Environmental Science and Pollution Research (2023) 30:44400–44414
https://doi.org/10.1007/s11356-023-25518-3

RESEARCH ARTICLE

Mixture toxicity of pyraclostrobine and metiram to the zebrafish (*Danio rerio*) and its potential mechanism

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Received: 11 July 2022 / Accepted: 19 January 2023 / Published online: 24 January 2023

Abstract

The interplay between pesticides plays a critical role in ecotoxicology since these chemicals rarely emerge as single substances but rather in mixtures with other chemicals. In the present work, we purposed to clarify the combined toxic impacts of pyraclostrobine (PYR) and metiram (MET) on the zebrafish by using numerous indicators. Results exhibited that the 4-day LC_{50} value of MET to fish embryos was 0.0025 mg a.i. L^{-1}, which was lower compared with PYR (0.019 mg a.i. L^{-1}). Combinations of PYR and MET presented a synergetic impact on fish embryos. Contents of POD, CYP450, and VTG were drastically increased in the plurality of the single and joint treatments relative to the baseline value. Three genes, including *vtg1*, *crh*, and *il-8*, related to the endocrine and immune systems, were also surprisingly up-regulated when fish were challenged by the individual and mixture pesticides compared with the baseline value. These results afforded valuable information on the latent toxicity mechanisms of co-exposure for PYR and MET in the early growth stage of fish. Moreover, our data also revealed that frequent application of these two pesticides might exert a potentially ecotoxicological hazard on aquatic ecosystems. Collectively, the present study provided valuable guidance for the risk evaluation of chemical combinations.

Keywords Combined toxicity · Aquatic toxicology · Toxic mechanism · Synergistic action

Introduction

Fungicides have been reported as fundamental elements of plant disease management plans for agronomic crops since fungal infections can severely wreck crops (Klittich et al. 2020; Ons et al. 2020). However, these chemicals have the potential to enter aquatic environments through various pathways rendering the accumulation of biologically consequential concentrations (Zubrod et al. 2019). Such accumulation can negatively affect the environment, severely threatening the safety of aquatic organisms (Bhagat et al. 2021). Moreover, pesticide contamination of surface waters is most commonly correlated to a combination of substances rather than an individual chemical because different active ingredients are usually applied simultaneously to one type of crop (Vu et al. 2017). The strobilurin fungicide pyraclostrobine (PYR) and polymeric dithiocarbamic fungicide metiram (MET) are frequently applied in agriculture to reduce the damage from fungal infections all over the world (Piel et al. 2019; Zhang et al. 2020). Moreover, these two compounds are usually used in tank mixtures, resulting in their coexistence in identical environmental specimens due to spray drift or surface transport (Vu et al. 2017). Co-occurrence of PYR and MET (the concentrations for the two pesticides range from 10 to 1000 ng a.i. L^{-1}) has been usually detected in aquatic ecosystems, especially the area surrounding intensive agricultural sites (Xu et al. 2016; Li et al. 2021). A

Responsible Editor: Bruno Nunes

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previous study has demonstrated that a commercial formulation containing PYR and MET possesses the ability to cause DNA damage and cytotoxicity in human cells (Çayır et al. 2016). Because of an increasing trend for pyraclostrobine and metiram concentrations in surface water, the widespread prevalence of PYR and MET mixture-associated ecological risk has garnered much attention.

The contamination of aquatic environments by pesticides may adversely affect non-target organisms, such as fish (El-Nahhal 2018). As a widely used model organism, zebrafish (*Danio rerio*) are often adopted in water quality evaluations since they have a small size, high fecundity, and transparent embryos (Novoa and Figueras 2012). Moreover, zebrafishes of the early life stage are especially sensitive to environmental stimulators, such as pollutants (Abe et al. 2021). Exposure to low levels of pollutants during their early life stage may cause injury to biology and physiology in their later developmental stages (Di Paolo et al. 2015). Therefore, the toxicity evaluation derived from these early life stage experiments of *D. rerio* can highly imply the range of potential biological impacts of toxicant action (Martínez et al. 2020). Taking this into consideration, the consequences of mixture effects of PYR and MET on the early life stage for *D. rerio* are not negligible, demanding an urgent risk assessment of their mixture compounds in fish early life stages.

Although zebrafish have been used in numerous toxicological studies in recent years, most of these investigations have only assessed the impacts of single pollutants (Ma et al. 2019; Sun et al. 2020). Nevertheless, pesticides are rarely found as single chemicals in the natural ecosystem (Covert et al. 2020). On the contrary, they are often found as mixtures (Mansano et al. 2020). Due to the widespread concurrence of PYR and MET in the ecosystem, there is a growing concern about the environmental organisms of their mixture. However, data on the combined toxic impacts of PYR and MET on aquatic organisms and potential mechanisms are not fully elucidated (Zhang et al. 2017; Wu et al. 2018; Mao et al. 2020). In the present study, variations in enzymatic activity and gene expression were examined when *D. rerio* were exposed to the mixture of PYR and MET. The systematic study would offer a scientific basis for the proper application and joint risk assessment of PYR and MET. Meanwhile, the findings also provided essential data for developing water quality standards in an aquatic ecosystem.

Materials and methods

Ethical statement

Our current work complied with the Experimental Animal Management Law of China and was authorized by the Independent Animal Ethics Committee of the Zhejiang Academy of Agricultural Sciences (2020, NO. 032).

Tested organisms and chemicals

Healthy zebrafish were obtained from Hangzhou Minghong Aquarium and used as breeders. The stock breeders were reared in clean well-aerated tanks (200 L) at 27 ± 1 ℃ under a 14–10 h/light–dark cycle. For oviposition, about 60 sexually mature adult fish at a female/male ratio of 1:2 were placed into a spawning box. The light induction triggered oviposition the following day. Embryos at 3 h post-fertilization (hpf) were adopted for the test.

PYR (98% purity) was provided by Jiangsu Henglong Crop Protection Co., Ltd. (Jiangsu, China). MET (90% purity) was purchased from Hebei Xingbai Agrochemical Technology Co., Ltd. (Hebei, China). The stock of each pesticide was dissolved in *N*,*N*-dimethylformamide (DMF) containing 100 g L^{-1} Tween-80 and then kept at 4 °C. Pesticide exposure solutions were prepared by diluting the stock solutions with standard water (ISO 1996). All other chemicals used in this study were of analytical grade.

Single and combined toxicity tests

Embryos at 3 hpf were stochastically picked up and discharged into 24-well plates, and there were one embryo and 2 mL of exposure solution in each well. Standard water (ISO 1996) was used as an untreated (blank untreated) control. The final concentration of DMF and Tween-80 was less than 0.01% (v/v) in all the exposure groups. At least four concentrations with a geometrical ratio that produced 10–90% mortality, according to the preliminary results, were determined for every pesticide. Each concentration was tested in triplicate. During the exposure period, the exposure solution was renewed for half of the day to keep the concentration consistent. Fish were regarded as dead if the embryo stopped developing or the nuclear material became coagulated. Mortality was only evaluated after 4 days of exposure.

The combined toxicity of PYR and MET was assessed using embryonic zebrafish, which was carried out at an equitoxic ratio. To explore the interactive effect of chemical mixture, fish were challenged to serial dilutions of each compound at a constant equitoxic ratio according to the LC_{50} values of a single chemical. Each treatment level was performed three times.

