-responsive CYP genes.

We next asked whether AhR activity is required for FMO-mediated CYP induction. To address this question, flies carrying inducible FMO overexpression constructs were treated with the AhR antagonist SR1 prior to FMO induction. As expected, FMO induction increased the expression of *spineless* and its downstream CYP targets. However, inhibition of AhR signaling by SR1 substantially attenuated the induction of *Cyp4p2*, *Cyp6d2*, and *Cyp6a14* in both *FMO1*- and *FMO2*-overexpressing flies (Fig. 3F-I). Notably, SR1 exerted minimal effects on control flies, indicating that AhR activity is specifically required for the transcriptional response elicited by elevated FMO activity.

Having established that AhR signaling is necessary for FMO-induced CYP expression, we next examined whether AhR also mediates the corresponding insecticide tolerance phenotype. If AhR functions downstream of FMO, activation of AhR should rescue the hypersensitive phenotype caused by FMO deficiency, whereas inhibition of AhR should suppress the tolerant phenotype caused by FMO overexpression. Consistent with this prediction, pretreatment with Kyn or KynA significantly reduced metaflumizone-induced mortality in FMO knockdown flies (Fig. 3J-M). Importantly, the rescue effect was substantially stronger in FMO-deficient flies than in their corresponding controls, indicating that activation of AhR signaling can compensate for the loss of FMO activity.

Conversely, pharmacological inhibition of AhR signaling by SR1 markedly increased metaflumizone susceptibility in FMO-overexpressing flies (Fig. 3N,O). While SR1 exerted little effect on control flies, it largely abolished the enhanced tolerance conferred by FMO overexpression, demonstrating that AhR activity is required for the manifestation of the FMO-dependent resistance phenotype.

To determine whether AhR signaling specifically mediates the indirect detoxification pathway identified above, we performed parallel experiments using cartap, an insecticide that can be directly metabolized by FMO. In contrast to metaflumizone, neither KynA supplementation nor SR1 treatment significantly altered the susceptibility of FMO-manipulated flies to cartap (Fig. 3P-S). Thus, AhR signaling is dispensable for FMO-mediated direct detoxification but is essential for the indirect resistance mechanism observed with metaflumizone.

Taken together, these findings establish AhR as the missing mechanistic link between FMO activity and CYP-dependent detoxification. Elevated FMO activity promotes the accumulation of endogenous kynurenine pathway metabolites, which activate AhR signaling and subsequently induce a subset of detoxification-associated CYP genes. Through this signaling cascade, metabolic changes initiated by FMO are translated into enhanced insecticide tolerance, revealing a previously unrecognized FMO-AhR-CYP regulatory axis.

The FMO-AhR-CYP signaling axis contributes to field-evolved metaflumizone resistance in *S. exigua*

To determine whether the FMO-AhR-CYP signaling axis identified in *Drosophila* is conserved in *S. exigua*, we first examined the expression of the AhR homolog *SeAhR* in metaflumizone-resistant (HZ24) and susceptible populations. Compared with the susceptible strain, *SeAhR* expression was significantly upregulated in HZ24 larvae at both developmental stages analyzed, with 2.3-fold and 5.6-fold increases observed in third- and fifth-instar larvae, respectively (Fig. 4A). The greater increase in *SeAhR* expression in late instar larvae suggests a stage-dependent activation of AhR signaling in resistant populations.

To assess the functional relevance of AhR signaling in resistance, we next evaluated the effects of pharmacological inhibition of AhR and FMO activity in the resistant population. Neither the AhR antagonist SR1 nor the FMO inhibitor methimazole (MMI) significantly affected metaflumizone susceptibility in the laboratory susceptible strain (Fig. 4B). In contrast, both compounds markedly increased the susceptibility of the resistant HZ24 population. SR1 reduced the LC_{50} of metaflumizone from 2818.3 mg/L to 716.1 mg/L, representing a 3.9-fold synergistic effect, while MMI reduced the LC_{50} to 521.7 mg/L, corresponding to a 5.4-fold increase in toxicity (Fig. 4C). Combined treatment with SR1 and MMI further decreased the LC_{50} to 371.8 mg/L, although no significant additive effect was observed between single and combined treatments, suggesting that both compounds act on overlapping components of the same detoxification network. In contrast, SR1 had minimal impact on chlorpyrifos susceptibility (Table S5), indicating specificity of the AhR-associated pathway in mediating metaflumizone resistance.

To investigate whether SR1 directly interacts with SeAhR, molecular docking analyses were performed. SR1 was predicted to bind stably within the PAS-B ligand-binding domain of SeAhR, forming hydrogen bonds with Y240 and S256, π - π interactions with H181, and hydrophobic contacts with multiple residues including F177, L198, V222, Y240, and S270. The predicted binding affinity was -8.8 kcal/mol, indicating a favorable interaction between SR1 and SeAhR (Fig. 4D).

Molecular dynamics simulations further supported the stability of the SR1-SeAhR complex. Over a 100 ns simulation, the complex remained structurally stable, with RMSD values stabilizing around 0.4 nm after equilibration and a consistent radius of gyration of approximately 2.4 nm, indicating a compact and stable binding conformation (Fig. 4E,F). Consistently, MM/GBSA analysis estimated a total binding free energy of -62.20 kcal/mol, with van der Waals interactions being the dominant favorable contributor (Fig. 4G). These results support the likelihood of a stable interaction between SR1 and SeAhR.

