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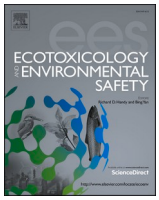

Sublethal effects of chlorantraniliprole on growth, biochemical and molecular parameters in two chironomids, *Chironomus kiiensis* and *Chironomus javanus*

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ABSTRACT

Pesticide residues have serious environmental impacts on rice-based ecosystems. In rice fields, *Chironomus kiiensis* and *Chironomus javanus* provide alternative food sources to predatory natural enemies of rice insect pests, especially when pests are low. Chlorantraniliprole is a substitute for older classes of insecticides and has been used extensively to control rice pests. To determine the ecological risks of chlorantraniliprole in rice fields, we evaluated its toxic effects on certain growth, biochemical and molecular parameters in these two chironomids. The toxicity tests were performed by exposing third-instar larvae to a range of concentrations of chlorantraniliprole. LC_{50} values at 24 h, 48 h, and 10 days showed that chlorantraniliprole was more toxic to *C. javanus* than to *C. kiiensis*. Chlorantraniliprole significantly prolonged the larval growth duration, inhibited pupation and emergence, and decreased egg numbers of *C. kiiensis* and *C. javanus* at sublethal dosages (LC_{10} = 1.50 mg/L and LC_{25} = 3.00 mg/L for *C. kiiensis*; LC_{10} = 0.25 mg/L and LC_{25} = 0.50 mg/L for *C. javanus*). Sublethal exposure to chlorantraniliprole significantly decreased the activity of the detoxification enzymes carboxylesterase (CarE) and glutathione *S*-transferases (GSTs) in both *C. kiiensis* and *C. javanus*. Sublethal exposure to chlorantraniliprole also markedly inhibited the activity of the antioxidant enzyme peroxidase (POD) in *C. kiiensis* and POD and catalase (CAT) in *C. javanus*. Expression levels of 12 genes revealed that detoxification and antioxidant abilities were affected by sublethal exposures to chlorantraniliprole. There were significant changes in the expression levels of seven genes (*CarE6*, *CYP9AU1*, *CYP6FV2*, *GSTo1*, *GSTs1*, *GSTd2*, and *POD*) in *C. kiiensis* and ten genes (*CarE6*, *CYP9AU1*, *CYP6FV2*, *GSTo1*, *GSTs1*, *GSTd2*, *GSTu1*, *GSTu2*, *CAT*, and *POD*) in *C. javanus*. These results provide a comprehensive overview of the differences in chlorantraniliprole toxicity to chironomids, indicating that *C. javanus* is more susceptible and suitable as an indicator for ecological risk assessment in rice ecosystems.

1. Introduction

Pesticide residues can cause serious environmental problems and are important keys to biodiversity loss in aquatic ecosystems (Yadav et al., 2015; Liu et al., 2018; Jiang et al., 2018). Rice fields are one of the most important aquatic micro-ecosystems. To increase rice yields, pesticides are widely and frequently used to control insect pests and weeds (Fang et al., 2015; Song et al., 2019). Previous studies have shown that only 10–15 % of the pesticide applied reaches the target pests (Yang et al., 2021). The efficiency of pesticides is thus low, and large proportions of the pesticides remain in the soil and water, posing a threat to aquatic organisms (Zhang, 2021).

Chlorantraniliprole belongs to the anthranilic diamide class of insecticides and is highly toxic to lepidopteron pests (Li et al., 2021). It acts by binding to the ryanodine receptors, leading to an uncontrolled release of stored calcium causing feeding cessation, paralysis, and death (Ashfaq et al., 2011; Lu et al., 2017a). Recently, chlorantraniliprole has been used as a substitute for older classes of insecticides and is extensively used to control lepidopteron pests of rice, including *Chilo suppressalis* (Lu et al., 2017a; Yang et al., 2021), *Sesamia inferens* (Yang

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et al., 2021), and *Cnaphalocrocis medinalis* (Zhang et al., 2014). Owing to spray drift, soil adsorption, and water movement in rice fields, chlorantraniliprole applications often result in poor contact with rice plants, leaving high concentrations of residues in the rice water (Yang et al., 2021). A previous study found that only 10.27 % of chlorantraniliprole applied for the control of lepidopteron insects stay on plants (Song et al., 2019). In this paper, we evaluate the toxic effects of chlorantraniliprole on non-target species in the rice ecosystems.

Paddy fields have long been recognized as the main habitats for chironomids. The midges *Chironomus kiiensis* and *Chironomus javanus*, are most common in rice fields (Al-Shami et al., 2012). They reduce the eutrophication of the water and serve as alternative food sources for numerous predatory natural enemies especially when pests are low. *C. kiiensis* and *C. javanus* belong to the chironomidae family and possess characteristics such as a short life cycle, an aquatic larval stage, and high sensitivity to aquatic contaminants, which make them ideal bioindicator species (Cao et al., 2013). Previous studies have reported that some pesticides negatively affect these midges at the physiological, biochemical and molecular levels (Ibrahim et al., 1998; Crane et al., 2002; Schuler et al., 2005; Faria et al., 2007; Liu et al., 2021).