Evaluations at the cellular and gene levels

Specimen preparation

Briefly, 350 embryonic zebrafish at about 3 hpf were placed into a beaker, which was composed of 400 mL substance

solution as a replicate. Three beakers were set up for each test level. According to the acute toxicity results of the embryos, the low, medium, and high concentrations were defined as concentrations of 1/400, 1/100, and 1/25 of 4-day LC_{50} for PYR and MET, respectively. In the combined exposure (COT) experiments, low, middle, and high concentrations of PYR and MET were mixed. Chemical solutions were changed half of the day to maintain adequate concentrations. After 4 days of challenge, treated fish from each exposure level were harvested and rinsed twice using standard water. Harvested fish were kept at − 80 °C before the subsequent cellular and gene level tests.

Evaluations at the cellular level

Approximately 200 zebrafish were homogenized (1:10, w/v) in cold phosphate buffer (PBS, pH 7.4). The tissue lysates were centrifuged at 3,000 × g for 30 min at 4 °C, and the activities of antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ferric reducing antioxidant power (FRAP)] using the supernatant. SOD activity was assessed according to the 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) reduction rate (Lewandowski et al. 2018). CAT activity was reflected using the decreased rate of H_2O_2 (Heck et al. 2010). POD activity was determined by the change of absorbance at 420 nm when catalyzing H_2O_2 (Soffan et al. 2014).

The activities of detoxification enzymes [glutathione-S-transferase (GST), carboxylesterase (CarE), and CYP450] were also examined. Briefly, the weighed specimens were subjected to homogenization in cold PBS (pH 7.4). The tissue lysates were centrifuged at 12,000 × g for 20 min at 4 °C, and supernatants were collected and placed in new tubes. GST activity was assessed using 1-chloro-2,4-dinitrobenzene as the substrate (Güvercin et al. 2008), and CarE activity was evaluated using α-naphthyl acetate as the substrate (de Lima et al. 2013). CYP450 activity was examined by monitoring the fluorescence of 7-hydroxycoumarin at excitation and emission wavelengths of 368 nm and 456 nm, respectively (Lin et al. 2014). The protein concentration was determined using a Bradford method based on a standard curve generated with bovine serum albumin (Bradford 1976).

Enzyme-linked immunosorbent assay was adopted to determine the levels of vitellogenin (VTG) and thyroid hormones (THs), such as triiodothyronine (T3) (Shen et al. 2021). Briefly, the specimens were subjected to homogenization in 300 µL cold PBS (pH 7.4) and then centrifuged at 5000 × g for 20 min at 4 °C. The tissue supernatants were preserved at − 20 °C until analysis. The reported detection limits for T3 and VTG are 0.05 ng a.i. mg^{-1} and 0.05 µg a.i. mg^{-1}, respectively. The protein concentration was employed as an internal reference, and the final levels of THs and VTG were expressed in ng a.i. mg^{-1} and µg a.i. mg^{-1}, respectively.

A colorimetric assay was adopted to determine the caspase 3 activity. About 70 embryos were rinsed with cold PBS two times, homogenized in lysis buffer, and then centrifuged at 3,000 × g for 15 min at 4 °C, followed by the collection of the supernatant (Shen et al. 2021). The caspase-3 activity was reported as the percentage of enzyme activity normalized to the control.

Evaluations at the gene level

About 30 fish from each beaker were harvested and homogenized. Total RNA was extracted from the tissue homogenates employing the TransZol Up Plus RNA Kit (TransGen Biotech Ltd., China), and OD_{260nm}/OD_{280nm} of 1.8–2.0 indicated that the RNA quality was suitable for cDNA synthesis. Next, cDNA synthesis was carried out using the purified RNA with commercial kits (PrimeScript™ RT Master Mix, Takara, Dalian, China). Quantitative real-time PCR amplification was conducted using the SYBR Green reagents (Takara, Dalian, China) on a CFX Real-Time PCR Detection System (Bio-rad, USA). In addition, *β-actin* was selected as a housekeeping gene. Table S1 lists the primer sequences. Briefly, after an initial denaturation step at 95 °C for 30 s, the amplifications were conducted with 39 cycles at a melting temperature of 95 °C for 5 s, an annealing temperature of 60 °C for 30 s, and an extension temperature of 95 °C for 10 s, followed by a melting curve analysis.

The classic $2^{-\Delta\Delta Ct}$ method was adopted to calculate the relative expressions of target genes using the formula as follows (Livak and Schmittgen 2001):

$$\text{Amount of target} = 2^{-\Delta\Delta^{Ct}}$$

where $\Delta\Delta C_t = (C_{t,\ \text{target gene}} - C_{t,\ \text{reference gene}})_{\text{Time } x} - (C_{t,\ \text{target gene}} - C_{t,\ \text{reference gene}})_{\text{Time } 0}$. Time x is any time point, and Time 0 indicates the 1 × expression of the target gene normalized to *β*-actin.

IBR index

The integrated biomarker response (IBR) is an indicator widely used to examine the composite impact of all the measured biomarker responses to the target animal. We used IBR to reflect the toxicity of MYC and THI to zebrafish embryos in the present work in the following steps. Briefly, (1) the mean (m) and SD (s) of each biomarker were obtained for each treatment; (2) the formula $Y = (x\text{-}m)/s$ was used to estimate the standardized data (Y), where x indicates the value of each marker for each treatment; (3) Z value was obtained based on the biological impact, $Z = Y$ (activation effect) or $Z = -Y$ (inhibition effect); (4) the score (S) value

was determined by the formula $S = Z + |\min|$, where |min| indicates the absolute value of the minimum of the standardized data; (5) the IBR index was obtained using the formula: $[(S_1 \times S_2) + (S_2 \times S_3) + \ldots + (S_{n\text{-}1} \times S_n) + (S_n \times S_1)]$; and (6) the IBR was divided by the number of biomarkers, which was termed as IBR/n (Shen et al. 2021).

Chemical analysis

To determine the actual concentrations of PYR, MET, and their mixtures, water samples were detected at the beginning (0 h) and before water renewal (12 h) during the experimental period. The water samples (10 mL) were extracted with acetonitrile (10 mL) by vortexing (5 min), followed by the addition of NaCl (5 g), and then the mixture was vortexed (1 min). Subsequently, 1 mL supernatant was dried with a stream of nitrogen in a test tube after centrifugation (3000 × g, 3 min) and re-dissolved with 1 mL acetonitrile. The samples containing PYR were analyzed using liquid chromatography-tandem mass spectrometry performed (SHIMADZU, LCMS-8050) on an ACE C18 column (2.1 mm × 100 mm, 1.7 μm) with a mobile phase composed of aqueous solution (2 mmol/L ammonium acetate and 0.05% formic acid, A) and methanol (B). The separation was carried out by a gradient elution program as follows: 0–1.0 min, 90% A; 1.0–3.0 min, 50% A; 3.0–6.0 min, 25% A; 6.0–10.0 min, 5% A; 10.0–11.0 min, 90% A. Mass spectrometric detection was conducted in positive electrospray ionization (ESI) with multiple reaction monitoring (MRM) mode. For PYR, the precursor ion was m/z 388.2, the product ions were m/z 194.2 for quantization and m/z 163.2 for confirmation, and the collision energy was 18 and 36 V, respectively. The water sample (5 mL) of MET was supplemented with ascorbic acid (50 mg), n-hexane (5 mL), and hydrochloric acid solution (3% $SnCl_2$, 10 mL) and then sealed into the headspace bottle. The bottle was shaken in a water bath (80 ℃) for 2 h and then cooled at room temperature. The upper n-hexane was analyzed for the determination of CS_2 using a GC (Agilent 7890A-μECD) with a GS-GASPRO column (30 m, 0.32 mm i.d.; Agilent). GC conditions were set as follows: injection port temperature 130 °C (splitless injection), column temperature 100 °C (20 min, invariably), electron capture detector temperature 190 °C, make-up gas (nitrogen) 30 mL/min, and carrier gas (nitrogen) 2.0 mL/min. Analysis results revealed that the deviations between the nominal and actual concentrations of PYR, MET, and their mixtures were less than 20%. Therefore, the nominal concentration was used as the actual concentration in this study.

