Article

Resistance Monitoring of *Nilaparvata lugens* to Pymetrozine Based on Reproductive Behavior

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Simple Summary: Pymetrozine is one of the most common insecticides used to control the rice pest *Nilaparvata lugens* in China. Because of the unique mechanism of action and the fact that there is no obvious lethal effect after pymetrozine treatment, it is unreasonable to determine the sensitivity of pymetrozine by only calculating the mortality rate. In the context of traditional bioassay methods, which do not reflect pymetrozine's control effect on pest populations in the field, we established fecundity assay bioassay methods to monitor the resistance level of *N. lugens* to pymetrozine, and systematically evaluated pymetrozine's effect on the fecundity of *N. lugens*. Treatment with pymetrozine affected male courtship, female receptivity, and oviposition in both *N. lugens* nymphs and adults.

Abstract: To explore the effects of pymetrozine on the reproductive behavior of *N. lugens*, we established a bioassay method to accurately evaluate the toxicity of pymetrozine in *N. lugens* and establish the level of pymetrozine resistance of *N. lugens* in the field. In this study, pymetrozine's effects on the fecundity of *N. lugens* were evaluated using the topical application method and rice-seedling dipping method. Moreover, the resistance of *N. lugens* to pymetrozine in a pymetrozine-resistant strain (Pym-R) and two field populations (YZ21 and QS21) was determined using the rice-seedling dipping method and fecundity assay methods. The results showed that treatment of *N. lugens* third-instar nymphs with LC_{15}, LC_{50}, and LC_{85} doses of pymetrozine resulted in significantly reduced male fertility and female fecundity. In addition, *N. lugens* adults treated with pymetrozine using the rice-seedling dipping and topical application method also exhibited significantly inhibited male courtship, fertility, and female receptivity. Using the rice-stem dipping method, pymetrozine resistance was shown to be at high levels in Pym-R (194.6-fold), YZ21 (205.9-fold), and QS21 (212.8-fold), with LC_{50} values of 522.520 mg/L (Pym-R), 552.962 mg/L (YZ21), and 571.315 (QS21) mg/L. However, when using the rice-seedling dipping or topical application fecundity assay method, Pym-R (EC_{50}: 14.370 mg/L, RR=12.4-fold; ED_{50}: 0.560 ng/adult, RR=10.8-fold), YZ21 (EC_{50}: 12.890 mg/L, RR=11.2-fold; ED_{50}: 0.280 ng/adult; RR=5.4-fold), and QS21 (EC_{50}: 13.70 mg/L, RR=11.9-fold) exhibited moderate or low levels of resistance to pymetrozine. Our studies show that pymetrozine can significantly inhibit the fecundity of *N. lugens*. The fecundity assay results showed that *N. lugens* only developed low to moderate levels of resistance to pymetrozine, indicating that pymetrozine can still achieve effective control on the next generation of *N. lugens* populations.

Keywords: *Nilaparvata lugens*; pymetrozine; reproductive behavior; bioassay method; resistance monitoring

1. Introduction

The brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), is an important migratory rice pest in the Yangtze River Valley, in South and Southwest China [1,2]. There are three main ways in which BPH damages rice plants. Firstly, BPH ingests the rice sap through the mouthparts, which hinders the transport of nutrients and water in the rice plants. Secondly, females pierce the rice leaf sheaths with their spawning

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needle to dissipate plant water when spawning. Thirdly, BPH transmits rice virus diseases such as rice grassy stunt virus and rice ragged shunt virus [3,4]. BPH has high innate capacity for proliferation, strong adaptability to the environment, and a long-distance migration ability, which make it insidious, sudden, violent, and destructive. Therefore, BPH presents a significant challenge for rice production in many Asian countries [5,6]. Chemical pesticides are still the main methods with which to control *N. lugens*. However, as a result of the long-term, widespread, and unscientific use of pesticides, *N. lugens* has developed high resistance levels to neonicotinoids, organophosphates, carbamates, and insect growth regulators [7-9]. Among them, imidacloprid, buprofezin, and thiamethoxam have been suspended for the control of *N. lugens* owing to the high resistance levels in China.

