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Secondary metabolites in host pears defense against two fruit borers and cytochrome P450-mediated counterdefense

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Summary

Herbivores insects have evolved metabolic strategies to survive the challenges posed by plant secondary metabolites (SMs). This study reports an exploration of SMs present in pears, which serve as a defense against invasive *Cydia pomonella* and native *Grapholitha molesta*, and their counterdefense response. The feeding preferences of fruit borers are influenced by the softening of two pear varieties as they ripen. The content of SMs, such as quercetin and rutin, increasing due to feeding by fruit borers. Notably, quercetin levels only increase after *C. pomonella* feeding. The consumption of SMs affects the growth of fruit borers populations differently, potentially due to the activation of P450 genes by SMs. These two fruit borers equipped with specific P450 enzymes that specialize in metabolizing quercetin and rutin, enabling them to adapt to these SMs in their host fruits. These findings provide valuable insights into the co-evolution of plants and herbivorous insects.

Key Word: herbivorous insects-plants co-evolutionary, defense, counter defense, plant secondary metabolites, cytochrome P450

1. Introduction

China is a prominent global contributor to pear industry, holding the largest cultivated area and highest output. Notably, Liaoning Province possesses a remarkable and competitive edge in terms of scale for pear production within China. In this province, *Pyrus ussuriensis* (Maxim) (known as Nanguo Pear in Chinese) and *Pyrus bretschneideri* (Rehd) (known as Pingguo Pear in Chinese) are the two primary varieties of pears cultivated. Both *P. ussuriensis* and *P. bretschneideri* belong to the Pyrus genus. *P. ussuriensis* is the most important cultivated pear in the northeastern, cold areas of China (Qiu et al. 2018). This fruit is round or oblate, yellowish green in color, and has a persistent calyx (Liu et al. 2019). *P. ussuriensis* is typically harvested in September when they are still green, firm, and less juicy. Following harvest, the fruit ripens at room temperature and transforms into golden yellow, soft, juicy, and uniquely aromatic state (Shi et al. 2018; Li et al. 2019). On the other hand, *P. bretschneideri* belongs to the white pear system, which boasts excellent cold tolerance, storage capabilities, high yield, and high quality (Liu et al. 2019). *P. bretschneideri* are crucial parent plants in breeding program, as they have contributed to the creation of 68 different varieties within the *P. bretschneideri* family. Consequently, both varieties of fruit trees hold significant economic value.

Both the codling moth, *Cydia pomonella* (Linnaeus) and the oriental fruit moth, *Grapholitha molesta* (Busck) are prominent members of the Lepidoptera family Tortricidae, and are recognized as significant pests of fruit trees worldwide. While *C. pomonella* is the major agricultural invasive pest in China, its initial detection

53 occurred in Xinjiang, China, in 1957 (Zhang, 1957). *C. pomonella* has spread to
54 approximately 70 countries since 1900, owing to the rapid growth of global trade and
55 international travel, leading to an annual global fruit loss of around US$10 million
56 (Willett et al., 2009). *G. molesta* also poses a substantial threat in this region (Ju et al.
57 2021). These two fruit borers have a similar host range, which includes pears, apples
58 and other nut fruits (Zhao et al. 2016). Previous studies have shown that there is a
59 competition between these two fruit borers. Notably, C. pomonella was able to rapidly
60 increase its population after exposure to quercetin by adopting an 'accelerated burst' of
61 oviposition strategy (Bai et al., 2023). *C. pomonella* and *G. molesta* consume fruit
62 with their larvae, causing a significant number of fruit drops, severely impacting fruit
63 quality, and resulting in substantial economic losses to the global pear fruit industry
64 (Yang and Zhang 2015). The safety and security of *P. ussuriensis* and *P.*
65 *bretschneideri* are seriously compromised by both of these fruit borers.

66 Plants have developed intricate defense mechanisms to safeguard themselves
67 against insect herbivory. These mechanisms can be categorized as constitutive or
68 inducible defenses (Erb and Reymond 2019). Constitutive defense refers to the
69 inherent physicochemical traits of plants that impede the feeding activities of
70 phytophagous insects, such as fruit fuzz, surface waxes, hardness (Moreira et al.
71 2018a). For instance, the trichomes found on *Arabidopsis* leaves have been observed
72 to negatively influence the feeding and egg-laying behaviors of *Plutella xylostella*
73 (Handley et al. 2005). Additionally, an increase in silica cell content has been linked
74 to enhanced plant resistance against insect pests by host plant hardness (Gouranga et

al. 2023). When faced with insect herbivory, plants synthesized a diverse array of secondary metabolites (SMs) or release volatiles as a part to their induced defense. These SMs can be broadly classified into phenolics (e.g., lignin, flavonoids, and tannins), terpenes, and sulfur (S)- (e.g., glutathione, glucosinolates, and defensin) and nitrogen (N)-containing compounds (e.g., alkaloids, cyanogenic glycosides) (Mostafa et al. 2022). In recent years, several studies have indicated that flavonoids can impact the nutrient accumulation of Lepidoptera larvae, leading to their inability to complete the moulting process and resulting in their death (Bentivenha et al., 2018). Plants produce important flavonoids such as quercetin and rutin, which have diverse effects on herbivorous insects. These effects include regulating growth and development, inhibiting immune defense, and inducing detoxification enzymes (Zhang et al. 2012). After feeding on branches containing quercetin and rutin, mortality rates for *Eriosoma lanigerum* larvae reached up to 80% (Ateyyat et al., 2012). Furthermore, rutin has been found to decrease the survival rate of *Ostrinia nubilalis* and has the ability to reduce pupal weight and prolong the pupal period in the *Spodoptera litura* (Simmonds, 2003). However, the effects of consuming quercetin and rutin on the development and growth of *C. pomonella* and *G. molesta* populations are less known.

In order to cope with SMs, insects have developed a range of adaptive mechanisms, and metabolic detoxification is usually one of the most important ways in which insects adapt to exogenous substances (Ju et al., 2021). One such mechanism involves the utilization of cytochrome P450 monooxygenases (P450), which are widely distributed throughout the organism and play a crucial role in the primary

97 metabolism of exogenous substances, leading to the adaptation of insects to SMs
98 (Feyereisen, 2015). As a phase I detoxification enzyme, P450 directly participates in
99 the metabolism of secondary plant metabolites. In the case of Lepidoptera, Clan 3
100 represents the largest gene family of P450, within which the CYP6, CYP9, and
101 CYP321 gene subfamilies have demonstrated the ability to metabolize various
102 phytotoxins (Joußen et al., 2012). For instance, research revealed the potential of
103 *CYP6AB14* from *Spodoptera litura* in the detoxification of plant allelochemicals
104 (Wang et al. 2015). In *Spodoptera frugiperda*, numerous P450 genes in the CYP6B,
105 CYP321A, and CYP9A subfamilies are responsive to phytochemicals and are
106 involved in the detoxification (Giraudo et al., 2015). Additionally, it was observed that
107 xanthotoxin and flavone induced the expression of *CYP321A1* in *Helicoverpa zea*
108 (Zhang et al. 2014). However, knowledge on the role of individual P450 gene in
109 response to the presence of SMs is lack in *C. pomonella* and *G. molesta*.

110 To gain valuable insights into the co-evolution of host fruit and phytophagous
111 insects, in this study we focused on investigating the mechanisms of fruit defense and
112 counter defense by phytophagous insects. To test our hypothesis, we conducted an
113 assessment of the impact of quercetin on the growth and development of fruit borers,
114 as well as the expression level of the P450 Clan 3 gene. This assessment involved a
115 comparison of the fruit hardness and the content of major phytosubstance in *P.*
116 *ussuriensis* and *P. bretschneideri*, with quercetin serving as an illustrative example.

2. Materials and Methods

2.1 Fruits and Chemicals

The *P. ussuriensis* and *P. bretschneideri* used in the experiment were gathered from the orchard located in Xiha Village, Hartau Town, Zhangwu County, Liaoning Province, China (122.14°E, 42.48°N). The orchard spans an area of 13,340 m^2, with plant spacing set as 6 m×4 m.

The chemicals used for the extraction and detection of SMs were of chromatographic grade. The standards, namely gallic acid (98% purity), isoquercitrin (98% purity), rutin (98% purity), quercetin (98% purity), chlorogenic acid (98% purity), epigallocatechin (98% purity), and catechin (98% purity), were purchased from Solarbio (China). Methanol (chromatography grade) was purchased from Concord Technology (China).

Large fragment DNA polymerase I (9 U/μL), Taq DNA polymerase (5 U/μL), cloned Pfu DNA polymerase (2.5 U/μL), BamH I/Kpn I (10 U/μL), Sf9 insect cells, SF-900 serum-free medium, fetal bovine serum (FBS), and TRIzol Reagent were purchased from Invitrogen (Carlsbad, CA, USA). Penicillin/streptomycin was purchased from Bio-Whittaker (Walkersville, MD, USA). D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase (360 U/mg protein) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Insects

The susceptible strain (SS) of *C. pomonella* and *G. molesta* were bred for more than 50 generations in a controlled environment without exposure to any insecticide

or plant secondary material. The rearing method used in this study follows the protocol described by Hu et al. (2023). Both species were raised in a climatic chamber maintained at a temperature of 26±1°C, with a photoperiod of 16:8 (L: D) and a relative humidity of 60±5%. Adults were provided with honey water at a concentration of 10% as their food source.