Statistical analysis

The LC_{50} values of pesticides were assessed as previously reported (Chi 1997). Toxicity was deemed dramatically different if the 95% fiducial limits of two LC_{50} values did not overlap ($P < 0.05$). Combined toxicity was explored using Marking's additive index (AI) method with minor modifications (Marking 1985). Details about the AI method were placed in the Supplemental Information. The SPSS software (SPSS version 18.0, USA) was employed for all statistical comparisons, and the data were presented as mean ± standard deviation (SD). The homogeneity of variance of the data was assessed by Levene's test, and Dunnett's post hoc test was performed to assess significant differences among different groups. A $p < 0.05$ was considered statistically significant. Spearman's test was adopted to evaluate the correlation between measured arguments.

Results

Single and mixture toxicity determinations

Table 1 presents the toxicities of PYR and MET to embryonic fish. Our data showed that the 4-day LC_{50} value of MET was 0.0025 mg a.i. L^{-1}, which was lower relative to PYR (0.019 mg a.i. L^{-1}). By contrast, the 4-day LC_{50} value of PYR and MET to the embryonic fish was 0.0055 and 0.00073 mg a.i. L^{-1} in the combination, respectively, and their mixture displayed a synergistic response with an AI value of 0.72.

Table 1 Individual and mixture toxicities of pyraclostrobine and metiram to the embryos of *Danio rerio*

LC_{50} (95% FL)[a] mg a.i. L^{-1}		LC_{50} (95% FL)[b] mg a.i. L^{-1}		AI value
Pyraclostrobine	Metiram	Pyraclostrobine	Metiram	
0.019 (0.014 ~ 0.025)	0.0025 (0.0019 ~ 0.0034)	0.0055 (0.0041 ~ 0.0078)	0.00073 (0.00056 ~ 0.0010)	0.72

AI additive index

[a]The LC_{50} (95% fiducial limit) for pyraclostrobine or metiram individually

[b]The LC_{50} (95% fiducial limit) for pyraclostrobine or metiram in the mixture

Biochemical index examination

Oxidative stress- and cell apoptosis-related indexes

The content of ROS was remarkably raised in the low and middle levels of the MET group relative to the baseline value ($p = 0.010$ and 0.008, respectively). A significantly higher content of ROS was found in the low concentration of the MET group relative to the corresponding PYR group ($p = 0.033$). Besides, a dramatic up-regulation was also observed in the high concentration of the COT group relative to the corresponding PYR and MET groups ($p = 0.044$ and 0.048, respectively) (Fig. 1A). The MDA content was substantially raised in the middle and high concentrations of the COT group relative to the baseline value ($p = 0.027$ and 0.004, respectively) and the corresponding PYR group ($p = 0.029$ and 0.008, respectively). Furthermore, apparent induction was seen in the high concentration of the COT group relative to the corresponding MET group ($p = 0.027$) (Fig. 1B). The POD activity in all the groups (except for the middle concentration of the MET group and the high concentration of the COT group) was pronouncedly raised relative to the baseline value (the p value ranged from 0.009 to 0.048). In contrast, a substantially lower POD activity was detected in the high concentration of the COT group relative to the corresponding PYR group ($p = 0.046$) (Fig. 1C).

The SOD activity was markedly enhanced when exposed to MET and COT (except for the high concentration of the MET group and the middle concentration of the COT group) relative to the baseline value (the p value ranged from 0.013 to 0.039). Besides, marked enhancement was also discovered in the low concentration of the COT group relative to the corresponding PYR group ($p = 0.016$) (Fig. 1D). The CAT activity was notably enhanced in the middle concentration of the PYR group and the high concentration of the MET group relative to the baseline value ($p = 0.021$ and 0.026, respectively) and COT group ($p = 0.038$ and 0.037, respectively), respectively. Additionally, the CAT activity was also raised in the middle concentration of the COT group relative to the corresponding MET group ($p = 0.014$) (Fig. 1E). The FRAP level was noticeably up-regulated in the low concentration of the MET group and the middle concentration of the COT group relative to the baseline value ($p = 0.029$ and 0.042, respectively). Moreover, a substantial elevation was also disclosed in the high concentration of the COT group relative to the baseline value ($p = 0.043$) and the corresponding PYR group ($p = 0.049$) (Fig. 1F). The caspase-3 activity was substantially raised in the middle concentration of PYR and MET groups relative to the baseline value ($p = 0.049$ and 0.018, respectively) and COT group ($p = 0.027$ and 0.013, respectively), respectively. Besides, a significant increase was also noticed in the low concentration of the MET group and the high concentration of the COT group relative to the baseline value ($p = 0.016$ and 0.035, respectively) (Fig. 1G).

Detoxification enzyme and endocrine system-related indexes

The CarE activity was apparently up-regulated in all the MET and COT groups (except for the high concentration of the MET group and the middle concentration of the COT group) relative to the baseline value (the p value ranged from 0.006 to 0.042). Furthermore, it was substantially raised in the high concentration of the PYR group relative to the baseline value ($p = 0.044$). Nevertheless, its activity was substantially down-regulated in the middle concentration of the COT group relative to the corresponding MET group ($p = 0.041$) (Fig. 2A). The GST activity was pronouncedly elevated in all the PYR groups and the high concentration of the MET group relative to the baseline value (the p value ranged from 0.005 to 0.028). However, a substantial down-regulation was seen in the high concentration of the COT group relative to the corresponding PYR and MET groups ($p = 0.003$ and 0.003, respectively). Its activity was also pronouncedly weakened in the low concentration of the COT group relative to the corresponding PYR group ($p = 0.032$) (Fig. 2B). The CYP450 activity was markedly up-regulated in all the PYR and MET groups (except for the high concentration of the PYR group) relative to the baseline value (the p value ranged from 0.015 to 0.048). Additionally, marked up-regulation was also seen in the low concentration of the COT group relative to the baseline value ($p = 0.010$ and 0.008, respectively), whereas its activity was substantially diminished in the middle concentration of the COT group relative to the corresponding PYR and MET groups ($p = 0.042$ and 0.030, respectively) (Fig. 2C).