Although chlorantraniliprole exhibits remarkable selectivity and is considered to present a low risk for mammals (Lahm et al., 2007; Mandal et al., 2014), it is highly toxic to several soil and aquatic invertebrates. Lavtižar et al. (2015) measured chlorantraniliprole toxicity to non-target soil invertebrates, including *Porcellio scaber*, *Folsomia candida*, *Oppia nitens*, and *Enchytraeus crypticus*; and they found that chlorantraniliprole was a threat to these non-targets and related soil arthropods. It has also been reported that chlorantraniliprole has extremely high toxicity to aquatic invertebrates such as *Centroptilum triangulifer*, *Chimarra aterrima*, *Chironomus riparius*, *Daphnia magna*, *Gammarus pseudolimnaeus*, and *Procambarus clarkii* (Barbee et al., 2010; Lavtižar et al., 2015). Rodrigues et al. (2015) also evaluated the toxicity of chlorantraniliprole on the life history and biochemical indices in *C. riparius*. Maloney et al. (2020) found that 96 h toxicity of chlorantraniliprole to *Chironomus dilutus* was 1.5-fold higher than that of imidacloprid. In addition, Kobashia et al. (2017) compared the toxicity of imidacloprid and dinotefuran to aquatic insect assemblages, including *Crocothemis servilia mariannae*, *Lyriothemis pachygastra*, and *Orthetrum albistylum speciosum* in rice fields. Studies on the potential adverse effects of chlorantraniliprole on midges in rice ecosystems is however limited.

In general, xenobiotic detoxification in insects can be divided into three phases: phase I (solubilization), phase II (conjunction), and phase III (excretion) (David et al., 2013). Among the various detoxification enzyme systems, carboxylesterases (CarEs), multifunctional oxidase (MFO), and glutathione S-transferases (GSTs) in phase I and phase II have been most widely studied owing to their genetic diversity and broad substrate specificity. In addition, previous studies have reported that pesticides induce oxidative stress and genotoxic damage caused by an imbalance between oxidants and antioxidants at the cellular level. Reactive oxygen species (ROS) produced after exposure to pesticides may cause apoptosis (Hong et al., 2020).

Our study aims to determine the ecological risk of chlorantraniliprole in rice fields by evaluating the toxic effects, such as on growth, biochemical and molecular parameters of chlorantraniliprole on *C. kiiensis* and *C. javanus*. Our results can provide key insights into chlorantraniliprole toxicity to chironomids which might serve as a guide for the rational applications.

2. Materials and methods

2.1. Midge rearing

C. kiiensis and *C. javanus* adults were collected using a sweep net from a paddy field at the Agricultural Experiment Station of the Zhejiang Academy of Agricultural Sciences and cultured using standard protocols (USEPA, 2000; Cao et al., 2013). Midges were reared in a glass tank containing acid-washed sand and aerated dechlorinated tap water at 25 ± 1 °C with a 16/8 h light/dark cycle. Midges were fed powdered fish food containing ≥ 45 % protein, ≥ 4% fat, ≤ 5 % fiber, and ≤ 12 % ash (Penison-Made, Xiamen, China). The short-term (24 h and 48 h) toxicity tests and biomarker tests were conducted on third-instar midge larvae; long-term toxicity tests were conducted on newly hatched larvae. All experimental treatments were performed under standard temperature and light conditions.

2.2. Chemicals and reagents

Chlorantraniliprole (95 % TC, technical grade; Shanghai Acmec Biochemical Co., Ltd, China) was kindly provided by Prof. Xiwu Gao of China Agricultural University. The chlorantraniliprole stock solution was prepared in analytical grade dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and stored at 4 °C prior to further dilution.

All biochemical reagents, including ethylene diamine tetra-acetic acid (EDTA), DL-dithiothreitol (DTT), phenylthiourea (PTU), phenylmethanesulfonyl fluoride (PMSF), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.3. Short-term toxicity tests

Short-term toxicity was evaluated using the method described by Liu et al. (2021) with some modifications. The midges were cultured in moderately hard water (RMDW), which was generated by adding $NaHCO_3$ (96 mg/L), $CaCl_2$ (50 mg/L), $CaSO_4$ (50 mg/L), $MgSO_4$ (30 mg/L), and KCl (4 mg/L) to deionized water. RMDW was aerated for > 24 h before use. Chlorantraniliprole solution used was two-fold serially diluted in RMDW to five required concentrations (10, 20, 40, 80, and 160 mg/L for *C. kiiensis* and 2.5, 5, 10, 20, and 40 mg/L for *C. javanus*), which were placed into sterile petri dishes (diameter = 12 cm) and containing 60 mL chlorantraniliprole solution each. RMDW without chlorantraniliprole was used as control. Twenty midge larvae (third instars) were introduced into each dish; there were three biological replicates per treatment, for a total of 60 midge larvae per chlorantraniliprole concentration and 360 midge larvae per toxicity test. Larval mortalities (signified by immobilized larvae) were observed daily and evaluated after 24 h and 48 h. Immobilization was defined as the cessation of all visible signs of movement or activity when viewed under the microscope (Cao et al., 2014). The third-instar larvae were not fed during the short-term toxicity tests.

2.4. Long-term toxicity and sublethal effect assays

Long-term toxicity tests were performed using the method described by Ma et al. (2019) and Liu et al. (2021) with some modifications. One group was used to determine the sublethal concentrations (LC_{10} and LC_{25}) and median lethal concentration (LC_{50}) over a 10 day treatment period. Chlorantraniliprole solution (test solution) was two-fold serially diluted in RMDW to five concentrations (1.25, 2.5, 5, 10, and 20 mg/L for *C. kiiensis* and 0.25, 0.5, 1, 2, and 4 mg/L for *C. javanus*). For each concentration, 300 mL was placed into a 500 mL glass beaker with a layer of quartz sand (0.5 cm thick) covering the bottom. RMDW without chlorantraniliprole was used as control, and three biological replicates were used for each treatment. One hundred newly hatched larvae (< 24 h old) selected at random and were transferred to each beaker. Midge larvae were fed with powdered fish food every 2 days and the solution was replaced after 7 days. Long-term exposure was terminated after 10 days to evaluate the LC_{10}, LC_{25}, and LC_{50}.