2.3 Fruit hardness and infestation rate investigation

To assess fruit hardness and infestation rates, a survey was conducted every 20 days between May and September 2021, encompassing the stages of fruit expansion (I-IV) and fruit ripening (V-VI), as defined by Nan (2014). Six pear trees of each species were randomly selected at each time point, then five fruits were randomly chosen from each tree, resulting in a total of 30 fruits selected for fruit hardness testing. Fruit hardness was measured using a GY-4 fruit hardness tester (Saiyas, China), with each fruit being tested four times. Additionally, 2 grams of each fruit, including the flesh, peel, and kernel, were rapidly frozen in liquid nitrogen and stored at -80°C for further analysis.

In order to record the fruit infestation rate caused by the two fruit borers, five trees of both Pear species were randomly selected in the orchard, and five fruits of each tree were surveyed at each of the four cardinal directions (east, west, south, and north).

2.4 Extraction and detection of SMs

To investigate the alterations in SMs present in fruits after being infested, three *P. bretschneideri* and three *P. ussuriensis* that were harmed by *C. pomonella* and *G.*

molesta were selected for analysis. This experiment was repeated three times, with unaffected fruits serving as the control group. Upon returning to the laboratory, the damaged sections of the fruit, including the pulp, skin, and core, were excised and rapidly frozen using liquid nitrogen, and stored at -80°C for further analysis. The extraction and detection of SMs were conducted according to Zhang et al. (2017) with slight modification. Detailed descriptions of the detection of SMs were given in Supplementary Information.

2.5 Construction of population parameters of *C. pomonella* and *G. molesta* fed with quercetin and rutin

In order to construct a life table, an artificial diet was prepared for the rearing of larvae of *G. molesta* and *C. pomonella* using different concentrations of quercetin (10 μg/g, 100 μg/g, 1000 μg/g, and 5000 μg/g) and rutin (20 μg/g, 100 μg/g, 200μg/g) according to quercetin and rutin contents measured in *P. ussuriensis* and *P. Bretschneideri*, respectively. The first instar larvae of both species that hatched within 24 hours were reared in separate diets until they reached the pupal stage. To ensure proper ventilation and prevent escape, the rearing tubes were plugged with absorbent cotton. The development stages and survival rates of each species were recorded. During the pupal stage, the males and females were distinguished based on their pupal morphology (Feng et al. 2019). After the emergence of adults, ten individuals consisting of five males and five females were placed together in an inverted clear plastic cup (7.2 cm in diameter and 7.9 cm in height) for mating. The cup was covered with plastic wrap to facilitate oviposition and sealed with a rubber band. To ensure air

permeability, holes were made in the plastic cups, and cotton was placed at the bottom to provide a 10% honey solution. The fecundity and survival rate of the adults were recorded on a daily basis until the death of all individuals. Each concentration of quercetin and rutin was treated with 100 larvae and the experiment was repeated three times for each concentration. A control group without quercetin and rutin were also included.

2.6 Determination of P450 enzyme activity

Fifteen fourth instar larvae were collected from each treatment, and their intestines were dissected to detect P450 enzyme activity. This process was repeated three times for each sample. To extract the P450 enzyme, the test insects were ground into powder and placed in a 1.5 mL centrifuge tube. Then, 1 mL of pre-cooled P450 enzyme extraction buffer was added and mixed thoroughly. The mix was centrifuged at 14000 rpm for 30 min at 4°C. The supernatant of each sample was carefully transferred to a new enzyme-free centrifuge tube for further use.

The protein concentration was determined using the Takara BCA Protein Assay Kit (Takara, China) according to the provided instructions. The P450 enzyme activity was assessed following the method described in Li et al. (2023) with some minor adjustments. In brief, the extracted P450 enzyme solution was mixed with 7-ethoxy-coumarin substrate (2 mM) and NADPH (10 mM) in a centrifuge tube. The volume of the mixture was then increased to 200 μL by adding 100 mM sodium phosphate buffer (pH 7.8). After an incubation period of 10 minutes at 30 °C, trichloroacetic acid (15%) was added to the mixture (60 μL) to stop the reaction. The

resulting mixture was then centrifuged at 4 °C for 10 minutes, and 200 μL of the supernatant was transferred to opaque microplate wells containing glycine/sodium hydroxide (1.6 mM, pH = 10.5) in a volume of 90 μL. The absorption value of the mixture was measured at an excitation wavelength of 358 nm and an emission wavelength of 456 nm. The P450 enzyme activity was determined by quantifying the amount of 7-hydroxycoumarin (ECOD) produced within a 10-minute timeframe. A standard curve was established with various concentrations of the ECOD standard, and their absorbance values were correlated.

2.7 Total RNA Extraction, cDNA Synthesis, and RT-qPCR

The RNA extraction procedure was performed using the RNAiso Plus kit (Takara, China) according to the manufacturer's instructions. The concentration of the extracted RNA samples was determined using the NanoDrop 2000 (ThermoFisher Scientific, USA). Subsequently, the first strand cDNA synthesis was performed using 1 μg of total RNA, following the instructions provided by the PrimeScriptTMRT reagent Kit with gDNA Eraser (Takara, China).

There are 72 and 77 P450 genes in *C. pomonella* and *G. molesta*, respectively. *C. pomonella* possessed 31 Clan 3 genes (Ju et al., 2023), whereas *G. molesta* had 30 (Lv et al., 2022). In this study, the expression levels of all P450 Clan 3 genes in *C. pomonella* and *G. molesta* were analyzed. The expression levels of P450 genes were quantified using real-time quantitative PCR (RT-qPCR) on a Bio-Rad CFX96 (BioRad, USA). The reaction mixture consisted of 1 μL of cDNA template, 10 μL of TB Green Premix Ex Taq 2 (Takara, China), 0.8 μL of each primer, and 7.4 μL of sterile water.

The *EF-1α* and *β-actin* genes were set as internal reference genes for *C. pomonella* (Wei et al. 2020) and *G. molesta* (Zhang et al., 2023), respectively. The specific primers used in the RT-qPCR are listed in Table S1.The reaction conditions included an pre-denaturation step at 95°C for 30 s, followed by denaturation at 95°C for 10 s, annealing at a temperature determined by primer requirements for 30 s, and extension at 72°C for 30 s. The amplification was carried out for a total of 40 cycles. A no template control was included by replacing the cDNA in the reaction mixture with ddH_2O. Each sample was analyzed in triplicate. The gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.8 RNA Interference (RNAi) of P450 genes

Double-stranded RNA (dsRNA) synthesis was performed following the instructions provided by the T7 RiboMAXTM Express RNAi System (Promega, USA). The resulting dsRNA solution was diluted to a concentration of 2000 ng/μL. Prior to injection, both test fourth-instar larvae were briefly exposed to ice for 5 min to induce temporary anesthesia, after which they were promptly transferred to a Petri dish for injection. A volume of 1 μL of dsRNA was injected into the three-to-five segment at the end of the posterior end of each larva′ abdomen, while an equal amount of dsGFP was injected as a control. Subsequently, the injected larvae were returned to their diet without quercetin and kept under appropriate rearing conditions. At time intervals of 6 h, 12 h, 24 h, and 48 h post-injection, samples were collected from each treatment group. For each time point, three larvae were selected from each group for analysis.

2.9 Effect of knock down of P450 genes on the growth and development *C. pomonella* and *G. molesta* under quercetin stress

Fourth-instar larvae of *C. pomonella* and *G. molesta* underwent a period of 12 h of fasting before being subjected to injection with *dsRNA* and *dsGFP* of the target genes. These injected larvae were then provided with artificial diets containing 100 μg/g quercetin, which similar to the content in their host pears. The weight of the insects was measured at 12 h intervals over a duration of for 48 h. For each treatment, a total of fifteen larvae were selected and the experiment was replicated three times to ensure reliability. Prior to and after the experiment, the weights of diets, feces, and larvae were recorded after undergoing a drying process. The Relative growth rate (RGR), Relative consumption rate (RCR), Efficiency of the conversion of ingested food (ECI), and Efficiency of the conversion of digested food (ECD) were calculated using the formulas presented in Table S2.

2.10 Construction of the recombinant plasmids

The open reading frame sequence of *CYP6K1B*, *CYP6AW1*, *CYP6B74*, *gm_13876*, and a NADPH-dependent cytochrome P450 reductase (CPR) were synthesized directly by Tsingke Science (Beijing, China) following the method described in Li et al. (2009). They were then subcloned into the pFastBac1 vector using restriction enzyme digestion. The resulting recombinant plasmids were analyzed and verified through PCR and sequencing, and were subsequently stored at −20°C. To generate the recombinant Bacmid DNA, the recombinant pFastBac1 constructs were transformed into the MAX Efficiency® DH10Bac ™ chemically competent cells

(Thermo Fisher Scientific, Langenselbold, Germany). Positive DH10Bac cells containing the recombinant bacmid DNA were confirmed through PCR using M13 forward and reverse primers. The recombinant bacmid DNA was then isolated, quantified, aliquoted, and stored at −20°C in TE Buffer at a concentration of approximately 500 ng/μL .