Pymetrozine is a pyridine azomethine insecticide with an excellent control effect against sucking pests [10]. In 2015, Nesterov et al., using *Drosophila* as the research object, found that pymetrozine can interfere with the gravity sense and hearing of *Drosophila*, and can also evoke calcium responses in chordotonal stretch receptor neurons co-expressing *Nanchung* (Nan) and *Inactive* (Iav) proteins. Further in vitro expression experiments showed that the molecular target of pymetrozine is a transient receptor potential vanilloid (TRPV) ion channel complex [11]. Wang et al. confirmed that pymetrozine can activate the TRPV channels, i.e., the *nan* and *iav* complexes, of *N. lugens* and inhibit the reproductive behavior of *N. lugens* and *Drosophila melanogaster* [12,13]. However, the effects of pymetrozine's systemic and contact activities on the male courtship, female receptivity, and oviposition of *N. lugens* have not been systematically studied.

Pymetrozine has unique mode of action. Its molecular target is the TRPV channel, which participates in the hearing and gravity senses in insects [14,15]. Moreover, hearing plays an important role in the reproductive behavior of brown planthopper. The mating of *N. lugens* relies on abdominal vibrations, which are transmitted by the vibration of the rice stem. Females can emit an invitation signal (IS) that consists of a steady series of pulses, which attract male courtship [16]. Males can emit two types of vocalizations. The first male vibrational signal (FMVS), also known as the calling signal (CS), consists of four different pulses per syllable. The second male vibrational signal (SMVS) can be divided into the pre-aggressive signal (pre-AS) and aggressive signal (AS). The pre-AS is a short string of pulses that are replaced by the AS a short time after being emitted [17,18]. Ichikawa found that males communicate with their counterparts by emitting a CS that is transmitted to the local 60-80 cm range through the vibration of the rice stem. Females suitable for mating receive this emission and respond with the IS. Males rely on the IS emission to locate and pursue females on rice plants, approaching the end of the female abdomen and emitting courtship signals to females by vibrating the wings and abdominal jitters, while the hind leg of females is lifted upwards to provide space for males to mate. After the male unilateral middle legs rub the female abdomen for 1-2 s, males cross alongside their body to attempt mating. The male genitals are fixed above the female spawning needle. Then, the female spawning needle is tilted about 15°, indicating the onset of mating. The time of copulation generally lasts 1 to 2 min. Males are kicked off by the female after the completion of mating using her hind legs. If females are immature or have been mating within a week, they will not respond to the CS emitted by males (unpublished data from our lab). Males are not certain to attract conspecific females when they emit the CS for courtship, and if another male arrives, rejection behavior (RB) occurs. In addition to RB, two males are prone to emit the SMVS and perform a direct body attack (DBA) after recognizing each other [18]. Typically, males that emit the SMVS are more competitive in mating with females. However, short-term SMVS playback results in significantly lower mating success, while long-term playback results in significantly smaller male spermatophore [19]. Therefore, pymetrozine may significantly inhibit the reproductive behavior of *N. lugens* by interfering with hearing, thus effectively inhibiting the number of next-generation insects.

In order to ensure the continuity of resistance monitoring and take into account the actual operability of monitoring a large number of field populations, scientific research

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institutes in China have long been using the rice-stem dipping method to monitor pymetrozine resistance in BPH. This method evaluates BPH's toxicity to pymetrozine by introducing the third-instar nymphs onto rice stems treated with different pymetrozine concentrations and then assessing the mortality after 7 days. The monitoring results showed that during 2012-2021, *N. lugens* field populations in China reached moderate to high resistance levels to pymetrozine, except for a few populations in 2012, which were at sensitive to low resistance levels [9,20,21]. The LC_{50} values determined by the rice-stem dipping method could only reflect the sensitivity of contemporary *N. lugens* to pymetrozine, but could not reflect the pymetrozine inhibition in the next-generation *N. lugens* population. Hence, pymetrozine toxicity and *N. lugens* resistance could not be fully assessed. Consequently, the Insecticide Resistance Action Committee (IRAC) and scientists from Japan have established bioassay methods to evaluate the activity of pymetrozine against *N. lugens* based on treating contemporary *N. lugens* with pymetrozine while investigating the number of next-generation nymphs [22]. The IRAC no.005 method (IRAC no.005) introduces adult BPH females and males onto potted rice plants dipped in different concentrations of pymetrozine solution. Then, they are removed after 7 days of spawning, and the number of offspring are counted after 18 days. This method can investigate pymetrozine's effect on contemporary and next-generation test insects under both systemic and contact modes of action. However, this method has a large space demand, and a long test cycle due to the preparation of potted rice plants. Tsujimoto et al. only examined the contact effect of pymetrozine. Different doses of pymetrozine were topically applied to female brown planthoppers using a micro-applicator, and the males were not treated. Thereafter, they were transferred to rice seedlings in a test tube to spawn for 7-8 days and then removed. After 15-16 days, the number of offspring was counted. This method can only detect pymetrozine's effect on female receptivity and oviposition behavior, but cannot reflect the pymetrozine's effect on male courtship, male fertility, or the systemic activity of pymetrozine. Accordingly, there is still a relative lack of effective resistance monitoring technology to assess *N. lugens'* resistance to pymetrozine. In addition, the real resistance level of field *N. lugens* populations to pymetrozine still needs to be established, and these data, when available, will significantly affect resistance monitoring and the management of pymetrozine in relation to *N. lugens*.