After the LC_{10}, LC_{25}, and LC_{50} values were determined, the second group was treated with the LC_{10} and LC_{25} dosage to determine certain growth parameters of *C. kiiensis* and *C. javanus*. Chlorantraniliprole solution was prepared using the method described for the first group. Treated midges were assessed daily to determine the developmental duration, pupation rate, emergence rate, and sex ratio. Because male

and female midges cannot be distinguished before eclosion, newly emerged adults were classified into males and females to determine the sex ratio and then paired for mating and reproduction; egg numbers were then recorded. The egg numbers and hatching rates were determined using an electric stereomicroscope (VHX-950F, Keyence, USA).

2.5. Biochemical assays

Sublethal concentrations of chlorantraniliprole were used to determine the effects on enzyme activity levels in *C. kiiensis* and *C. javanus*. For *C. kiiensis*, the LC_{10} was 1.50 mg/L, and LC_{25} was 3.00 mg/L; for *C. javanus*, the LC_{10} was 0.25 mg/L, and LC_{25} was 0.50 mg/L. Newly hatched midge larvae were treated with each of these sublethal chlorantraniliprole concentrations for 10 days (reared under the conditions of 25 ± 1 °C and L: D = 16: 8 h) and then collected and flash-frozen in liquid nitrogen. Each treatment contained three replicates with 10 larvae per replicate. Samples were manually homogenized in 1 mL of cold extracting solution (0.1 M; pH 7.6; containing 1 mM EDTA, 1 mM DTT, 1 mM PTU, 1 mM PMSF and 20 % glycerol) and then centrifuged at 4 °C and 10000 × *g* (Thermo Scientific Legend micro 17 R) for 15 min. The supernatants were collected and used to measure enzyme activities (Lu et al., 2017a, 2017b) with assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Protein concentrations were analyzed using BSA as the standard as described by Bradford (1976). All samples were measured with a SpectraMax 190 microplate reader (Molecular Devices, USA) with three technical replicates. Specific activity of carboxylesterases (CarE), mixed function oxidases (MFO), glutathione *S*-transferase (GSTs), catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) were calculated in nmol/min/mg protein.

2.6. Gene expression analysis

For gene expression analysis, samples were collected as described for enzyme activity measurements. Larvae were treated for 10 days with sublethal concentrations of chlorantraniliprole. Total RNA was isolated from these samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was quantified on a NanoDrop 2000 and then treated with DNase I (ThermoFisher Scientific, Waltham, MA, USA). First-strand cDNA templates were synthesized using a First-Strand cDNA Synthesis Kit (ThermoFisher Scientific). Reverse transcription quantitative PCR (RT-qPCR) was performed on a Bio-Rad CFX96 System using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) (Lu et al., 2017b). There were three technical replicates per biological replicate. Relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using ribosomal protein S3 (*RPS3*) as the internal reference gene. Primers for RT-qPCR are listed in Table 1. We measured the expression of genes related to detoxification metabolism (*CarE*, the mixed-function oxidase genes *P450*, and *GSTs*) and antioxidant and stress response related genes (*CAT*, *SOD*, and *POD*).

2.7. Statistical analysis

Bioassay data were analyzed, and LC_{10}, LC_{25}, and LC_{50} values were calculated using PoloPlus software (LeOra software, 1997). Predicted mortality and the chi square value (χ^2) for each assay were estimated using Probit analysis (Finney, 1971). All the other data were analyzed using SPSS v 16.0 (SPSS Inc., Chicago, IL, USA). Results were calculated as the mean ± standard error (SE, $n = 3$). Significant differences between the control and treatment groups were evaluated with one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test ($p < 0.05$).

3. Results

3.1. Short-term toxicity of chlorantraniliprole to Chironomus kiiensis and C. javanus

We estimated the LC_{10}, LC_{25}, and LC_{50} of chlorantraniliprole at different endpoints for each chironomid species (Table 2). The results of the short-term toxicity tests showed that chlorantraniliprole was 10.23- and 4.98-fold more toxic to *C. javanus* than to *C. kiiensis* at the 24 h and 48 h endpoints, respectively. The LC_{50} values were 11.39 and 7.83 mg/L (at 24 h and 48 h, respectively) for *C. javanus* and 116.51 and 39.00 mg/L (at 24 h and 48 h, respectively) for *C. kiiensis*. At 24 h, the sublethal

Table 1
All the primers used in this study.