The VTG content was notably raised in all the PYR groups relative to the baseline value (the p value ranged from 0.007 to 0.043). Additionally, notable elevation was also disclosed in the high concentration of the MET group relative to the baseline value ($p = 0.049$). Its activity was also notably raised in the low and middle concentrations of the COT group relative to the baseline value ($p = 0.009$ and 0.022, respectively) and the corresponding MET group ($p = 0.003$ and 0.046, respectively) (Fig. 2D). The T3 level was noticeably raised in all the PYR and MET groups (except for the low concentration of the PYR group and the high concentration of the MET group) relative to the baseline value (the p value ranged from 0.015 to 0.046). In addition, a noticeable rise was also detected in the middle concentration of the COT group relative to the baseline value ($p = 0.043$) (Fig. 2E). The T4 level was dramatically induced in all the MET and COT groups (except for the high concentration of the MET group and the middle concentration of the COT group) relative to the baseline value (the p

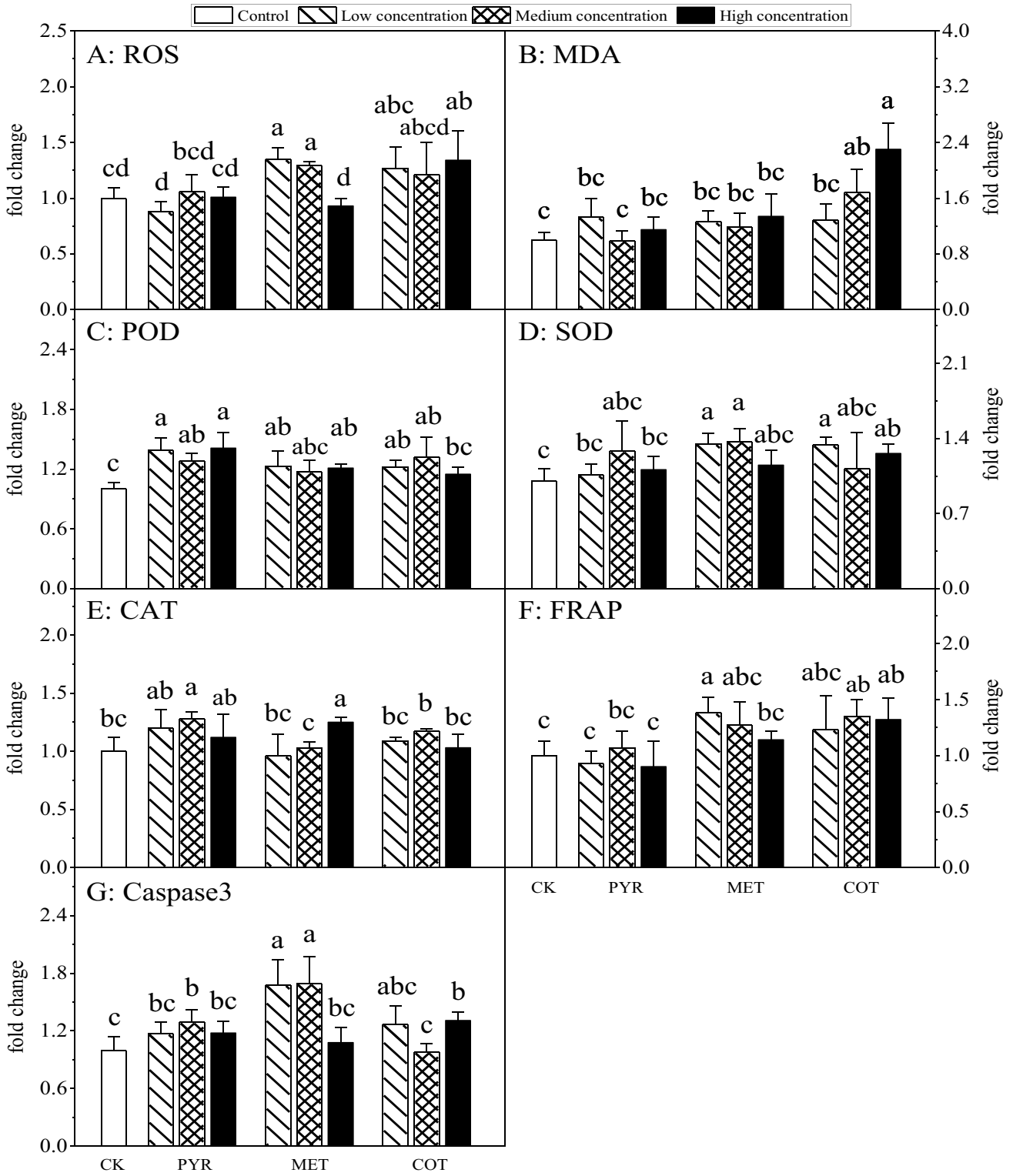

Fig. 1 The changes of oxidative stress- and cell apoptosis-related indexes in fish exposed with PYR, MET, and their combinations. Each bar expresses the mean ± standard deviation of the mean of n = triplicates. Different letters above columns imply significant differences among treatments ($p < 0.05$). Abbreviations: L, low concentration; M, moderate concentration; H, high concentration; PYR, pyraclostrobine; MET, metiram; COT, combined exposure