We next examined whether AhR inhibition affects the expression of detoxification genes in the resistant population. Based on previous transcriptomic analyses of HZ24, several detoxification genes were selected, including *GSTE6*, *GSTO2*, *CYP6AE70*, *CYP321A16*, and *CYP321A8*, all of which were significantly upregulated in resistant larvae (ranging from 3.7- to 26.7-fold compared with susceptible larvae) (Fig. 4H). Treatment with SR1 significantly reduced the expression of *GSTO2*, *CYP321A16*, and *CYP321A8*, whereas *GSTE6* and *CYP6AE70* were not significantly affected (Fig. 4I). Similarly, MMI treatment resulted in a significant reduction only in *CYP321A16* expression, with a weaker effect on *CYP321A8*, and no detectable effect on the remaining genes (Fig. 4J). These results indicate that only a subset of detoxification genes is jointly regulated by FMO and AhR signaling in the resistant population.

To validate the functional relevance of downstream CYP genes, *CYP321A16* and *CYP321A8* were heterologously expressed in *Drosophila*. Flies expressing either gene exhibited significantly reduced mortality under metaflumizone exposure, indicating enhanced tolerance relative to control flies (Fig. 4K). Importantly, this tolerant phenotype was abolished by PBO treatment, confirming that CYP-mediated detoxification activity is required for the observed resistance.

Collectively, these results demonstrate that inhibition of either FMO or AhR activity reduces metaflumizone resistance in field-derived HZ24 populations, with no additive effect observed between the two inhibitors. Together with the downstream induction of *CYP321A16* and *CYP321A8*, these findings provide strong evidence that the FMO-AhR-CYP signaling axis operates in field-evolved resistance. This axis links metabolic reprogramming and transcriptional detoxification networks and represents a conserved mechanism underlying metaflumizone resistance in *S. exigua*.

Discussion

FMOs have long been regarded as auxiliary phase I detoxification enzymes involved in the oxidation of soft nucleophilic xenobiotics^{16, 43}. However, the present study challenges this conventional view and reveals a fundamentally different functional paradigm for FMOs in insecticide resistance. Although

FMOs are evolutionarily conserved, insect genomes encode only a limited number of FMO genes compared with the extensive CYP superfamily^{16, 17}. This apparent discrepancy between limited gene number and broad resistance phenotypes has remained unresolved. Our results demonstrate that FMO overexpression does not directly metabolize metaflumizone, but instead regulates resistance through a higher-order metabolic signaling mechanism. This finding redefines FMO not as a terminal detoxification enzyme, but as a metabolic signaling hub that links endogenous metabolic states to xenobiotic response systems.

A key conceptual advance of this study is the identification of a hierarchical organization underlying insecticide resistance. Rather than being driven solely by enzymatic detoxification capacity, resistance emerges from transcriptional reprogramming of detoxification networks^{5, 44, 45, 46}. FMO modulates the tryptophan-kynurenine metabolic pathway, leading to the accumulation of endogenous metabolites such as kynurenine and kynurenic acid. These metabolites function as signaling molecules capable of activating AhR, a conserved ligand-dependent transcription factor that regulates xenobiotic response genes^{31, 47}. Activated AhR subsequently induces a subset of cytochrome P450 genes, forming a transcriptional detoxification program that contributes to insecticide resistance^{38, 48, 49}. In this framework, CYP enzymes represent downstream executors of a transcriptionally programmed detoxification response rather than isolated metabolic determinants. Thus, insecticide resistance should be viewed as an emergent property of metabolic-transcriptional coupling rather than a simple enzyme abundance phenotype.

Our cross-species validation using both *Drosophila* and *S. exigua* suggests that the FMO-AhR-CYP regulatory axis is evolutionarily conserved. Although AhR signaling has been extensively characterized in mammals^{12, 24, 28, 50, 51}, its role in insects has remained less defined^{16, 52}. Our data support the notion that insect AhR homologs retain functional responsiveness to endogenous metabolic ligands derived from tryptophan metabolism^{31, 32, 37, 53, 54, 55}. Importantly, pharmacological modulation of AhR activity consistently altered CYP expression and insecticide susceptibility in both model and field-derived systems. These findings indicate that the metabolic signaling architecture uncovered here is not species-specific, but may represent a conserved regulatory module linking endogenous metabolism with xenobiotic adaptation across insects. This conservation suggests that metabolic signaling, rather than direct enzymatic detoxification alone, may be a general evolutionary strategy for coping with environmental chemical stress.

An important implication of our findings is the resolution of a long-standing paradox in insect toxicology: how a small FMO gene family can contribute to broad-spectrum resistance. Previous studies have documented direct FMO-mediated oxidation of certain insecticides^{56, 57, 58, 59, 60}; however, such enzymatic activity cannot fully account for resistance to structurally diverse compounds such as metaflumizone or diamide-class insecticides^{8, 20}. Our data reconcile this discrepancy by demonstrating that FMO exerts dual functionality: a limited direct detoxification capacity for specific substrates, and a dominant indirect role through metabolic signaling and transcriptional regulation. This dual-mode

function explains how a numerically small enzyme family can exert disproportionately large effects on detoxification capacity by acting upstream of transcriptional regulatory networks.

From an applied perspective, the identification of the FMO-AhR-CYP axis provides new insight into the regulation of field-evolved insecticide resistance. Pharmacological inhibition of FMO or AhR significantly restored susceptibility in resistant populations, indicating that targeting upstream metabolic signaling may represent a viable strategy for resistance management⁴⁵. Unlike conventional approaches focused on inhibiting individual detoxification enzymes, disruption of signaling-level regulation may offer broader efficacy by simultaneously suppressing multiple downstream detoxification pathways. Therefore, metabolic signaling represents a previously underappreciated target space for insecticide resistance mitigation strategies.