In this study, we found that pymetrozine could significantly inhibit the reproductive behavior of *N. lugens*. Pymetrozine treatment can significantly inhibit male courtship, female receptivity, and oviposition in *N. lugens* nymphs and adults. On this basis, a more accurate bioassay method, i.e., the fecundity assay method, was established to reflect the pymetrozine resistance of *N. lugens*. In addition, this method was used to monitor pymetrozine resistance in *N. lugens* both in laboratory and field populations. These results are valuable for the monitoring and management of pymetrozine resistance.

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2. Materials and Methods

2.1. Insects

The susceptible strain (Pym-S) of BPH was initially collected from Hangzhou, Zhejiang Province, in 1995 and was maintained in the laboratory without exposure to any insecticides since. The pymetrozine-resistant strain (Pym-R) was collected from Jinhua, Zhejiang Province, in 2013 and was selected for resistance to pymetrozine for more than 90 generations. Two field populations (QS21 and YZ21) were collected from Qianshan (30°37′N, 116°34′E), Anhui Province, and Yizheng (32°16′N, 119°11′E), Jiangsu Province, in September 2021, respectively. All the populations were reared on indica rice seedlings (Taichung Native 1, TN1) under standard conditions of 27 ± 1 °C, a 70–80% relative humidity, and a 16 h light/8 h dark photoperiod.

2.2. Insecticides

Technical-grade pymetrozine (95%) was supplied by Jiangsu Anpon Electrochemical Co., Ltd. (Jiangsu, China). The technical-grade pymetrozine was dissolved in acetone (for topical application) or *N,N*-dimethylformamide (for systemically application) as stock solution. Then, a serial dilution was prepared using acetone for the topical application bioassay and water containing 0.1% Triton X-100 for the rice-stem dipping bioassay.

2.3. Bioassays

2.3.1. Rice-stem dipping bioassay method

The rice-stem dipping bioassay method was used to evaluate the LC_{50} estimations [23]. Briefly, three rice stems were grouped together and dipped in insecticide solutions for 30s and then air-dried at room temperature. Fifteen insects (third-instar nymphs) were introduced onto rice stems into a plastic cup for each replicate. There were 3-4 replicates for each concentration and 4-5 doses of pymetrozine. Control rice stems were treated with 0.1% Triton X-100 water solution only. All treatments were maintained under standard conditions of 27 ± 1°C and a 70-80% relative humidity, with a 16 h light/8 h dark photoperiod. Mortality was assessed after 120h of exposure to pymetrozine.

2.3.2. Fecundity assay bioassay method

The rice-seedling dipping fecundity assay method was used to evaluate the EC_{50} estimations. The no.005 method was referred to and modified (IRAC no.005). Firstly, 30-day-old rice seedlings (approximate) were dipped in a series of concentrations of pymetrozine for 30s each. Then, each newly emerged virgin female adult was paired with a single male onto the rice seedlings. After 7 days, the adults were removed and their survival was counted. After 15 days, the number of nymphs (only counting the replicates of female adults surviving after 7 days) was counted, and the inhibition rate of offspring under the treatment of the corresponding pymetrozine concentration was calculated. There were 30 replicates for each concentration and 4-5 doses of pymetrozine. Control rice stems were treated with 0.1% Triton X-100 water solution only. All treatments were maintained under standard conditions of 27 ± 1°C and a 70-80% relative humidity, with a 16 h light/8 h dark photoperiod.

The topical application fecundity assay method was used to evaluate the ED_{50} estimations. The method by Katsuhiko Tsujimoto that combines the topical application and the determination of offspring number was referred to and modified [22]. Newly emerged virgin female adults were anesthetized with carbon dioxide for 10s. A droplet (0.2μL) of insecticide acetone solution was applied topically to the prothorax notum of each individual female with a hand-held micro-applicator (Hamilton Repeating Applicator, Burkard Manufacturing Co. Ltd., Rickmansworth, UK). Males were not treated and control groups were treated with acetone. Then, each treated female adult was paired with a single male onto the 30-day-old rice seedlings (approximate). The remaining steps and experiment conditions were consistent with rice-seedling dipping fecundity assay method.