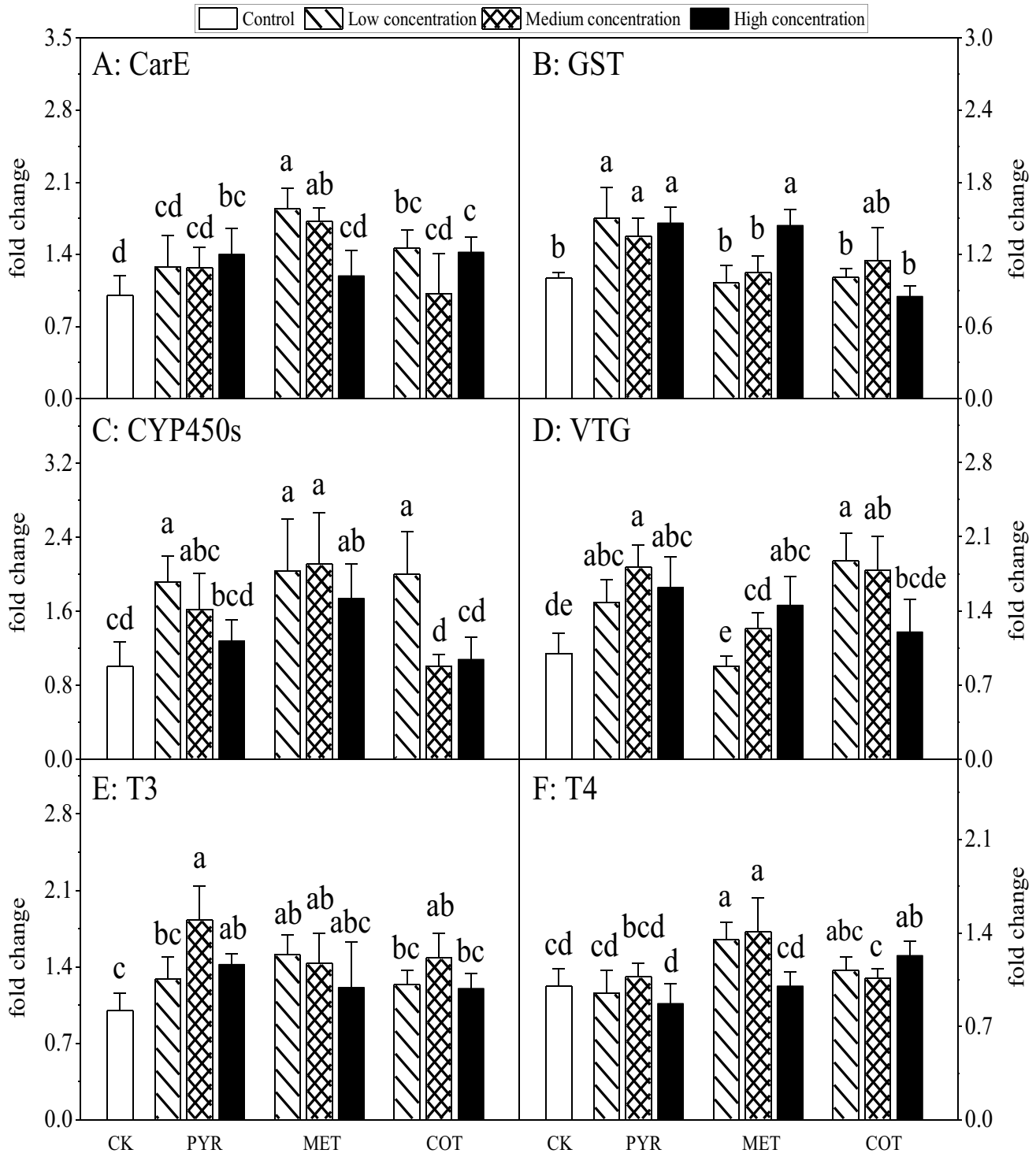

Fig. 2 The changes of detoxification enzymes and endocrine system-related indexes in fish exposed with PYR, MET, and their combinations. Each bar expresses the mean ± standard deviation of the mean of n = triplicates. Different letters above columns imply significant differences among treatments ($p < 0.05$). Abbreviations: L, low concentration; M, moderate concentration; H, high concentration; PYR, pyraclostrobine; MET, metiram; COT, combined exposure

value ranged from 0.032 to 0.048). Besides, a dramatical induction was also disclosed in the high concentration of the COT group relative to the corresponding PYR group ($p = 0.028$). However, its level was remarkably inhibited in the middle concentration of the COT group relative to the corresponding MET group ($p = 0.049$) (Fig. 2F).

Analysis of gene expression

Antioxidation and apoptosis-related gene expressions

The expression of *Mn-sod* at the transcription level was somewhat varied in all the single and COT exposures, except for the high concentration of the MET group, in which the *Mn-sod* expression was obviously increased relative to the baseline value ($p = 0.011$) (Fig. 3A). The expression of *cat* at the transcription level was slightly varied in all the single and COT groups relative to the baseline value (Fig. 3B). Similar to *Mn-sod*, the expression of *ucp* at the transcription level in most single and COT groups only showed slight changes, whereas its activity was distinctly enhanced in the high concentration of the MET group relative to the baseline value ($p = 0.039$) (Fig. 3C). Similar to the expression of *cat*,

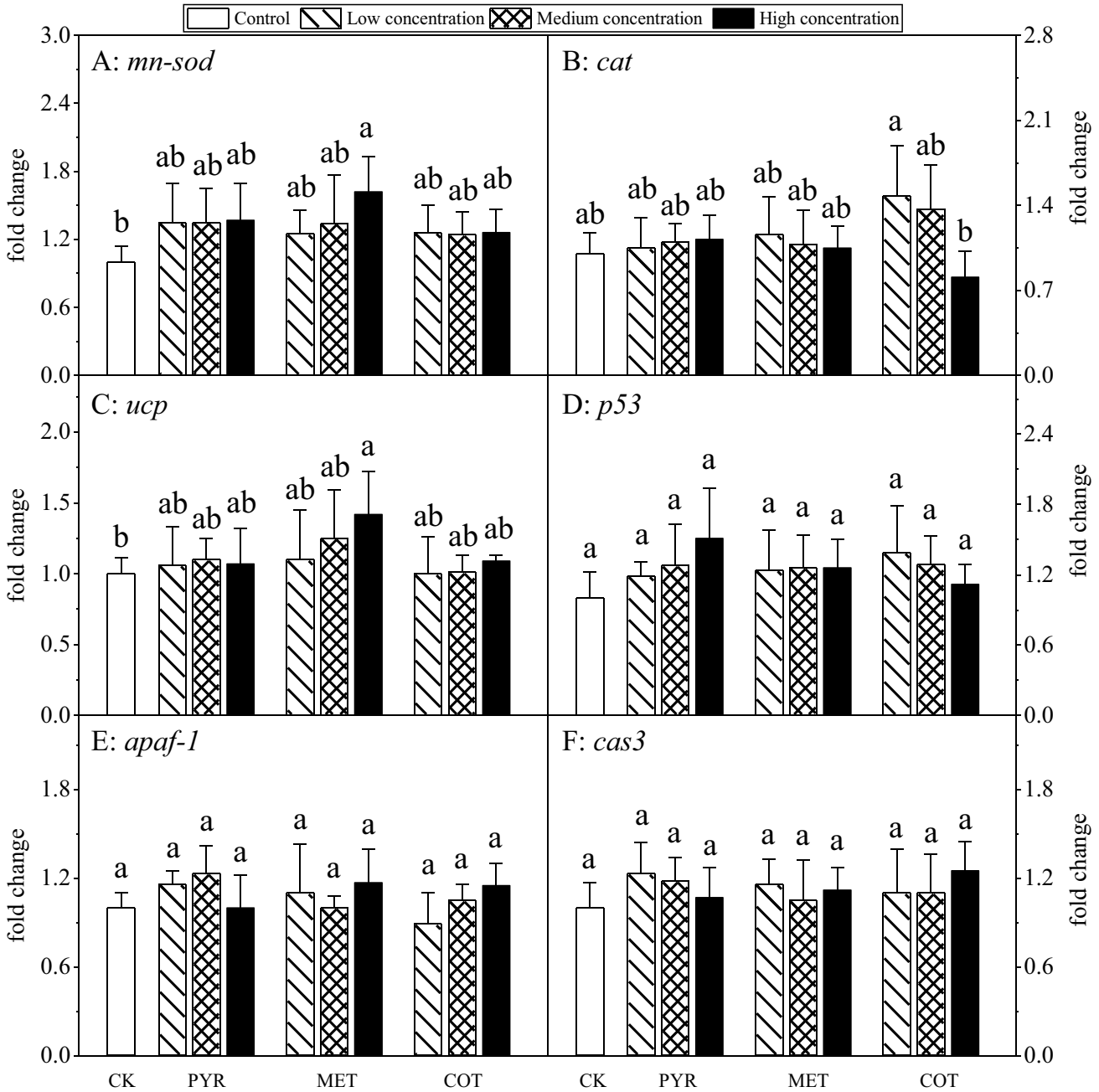

Fig. 3 Influences upon gene expressions of involved in antioxidation and apoptosis-related in fish exposed with PYR, MET, and their combinations. Each bar expresses the mean ± standard deviation of the mean of n = triplicates. Different letters above columns imply significant differences among treatments ($p < 0.05$). Abbreviations: L, low concentration; M, moderate concentration; H, high concentration; PYR, pyraclostrobine; MET, metiram; COT, combined exposure

the expressions of *P53*, *apaf-1*, and *cas3* in all the single and COT groups tended to change slightly relative to their baseline values (Fig. 3D–F).

Endocrine system and immunity-related gene expressions

The expression of *tra* at the transcription level was markedly enhanced in the high concentration of MET and COT groups relative to the baseline value ($p = 0.004$ and 0.002, respectively) (Fig. 4A). The expression of *dio1* at the transcription level was markedly raised in the high concentration of the MET group and the middle concentration of the COT group relative to the baseline value ($p = 0.015$ and 0.019, respectively) (Fig. 4B). The expressions of *tshb* and *cyp19a* at the transcription level were not prominently altered in all the single and COT groups relative to the baseline value. Moreover, no prominent alteration was found in the COT group relative to the baseline value and the corresponding individual groups (Fig. 4C, F). On the other hand, the expression of *UGT1ab* at the transcription level was remarkably elevated in the middle concentration of PYR and MET groups relative to the baseline value ($p = 0.003$ and 0.039, respectively). Besides, remarkable elevation was also found in the high concentration of the MET group relative to the baseline value ($p = 0.008$). Nevertheless, its expression was markedly suppressed in the high concentration of the COT group relative to the corresponding MET group ($p = 0.003$) (Fig. 4D). The expression of *vtg1* at the transcription level was noteworthy induced in all the single and COT groups (except for the high concentration of the PYR group and the low concentration of the COT group) relative to the baseline value (the p value ranged from 0.002 to 0.045) (Fig. 4E).