In summary, we propose a conceptual framework in which insecticide resistance is governed by a metabolic signaling cascade: FMO → tryptophan–kynurenine metabolism → AhR signaling → CYP transcriptional reprogramming → insecticide resistance. This model shifts the interpretation of insecticide resistance from enzyme-centric detoxification to signaling-driven transcriptional adaptation. Future studies should investigate the upstream regulators of FMO expression in field populations and determine whether similar metabolic signaling architectures exist for other detoxification enzyme families, such as GSTs and carboxylesterases. Such work will be essential to establish whether metabolic signaling represents a general organizing principle of xenobiotic adaptation.

Materials and Methods

Insects

Two field populations of *S. exigua*, HZ21 and HZ24, were collected from scallion (*Allium fistulosum* L.) fields in Huiyang District, Huizhou, Guangdong Province, China, in October 2021 and October 2024, respectively. The sampling area represents an intensively managed vegetable production region where insecticides are routinely applied approximately one to two times per month and the field populations had developed high levels of resistance to commonly used insecticides including chlorantraniliprole, emamectin benzoate, lambda-cyhalothrin, and metaflumizone^{8, 46}. The laboratory susceptible strain (Sus), originally provided by Wuhan Kernel Biotechnology Co., Ltd. in 2001, has been maintained under insecticide-free conditions⁸.

The Gal4/UAS system was used to manipulate FMO and CYP gene expression in *D. melanogaster*. UAS-FMO and UAS-CYP transgenic fly lines were generated by Zhuhai United Huayi Technology Co., Ltd. (Zhuhai, China). Coding sequences of the target genes were cloned into UAS-attB vectors and integrated into the same attP40 landing site by phiC31-mediated site-specific recombination. FMO1 and FMO2 overexpression and knockdown lines were generated as described in detail previously⁶¹. To minimize genetic background effects, all transgenic and RNAi lines were backcrossed to the same laboratory w1118 strain for six generations; The resulting UAS lines were crossed with the indicated Gal4 drivers,

with progeny from crosses between the same drivers and w¹¹¹⁸ serving as controls. Overexpression or knockdown of the corresponding target genes in adult F1 progeny was verified by RT-qPCR before insecticide susceptibility assays. Temporal induction of transgene expression was achieved using a temperature-sensitive Gal80^{ts} system by shifting flies from 24°C to 30°C in specific experiments. Detailed information on transgenic line construction and driver stocks is provided in Supplementary Table S1.

Reagents and insecticides

All insecticides used in this study were technical-grade active ingredients. Detailed information on insecticides and reagents, including their purity, source is provided in Supplementary Table S2. Stock solutions were prepared according to the manufacturers' instructions, protected from light, and stored at -80°C until use. Working solutions were freshly prepared before each experiment.

Bioassays

The resistance level of *S. exigua* was bioassayed using a diet-incorporation method to assess larval susceptibility to the tested insecticides listed in Supplementary Table S3 according to the previous protocol⁸. Early third-instar larvae were exposed to insecticide-treated artificial diet, with ten larvae per replicate and five biological replicates per treatment. Mortality was recorded after 72 h for lambda-cyhalothrin and chlorpyrifos, and after 96 h for the remaining insecticides.

Fly bioassays were conducted using a diet-incorporation method, as described previously⁵⁶. Mortality was assessed after at least 60 h of exposure to cartap, 72 h of exposure to chlorpyrifos, and at least 96 h of exposure to metaflumizone or indoxacarb. Cartap, previously shown to be directly metabolized by FMO⁵⁶, was included as a positive reference for FMO-mediated insecticide metabolism. For single-concentration bioassays, working concentrations were selected based on preliminary assays and are listed in Supplementary Table S2. For PBO synergism assays in fly, piperonyl butoxide (PBO), a P450 inhibitor, was incorporated into standard fly diet at 200 mg/L. Flies were pretreated with PBO for 1 d and then exposed to diet containing both PBO and the corresponding insecticide, with mortality recorded at the same time points described above.

For MMI synergism assays, methimazole (MMI), a commonly used FMO substrate and competitive inhibitor, was used to inhibit FMO activity before insecticide exposure^{56, 62}. For *S. exigua*, late first-instar larvae were fed artificial diet containing 2 mM MMI for 3-4 d, while control larvae were fed MMI-free diet. Early third-instar larvae were then subjected to diet-incorporation bioassays. Preliminary tests confirmed that 2 mM MMI did not significantly affect larval growth. For *Drosophila*, FMO1 or FMO2 expression was transiently induced using the Gal80^{ts}/Act5C-Gal4 system. Adult flies were pretreated with 30 mM MMI for 2 d before heat activation and then maintained on MMI-containing diet at 30°C for 4 d. Flies were subsequently transferred to metaflumizone-containing diet for mortality assays. Preliminary tests showed that continuous exposure to 30 mM MMI for 6 d caused no obvious adverse effects on flies.