Gene name (Abbreviation)	Unigene number	Primer sequences (5′–3′)[a]	Product length (bp)	Amplification efficiency (%)	R^2[b]
Carboxylesterase-6 (*CarE6*)	Cluster-9703.51160	F: GTCAATCAGCTGGTTCAGCA R: ATGCCAATCCAACGTTCTTC	285	103.5	0.991
cytochrome P450 4DG2 (*CYP4DG2*)	Cluster-9703.58987	F: AAAAGAAGCGTGCAGCAAT R: TGTGCTTCTGGATGATGAGC	173	96.7	0.984
cytochrome P450 9AU1 (*CYP9AU1*)	Cluster-9703.59786	F: GCAGTTCTTGGCTTCAAAGG R: CTGCTCACGTTCCTTCATCA	222	107.2	0.995
cytochrome P450 6FV2 (*CYP6FV2*)	Cluster-9703.57633	F: GGGATGCAAAGAGATCCAGA R: GCTGCAACAAGACCCAATTT	185	98.1	0.980
glutathione S-transferase omega-1 (*GSTo1*)	Cluster-9703.45715	F: CGAAATTTTTCGGAGGATCA R: TCCTGCAAGTCTCGATGATG	230	95.4	0.992
glutathione S-transferase sigma-1 (*GSTs1*)	Cluster-9703.58025	F: TGCATTCCCATCTGTCGTTA R: TTTCCACCATTTTCCTTTGC	236	97.6	0.994
glutathione S-transferase delta-2 (*GSTd2*)	Cluster-9703.16861	F: GCACCATGTCGTGCTGTATT R: GCGTTGCCATGTCAAAGTAA	283	102.7	0.987
glutathione S-transferase unclassified-1 (*GSTu1*)	Cluster-9703.24508	F: AAAATTGGGTGCACTTGAGC R: TGATTCCCTTCGATTTCAGG	245	105.4	0.985
glutathione S-transferase unclassified-2 (*GSTu2*)	Cluster-9703.16155	F: AACCCACTGCATCAAGTTCC R: CGAGATGCTGATGGGATTTT	242	109.2	0.997
Catalase gene (*CAT*)	Cluster-9703.39682	F: TCCCAAGTTTCATCCACACA R: TTTGAATTCCTTGGGCAGTC	249	104.9	0.983
Manganese superoxide dismutase gene (*SOD*)	Cluster-9703.45380	F: CTGATGCACTCCAAAAAGCA R: ATGGAACTAAGCCGGTTGTG	195	106.3	0.986
Peroxidase gene (*POD*)	Cluster-9703.41197	F: CCAGCAGCTGATGTTGAGAA R: CAATTGCGGTCGTGTAAATG	219	98.6	0.992
Ribosomal protein S3 (*RPS3*)	Cluster-9703.39643	F: GCTGATTTGCAGAACGACAA R: GAGGTTTGGGCATAGCATGT	272	97.3	0.993

[a] F and R refer to forward and reverse primers, respectively.
[b] R^2 refers to the coefficient of determination.

Table 2
Toxicity tests of chlorantraniliprole against the third instar larvae of *C. kiiensis* and *C. javanus*.

Insects	Endpoint	LC_{10} (95 %CL, mg/L)	LC_{25} (95 %CL, mg/L)	LC_{50} (95 %CL, mg/L)	Slope ± SE	χ^2 (DF)
C. kiiensis	24 h mortality	34.94 (21.47–47.63)	61.81 (44.82–78.50)	116.51 (92.63–149.91)	2.45 ± 0.35	0.59 (3)
	48 h mortality	7.41 (3.15–11.95)	16.27 (9.50–22.79)	39.00 (28.75–52.66)	1.78 ± 0.29	0.75 (3)
	10 d mortality	1.66 (1.20–2.12)	2.98 (2.38–3.57)	5.69 (4.84–6.73)	2.40 ± 0.24	1.15 (3)
C. javanus	24 h mortality	3.17 (1.56–4.77)	5.81 (3.59–7.86)	11.39 (8.53–14.49)	2.31 ± 0.35	1.17 (3)
	48 h mortality	2.40 (1.40–3.37)	4.20 (2.90–5.43)	7.83 (6.15–9.82)	2.50 ± 0.35	0.13 (3)
	10 d mortality	0.26 (0.17–0.35)	0.54 (0.41–0.68)	1.24 (1.02–1.52)	1.89 ± 0.21	1.51 (3)

LC_{10} and LC_{25} values were 34.94 and 61.81 mg/L, respectively, for *C. kiiensis* and 3.17 and 5.81 mg/L, respectively, for *C. javanus*. At 48 h, the sublethal LC_{10} and LC_{25} values were 7.41 and 16.27 mg/L, respectively, for *C. kiiensis* and 2.40 and 4.20 mg/L, respectively, for *C. javanus*.

3.2. Long-term toxicity of chlorantraniliprole in Chironomus kiiensis and C. javanus

In the long-term toxicity tests, the mortality rates of *C. kiiensis* and *C. javanus* were < 10 % in the control samples. The survival rates for both chironomid species decreased significantly with increasing chlorantraniliprole concentrations (Fig. 1). The 10-day LC_{50} values of

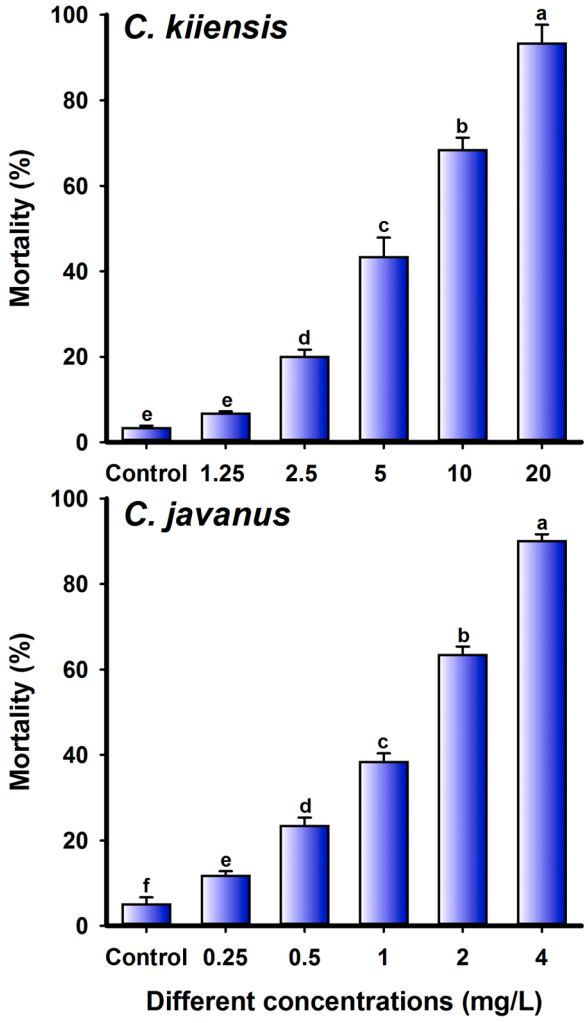

Fig. 1. The mortality of third instar larvae of *C. kiiensis* and *C. javanus* after the newly hatched larvae 10 days exposure to different concentrations chlorantraniliprole. Each bar represents the mean ± SE of three replicates. Different lowercase letters above the SE bars indicated significant differences among the different treatments based on the one-way ANOVA followed by Tukey's HSD tests ($P < 0.05$).