2.11 Heterologous expression of P450s and microsome isolation

Recombinant P450 proteins were produced using Sf9 cells. The bacmids containing CYP6K1B, CYP6AW1, CYP6B74, and gm_13876 were transfected into the Sf9 insect cells using a Bac-to-Bac baculovirus expression system (Thermo Fisher Scientific, Langenselbold, Germany) following the manual instructions. The titer of the recombinant virus was determined following the manufacturer's instructions. Sf9 cells were co-infected with recombinant baculoviruses expressing P450s and CPR, with a multiplicity of infection (MOI) of 1 and 0.1, respectively. The Sf9 cells were maintained at 27 °C using Sf-900 II SFM medium (LifeTechnologies, Carlsbad, CA, USA), supplemented with 2.5 μg/ml hemin and 0.3% (v/v) fetal bovine serum. To detect successful expression of the recombinant protein using western blot, a positive recombinant control CYP341B14/CPR (accession number: LC326250.2) underwent the same experimental procedure. After 72 h, cells were harvested to isolate the microsomal fraction, which was then aliquoted and stored at −80 °C after protein quantification using the Bradford Protein Assay Kit (Beyotime, Shanghai, China).

2.12 Western blot

293 Microsomal fractions containing the recombinant CYP341B14/CPR positive
294 control, fused with the C-terminal overhang of the His tag, were denatured by
295 incubation at 70 °C for 5 min and separated using SDS PAGE. The membrane proteins
296 were then blotted onto a polyvinylidene difluoride (PVDF) membrane (Merck
297 Millipore, Darmstadt, Germany) using a Bio-Rad blotting system. The membrane was
298 blocked with a 5% (w/v) non-fat dry milk in Tris-buffered saline with Tween-20
299 (TBST) buffer) for 1 h, followed by overnight incubation with Anti-His antibody
300 (1:2000; Beyotime, Shanghai, China) in a 0.25% (w/v) non-fat dry milk TBST
301 buffer at 4 °C.After three washes with TBST buffer, the membrane was briefly
302 incubated with SuperKine enhanced chemiluminescence (ECL) solution (Abbkine,
303 Wuhai, China), and visualized using the Tanon 5200 chemiluminescent imaging
304 system (Tanon, Shanghai, China).
305 2.13 Metabolism Assay
306 The metabolism assay was assessed following the method described in Mao et al.
307 (2009) with some minor adjustments. Reaction mixtures for quercetin were set up
308 with 100 pmol P450, 5 μL of 5 mM stock solution for tested quercetin, 5 μL of 2.5
309 mM stock solution for tested rutin, 0.5 mg of D-glucose-6-phosphate, 0.5 μL of
310 glucose-6-phosphate dehydrogenase, 50 μL of NADPH (1 mg/mL in 0.1 M phosphate
311 buffer (pH 7.8) or 50 μL of phosphate buffer (for the no NADPH control). The total
312 volume was adjusted to 500 μL with 0.1 M phosphate buffer (pH 7.8). The reaction
313 mixtures were incubated at 30 °C for 90 min in a shaking metallic bath. Each 500 μL
314 reaction was then combined with an equal volume of acetone and centrifuged at

315 10,000g for 10 min at room temperature. The reaction products were analyzed with a
316 reverse phase XDB- C18 column (AG120, 5 μm, 4.6×150 mm; Agilent Technologies
317 Ltd., USA). For quercetin, a mobile phase of 70% water containing 0.1% phosphoric
318 acid and 30% acetonitrile was used. For rutin, a mobile phase of 83% water
319 containing 0.1% phosphoric acid and 17% acetonitrile was used . The absorbances of
320 quercetin were monitored at 372 nm and rutin at 376 nm using a Waters 996
321 photodiode array detector. The analyses for quercetin and rutin metabolism were
322 repeated three times. Quercetin standard was dissolved diluted to five different
323 concentrations (5, 10, 20, 50, and, 100 μg/mL) in methanol, while rutin standard was
324 dissolved diluted to five different concentrations (12.5, 37.5, 50, 62.5, and 87.5 μg/mL)
325 in methanol for kinetics assay.
326 2.14 Data Analysis
327 The intrinsic rate of increase (r), net reproductive rate (R_0), finite rate of increase
328 (λ), and mean generation time (T) were compared between *G. molesta* and *C.*
329 *pomonella* under different concentrations of quercetin using the paired bootstrap test
330 ($P < 0.05$). Fruit infestation rate and nutrient utilization index of *G. molesta* and *C.*
331 *pomonella* were converted to inverse sine values and then tested for normal
332 distribution using the Shapiro-Wilk analysis. The fruit infestation rate and nutrient
333 utilization index of *G. molesta* and *C. pomonella* were analyzed separately for
334 significance using a one-way analysis of variance (ANOVA) with Tukey's honest
335 significant difference (HSD) tests. The software SAS 8.1 was employed for this
336 analysis. All data were presented as the mean of three replicates ± standard error (SE)

and plotted with SigmaPlot 12.5. The P450 enzyme activity, gene expression levels, and peak area were examined through using one-way analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) tests ($P < 0.05$) using SPSS Statistics 22 (IBM, Chicago). Student's *t*-test (*, $P < 0.05$) was used to assess the distinctions in SMs content between two samples. The data were presented as the mean of at least three independent experiments ± SE and visualized using GraphPad Prism 5 software (GraphPad Software, CA).

3. Results

3.1 Fruit hardness and infestation rates of *P. ussuriensis* and *P. bretschneideri*

During the fruit enlargement stage to the ripening, there was a significant decrease in fruit hardness observed in both *P. ussuriensis* and *P. bretschneideri*. Conversely, the rate of fruit decay showed an opposite trend (Figure 1). *P. ussuriensis* exhibited higher fruit firmness compared to *P. bretschneideri* during the fruit enlargement period (P <0.0001) and fructescence (V) (P <0.001) (Figure S1). The fruit infestation rate of *C. pomonella* was significantly higher than in both fruits compared to *G. molesta* in all periods. The fruit infestation rate of both borers increased rapidly as fruit hardness decreased. Correlation analysis revealed a negative correlation between fruit hardness and infestation rate of the two borers, indicating that a harder fruit host resulted in less damage caused by the borers (Table S3).

3.2 Changes in plant secondary metabolite content in fruit development stages

In *P. ussuriensis*, except for quercetin, the content of chlorogenic acid,

359 epigallocatechin, catechin, rutin, gallic acid, and isoquercitrin continued to decrease
360 from fruit expansion stage I to stage III. Among these compounds, chlorogenic acid,
361 epigallocatechin, gallic acid, and isoquercitrin content increased in stage IV, decreased
362 in stage V, and increased in stage VI. Rutin exhibited in an opposite trend from stage
363 IV to stage VI. Catechin content increased in stage IV and then began to decrease
364 until maturity stage VI. Quercetin content increased initially and then decreased from
365 stage I to stage VI, peaking in stage III (Figure 2 A). In *P. bretschneideri*, there was a
366 trend of increasing chlorogenic acid, quercetin, and rutin content and then decreasing
367 throughout the growth and development period. Gallic acid content showed a
368 decreasing trend throughout the period, while epigallocatechin, catechin, and
369 isoquercitrin content exhibited a fluctuating trend (Figure 2 B).

370 3.3 Changes in plant secondary metabolite content after borers feeding

371 SMs were examined before and after fruit borer damage at stage IV. The results
372 showed that the content of chlorogenic acid, gallic acid, isoquercitrin, rutin, and
373 quercetin in *P. ussuriensis* increased by 1.85-fold, 1.92-fold, 2.32-fold, 1.60-fold, and
374 1.85-fold, respectively, when induced by *C. pomonella* infection compared to
375 uninfested fruit. Similarly, the levels of chlorogenic acid, gallic acid, and rutin
376 increased by 1.71-fold, 1.41-fold, and 1.89-fold in *P. ussuriensis* induced by *G.*
377 *molesta* damage. Epigallocatechin and catechin content did not show significant
378 changes (Figure 2C). Furthermore, in *C. pomonella* damaged *P. bretschneideri* fruit,
379 the substances chlorogenic acid, epigallocatechin, gallic acid, rutin, and quercetin
380 were significantly elevated by 1.81-fold, 2.57-fold, 2.73-fold, 1.36-fold, and 1.75-fold,

respectively. However, the isoquercitrin content did not show significant changes after feeding by *C. pomonella*. In *P. bretschneideri* damaged by *G. molesta*, only gallic acid and isoquercitrin levels were significantly increased by 3.27-fold and 1.45-fold (Figure 2D).These results indicate that the content of quercetin in both pears was only induced by *C. pomonella* damage, not by *G. molesta* damage. Consequently, quercetin will be used as an example to explore the effects of SMs on the growth and development of two insect species.