Pharmacological manipulation of AhR-related signaling

The kynurenine pathway metabolites KynA, Kyn, and XA were used to evaluate their effects on AhR-related responses. StemRegenin 1 (SR1) was used as an AhR antagonist⁶³. For fly experiments, KynA, Kyn, XA, and SR1 were incorporated into standard fly diet for compound treatment. The final dietary concentrations were 500 μ M for KynA, 200 μ M for Kyn, 500 μ M for XA, and 200 μ M for SR1. For KynA, Kyn, and XA treatments, flies were maintained on compound-containing diet for 6 d. For SR1-related experiments involving FMO induction, Gal80^{ts}/Act5C-Gal4 > UAS-FMO flies were pretreated with SR1 at 25°C for 2 d and then shifted to 30°C for 4 d on SR1-containing diet to induce FMO expression. After treatment, flies were either subjected to bioassays or collected for RNA extraction and gene expression analysis.

To inhibit AhR signaling in *S. exigua*, late first-instar larvae from the HZ24 population were pretreated with SR1 before insecticide susceptibility assays. To ensure sustained exposure to SR1 and facilitate its inhibitory effect on AhR signaling, SR1 was applied using a leaf-dip method. Cabbage leaves were immersed for 10 min in a solution containing 500 μ M SR1 and 0.2% Triton X-100, while control leaves were treated with the corresponding background solution. After air-drying, treated leaves were provided to larvae for 3-4 d until they reached the third-instar stage, followed by metaflumizone exposure using the diet-incorporation method. For experiments comparing SR1, MMI, or their combined effects, the same leaf-dip procedure was used. Treatment solutions contained 500 μ M SR1, 2 mM MMI, or both compounds at the same concentrations. After treatment, larvae were used for bioassays or collected for RNA extraction and gene expression analysis.

Real-Time Quantitative PCR

Gene expression was analyzed by RT-qPCR. Total RNA was extracted from the indicated *Drosophila* or *S. exigua* samples and reverse-transcribed into cDNA. qPCR was performed using SYBR Green chemistry on a QuantStudio 5 Real-Time PCR System. Actin5C was used as the reference gene for *Drosophila*, whereas β -Actin and GAPDH were used for *S. exigua*. Relative expression levels were calculated using the $2^{-\Delta\Delta C_T}$ method⁶⁴. Detailed sample preparation, reaction conditions, and primer information are provided in the Supplementary Table S4.

Prokaryotic expression of SeFMO and in vitro metabolism assay

The prokaryotic expression system for SeFMO has been described in detail in our previous study⁵⁶. In this study, an *in vitro* enzymatic reaction system was used to assess the metabolic activity of SeFMO toward insecticides. The reaction was performed in a total volume of 500 μ L using 1 $\times$ PBS as the reaction buffer, containing 160 μ g of SeFMO protein, an NADPH regeneration system, and 10 μ M metaflumizone. A reaction mixture without enzyme was used as the control, with all other components identical to those in the treatment group. Reactions were incubated at 37°C for 12 h. After incubation, reaction products were extracted, and the remaining metaflumizone was quantified by HPLC.

Transcriptome sequencing and data analysis

FM01 and FM02 were ubiquitously overexpressed in *Drosophila* using the Gal4/UAS system with the Act5C-Gal4 driver. Flies generated by crossing Act5C-Gal4 to W1118 were used as controls. Newly emerged male flies were collected within 12 h after eclosion and maintained on standard diet for 3 d. For each genotype, three biological replicates were prepared, with 10–15 flies per replicate. Total RNA was extracted from the flies, and qualified RNA samples were submitted to LC-Bio Technology Co., Ltd. (Hangzhou, China) for library preparation and paired-end 150-bp sequencing on an Illumina NovaSeq 6000 platform. Detailed procedures are provided in the Supplementary Materials and Methods. Heatmap visualization was performed using the Advanced Heatmap Plots module in OmicStudio (<https://www.omicstudio.cn>). Original FPKM values were subjected to gene-wise Z-score normalization. The resulting Z-scores were used for heatmap visualization.

Homology Modeling and Molecular Docking

The three-dimensional structure of SeAhR (GenBank: AYI99241.1) was predicted using ProteinX and optimized with Rosetta Relax. Ligand structures were obtained from PubChem, converted to three-dimensional conformers, and energy-minimized before docking. Potential binding pockets were identified by CB-Dock2⁶⁵, followed by docking refinement with AutoDock Vina. The best-scoring docking pose was selected for interaction analysis using PLIP and visualization in PyMOL. Molecular dynamics simulations of the selected protein-ligand complex were performed using GROMACS 2023.3 for 100 ns. Complex stability was evaluated by RMSD and radius of gyration analyses, and binding free energy was calculated from trajectory frames extracted from the final 10 ns.

Data Analysis

Bioassay data were analyzed based on dose–mortality responses. LC₅₀ values, 95% fiducial limits, slopes, and standard errors were estimated by probit analysis using PoloPlus. Differences in insecticide susceptibility between groups were considered significant when the 95% fiducial limits of LC₅₀ values did not overlap. Synergism ratios (SRs) were calculated as the LC₅₀ of the insecticide alone divided by the LC₅₀ of the same insecticide in combination with MMI or SR1. The 95% confidence intervals of SRs were estimated on the log scale using the 95% fiducial limits of LC₅₀ values. SRs were considered significant when their 95% confidence intervals did not include 1⁶⁶.

Other statistical analyses are described in the corresponding figure legends. For repeated-measures ANOVA, significance levels were indicated as ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. For all other analyses, * $p < 0.05$ was considered statistically significant unless otherwise indicated.

Declarations

Data availability

All data generated or analyzed during this study are included in the manuscript and supporting files; source data files have been provided for all figures.