C. kiiensis and *C. javanus* were 5.69 mg/L (range = 4.84–6.73 mg/L) and 1.24 mg/L (range = 1.02–1.52 mg/L), respectively (Table 2). The sublethal 10-day LC_{10} and LC_{25} values were 1.66 and 2.98 mg/L, respectively, for *C. kiiensis*, and 0.26 and 0.54 mg/L, respectively, for *C. javanus* (Table 2).

3.3. Sublethal toxicity effects of chlorantraniliprole on Chironomus kiiensis and C. javanus growth

The developmental durations of newly hatched larvae to adults in control samples were 18.2 and 16.5 days for *C. kiiensis* and *C. javanus*, respectively. When exposed to LC_{10} and LC_{25} concentrations, the developmental durations significantly increased, suggesting that sublethal concentrations prolonged the development in both *C. kiiensis* and *C. javanus* larvae (Fig. 2). Pupation rates of *C. kiiensis* and *C. javanus* were 89.2 % and 87.7 %, respectively, in the control groups and significantly lower for *C. kiiensis* at the LC_{25} dosage and *C. javanus* at both the LC_{10} and LC_{25} dosages (Fig. 2). Similarly, emergence rates of *C. kiiensis* and *C. javanus* were 95.4 % and 93.8 %, respectively, in the control groups and significantly lower in *C. kiiensis* at the LC_{25} dosage and *C. javanus* at both the LC_{10} and LC_{25} dosages (Fig. 2).

3.4. Sublethal toxicity of chlorantraniliprole on the sex ratios, egg numbers and hatching rates of Chironomus kiiensis and C. javanus

For both *C. kiiensis* and *C. javanus*, there were no significant differences in the sex ratios in all dosages (Fig. 3). The mean egg numbers of *C. kiiensis* and *C. javanus* were 441.6 ± 33.7 and 382.8 ± 30.6 per pair, respectively, in the control groups were significantly lower than the observed in *C. kiiensis* at both the LC_{10} and LC_{25} dosages and *C. javanus* at the LC_{25} dosage (Fig. 3). Hatching rates were also markedly lower at both the LC_{10} and LC_{25} dosages in *C. kiiensis* and *C. javanus* (Fig. 3).

3.5. Sublethal toxicity of chlorantraniliprole on detoxification and antioxidant enzyme activity

The activities of detoxification enzyme (CarE and GSTs) were significantly lower in the 10-day LC_{10} and LC_{25} treatment groups than in the control groups in both *C. kiiensis* and *C. javanus* (Fig. 4). Furthermore, the activity of the antioxidant enzyme POD was lower in *C. kiiensis*, and the activities of both CAT and POD were markedly lower in the 10-day LC_{10} and LC_{25} treatment groups in *C. javanus* (Fig. 4).

3.6. Sublethal toxicity of chlorantraniliprole on expression of detoxification and antioxidant enzyme genes

The expression levels of *CarE6*, *GSTo1*, *GSTs1*, *GSTd2*, and *POD* were significantly reduced in *C. kiiensis* exposed to sublethal LC_{10} and LC_{25} dosages compared to the control. However, expression levels of the P450 genes *CYP9AU1* and *CYP6FV2* were significantly increased in *C. kiiensis* treated with the LC_{25} and LC_{10} dosages, respectively (Fig. 5). *CarE6*, *GSTo1*, *GSTs1*, *GSTd2*, *GSTu1*, *CAT*, and *POD* expression levels were significantly inhibited by both the LC_{10} and LC_{25} dosages in *C. javanus*. Furthermore, in *C. javanus*, the expression levels of *CYP9AU1* and *GSTu2* were significantly decreased in the LC_{25} treatment group compared to