3.4 Effects of quercetin and rutin on the population parameters of two borers

In comparison to the control group, the presence of quercetin in the diet had a significant impact on various population parameters in *C. pomonella*. The group treated with 10 μg/g quercetin exhibited the highest values for intrinsic rate of population increase (r), finite rate of increase (λ), mean generation time (T), while the control group (0 μg/g quercetin) had the highest net reproductive rate (R_0). The largest decrease in λ was observed in the 100 μg/g quercetin group, whereas the greatest reductions in r, R_0, and T of *C. pomonella* populations were observed in the 5000 μg/g quercetin group compared to the control.

For *G. molesta*, the r, λ, and R_0 values were higher in the 10 μg/g and 100 μg/g quercetin groups compared to the other groups. No significant differences in r, λ, and R_0 were observed between the 1000 μg/g quercetin group and control, except for a prolonged T in the former. The 5000 μg/g quercetin group exhibited significantly reduced values for r, λ, and R_0, as well as significantly prolonged T when compared to the control (Table 1).

Feed on diet containing varying concentrations of rutin (20 μg/g, 100 μg/g, 200 μg/g) , all *G. molesta* individuals died within 9 days. Although not all *C. pomonella* individuals died, rutin had a significant inhibitory effect on their development. All *C. pomonella* larvae were in the second and third instars and were unable to undergo pupation (Figure S2).

3.5 Effect of quercetin feeding on P450 enzyme activity of two borers

In comparison to the control group (0 μg/g quercetin), the activity of P450 enzyme in the midgut of the fourth instar larvae significantly increased when they consumed an artificial diet containing quercetin. Notably, the larvae of *C. pomonella* exhibited the highest P450 enzyme activity in their midgut when fed a diet with 1000 μg/g quercetin, which was 1.98 times higher than the control group (Figure 3A).

In contrast to the control group, apart from the artificial diet containing 1000 μg/g quercetin, the activity of the P450 enzyme in the midgut of the fourth instar larvae of *G. molesta* was significantly increased in all other treatments. The greatest increase in P450 enzyme activity in the midgut of larvae was observed when they consumed an artificial diet containing 5000 μg/g quercetin, which was 1.53 times higher than the control (Figure 3B).

3.6 Expression patterns of P450 genes in two borers

The results of the RT-qPCR showed that the expression levels of 20 genes belonging to the P450 Clan 3 of *C. pomonella* were found to be induced by quercetin. Out of these up-regulated genes, 10 demonstrated significant up-regulation across all concentrations of quercetin, while the remaining 10 genes showed up-regulation only

425 at specific concentrations (Figure 3C). Similarly, in the P450 Clan 3 of *G. molesta*, the
426 expression levels of 22 genes were induced to be up-regulated by quercetin. Among
427 these genes, seven exhibited up-regulation at all concentrations of quercetin, whereas
428 the remaining 15 genes showed up-regulation at their respective concentrations
429 (Figure 3D).

430 Based on the quantification of quercetin content in two pear samples, further
431 investigation was carried out on the P450 genes (*CYP354A22, CYP6AW1, CYP6K1b,*
432 *CYP9e2a* of *C. pomonella; CYP6A17, CYP6B74*, gm_13876, gm_16205 of *G.*
433 *molesta*) that were significantly induced by 100 μg/g quercetin (Figure 3C & 3D). The
434 expression patterns of four P450 genes in *C. pomonella* and *G. molesta* were
435 investigated at different developmental stages. The results indicated that *CYP345A22,*
436 *CYP9E2a*, and *CYP6K1b* in *C. pomonella* exhibited the highest expression levels of
437 expression during the fourth instar larvae stage, while *CYP6AW1* showed the highest
438 level of expression during the first instar larvae stage (Figure 4A-D). In the case of *G.*
439 *molesta*, the expression patterns of *CYP6B74*, *gm_13876*, *gm_16205*, and *CYP6A17*
440 were observed throughout all developmental stages, with the highest expression
441 occurring during the fourth instar larvae stage (Figure 4E-H)

442 Furthermore, the results showed that these P450 genes were expressed in all
443 tissues of the fourth instar larvae of both *C. pomonella* and *G. molesta* (Figure 4). The
444 expression level of *CYP345A22*, *CYP9E2a*, and *CYP6K1b* in the midgut of *C.*
445 *pomonella* was significant higher than in other tissues (Figure 4I-K). Conversely,
446 *CYP6AW1* demonstrated higher expression in the fat body, Malpighian tubes, and

midgut relative to the head and cuticle (Figure 4I). On the other hand, the midgut of *G. molesta* displayed significantly higher expression levels of four P450 genes (*CYP6B74*, gm_13876, gm_16205, and *CYP6A17*) compared to other tissues (Figure 4M-P).

3.7 Functional analysis of P450 genes associated with quercetin metabolism of *C. pomonella* and *G. molesta*

To verify the metabolic function of P450 genes, the gene silencing technique in both borers was applied. The expression levels of *C. pomonella CYP6AW1* and *CYP6K1b* were observed to decrease significantly by 53.53% and 51.68%, and 41.31% and 36.64% respectively, after the injection of dsRNAs for 6 and 12 hours (Figure 5A-B). Similarly, the expression of *G. molesta CYP6B74* and *gm_13876* was found to decrease significantly by 59.67% and 56.41%, and 56.62% and 62.31% respectively, after the injection of dsRNAs for 6 and 12 hours (Figure 5D-E).

There was no significant difference in body weight gain observed in *C. pomonella* (Figure S4A) and *G. molesta* (Figure S4B) larvae within 48 h when treated with 0 μg/g quercetin (control) and 100 μg/g quercetin. Furethermore, when *dsGFP* was injected into the aforementioned treatments, the body weight gain of *C. pomonella* (Figure S5A) and *G. molesta* (Figure S5B) larvae did not differ significantly from the control group within 48 hours.

The control groups consisted of dsGFP-injected larvae of *C. pomonella* and *G. molesta* fed on artificial diets containing 100 μg/g quercetin. In comparison to the control, *C. pomonella* larvae injected with *dsCYP6AW1* and *dsCYP6K1b* exhibited

significantly lower body weight gain within 48 h after consuming artificial diets containing 100 μg/g quercetin (Figure 5C). The same pattern was observed for *G. molesta* larvae injected with *dsCYP6B74* and *dsgm_13876* (Figure 5F).

As controls, *C. pomonella* and *G. molesta* larvae injected with *dsGFP* and fed with artificial diet containing 100 μg/g quercetin were used. The relative growth rate (RGR) and relative consumption rate (RCR) of *C. pomonella* larvae injected with *dsCYP6K1b* showed a significant decrease compared to the control, while no significant differences were observed in the efficiency of the conversion of ingested food (ECI) and efficiency of the conversion of digested food (ECD). The RCR, RGR, ECI, and ECD of *C. pomonella* larvae were significantly reduced compared to the control after *dsCYP6AW1* injection. Similarly, the RCR, RGR, ECI, and ECD of *G. molesta* larvae were significantly reduced compared to the control after *dsCYP6B74* and *dsgm_13876* injection (Table 2).

3.8 Verification of the recombinant bacmid DNA

Bacmid DNA from five colonies was utilized to perform PCR verification. The PCR products for CYP6K1B, CYP6AW1, CYP6B74, and CYP341B14 from all five colonies exhibited the expected size, indicating successful transposition. Additionally, four colonies of gm_13876 were confirmed to contain the correct recombinant bacmid (Figure S6). Thus, the recombinant bacmid DNA harboring the P450s was suitable for subsequent recombinant protein expression.

The western blot analysis (Figure S7) revealed the successful detection of CYP341B14 and CPR, with molecular mass of 58.9kDa and 77.9kDa, respectively, in Sf9 insect cells, indicating their successful expression. Considering that CYP6K1B,

CYP6AW1, CYP6B74, and gm_13876 were co-transfected with the same CPR lacking a His tag, parallel to the positive control, it can be inferred that these four P450s and the CPR lacking a His tag were also successfully expressed. Consequently, the microsomal fractions containing these four P450s and the corresponding CPR were prepared for enzymatic assays.

3.9 Metabolic analysis of recombinant P450 proteins of *C. pomonella* and *G. molesta* against quercetin and rutin

The retention time for quercetin was observed to be between 6.227 and 6.341 min, while for rutin it was between 6.92 to 7.138 min. Quercetin was detected with a peak area of 257.18±4.47, 227.36±4.74, 212.13±4.47, and 391.75±4.88 mAU*s, after incubation with recombinant CYP6AW1, CYP6K1b, CYP6B74, and gm_13876 for 90 min, respectively. However, except for CYP6AW1, which showed no significant difference in peak area with or without NADPH, all other P450s exhibited a significant decrease in peak area when NADPH was present (Figure 6A-D). All P450s demonstrated metabolic activity towards rutin (Figure 6E-H). The depletion rates of quercetin by CYP6AW1, CYP6K1b, CYP6B74, and gm_13876 were 1.56%±0.04%, 13.72%±0.24%, 22.39%±0.37%, 5.07%±0.07%, with CYP6B74 being the most efficient (Figure 6 I). The depletion rates of rutin by CYP6AW1, CYP6K1b, CYP6B74, and gm_13876 were 4.48%±0.53%, 27.78%±0.10%, 7.90%±1.69%, 4.23%±0.31%, with CYP6K1b being the most efficient (Figure 6 J).