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Author contributions

C.R. and J.S. designed the research. C.R., H.G., K.L., W.H. and B.H. performed the experiments. C.R. and B.H. analyzed the data. C.R., B.H. and J.S. wrote and revised the manuscript.

Competing interests

The authors declare no competing interests.

Inclusion & Ethics

Insects were used in this study and inclusion & ethics statement is not applicable to this study.

Additional information

The online version contains supplementary information

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Figures

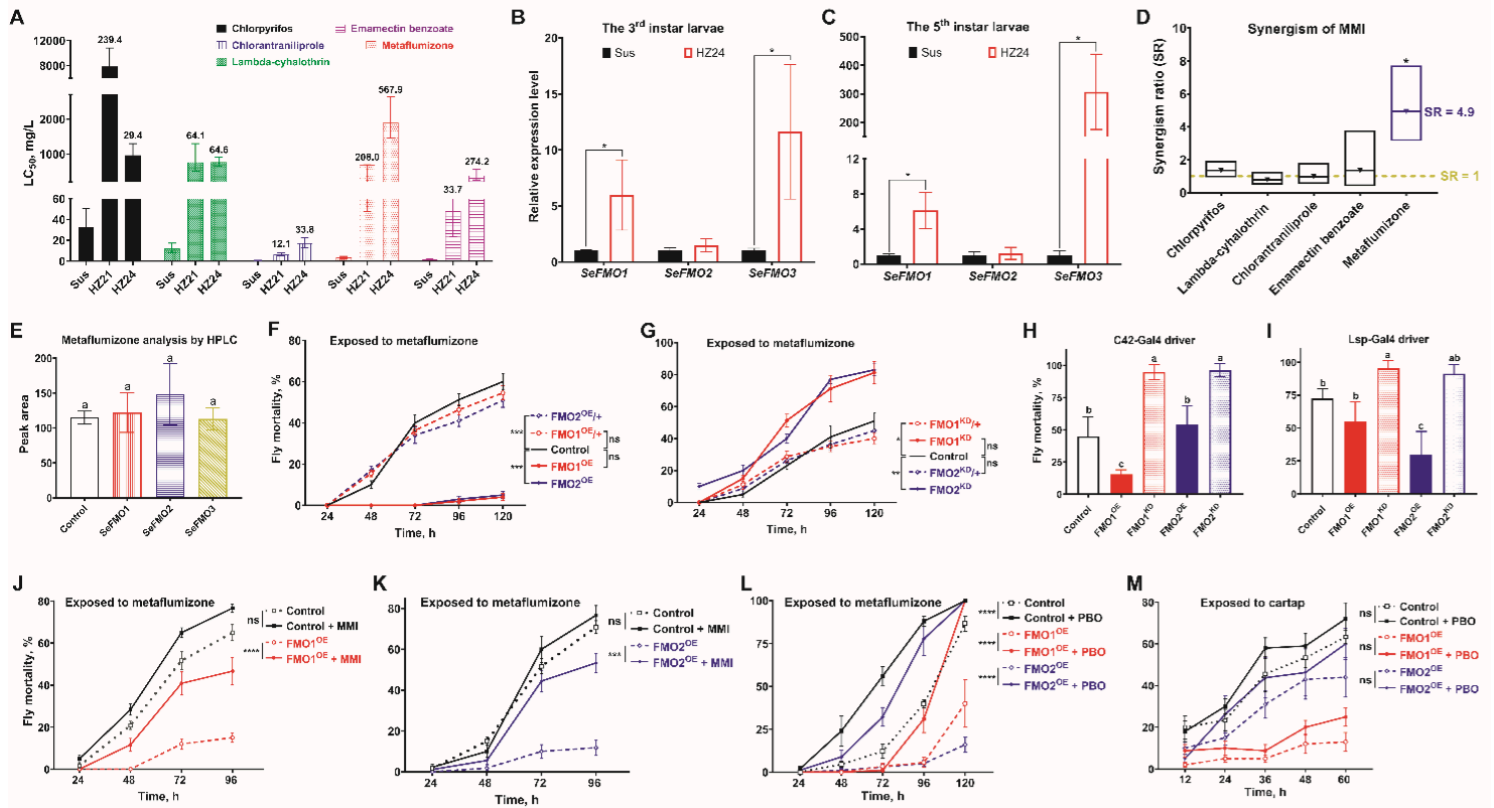


Figure 1

FMO is associated with field-evolved metaflumizone resistance in *S. exigua* and modulates metaflumizone susceptibility in *Drosophila*.

(A) Susceptibility of Lab-Sus, HZ21 and HZ24 populations to different insecticides. Numbers above bars indicate resistance ratios. (B-C) Relative expression levels of SeFMO genes in 3rd and 5th instar larvae of HZ24 compared with the susceptible strain. (D) Synergism ratios of MMI against different insecticides in HZ24. Center lines indicate SR estimates, and boxes indicate 95% confidence intervals. The dashed line indicates SR = 1. (E) *In vitro* metabolism assays show no detectable metabolism of metaflumizone by recombinant SeFMO proteins. Reaction mixtures without recombinant SeFMO were used as the control. (F-G) FMO overexpression and knockdown significantly decrease and increase the mortality of flies exposed to 350 mg/L metaflumizone, respectively. (H-I) Tissue-specific FMO expression in the Malpighian tubules (H) and fat body (I) of flies modulates metaflumizone tolerance. (J-K) MMI restores metaflumizone susceptibility in FMO1- and FMO2-overexpressing flies. (L-M) PBO abolishes FMO-mediated metaflumizone tolerance, but does not affect cartap tolerance. For panels F, G, and J–M, all UAS-FMO lines were backcrossed to the same w1118 background for six generations, and FMO expression was manipulated using Act5C-Gal4, with Act5C-Gal4 × w1118 progeny serving as background-matched driver-only controls. For panels H and I, C42-Gal4 × w1118 and Lsp-Gal4 × w1118 progeny, respectively, served as the corresponding controls. Statistical analyses were performed using two-tailed unpaired *t*-tests for panels B and C, one-way ANOVA followed by Tukey's HSD multiple-

comparison test for panels E, H and I, and repeated-measures ANOVA followed by Dunnett’s multiple-comparison test for panels F-G and J-M. For panel D, SRs were considered significant when the 95% confidence intervals of LC₅₀ values did not overlap. ns, not significant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