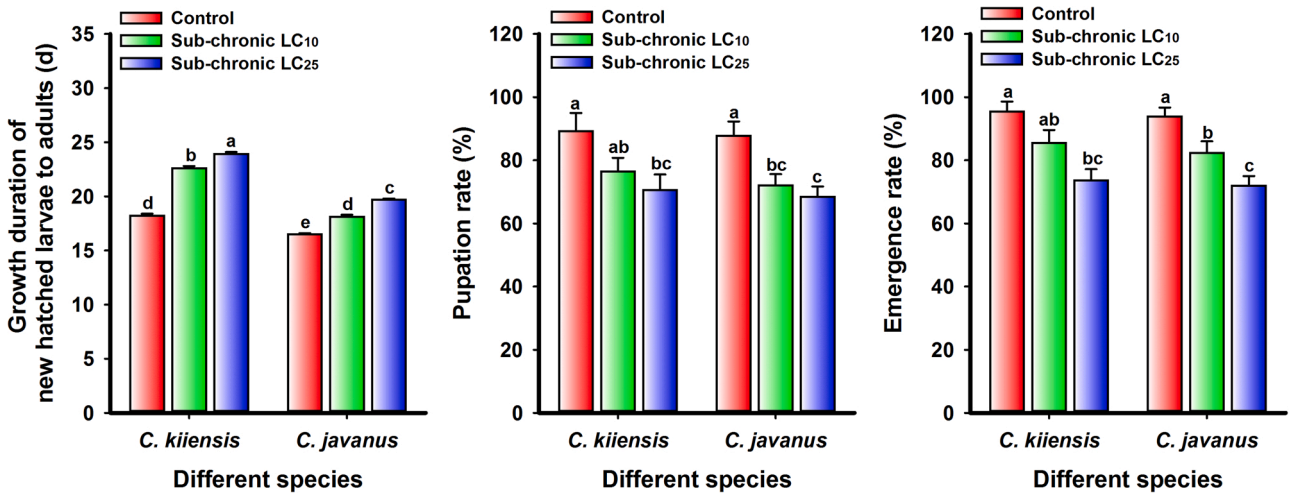

Fig. 2. Sublethal effects of chlorantraniliprole on the growth duration of newly hatched larvae to adults, pupation rate and emergence rate of *C. kiiensis* and *C. javanus*. Each bar represents the mean ± SE of three replicates. Different lowercase letters above the SE bars indicated significant differences among the different treatments based on the one-way ANOVA followed by Tukey's HSD tests ($P < 0.05$).

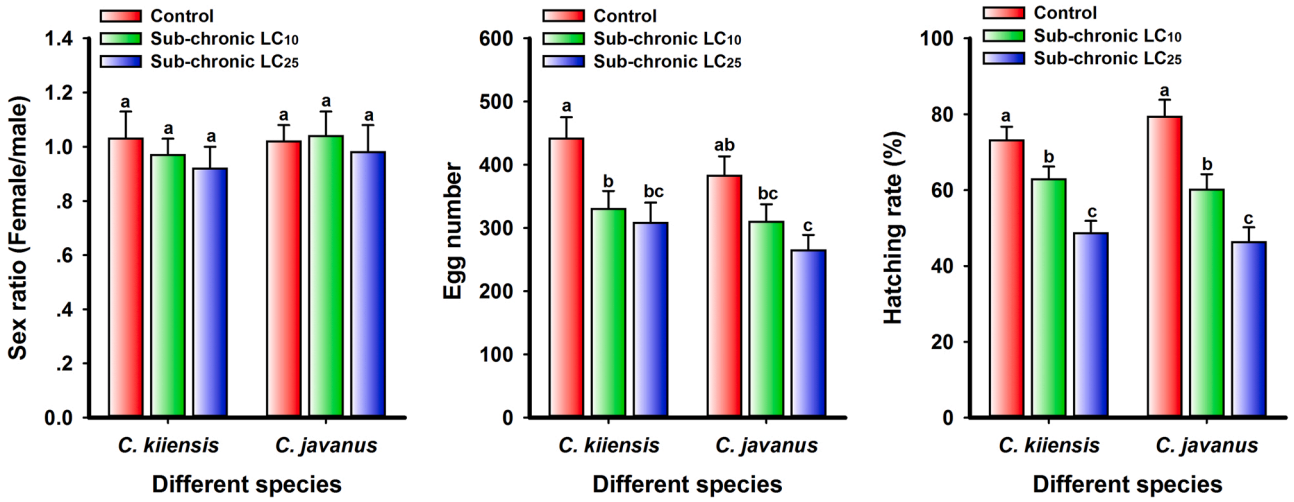

Fig. 3. Sublethal effects of chlorantraniliprole on the sex ratio, egg number and hatching rate of *C. kiiensis* and C. *javanus*. Each bar represents the mean ± SE of three replicates. Different lowercase letters above the SE bars indicated significant differences among the different treatments based on the one-way ANOVA followed by Tukey's HSD tests ($P < 0.05$).

the control, and *CYP6FV2* expression was significantly inhibited by LC_{10} dosage (Fig. 5).

4. Discussion

The extensive use of chlorantraniliprole in rice fields can pose potential risks to non-target aquatic organisms (Song et al., 2019). In our study, we found that the LC_{50} values at 24 and 48 h were 11.39 mg/L and 7.83 mg/L, respectively, for *C. javanus*, and 116.51 mg/L and 39.00 mg/L, respectively, for *C. kiiensis*. A previous study reported that chlorantraniliprole showed relatively higher toxicity in *C. dilutes* larvae (LC_{50} = 4.0 μg/L of 96 h exposure). Toxicological data for chlorantraniliprole can be highly variable in terms of endpoint, exposure durations, and in different species (Maloney et al., 2020). Our findings showed that *C. javanus* larvae were more susceptible than that of *C. kiiensis*. These two chironomid species typically coexist in paddy fields in China and some other Asian countries (Al-Shami et al., 2012). The difference in susceptibility may be attributed to activities and gene expression levels of detoxification and antioxidant enzymes.

Although pesticide toxicity to non-target insects is usually assessed using acute toxicity indices such as LC_{50} (Shan et al., 2020), current knowledge is insufficient to determine whether this approach fully represent pesticide toxicity. We further investigated the long-term sublethal toxicities of chlorantraniliprole to *C. kiiensis* and *C. javanus* in terms of certain growth, biochemical and molecular parameters. After exposure to sublethal dosages (LC_{10} and LC_{25}), growth and developmental durations of newly hatched larvae to adults for both *C. kiiensis* and *C. javanus* were significantly prolonged, indicating that chlorantraniliprole inhibited growth and development. These results are consistent with the previously reported effects in other insecticides. For instance, imidacloprid (0.625 μg/L) reduced the body length of *C. riparius* larvae after 10 days of exposure (Njattuvetty Chandran et al., 2018). Thiamethoxam also found to significantly inhibit larval growth in *Chironomus xanthus* and *C. riparius* (Ferreira-Junior et al., 2018; Saraiva et al., 2017) and sulfoxaflor significantly inhibited *C. kiiensis* larval growth at concentrations > 20 μg/L (Liu et al., 2021). Chironomid growth inhibition observed after insecticide exposure might be due the affected insects requiring more energy to resist insecticide toxin resulting in less energy available for growth and development (Nisbet et al., 2000). Our results also showed that pupations and emergence rates were