4. Discussion

Plants have developed intricate defense mechanisms to protect themselves

against phytophagous insects, which can be categorized into two types: constitutive and inducible defenses (Moreira et al. 2018b). Constitutive defense refers to the inherent physicochemical properties of the plant that counteract attacks from pests, which act as the initial barrier against phytophagous insects by inducing structural changes such as the formation of spines, trichomes, waxes, lignification, and thickened cell walls (Louis et al. 2023; Mello and Silva-Filho 2002). Sousa-Lopes et al. (2020) reported that the severity damage caused by the bean weevil is influenced by the softness, lightness, and size of the seeds. It was discovered that the hardness of pears is determined by the presence of stone cells within the fruit, and as the fruit matures, these stone cells are absorbed, resulting in a decrease in fruit hardness (Liu et al., 2011). Consequently, this led to a higher rate of infestation by *C. pomonella* and *G. molesta* (Figure 1). The infestation rate of both insects exhibited a negative correlation with fruit hardness. Both borers showed a preference for feeding on host fruits with lower hardness, possibly due to the larva′s ability to penetrate the pericarp (Wright and Samways, 1999). *C. pomonella* demonstrated a significantly higher infestation rate compared to *G. molesta* on both pear species (Figure 1), which could be due to attributed to the distinct mouthpart structure of the two fruit borers (Krenn, 2010). This further validates that fruit hardness is a crucial factor in deterring phytophagous insects.

The phenomenon of induced defense in plants has gained significance due to its ability to produce compounds that combat pathogens and insect herbivores (War et al. 2020). SMs play a crucial role in this induced defense mechanism (Yang et al. 2018).

Our finding indicate that the content of SMs in both *P. bretschneideri* and *P. ussuriensis* exhibited fluctuating changes during fruit ripening, which could potentially be influenced by abiotic factors such as moisture, temperature, and light, etc. (Verma and Shukla 2015). Except for epigallocatechin, all detected SMs were significantly impacted by damages caused by *C. pomonella* and/or *G. molesta*. This suggests that the effects of pests feeding on SMs are complex and vary depending on the specific pest and plant species. The content of quercetin in the host fruits increased significantly after feeding by *C. pomonella*, while there was no significant change when fed upon by *G. molesta* (Figure 2C and 2D). Quercetin accumulation was also observed in tea plants as a response to feeding by *Ectropis grisescens* (Jing et al., 2023). These findings imply that quercetin could potentially function as a crucial plant secondary metabolite involved in the defense of host plants against *C. pomonella*.

Flavonoids have a wide distribution in various plant species and play a significant role in biological processes, particularly in defense against phytophagous insects (Jain et al. 2019). One notable flavonoid, quercetin, has been found to enhance plant tolerance to phytophagous insects and exert effects on insect growth and development (Singh et al. 2021). The presence of quercetin on leaves treated with it has been shown to attract *Spodoptera frugiperda*, leading to increased feeding behavior (Georgina and Sara, 2015). Moreover, quercetin has been observed to impede the growth and development of *Spodoptera litura* larvae at low concentrations and significantly elevates larval mortality at high concentrations (Jadhav et al. 2012). Similarly, our findings indicate that the different concentrations of quercetin treatment

have an impact on the population parameters (r, λ, R_0 and T) of *C. pomonella* and *G. molesta* (Table 1). Specifically, the treatment with 100 μg/g quercetin has distinct effects on the populations of *C. pomonella* and *G. molesta*, suppressing the former while expanding the latter. These results suggest that quercetin is an important secondary metabolite in defending *C. pomonella* in pear fruits, but does not exhibit the same defense against *G. molesta*.

Insect P450 plays a crucial role in the detoxification of exogenous substances, including chemical insecticides and SMs. Its function is to enhance the reactivity and water solubility of toxic substances, thereby reducing the toxicity of phytotoxins and minimizing the harm caused to insects (Li et al. 2023; Rane et al. 2019). Previous studies have demonstrated that activity and gene expression of P450 enzymes, such as *CYP321A1*, *CYP6B6*, and *CYP6B8*, significantly increase when exposed to quercetin in *Helicoverpa armigera* (Chen et al. 2018). Similarly, the CYP6AS subfamily of *Apis mellifera* is capable of metabolizing quercetin found in pollen (Mao et al. 2009). Our findings also indicate that 20 and 21 P450 Clan 3 genes are highly expressed in *C. pomonella* and *G. molesta*, respectively, when subjected to quercetin treatment (Figure 3C & 3D). These results suggest that P450 genes in *C. pomonella* and *G. molesta* may be involved in responding to SMs, specifically quercetin.

The RNAi technology is a widely employed method for the analysis P450 function. The utilization of RNAi to silence specific target genes can enhance the susceptibility of insects to SMs (Zotti et al. 2018). For instance, Wang et al. (2015) observed an increase in the susceptibility of larvae to quercetin when the *S. litura*

CYP9A40 gene was knocked down using RNAi. Furthermore, injection of *dsCYP6AB60* and *dsCYP321A19* into fourth instar larvae of *S. litura* resulted in a decrease in tolerance to quercetin(Wang et al., 2020). In *H. armigera*, flavonoids have been found to induce significant up-regulation of the *CYP6B8* and *CYP321A1* genes (Wen *et al.,* 2009). This study demonstrated that interference with key P450 genes (*CYP6AW1* and *CYP6K1b* of *C. pomonella*; *CYP6B74* and gm_13876 of *G. molesta*) led to a significant decrease in the weight gain of fourth instar larvae when fed artificial diets containing quercetin within 12-48 h (Figure 6C & 6F). This decrease in weight gain indicated the adaptation of both insects to quercetin, as evidenced by a notable decline in relative consumption rate (RCR), relative growth rate (RGR), efficiency of conversion of ingested food (ECI), and efficiency of conversion of digested food (ECD). The involvement of both P450 genes in the detoxification of quercetin in the two fruit borers was established. However, in this study we employed RNAi technology to elucidate the role of specific P450 genes in the insect-mediated metabolism of plant toxins, focusing on *C. pomonella* and *G. molesta*. Given the limited effectiveness and short duration of Lepidopteran RNAi interference, it is essential to employ more intuitive research tools (Vandenhole et al. 2021).

Many studies have shown that SMs can be metabolized by P450s in insects (Mao et al., 2009; Li et al., 2021). For instance, CYP6B8 in *Helicoverpa zea* has the ability to metabolize xanthotoxin, flavone, quercetin, and other phytochemicals (Rupasinghe et al., 2007). *H. zea* also utilizes CYP321A1 to metabolize similar phytochemicals as CYP6B8, indicating that this insect species has developed systems

for detoxifying phytochemicals (Sasabe et al., 2004). However, the ability to detoxify toxic compounds is complex and not solely reliant on a single specialized P450 detoxification enzyme. Multiple up-regulated P450s are often responsible for enhancing the metabolic detoxification of phytochemicals and are implicated in the insect's adaptation to their host plant's defense mechanisms (Alyokhin and Chen, 2017). In this study, we discovered that *G. molesta* has two P450s (CYP6B74, and gm_13876) that metabolize quercetin, whereas *C.pomonella* has only one (CYP6K1b). Among these, CYP6B74 exhibited the highest metabolic capacity, depleting 22.39% ±0.37% of quercetin in 90 min. This may explain why *G. molesta* did not experience significant negative effects from quercetin and instead saw its population grow. Although it possesses CYP6AW1 with limited ability to metabolize quercetin, the presence of CYP6K1b, which exhibits a metabolic rate of 13.72%±0.24%, allows *C. pomonella* to avoid population extinction when exposed to quercetin. Interestingly, the ability of *C.pomonella* to metabolize rutin, as exhibited by the presence of the CYP6K1b, allows it to adapt to this plant secondary metabolite. However, *G. molesta* is unable to overcome the toxicity imposed by rutin, resulting in a decline in its population. The ability of insect populations to overcome toxicity conferred by SMs like quercetin and rutin determines their outbreak potential. The varying adaptations of insects to SMs can also lead to competition among different insect species in the same vicinity (Singh et al. 2021). These results imply that the capability of *C.pomonella* to metabolize both quercetin and rutin may contribute to its global invasiveness. However, it is crucial to recognize that this study only investigated a

few P450 protein metabolisms *in vitro* for restricted range of SMs. Consequently it remains unclear whether there are additional P450 enzymes capable of metabolizing quercetin and rutin, or if these four P450s have the capacity to metabolize other SMs.