The expression of *crh* at the transcription level was remarkably elevated in the middle concentration of PYR and MET groups relative to the baseline value ($p = 0.004$ and 0.041, respectively). Furthermore, a remarkable up-regulation was also detected in the high concentration of the COT group relative to the baseline value ($p = 0.004$) and the corresponding PYR group ($p = 0.018$). However, its expression was substantially reduced in the low and middle concentrations of the COT group relative to the corresponding PYR group ($p = 0.021$ and 0.009, respectively) (Fig. 4G). The expression of *il-8* at the transcription level was apparently enhanced in all the PYR and MET groups (except for the low concentration of the PYR group) relative to the baseline value (the p value ranged from 0.001 to 0.043). However, apparent weakening was seen in the middle and high concentrations of the COT group relative to the corresponding MET group ($p = 0.038$ and 0.009, respectively) (Fig. 4H). On the other hand, the expression of *cc-chem* at the transcription level was pronouncedly up-regulated in the middle and high concentrations of the MET group relative to the baseline value ($p = 0.041$ and 0.021, respectively). Besides, a pronounced up-regulation was also monitored in the middle concentration of the COT group relative to the baseline value ($p = 0.021$) (Fig. 4I).

Throughout transcription expression variations

The overall changes in gene expressions were compared using a heatmap assessment. All the single PYR exposures created a separate group. Besides, the low and middle concentrations of MET and COT exposures also created respective independent groups. The sites of the high concentration of MET and COT exposures crossed each other, indicating no distinct regularity (Fig. 5B).

IBR analysis

To identify the difference in toxicity of PYR, MET, and their combinations at various concentrations, we used IBR to integrate all the determined markers. We selected the results of zebrafish embryos exposed to various concentrations of pesticides and calculated the IBR index. The toxicity order of the single and combined pesticides at various concentrations was compared, and the results were shown as bar graphs. Among all the determined indexes, the most sensitive indexes for PYR, MET, and their combinations included apoptosis, immune response, and apoptosis indicators, respectively (Fig. 5A).

Throughout transcription expression variations

The overall changes of gene expressions were compared using a heatmap assessment. All the single PYR exposures created a separate group. Besides, the low and middle doses of MET and COT exposures also created respective independent groups. The sites of the high dose of MET and COT exposures crossed each other, indicating no distinct regularity (Fig. 5B).

Discussion

Acute toxicity assessment offers the primary approximation of the toxic impacts of emerging pollutants, and it can help determine concentration thresholds for the sequent investigation of sublethal effects (Majumder and Kaviraj 2019). A previous study has shown that PYR has a 4-day LC_{50} value between 0.061 and 0.085 mg a.i. L^{-1} (Mao et al. 2020), implying a higher toxic impact on zebrafish embryos relative to our data. Nevertheless, these data were quite similar, and the discrepancy might be attributed to population variations and different laboratories, indicating that PYR had high toxicity to zebrafish embryos. In addition, PYR also has other toxic effects on the zebrafish, including development

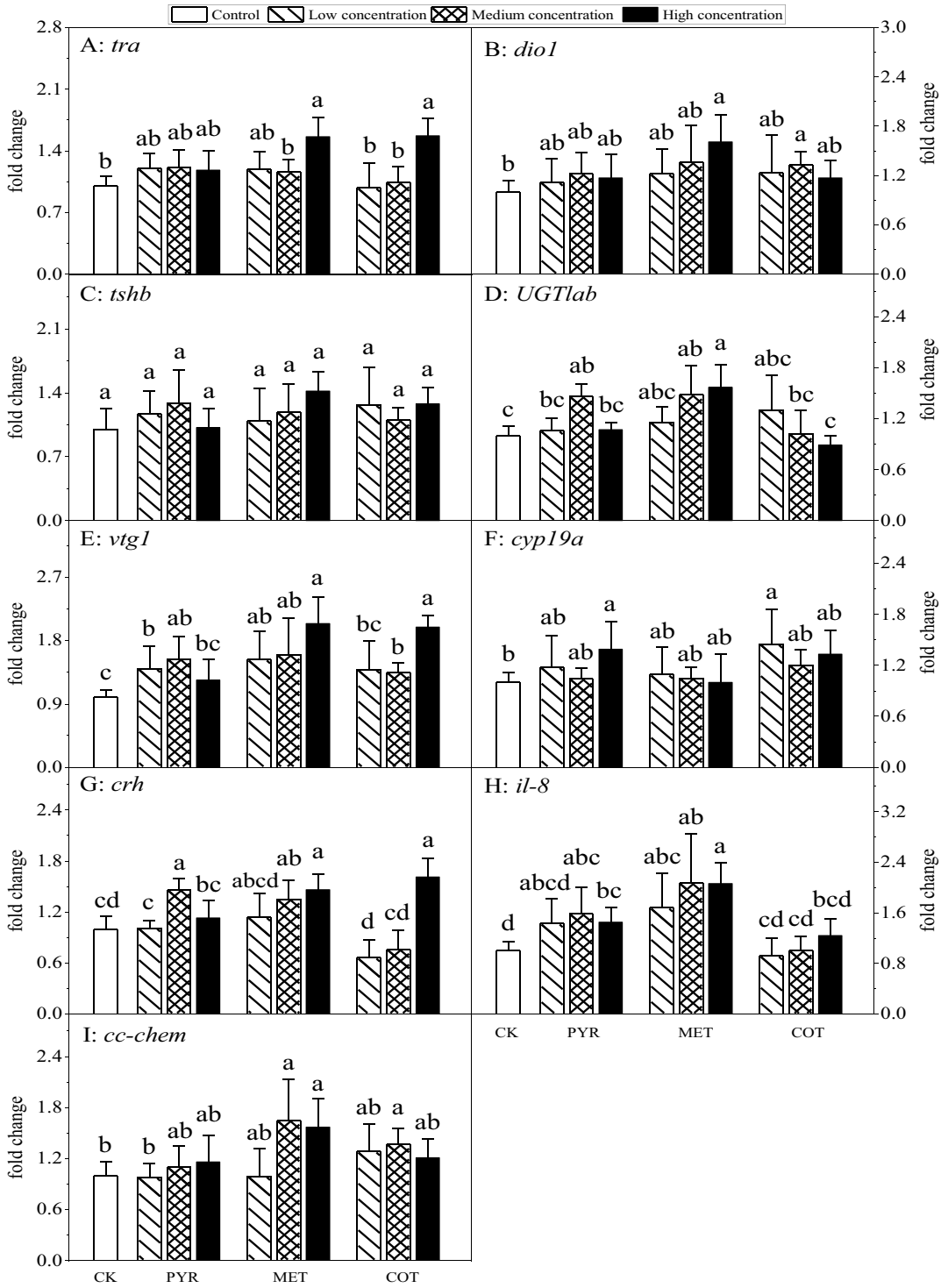

Fig. 4 Influences upon gene expressions of endocrine system and immunity-related in fish exposed with PYR, MET, and their combinations. Each bar expresses the mean ± standard deviation of the mean of n = triplicates. Different letters above columns imply significant differences among treatments ($P < 0.05$). Abbreviations: L, low concentration; M, moderate concentration; H, high concentration; PYR, pyraclostrobine; MET, metiram; COT, combined exposure

toxicity, oxidative stress, and DNA damage (Zhang et al. 2017; Mao et al. 2020). A previous study has exhibited that polyram (active ingredient MET) has low acute toxicity to harlequin fish *Othos dentex* (Serranidae) with a 4-d LC_{50} value of 1000 mg L^{-1} (Tooby et al. 1975). In contrast, the present study implied that MET could significantly affect