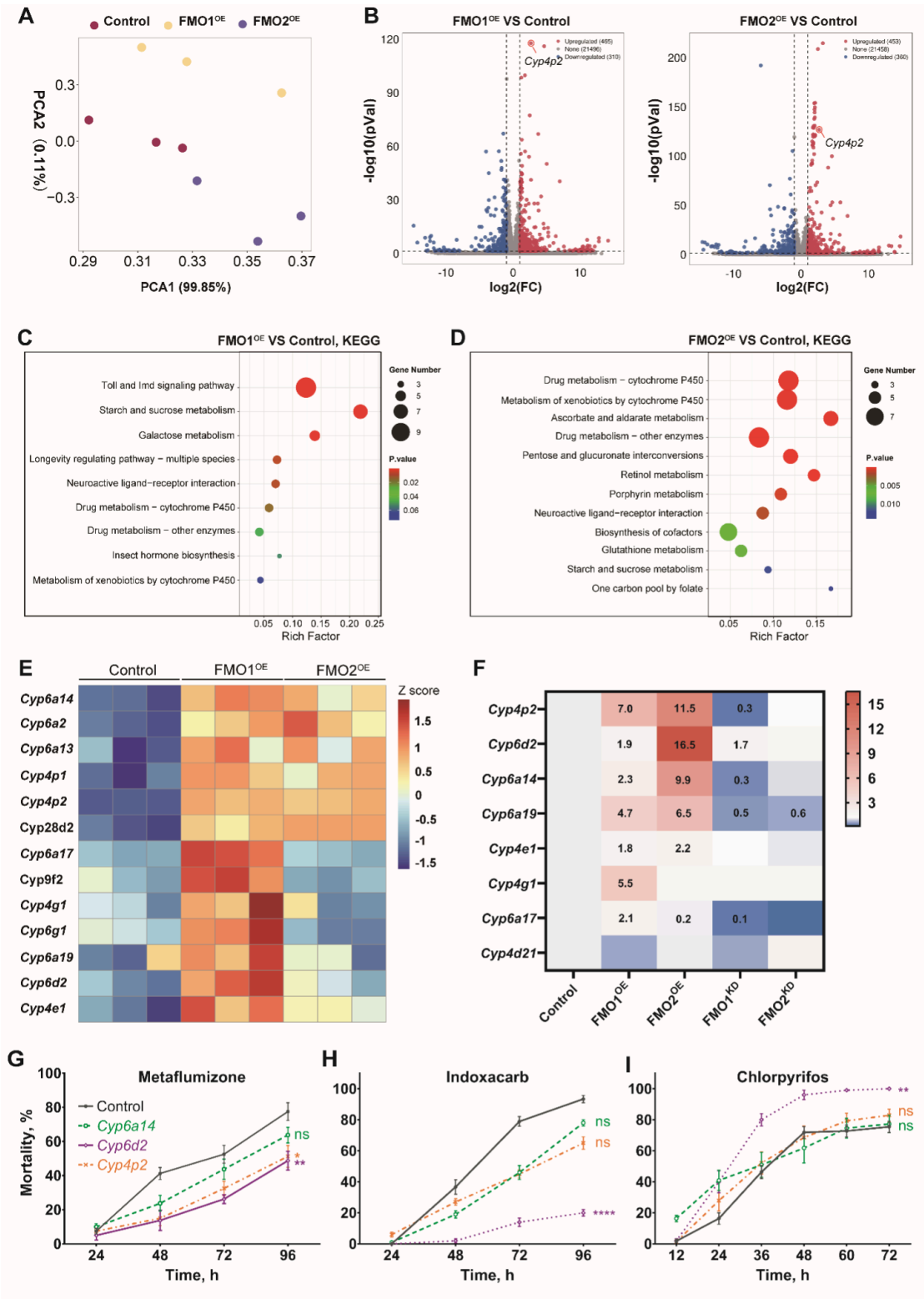


Figure 2

FMO promotes insecticide tolerance through transcriptional activation of CYP detoxification genes in *Drosophila*.