5. Conclusions

Plants possess the ability to protect themselves against herbivorous insects through the modification of their physical and chemical characteristics. Research has indicated that the firmness of a fruit plays a significant role in determining the extent of damage inflicted by these insects. In particular, *P. ussuriensis* and *P. bretschneideri* can regulate the concentration of defense substances, thereby influencing the population growth of two fruit borers. In response to the detrimental impacts of SMs, both *C. pomonella* and *G. molesta* have evolved their own detoxification metabolic mechanisms. The existence of varying quantities of P450 genes, which possess the ability to metabolize specific SMs, enables fruit borers to adapt to these compounds present in their host fruits. These finding shed light on the co-evolutionary relationship between plants and herbivorous insects.

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Author Contribution Statement

Shi-pan Zhang wrote the manuscript. Xue-qing Yang conceived and designed research. Bing Bai undertook the revision of the manuscript. Nan-Xia Fu and Xu-Fei Liu provided p450 protein. Yu-Ting Li, Ping Gao, Gao-Man Chen, Ya-Qi Wang, Chao Hu gave some suggestions for this paper. All authors read and approved the manuscript.

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Figure legends

Figure 1. The infestation rate of fruit at different stages caused by *C. pomonella* and *G. molesta*. The fruit hardness changes (red) within the fruit enlargement period (I-IV) and fructescence (V-VI), and the fruit infestation rate (blue) of two fruit borers in *P. ussuriensis* (A) and *P. bretschneideri* (B) were investigated. * represented the difference in the infestation rate of fruit caused by *C. pomonella* and *G. molesta.*

Figure 2. Content of SMs in Nanguo pears and Pingguo Pears. The contents of SMs (chlorogenic acid, epigallocatechin, catechin, quercetin, rutin, gallic acid, isoquercetin) were examined in *P. ussuriensis* (A) and *P. bretschneideri* (B) during fruit enlargement period (I-IV) and fructescence (V-VI) using HPLC. The results are the mean ± SD of three biological replicates. Moreover, SMs were examined in the *P. ussuriensis* (C) and *P. bretschneideri* (D) fruits collected before and after borers damage in stage IV, as μg equivalents per g of fresh weight. Asterisks above represent statistically significant differences analyzed by Student's *t* test (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

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= (peak area of without NADPH- peak area of with NADPH) / peak area of without NADPH*100%.

Table 1 Effects of different concentrations of quercetin on population parameters of *C. pomonella* and *G. molesta.*

Species	Concentrations	r	λ	R_0	T
Cydia pomonella	0 μg/g	0.092±0.005 b	1.096±0.006 b	27.274±4.715 a	35.798±0.353 b
	10 μg/g	0.099±0.006 a	1.104±0.006 a	19.700±3.973 c	35.997±0.152 a
	100 μg/g	0.080±0.005 d	1.092±0.006 d	17.337±3.006 d	35.534±0.210 c
	1000 μg/g	0.088±0.006 c	1.0947±0.005 c	23.256±4.378 b	35.717±0.229 b
	5000 μg/g	0.075±0.005 e	1.083±0.006 e	14.175±2.592 e	35.302±0.242 d
Grapholitha molesta	0 μg/g	0.102±0.008 c	1.107±0.009 c	19.010±4.104 b	28.912±0.283 d
	10 μg/g	0.107±0.008 b	1.113±0.009 b	23.100±4.936 b	29.341±0.303 c
	100 μg/g	0.127±0.013 a	1.136±0.014 a	53.950±24.897 a	31.314±0.688 b
	1000 μg/g	0.099±0.007 c	1.104±0.008 c	21.790±4.768 b	31.01±0.227 b
	5000 μg/g	0.084±0.009 d	1.087±0.010 d	14.420±3.769 c	31.84 ±0.445 a

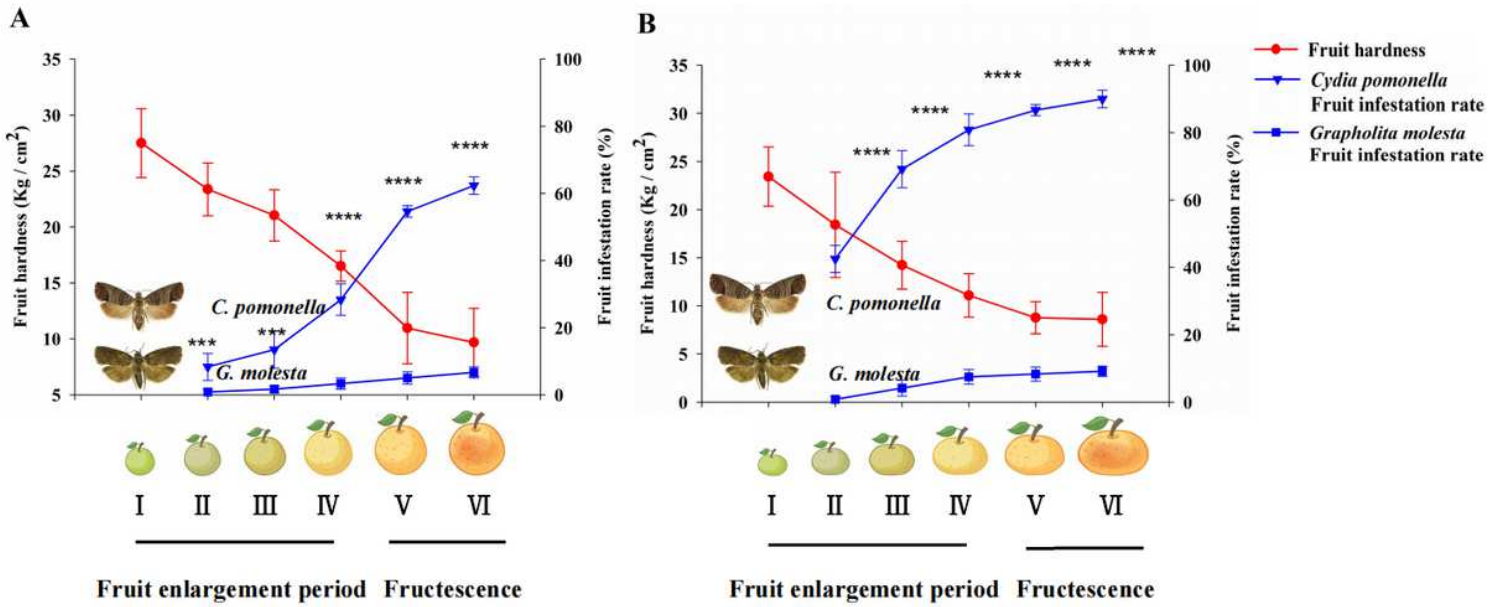
Note: r: Intrinsic rate of increase; λ: Finite rate of increase; R_0: Net reproductive rate; T: Mean generation time; The data in the table are represented as mean ± SE; Different letters indicate significant differences ($P < 0.05$) according to paired bootstrap test.

Table 2 Effect of feeding quercetin on nutrient utilization index of *C. pomonella* and *G. molesta* after knockdown of P450 genes by RNAi.

Species	Processing groups	Relative growth rate (RGR)%	Relative consumption rate (RCR)%	Efficiency of the conversion of ingested food (ECI)%	Efficiency of the conversion of digested food (ECD)%
Cydia pomonella	*dsGFP*	81.98±11.36 a	203.13±31.14 a	44.78±8.50 a	50.44±9.04 a
	dsCYP6K1b	47.73±10.14 b	104.23±13.58 b	37.68±8.17 ab	43.34±8.88 a
	dsCYP6AW1	30.84±8.75 c	88.69±9.97 b	31.52±8.14 b	36.01±9.39 b
Grapholitha molesta	*dsGFP*	88.02±7.73 a	203.73±36.82 a	50.44±5.85 a	64.05±7.59 a
	dsCYP6B74	36.56±5.39 b	151.13±17.97 b	26.08±5.54 b	26.42±2.22 b
	dsgm_13876	32.79±6.43 b	121.19±9.50 c	26.25±5.98 b	28.71±4.91 b

Note: The data in the table are represented as mean ± *SD*; Different letters showed significant differences.