2.4. Effects of pymetrozine treatment at third-instar nymph stage on the fecundity of N. lugens

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The *N. lugens* third-instar nymphs were introduced onto rice seedlings treated with pymetrozine solutions at LC_{15}, LC_{50}, and LC_{85} concentrations. After 7 days, the nymphs were transferred to new rice seedlings without pymetrozine treatment and reared until they emerged as adults. The newly emerged unmated *N. lugens* adults were paired according to the following combinations: ♀t (treated female) × ♂ck (untreated male); ♀ck (untreated female) × ♂t (treated male); and ♀t (treated female) × ♂t (treated male). Single pairs were introduced onto 30-day-old TN-1 rice seedlings to spawn for 7 days, and the number of offspring was counted after 15 days. ♀ck (untreated female) × ♂ck (untreated male) were regarded as the control group. There were no less than 30 replicates for each treatment.

2.5. Effects of pymetrozine treatment at adult stage on the fecundity of N. lugens

The rice-seedling dipping treatment was used to evaluate the systemic toxicity of pymetrozine in BPH. To this end, 30-day-old TN-1 rice seedlings (approximate) were dipped in 10 mg/L pymetrozine solution for 30s and then air-dried at room temperature. Control rice seedlings were treated with 0.1% Triton X-100 water solution only. Pymetrozine's effect on the fecundity of *N. lugens* was studied according to two treatments. The first treatment involved introducing single pairs of newly emerged unmated female and male adults in order to investigate the effects of pymetrozine on male courtship, female receptivity, and female oviposition in *N. lugens*. The second treatment involved introducing single *N. lugens* females that had emerged 2 to 3 days previous and were fully mated in order to investigate the effects of pymetrozine on oviposition in BPH. In both of the above treatments, we removed the adults after 7 days, and the number of offspring was counted after 15 days. There were no less than 30 replicates for each treatment.

The topical application treatment was used to evaluate the contact toxicity. *N. lugens* adults were anesthetized with carbon dioxide for 10s. A droplet (0.2μL) of 0.1 mg/L pymetrozine solution (0.02 ng/adult) was topically applied to the prothorax notum of the following adults with a hand-held micro-applicator (Hamilton Repeating Applicator, Burkard Manufacturing Co. Ltd., Rickmansworth, UK): 1) unmated females; 2) unmated males; 3) unmated females and males; 4) fully mated pregnant female adults. Control groups were treated with acetone only. We also removed the adults after 7 days, and the number of offspring was counted after 15 days. There were no less than 30 replicates for each treatment.

2.6. Data Analysis

The median lethal concentrations (LC_{50}) and their 95% fiducial limits (FL) were calculated using the POLO-plus program (Version 2.0) (LeOra Software 2008) for BPH. The median effective concentrations (EC_{50}) and the median effective dose (ED_{50}) were estimated using the GraphPad Prism 8 Software (GraphPad Software Inc, San Diego, USA). Data statistical analyses were conducted using the GraphPad Prism 8.0 software. The variance analysis of the data from more than two groups was assessed using one-way ANOVA with post hoc Tukey HSD, and the data between two groups was assessed using Student's *t*-test. The resistance ratio (RR) was calculated by dividing the LC_{50}, EC_{50}, or ED_{50} value of a resistant strain by that of the susceptible strain. Insecticide resistance of the field populations was classified as follows: RR < 5-fold as susceptible; RR = 5-10-fold as low resistance; RR = 10-100-fold as medium resistance; and RR >100-fold as high resistance [9].

3. Results

3.1. Pymetrozine toxicity determination in N. lugens *third-instar nymphs using the rice-stem dipping method*

Pymetrozine toxicity in the *N. lugens* third-instar nymphs was determined using the rice-stem dipping method. The results showed that the LC_{15}, LC_{50}, and LC_{85} values of pymetrozine to the pymetrozine-susceptible strain (Pym-S) were 0.422 mg/L, 2.685 mg/L, and 17.073 mg/L, respectively (Table 1). The LC_{50} values of the pymetrozine-resistant strain (Pym-R) and two field populations (QS21 and YZ21) were 522.520 mg/L, 571.315

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mg/L, and 552.962 mg/L, respectively. All three populations developed high levels of resistance to pymetrozine, with resistance ratios of 194.6-fold, 212.8-fold, and 205.9-fold, respectively (Table 1).