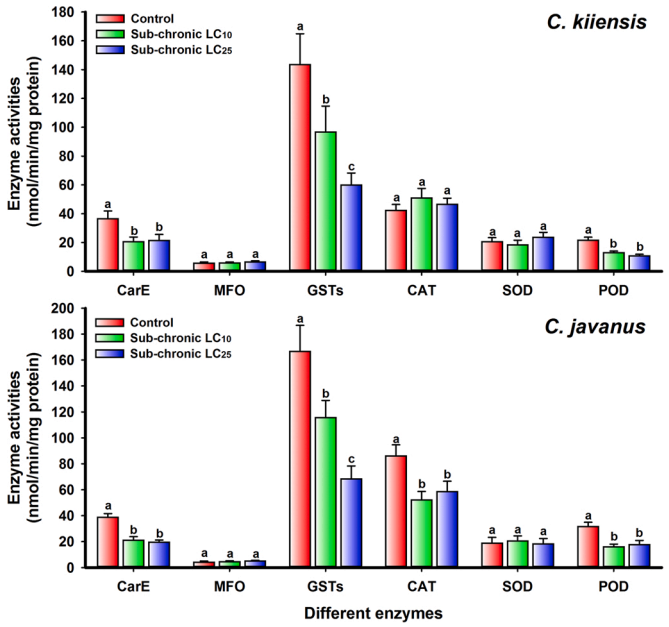

Fig. 4. Activities of detoxification enzyme carboxylesterase (CarE), P450, glutathione *S*-transferases and antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), peroxidase (POD) in the midge larvae of *C. kiiensis* and *C. javanus* exposed to sublethal chlorantraniliprole for 10 days. Each bar represents the mean ± SE of three replicates. Different letters above the SE bars indicated significant differences among the treatment of sublethal chlorantraniliprole for each enzyme based on the one-way ANOVA followed by Tukey's HSD tests ($P < 0.05$).

significantly reduced in *C. kiiensis* and *C. javanus* at sublethal dosages. Previous studies have reported that chlorpyrifos, imidacloprid, clothianidin, thiacloprid, and sulfoxaflor also inhibit chironomid emergence (Agra and Soares, 2009; Cavallaro et al., 2017; Raby et al., 2018; Liu et al., 2021). Lower pupation and emergence rates might also hinder adult midge mating, which would reduce the population density, eventually altering the population structure and eliminating the ecosystem functions provided by these species (Sibley et al., 1997). We found no significant difference in the sex ratios of emerged *C. kiiensis* and *C. javanus* adults after chlorantraniliprole exposure. However, the egg numbers and egg hatching rates were significantly decreased at some sublethal concentrations. The decreased emergence, egg numbers, and hatching rates would contribute to significant population reduction.

Changes in enzymatic activities and gene expressions signify organismal responses at the biochemical and molecular levels and therefore can serve as early warning indicators for exogenous stress (Velki et al., 2017). Enzymes such as CarEs, P450s, and GSTs are mainly involved in insecticide detoxification in insects and many other organisms (Wheelock et al., 2005; Gaaied et al., 2019). Overexpression of CarEs, which sequester insecticides and hydrolyze them into less harmful substances, facilitates external excretion on insecticides by the host insect (Feng and Liu, 2020). A previous study showed that CarE activity could serve as a sensitive endpoint in assessing toxicity (Wheelock et al., 2008). In our study, CarE activity and *CarE* expression levels were significantly lower in midge larvae treated with chlorantraniliprole at the LC_{10} and LC_{25} dosages than in the control larvae. Very similar results were reported in zebrafish treated with triazophos and imidacloprid (Wu et al., 2018). CarE thus played a key role in the toxicity of chlorantraniliprole to *C. kiiensis* and *C. javanus*. In addition to disrupting *CarE* expression, chlorantraniliprole also affected the *P450* gene expression in *C. kiiensis* and *C. javanus*. We found expression levels of two *P450* genes (*CYP9AU1* and *CYP6FV2*) markedly altered by chlorantraniliprole treatment. Similarly, Wei et al. (2021) reported that imidacloprid and azoxystrobin affected the expression of several *P450* genes in *C. dilutus*, including *CYP4*, *CYP12M1*, *CYP9AU1*, *CYP6FV1*, and *CYP315A1*. Azole fungicides have also been reported to inhibit cytochrome P450 activity in *C. riparius* (Gottardi and Cedergreen, 2018). GSTs are another type of key detoxification enzymes that biotransform various xenobiotics and catalyze the conjugation of electrophilic