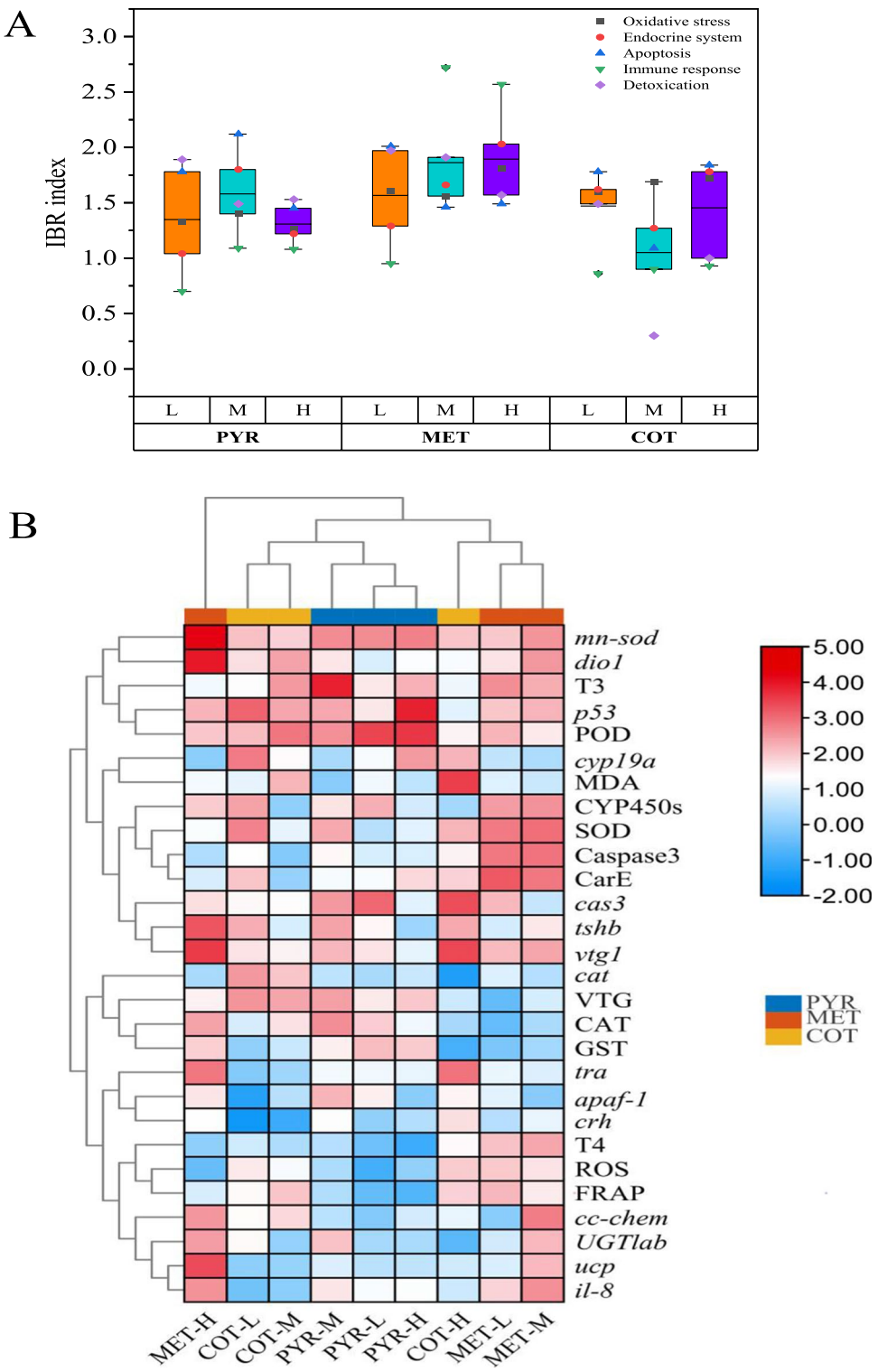

Fig. 5 IBR index and heat map evaluations for analyzing throughout variations of biochemical and molecular parameter. Abbreviations: IBR, Integrated biomarker response; L, low concentration; M, moderate concentration; H, high concentration; PYR, pyraclostrobine; MET, metiram; COT, combined exposure

the zebrafish with a 4-day LC_{50} value of 0.025 mg a.i. L^{-1}. The obvious differences in MET toxicity could be attributed to the different species of tested fish. However, many previous reports on PYR have only determined its single toxic effects on zebrafish, while the combined toxic impacts with other pesticides are unknown (Wu et al. 2018). Therefore, the combination of PYR and MET might exert possible risks to aquatic animals, and we should pay more attention to such hazards.

A comparative assessment of toxicities at different levels can facilitate the comprehensive poisonousness generated by the pollutants to environmental animals (Chagas et al. 2021).

Oxidative stress is of great attention in the investigation of ecotoxicology (Cong et al. 2020). Our findings showed that exposure to MET remarkably increased the reactive oxygen species (ROS) content, indicating that MET caused oxidative stress (Park et al. 2020). It is well known that SOD can catalyze superoxide radicals into hydrogen peroxide (H_2O_2), which is further broken down by CAT or POD into nontoxic H_2O and molecular oxygen (O_2) (Perumal et al. 2021). The distinct increase in SOD activity after exposure to MET and COT can protect the zebrafish against pesticide toxicity by clearing ROS (Cong et al. 2020). However, no distinct change in SOD activity was observed after exposure to PYR compared with the control, indicating that the organisms did not suffer from oxidative damage under the determined concentrations of PYR. In addition, the CAT activity was distinctly induced under the individual treatments, suggesting that the CAT enzyme was required to convert excess H_2O_2 into H_2O, thereby relieving the toxicity from PYR and MET exposures (Li et al. 2020). It was noteworthy that the activity of CAT was distinctly decreased under moderate and high concentrations of COT exposure relative to the corresponding PYR or MET exposure. Such reduction suggested that the oxidative system was destroyed by the combined pesticides, which might be one reason for the synergistic impact produced by the mixture of PYR and MET on the animals (Perumal et al. 2021).

MDA is the end-product of lipid peroxidation and a sensitive diagnostic index of oxidative injury in cells (Li et al. 2020). The drastically enhanced MDA level in the COT treatment showed that the zebrafish embryos were severely injured after exposure to the mixture of PYR and MET (Yan et al. 2015). In contrast, there was no drastic change in MDA level in all the single treatments relative to the baseline value. Such a result suggested that the induction of oxidative stress was not the main route of PYR- and MET-caused inhibition in the embryonic growth of zebrafish (Song et al. 2020). Nonetheless, the probability that PYR and MET produced slight oxidative stresses could not be excluded in our study, but such stresses were overcome by the antioxidant systems of embryos, finally showing no substantial augment of MDA content relative to the baseline value. Hence, the synergistic toxicity of PYR and MET was achieved through the increase of enzymatic activity of oxidative stress. The antioxidative response to COT exposure was more sensitive than that to individual exposures, suggesting the high resistance of *D. rerio* to PYR and MET exposures compared with their mixture.