Table 1. Pymetrozine resistance monitoring of *N. lugens* third-instar nymphs using the rice-stem dipping method.

Strains	Slope ± SE	χ^2(df)	*P* value	LC_{15} (95%F.L.) mg /L	LC_{50} (95%F.L.) mg /L	LC_{85} (95%F.L.) mg /L	RR[1]
Pym-S	1.290 ± 0.252	0.59 (3)	0.90	0.422 (0.101 - 0.843)	2.685 (1.610 - 4.022)	17.073 (9.840 - 50.096)	-
Pym-R	1.023 ± 0.236	1.20 (3)	0.75	50.647 (8.000 - 109.991)	522.520 (311.457 - 999.180)	5390.8 (2168.5 - 50752)	194.6
YZ21	1.263 ± 0.244	0.72 (3)	0.87	83.617 (27.819 - 146.078)	552.962 (374.727 - 892.761)	3656.7 (1852.5 - 14867)	205.9
QS21	1.328 ± 0.251	1.36 (3)	0.71	94.738 (34.384 - 160.658)	571.315 (390.675 - 911.574)	3445.3 (1801.1 - 12747)	212.8

[1] RR: resistance ratio; LC_{50} of Pym-R divided by LC_{50} of Pym-S.

3.2. Pymetrozine toxicity determination in N. lugens *adults using the fecundity assay method*

The fecundity assay method was developed based on the fact that pymetrozine can interfere with the reproductive behavior of *N. lugens* [13]. Pymetrozine's toxicity in *N. lugens* was determined by counting the inhibition rate of different pymetrozine doses on the number of *N. lugens* offspring. As shown in Table 2, the EC_{50} and ED_{50} values for the pymetrozine-susceptible strain (Pym-S) were 1.155 mg/L and 0.052 ng/adult, respectively, which were used as the baseline to monitor the resistance of BPH to pymetrozine using the fecundity assay method. Inhibition of the fecundity of *N. lugens* as a result of the systemic activity of pymetrozine was determined using the rice-seedling dipping fecundity assay method. The results indicated that Pym-R strain (EC_{50}: 14.370 mg/L, RR=12.4-fold), YZ21 (EC_{50}: 12.890 mg/L, RR=11.2-fold), and QS21 (EC_{50}: 13.70 mg/L, RR=11.9-fold) developed a moderate level of resistance to pymetrozine (Table 2). Inhibition of the fecundity of *N. lugens* resulting from the contact activity of pymetrozine was determined using the topical application fecundity assay method. The results indicated that Pym-R (ED_{50}: 0.560 ng/adult, RR=10.8-fold) and YZ21 (ED_{50}: 0.280 ng/adult; RR=5.4-fold) developed a low or moderate level of resistance to pymetrozine (Table 2). Our results indicate that both pymetrozine's systemic toxicity and contact toxicity can effectively inhibit the offspring of *N. lugens*.

Table 2. Susceptibility of *N. lugens* to pymetrozine using two different fecundity assay methods.

Populations	Rice-seedling dipping fecundity assay method					Topical application fecundity assay method				
	Slope ± SE	χ^2 (df)	*P* value	EC_{50} (95%F.L.) (mg/L)	RR	Slope ± SE	χ^2 (df)	*P* value	ED_{50} (95%F.L.) (ng /adult)	RR
Pym-S	0.85 ± 0.11	0.43 (3)	0.93	1.155 (0.882 - 1.513)	1.0	0.50 ± 0.07	0.34 (3)	0.95	0.052 (0.035 - 0.079)	1.0
Pym-R	0.89 ± 0.21	0.41 (3)	0.94	14.370 (9.474 - 21.790)	12.4	0.59 ± 0.08	0.26 (2)	0.97	0.560 (0.350 - 0.890)	10.8
YZ21	1.09 ± 0.12	0.67 (3)	0.88	12.890 (10.830 - 15.340)	11.2	0.56 ± 0.07	0.44 (3)	0.93	0.280 (0.200 - 0.400)	5.4
QS21	0.85 ± 0.35	0.17 (3)	0.98	13.700 (6.619 - 28.370)	11.9	-	-	-	-	-

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3.3. *Effects of pymetrozine treatment at third-instar nymph stage on the fecundity of* N. lugens