Figures



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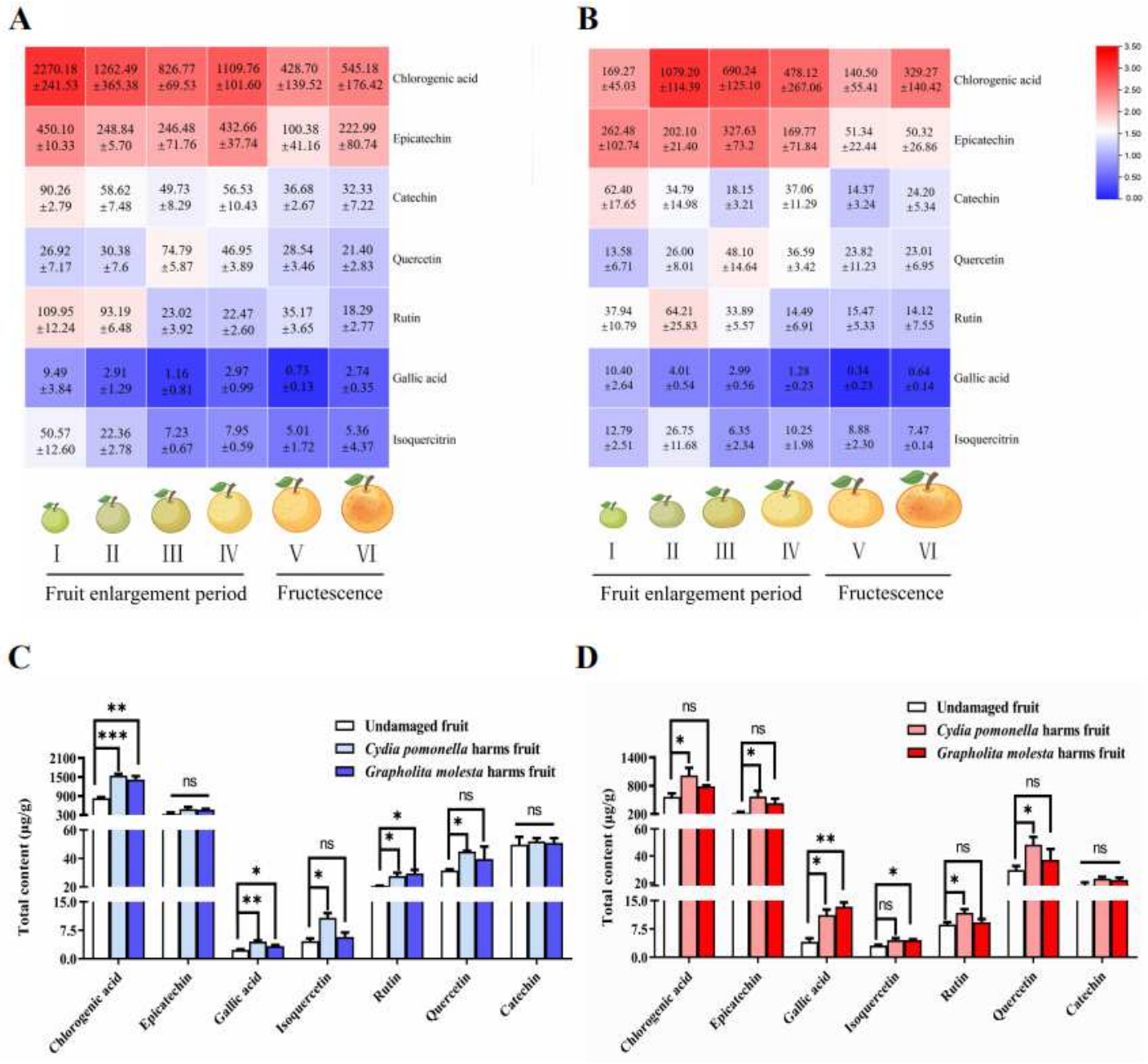

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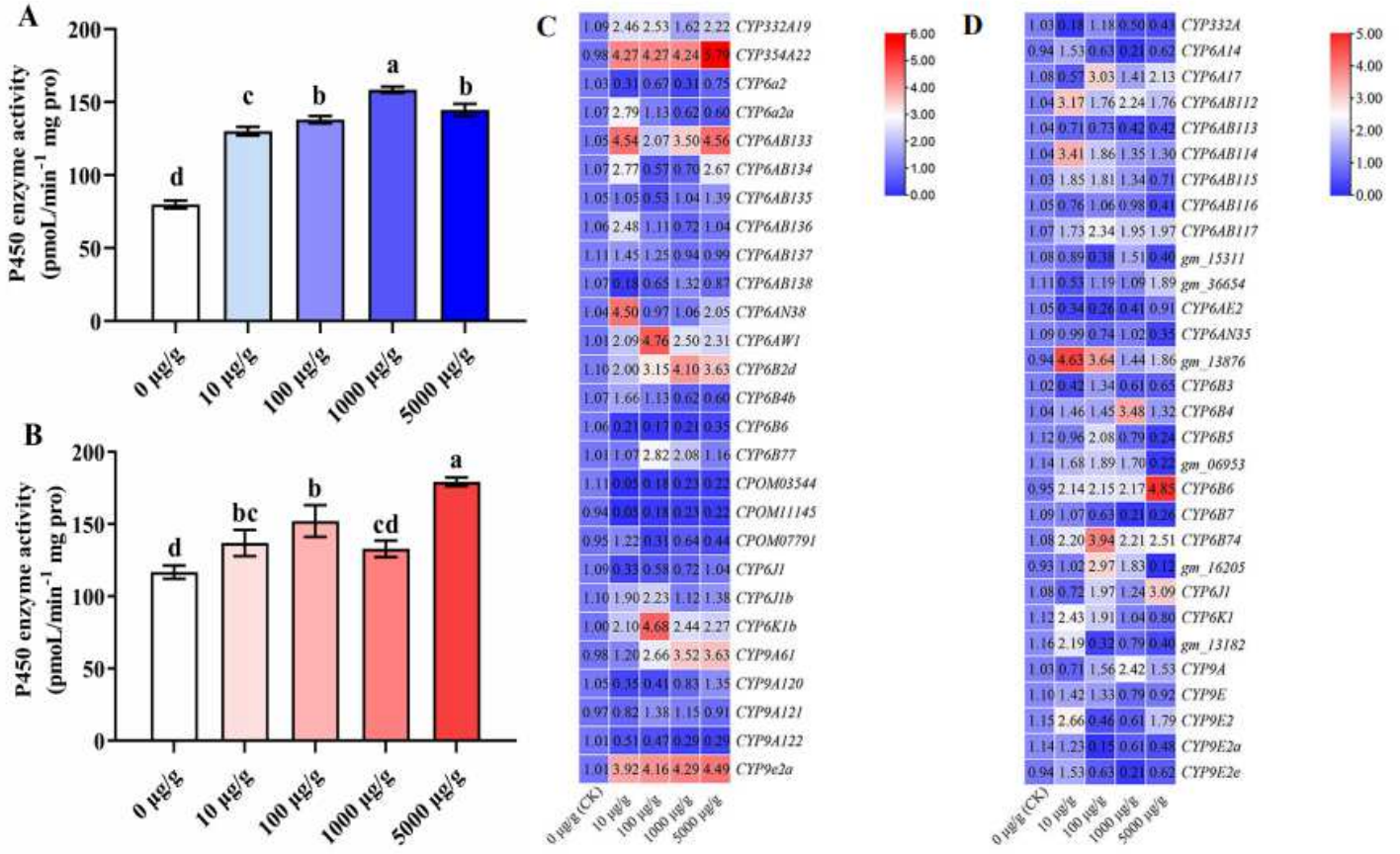

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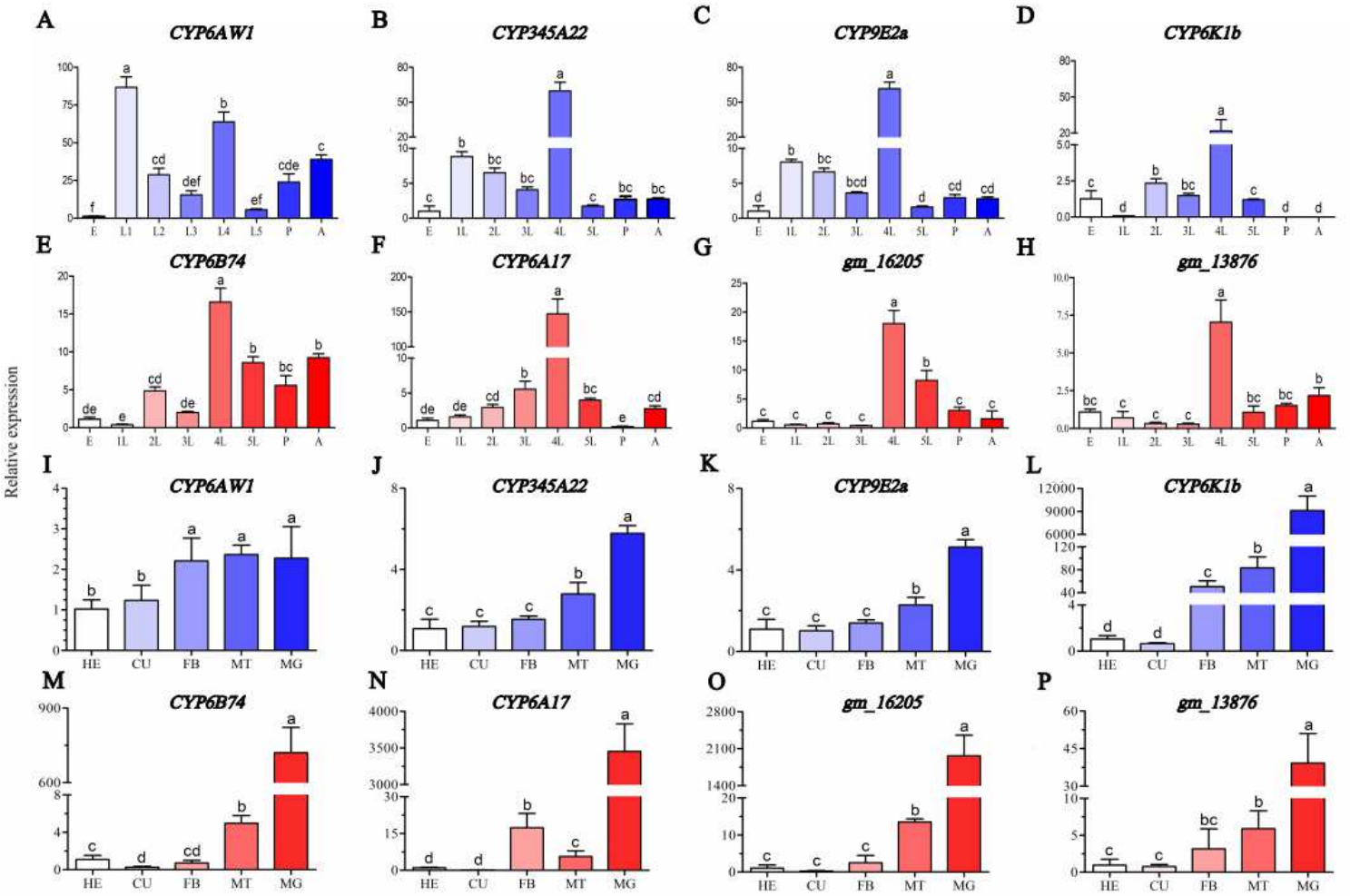