(A) Principal component analysis of RNA-seq profiles. (B) Volcano plots showing CYP genes induced by FMO1^{OE} and FMO2^{OE}. (C and D) KEGG enrichment analysis highlighting cytochrome P450-mediated drug metabolism in *Drosophila* with FMO1^{OE} (C) and FMO2^{OE} (D). (E) Heatmap of FMO-responsive CYP genes from RNA-seq based on gene-wise Z-score-normalized FPKM values. (F) qPCR validation of candidate CYP genes in FMO^{OE} and FMO^{KD} flies. Numbers shown in the heatmap indicate fold changes relative to the control group. (G-I) Overexpression of *Cyp4p2*, *Cyp6d2* and *Cyp6a14* reduces insecticide susceptibility. Act5C-Gal4 was used as the driver in all panels, with Act5C-Gal4 × w1118 progeny serving as the control. Statistical analyses were performed using two-tailed unpaired *t*-tests for panel F, in which each group was compared with the control, and repeated-measures ANOVA followed by Dunnett's multiple-comparison test for panel G, H, and I. ns, not significant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

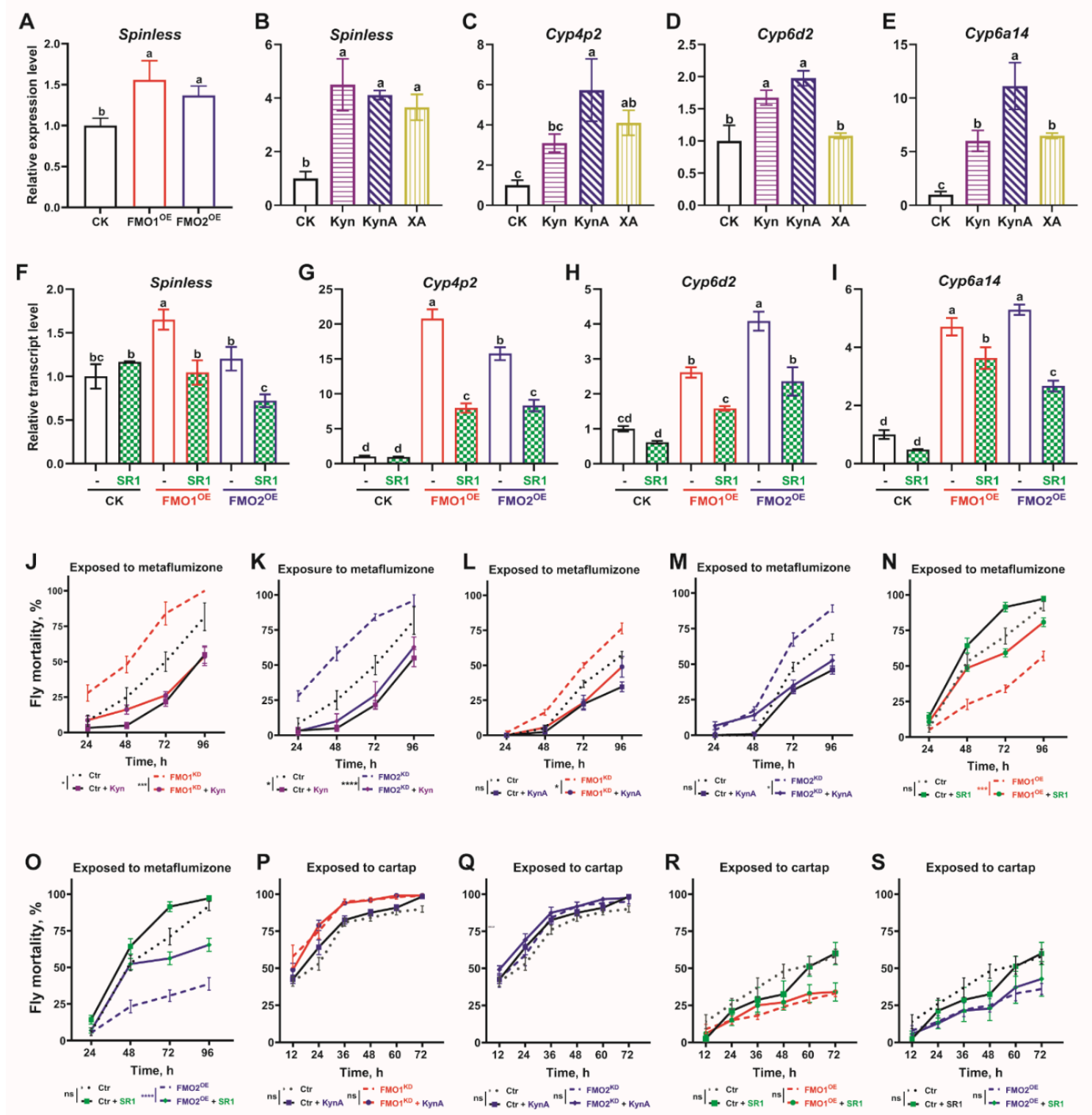


Figure 3

The FMO-AhR axis regulates CYP expression and metaflumizone susceptibility in *Drosophila*.

(A) FMO overexpression increases AhR transcript levels. (B-E) Kynurenine-pathway metabolites, including kynurenine (Kyn), kynurenic acid (KynA), and xanthurenic acid (XA), activate AhR–CYP signaling. Kynurenine metabolites activate AhR–CYP signaling. (F-I) The AhR inhibitor (SR1) suppresses CYP expression in FMO-overexpressing flies. (J-M) Kyn (200 μ M) and KynA (500 μ M) increase

metaflumizone tolerance in FMO-knockdown flies after 6 d of dietary supplementation before insecticide exposure. (N-O) SR1 (200 μ M) reverses FMO-conferred metaflumizone tolerance in FMO-overexpressing flies after 6 d of dietary supplementation before metaflumizone exposure. (P-S) KynA and SR1 do not alter susceptibility of fly to cartap, an insecticide directly metabolized by FMO. In panels F–I, N, O, R, and S, transgene expression was temporally induced using the Gal80^{ts}/Act5C-Gal4 system, with the corresponding Gal4/+ controls subjected to the same temperature-shift treatment; all other panels used Act5C-Gal4, with Act5C-Gal4/+ progeny serving as controls. Statistical analyses were performed using one-way ANOVA followed by Tukey's HSD multiple-comparison test for panels A–I, and repeated-measures ANOVA followed by Dunnett's multiple-comparison test for panels J–S. For panels labeled with different letters are significantly different from each other ($p < 0.05$). ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