In order to explore pymetrozine's effect on the reproductive behavior of *N. lugens*, sub-lethal (LC_{15}), median lethal (LC_{50}), and highly (LC_{85}) lethal concentrations of pymetrozine were used to treat third-instar pymetrozine-susceptible strain nymphs, as shown in Table 1. The brown planthoppers were transferred to fresh rice seedlings and reared until emergence after 7 days of treatment. Thereafter, *N. lugens* spawned for 7 days according to the combination of ♀ck (untreated female) × ♂ck (untreated male), ♀t (treated female) × ♂ck (untreated male), ♀ck (untreated female) × ♂t (treated male), and ♀t (treated female) × ♂t (treated male), and the number of offspring was counted. ♀ck (untreated female) × ♂ck (untreated male) was regarded as the control group. The results showed that, as compared with the control group (♀ck × ♂ck), the number of offspring in the LC_{15}, LC_{50}, and LC_{85} pymetrozine-treated female (♀t × ♂ck) groups was reduced by 40.7 %, 48.4 %, and 56.4 %, respectively (Figure 1A). In addition, the number of offspring in the pymetrozine-treated male (♀ck × ♂t) group was significantly reduced by 33.5 %, 42.3 %, and 49.7 %, respectively (Figure 1B), for the aforementioned concentrations. Moreover, the number of offspring in the pymetrozine-treated female and male (♀t × ♂t) groups was significantly reduced by 45.6 %, 50.3 % and 64.3 %, respectively (Figure 1C), for the aforementioned concentrations. Therefore, pymetrozine treatment at *N. lugens* third-instar nymph stage was shown to continuously inhibit male fertility and female fecundity and lead to a significant decrease in the number of offspring.

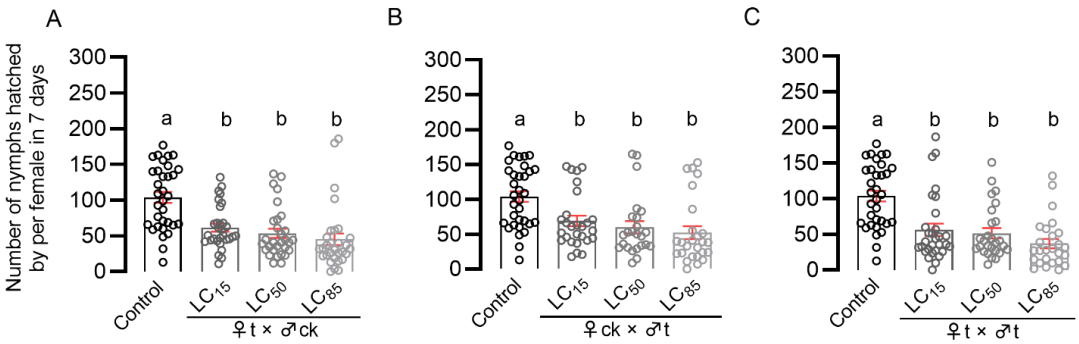

Figure 1. Effects of pymetrozine treatment at third-instar nymph stage on the fecundity of *N. lugens*. Single female fecundity after emergence of *N. lugens* nymphs treated with sub-lethal, median, and high concentrations of pymetrozine (A: ♀t × ♂ck; B: ♀ ck × ♂t; C: ♀t × ♂t). The variance analysis of all data was performed by one-way ANOVA with post hoc Tukey HSD. Error bars represent SE. Different lowercase letters showed significant differences at the 0.05 level ($P<0.05$).

3.4. *Effects of pymetrozine treatment at adult stage on the fecundity of* N. lugens *using the rice-seedling dipping method*

Previous studies demonstrated that pymetrozine can inhibit the number of offspring by interfering with the reproductive behavior of *N. lugens* [13]. However, there is no systematic study on the effects of pymetrozine treatment on male courtship, female receptivity, and female oviposition after mating. Therefore, in this study, unmated and fully mated adults were treated with pymetrozine to investigate pymetrozine's effects on male courtship, female receptivity, and female oviposition after mating.

The effect of pymetrozine's systemic activity on the fecundity of *N. lugens* was determined using the rice-seedling dipping method. Single pairs of unmated females and males or fully mated females 2-3 days after emergence were introduced onto rice seedlings treated with 10 mg/L pymetrozine to spawn for 7 days. The results showed that the number of offspring in the treatment group was significantly reduced by 84.3 % and 57.3 % as compared with the control group (Figure 2). These results indicate that pymetrozine can inhibit female oviposition.