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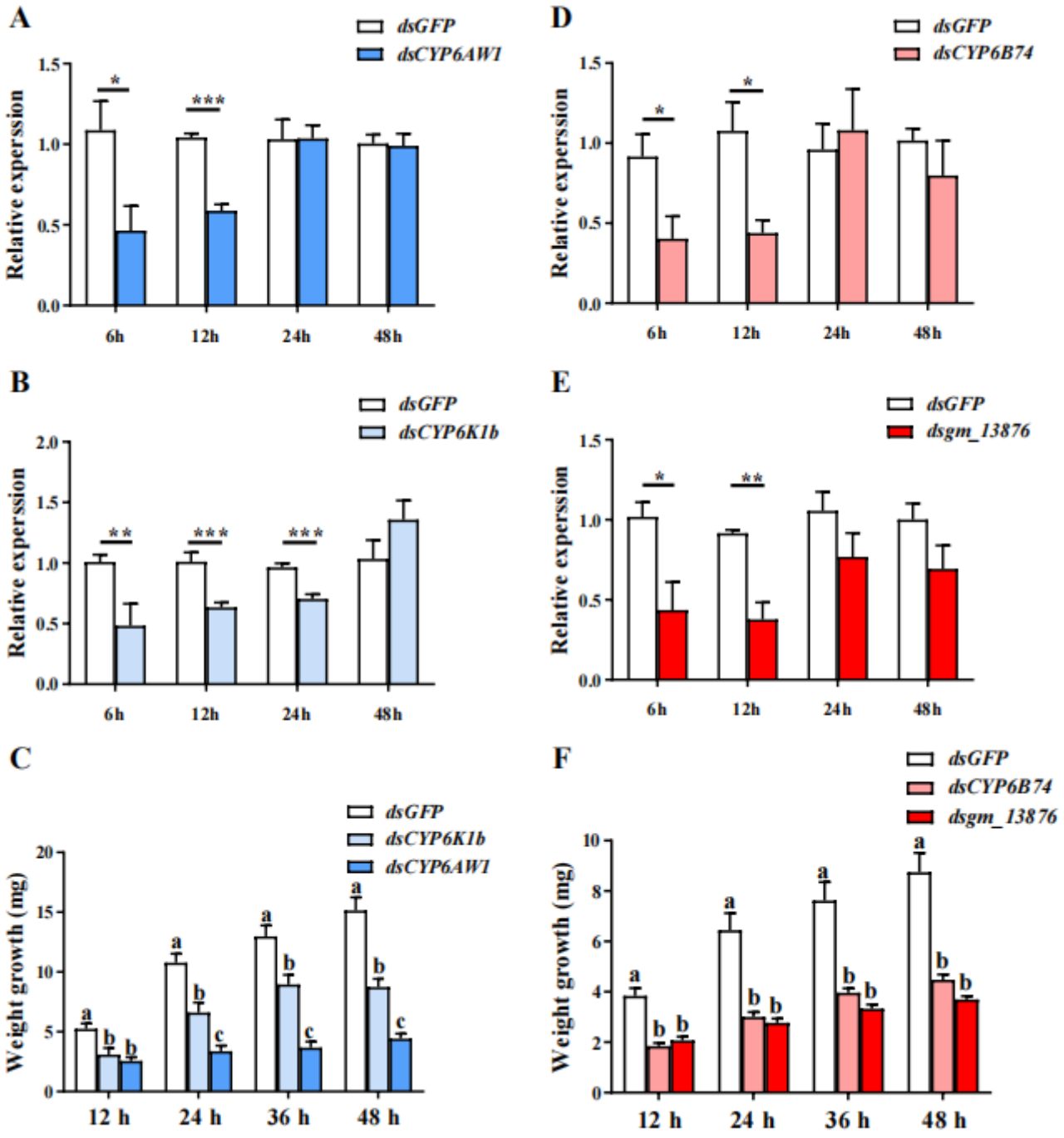

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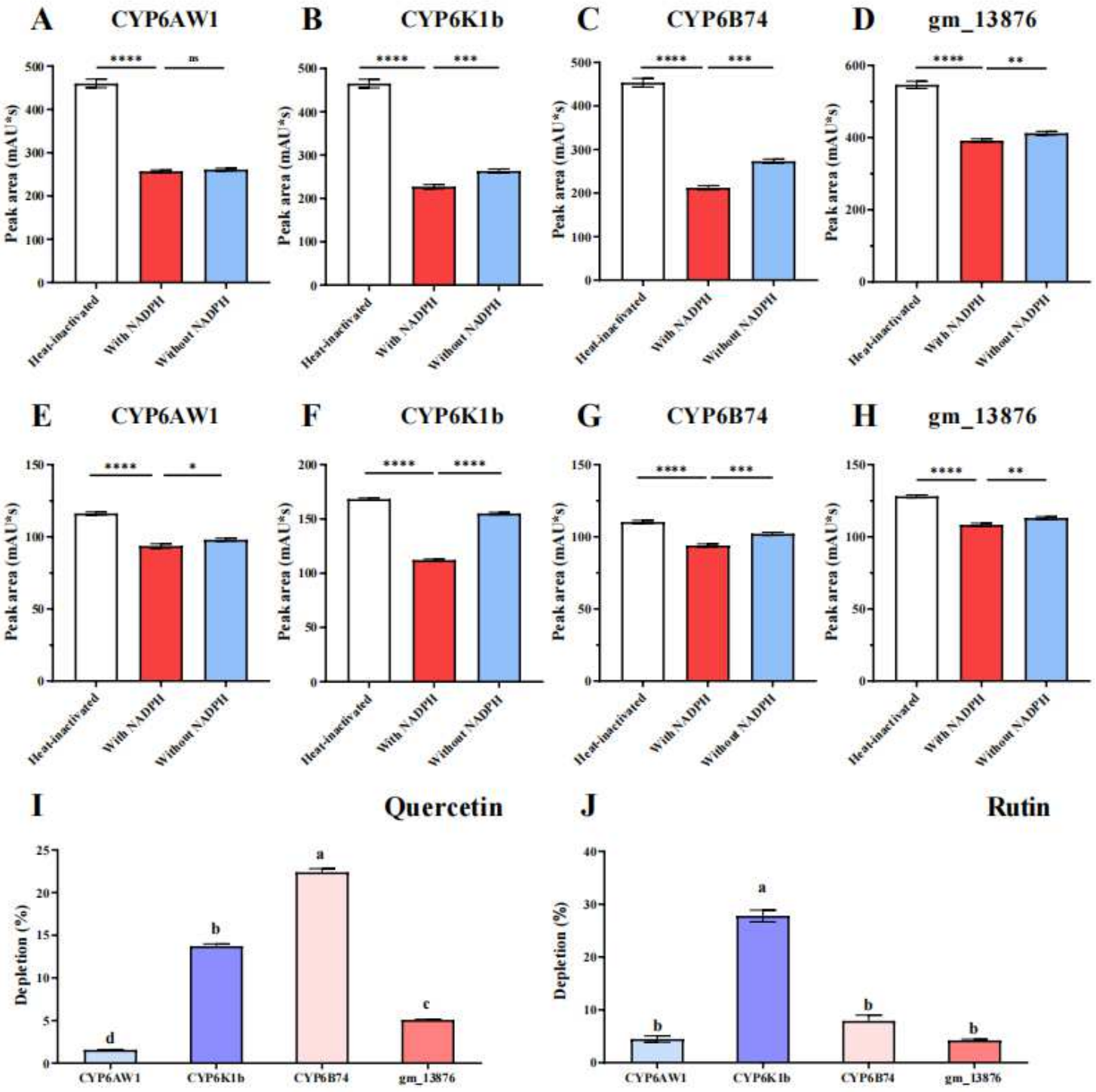

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