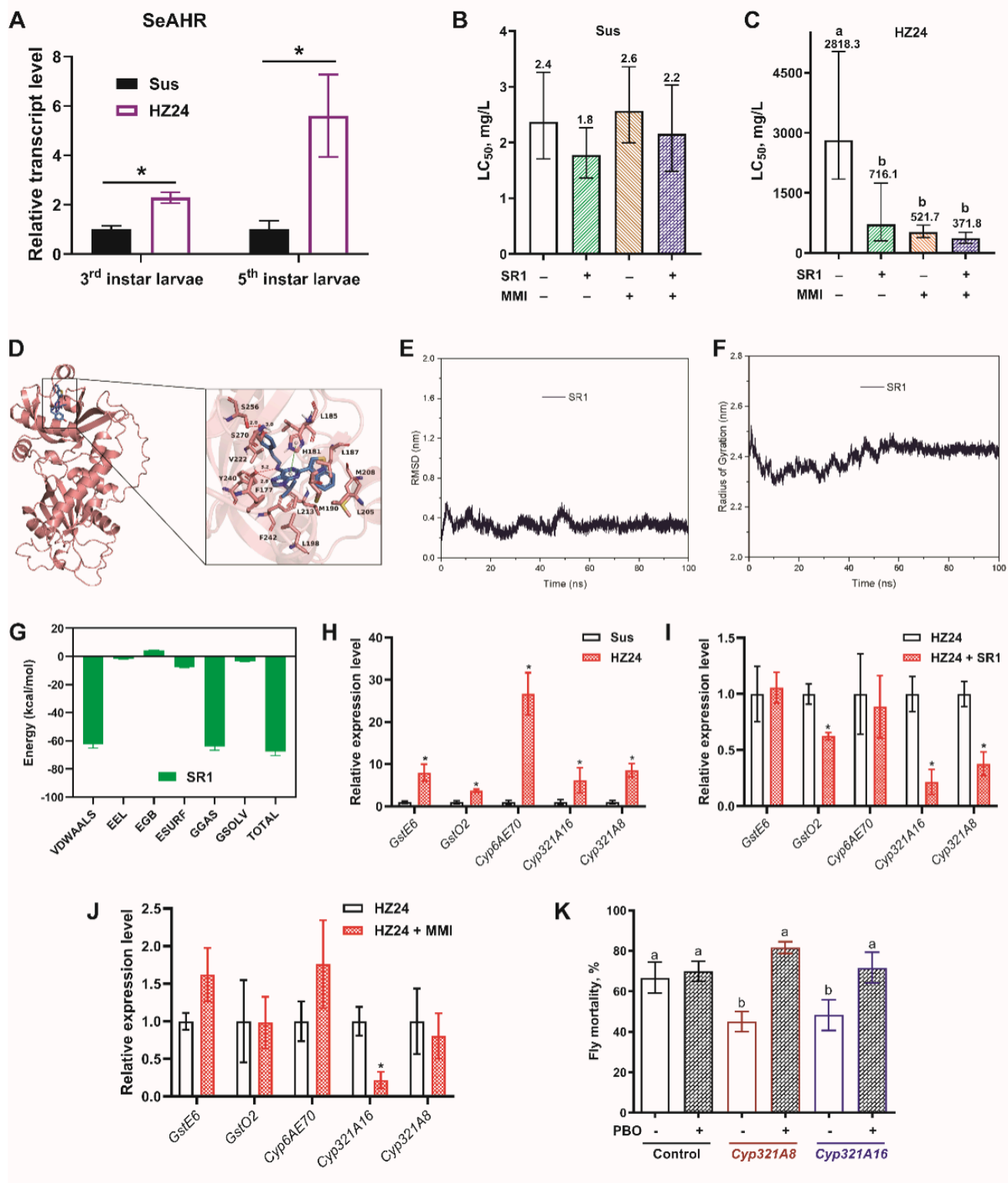


Figure 4

Validation of the AhR-CYP axis of FMO-associated metaflumizone resistance in *S. exigua*.

(A) SeAHR is upregulated in the metaflumizone-resistant HZ24 population. (B and C) SR1 and MMI reduce metaflumizone resistance in HZ24, not in Sus strain. (D) Predicted binding mode of SR1 within the SeAHR ligand-binding domain. (E-G) Molecular dynamics simulation supports the stability of the SR1-

SeAhR complex. (H) Detoxification-related genes are upregulated in HZ24. (I) SR1 treatment suppresses CYP gene expression in HZ24. (J) MMI treatment suppresses CYP gene expression in HZ24. (K) Heterologous expression of SeCYP genes in *Drosophila* reduces metaflumizone susceptibility. Act5C-Gal4 was used as the driver, with Act5C-Gal4 × w1118 progeny serving as the control. Statistical analyses were performed using two-tailed unpaired *t*-tests for panels A and H-J, and one-way ANOVA followed by Tukey's HSD multiple-comparison test for panel K. For panel B and C, LC₅₀ values were considered significantly different when their 95% fiducial limits did not overlap; different letters indicate significant differences among groups. **p* < 0.05.

Supplementary Files

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