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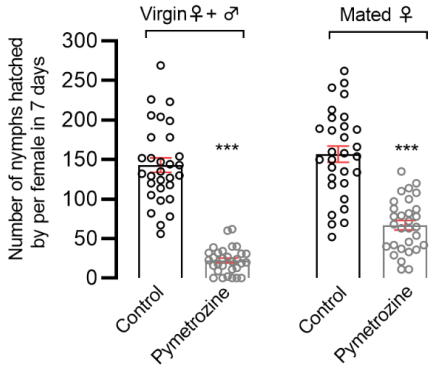

Figure 2. Effects of pymetrozine treatment on the fecundity of *N. lugens* adults using the rice-seedling dipping method. The variance analysis of all data was performed by Student's *t*-test. Error bars represent SE (*, p <0.05; **, p < 0.001; ***, p < 0.0001; ns, no significant).

3.5. Effects of pymetrozine treatment at adult stage on the fecundity of N. lugens *using the topical application method*

The effect of contact with pymetrozine on the fecundity of *N. lugens* was determined using the topical application method. Pymetrozine was topically applied in the following combinations: to unmated females paired with untreated males (♀t × ♂ck); unmated males paired with untreated females (♀ck × ♂t); unmated females paired with unmated males (♀t × ♂t); mated pregnant females (♀t). The results showed that the number of offspring in these four treatment groups was significantly reduced by 66.4 %, 35.8 %, 80.6 %, and 63.7 %, respectively, as compared with the control group (Figure 3).

Moreover, treating unmated adults with pymetrozine increased the number of no-offspring females whether using the topical application method or rice-seedling dipping method. These results indicate that pymetrozine can interfere with male courtship, female receptivity, and female oviposition after mating. Hence, pymetrozine exhibits strong inhibitory effects on next-generation BPH populations.

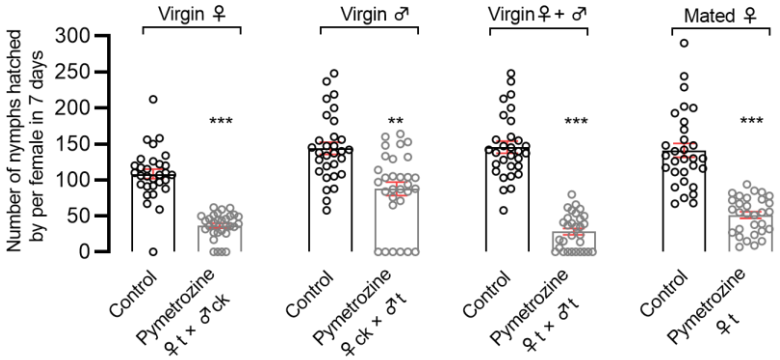

Figure 3. Effects of pymetrozine at adult stage treatment on the fecundity of *N. lugens* using the topical application method. The variance analysis of all data was performed by Student's *t*-test. Error bars represent SE (*, p <0.05; **, p < 0.001; ***, p < 0.0001; ns, no significant).

4. Discussion

Pymetrozine has been recommended as an alternative insecticide to control BPH, which has developed resistance to imidacloprid, thiamethoxam, and buprofezin [9,24]. Using the rice-stem dipping bioassay method, it was shown that BPH has developed strong resistance to pymetrozine [9,21]. However, pymetrozine still exhibits a good control effect against BPH in paddy fields. Therefore, there is a need to establish another bioassay method to evaluate the current resistance of BPH to pymetrozine. On the basis of

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the impact of pymetrozine on BPH reproductive behavior, we established a fecundity assay bioassay method and found that BPH only developed low to moderate resistance to pymetrozine. We also found that pymetrozine can inhibit male courtship, female receptivity, and oviposition.