Examining the expressions of antioxidative stress-related genes will facilitate the assessment of antioxidant capacity (Cong et al. 2020). It was worth mentioning that the expression of *Mn-sod* at the transcription level was only raised in the high concentration of the MET group, while the SOD activity was considerably induced in all the MET and COT groups (except for the high concentration of the MET treatment and the middle concentration of the COT treatment) (Hemalatha et al. 2020). Additionally, the expression of *cat* at the transcription level was somewhat varied in all the single and COT treatments relative to the baseline value, whereas the CAT activity was steeply elevated in the middle concentration of the PYR treatment and the high concentration of the MET treatment. Such unconformity between biochemical and molecular levels of SOD and CAT could be interpreted as follows: (1) the transcription only indicated the antioxidant enzyme activity at a single time point, and there was a delayed influence between transcription and translation; and (2) post-translation might predominantly regulate the enzyme activity (Gaaied et al. 2019).

As a good marker, caspase enzyme activity can monitor stress-produced apoptosis in the early stage of fish (Zhao et al. 2019; Jia et al. 2020). The expressions of *ucp*, *P53*, and *apaf-1* in most single and COT treatments were somewhat changed relative to their baseline values. Therefore, we hypothesized that PYR, MET, and their mixture could generate oxidative stress, which did not cause apoptosis in zebrafish embryos with the change of gene expression, including *ucp*, *P53*, and *apaf-1*. Besides, the present study showed that the single and COT treatments substantially raised the activity of caspase3 relative to the baseline value, implying that a caspase-dependent apoptotic route might be included in PYR-, MET-, and their mixture-produced apoptosis in fish (Félix et al. 2021). However, the expression of *cas3* was hardly varied in all the single and COT treatments, exhibiting that the caspase-3 enzyme was modulated by enzyme activation but not by the transcriptional way.

Xenobiotic metabolism enzyme, including CYP450, plays a fundamental function in the bioconversion of extraneous toxicants (Loerracher and Braunbeck 2020). Therefore, the CYP450 activity was considerably up-regulated in most single and COT exposures, and such influence might be regarded as a toxic mechanism upon exposure to PYR, MET, and their mixtures (Manikandan and Nagini 2018). However, considerable diminution of its activity was monitored in the COT treatment relative to the corresponding PYR and MET treatments, indicating that the CYP450 enzyme had an essential function in the synergistic response of PYR and MET on zebrafish embryos. The GST enzyme is the most critical phase II detoxification enzyme and can catalyze the conjugation of reduced glutathione (GSH) to the compounds (Gaaied et al. 2019). The activity of GST was distinctly increased after exposure to individual exposures, indicating that this enzyme was involved in the detoxification process of PYR and MET in zebrafish (Shen et al. 2021). However, the activity of GST declined under COT exposure compared with the corresponding individual exposures. We concluded that the inhibited enzymatic activity of GST after COT

exposure might be attributed to the synergistic impact of the mixture exposure of PYR and MET. The development of fish partly relies on their immune systems (Aksakal and Ciltas 2019). Our data exhibited that the expressions of *il-8* and *cc-chem* were markedly increased, showing that the immune system was attacked when embryonic fish were exposed to PYR, MET, or their mixtures, which could be a hidden mechanism (Campos-Sánchez and Esteban 2021).

In the present investigation, the transcription of *tra* was surprisingly enhanced in the MET and COT treatments, showing an effective feedback mechanism because of up-regulated T4 levels (Chen et al. 2018; Zhu et al. 2021). Raised *tra* transcription corresponding to elevated levels of T3 and T4 might be owed to that the MET and its combination with PYR bound to TRs and impaired the TR role by perturbing the co-activators mechanisms (Yao et al. 2020). The induction of *dio1* indicated the enhanced conversion of T4 to T3, leading to the accumulation of T4 (Lee et al. 2020; Liang et al. 2020). Alterations of TH proteins and transcriptions in the HPT axis implied that MET and its combination with PYR could produce thyroid disturbance to the embryonic growth of *D. rerio*.

The present study showed that the level of *vtg1* was significantly induced in most of the single and COT exposures, along with the markedly increased level of VTG (Sun et al. 2021). We concluded that exposure to PYR, MET, and their mixtures changed the level of vtg1, which impaired the generation of VTG, resulting in substantial absorption of nutrients (Guo et al 2021). Previous research has shown that cyp450 aromatase (*cyp19a*) functions as a significant indicator of environmental estrogen mimics (Kim et al. 2009). The estrogenic potential was further recognized by the substantial up-regulation of *cyp19a* in the PYR and its combination with MET treatments. Consequently, the provocation of *vtg1* and *cyp19a* elicited the estrogenic potential of PYR, MET, and their combinations in embryonic fish. In our current investigation, the transcription of *crh* gene in the HPA axis was vastly enhanced in most of the single and COT treatments, suggesting such a mechanism of action (Luo et al. 2020).

Exposure to PYR, MET, or their mixtures affected enzymatic activities and their corresponding gene expressions, indicating biochemical and molecular alteration on these pesticide exposures in the fish embryos (Santos et al. 2020; Wei et al. 2020). Our findings afforded helpful information on the mixture toxic mechanism of PYR and MET in water animals. In addition, the variations in enzyme activities reflected the extent of cellular harm (Li et al. 2020). Nonetheless, the sensitivities of various enzymes to pesticides are diverse. To validate our speculation, it is essential to examine the expressions of these enzymes at the protein level (Cong et al. 2020). Overall, a radical evaluation of stress index modes afforded a responsible forecast of harmful influence for the co-exposure of multiple compounds to the water ecosystem.

Conclusions

MET had a greater lethal toxic effect on the embryos of *D. rerio* relative to PYR. Mixtures of PYR and MET displayed a synergetic interplay on embryonic fish. The contents of POD, CYP450, and VTG were drastically changed in plenty of the single and combined treatments. Three genes, containing *vtg1*, *crh*, and *il-8*, related to endocrine-disrupting and immune systems were also surprisingly altered upon COT treatments compared with the corresponding single treatments, indicating that the mixture of PYR and MET exerted diverse toxicities during embryogenesis of *D. rerio*. Overall, the synergistic effects of the two pesticides highlight the importance of incorporating mixture toxicity studies into the ecological risk assessment of pesticides.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-023-25518-3.

Author contribution Yanhua Wang: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing-original draft, writing—review and editing. Zhongwen Gao: conceptualization, methodology, software, formal analysis, investigation, resources, data curation. Chuande Liu: investigation, data curation. Liangang Mao: investigation, data curation, writing—review and editing. Xinju Liu: writing—review and editing, administration, funding acquisition. Jindong Ren: investigation, resources. Zeqi Lu: conceptualization, methodology. Jie Yao: methodology, software, formal analysis. Xuan Liu: writing—review and editing, supervision, project administration, funding acquisition, corresponding author.

Funding The research was supported by the Scientific and Technological Innovation Project of Yantai City, China (Grant No. 2020MSGY053) and State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-products (Grant No. 2021DG700024-KF202106).

Data availability The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate The authors confirm that the national laws regarding animal protection were followed.

Consent to publish Not applicable.

Conflict of interest The authors declare no competing interests.

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