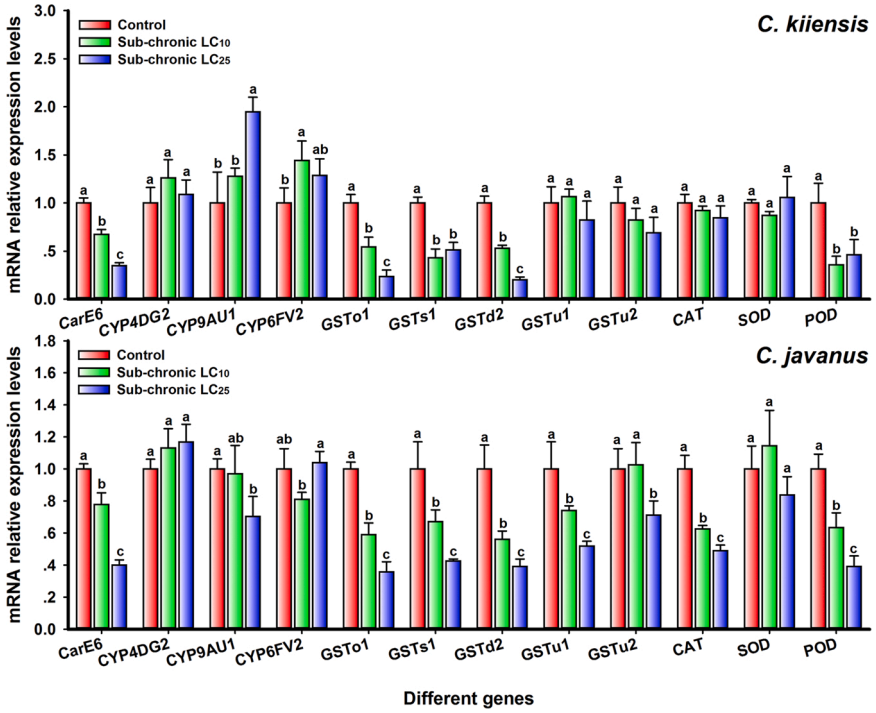

Fig. 5. The expressions of genes related to detoxification (*CarE*, *P450*, *GSTs*) and antioxidant (*CAT*, *SOD*, *POD*) in the larvae of *C. kiiensis* and *C. javanus* exposed to sublethal chlorantraniliprole for 10 days. Each bar represents the mean ± standard errors of three replicates. Different letters above the standard error bars indicated significant differences among the treatment of sublethal chlorantraniliprole for each gene based on the one-way ANOVA followed by Tukey's HSD tests ($P < 0.05$).

compounds with tripeptide glutathione (Ge et al., 2015). The expression of the *GST* genes *GSTo1*, *GSTs1*, and *GSTd2* was significantly lower in *C. kiiensis* as was that of *GSTu1* and *GSTu2* in *C. javanus*. Similarly, imidacloprid and azoxystrobin significantly altered *GST* gene expression in *C. dilutus* (Wei et al., 2021). Overall, GST activities and *GST* gene expressions were significantly lower in chlorantraniliprole treated *C. kiiensis* and *C. javanus*, which probably lead to accumulation of higher toxic metabolites and related to the severe damages caused by this pesticide. Taken together, the susceptibility of chironomids to chlorantraniliprole is thus attributed to the decreased detoxification enzyme activity and down regulation of corresponding genes (Jafir et al., 2021).

Antioxidant enzymes (CAT, SOD, and POD) have been used as biomarkers of pesticides that generate oxidative stress in aquatic animals (Ma et al., 2014). Exposure to insecticides can lead to imbalance between endogenous and exogenous reactive oxygen species (ROS) accumulation, reducing antioxidant defenses causing oxidative damages to organisms (Valavanidis et al., 2006). Ighodaro and Akinloye (2018) reported that CAT, SOD, and POD were primary antioxidant enzymes that defended against ROS and SOD catalyzes the conversion of superoxide anion to hydrogen peroxide (H_2O_2). In the present study, we found that chlorantraniliprole exposure decreased POD activity and *POD* expression in *C. kiiensis* and *C. javanus*. These effects might have aggravated H_2O_2 accumulation owing to a reduction in oxygen metabolism. In addition, chlorantraniliprole treatments at both LC_{10} and LC_{25} dosages inhibited CAT activity and *CAT* expression in *C. javanus*, partially explaining the higher susceptibility of *C. javanus* to chlorantraniliprole than that of *C. kiiensis*. The findings suggested that chlorantraniliprole induced excess ROS in *C. javanus* tissues, and CAT failed to timely eliminate the ROS, which in turn inhibited CAT activity. Previous studies have indicated that the expression levels of *SOD* and *CAT* in *C. dilutus* were significantly induced by imidacloprid and azoxystrobin (Wei et al., 2021). Similarly, Mu et al. (2016) found that CAT enzyme activity and the expression of the corresponding gene were decreased in zebrafish (*Danio rerio*) exposed to difenoconazole. Evaluation of oxidative stress through measuring changes in antioxidant enzyme activity and/or corresponding gene expression levels therefore proved to be valuable and necessary strategy (Velki et al., 2017). Furthermore, the results of previous studies and our findings together suggest that pesticides decrease the ability of cells to use functional antioxidant enzymes to resist oxidative stress.

The recommended dosage (59.38 mg a.i./L, Khan et al., 2021) of chlorantraniliprole for controlling rice pests is considerably higher than the sublethal dosages used in this study. Such high chlorantraniliprole concentrations in rice environment are potentially harmful to the survival of chironomids and other organisms.

5. Conclusion

Our studies suggest that sublethal concentrations of chlorantraniliprole disrupt growth, pupation, emergence, egg number, and egg hatching of *C. kiiensis* and *C. javanus*. Ten days of sublethal chlorantraniliprole exposure could induce significantly altered (mostly decreased) expressions of seven and ten genes in *C. kiiensis* and *C. javanus*, respectively, related to detoxification and oxidative stress. CarE, GSTs, and POD showed lower activity in *C. kiiensis*, whereas CarE, GSTs, CAT, and POD showed lower activity in *C. javanus*. Notably, these changes in enzyme activity were associated with changes in the expression levels of the corresponding genes, which might therefore serve as early molecular warning indicators for pesticide-induced damage.

CRediT authorship contribution statement

Yanhui Lu: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft. **Xusong Zheng:** Investigation, Formal analysis, Validation. **Xiaochan He:** Investigation, Formal analysis, Validation. **Qiming Fu:** Methodology, Investigation, Formal analysis. **Jiawen Guo:** Writing – review & editing. **Hongxing Xu:** Methodology, Investigation, Formal analysis, Writing – review & editing, Supervision, Funding acquisition. **Zhongxian Lu:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

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

Data Availability

No data was used for the research described in the article.

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