Establishing a standard bioassay method can help us to obtain the relationship between the insecticide dose and pest mortality, thus contributing to our understanding of the evolution of insecticide resistance and helping formulate strategies to delay the development of resistance [25]. The mortality rate is not the only criterion in the insecticide bioassay. In this study, the fecundity assay method was used to calculate insecticide toxicity according to the offspring inhibition rate. For certain kinds of carbamates, benzoylureas, and pyrethroids, people calculate toxicity values according to egg hatching inhibition rates to determine their ovicidal activity against pests such as *Plutella xylostella* (L.) and *Spodoptera frugiperda* [26,27]. However, an effective resistance monitoring method for pymetrozine needs assess both its systemic and contact activities. The rice-stem dipping bioassay method can reflect the two modes of action described above for pymetrozine, and the third-instar nymphs used in the test also meet the requirements concerning field prevention of lower-instar nymphs. The monitoring results of the rice-stem dipping method showed that pymetrozine was less active against the contemporary *N. lugens* and that *N. lugens* had reached high level of resistance to pymetrozine (194.6- to 212.8-fold). However, the results of this method did not fully reflect the field efficacy because of the long persistence of pymetrozine and the short duration of the treatment when rice-stem dipping. The rice-seedling dipping fecundity assay method examined both the systemic and contact effects of pymetrozine, taking into account its insecticidal mode and mechanism design. Using this method, we found that *N. lugens* only developed moderate resistance to pymetrozine (11.2- to 12.4-fold). In this method, pymetrozine's toxicity to *N. lugens* was determined by calculating the inhibition rate after different pymetrozine doses from the number of *N. lugens* offspring nymphs. Therefore, this method simultaneously evaluates pymetrozine toxicity in *N. lugens* from the newly hatched nymphs to the third-instar nymphs. The topical application fecundity assay method only examines pymetrozine's contact effect of. On the basis of this method, we found that *N. lugens* developed low to moderate resistance to pymetrozine (5.4- to 10.8-fold). Both fecundity assay bioassay methods demonstrated that pymetrozine had a significant inhibitory effect on brown planthopper fecundity, and the resistance level monitored by the two methods was basically consistent at low to moderate. This is consistent with the fact that, although the short-term efficacy of pymetrozine against BPH is decreasing in the current generation, it still has a good field control effect on the next generation due to its long persistence.

The fecundity assay method established in this study was improved based on the no.005 method released by the IRAC and the topical application method reported by Tsujimoto et al. [22]. The above two methods were used for test insects that had mated for many days and each replication introduced multiple females and males. However, there are certain limitations to the study. Firstly, the age and time of death of each female in each replication could not be determined, thus making it impossible to ensure that each female spawned for the same duration. Secondly, it was not possible to accurately determine whether each female mated or not, and thus, it was impossible to accurately assess the effects of pymetrozine on courtship, receptivity, and oviposition in brown planthopper. Accordingly, in order to comprehensively evaluate the effect of pymetrozine on *N. lugens* mating and female reproductive, the two fecundity assay methods established in our research used the newly emerged unmated *N. lugens* for single-pair pairing. In addition, only treatments with surviving females after 7 days were counted in order to exclude the effects of oviposition duration on the number of offspring and to improve the accuracy of the test results. The fecundity assay method can comprehensively reflect pymetrozine's persistence, its characteristics in terms of inhibiting reproductive behavior, and more objectively reflect pymetrozine's toxicity against BPH. On the other hand, as a result of the use of single-pair mating, the fecundity assay method had a large number of replications per concentration and required a large amount of work, which has certain limitations in

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terms of carrying large-scale resistance monitoring. Combined with the migration habits of *N. lugens*, it is recommended to select three to five field populations of *N. lugens* each year to monitor pymetrozine resistance using this method, so as to accurately grasp the resistance dynamics of *N. lugens* to pymetrozine.

In order to determine pymetrozine toxicity in BPH using the fecundity assay method, this study comprehensively evaluated the effects of pymetrozine's systemic and contact activity on male courtship, female receptivity, and female oviposition in *N. lugens* through rice-seedling dipping treatment and topical application. As can be seen in our findings, the number of female individuals without offspring increased after pymetrozine treatment, and pymetrozine was shown to inhibit the mating of female and male brown planthoppers. Furthermore, pymetrozine can significantly inhibit the oviposition in female *N. lugens*. Pymetrozine disrupts the normal function of the chordotonal mechanoreceptors, thereby affecting the insect's gravity sense, hearing, and coordination. This, in turn, affects the feeding and reproductive behavior of the target insects [11,28,29]. Our previous study demonstrated that pymetrozine inhibited the reproductive behavior of *N. lugens* by disrupting male initiative courtship, female abdominal vibration, and female oviposition. In addition, in *Drosophila*, a significant reduction in the male courtship index and female receptivity was observed after pymetrozine treatment [13]. However, the physiological, biochemical, and molecular mechanisms through which pymetrozine inhibits male courtship, female receptivity, and oviposition remain to be further studied.

In conclusion, pymetrozine can effectively inhibit the next-generation *N. lugens* population. Combined *N. lugens* control emphasizes the strategy of "suppressing the early population and controlling the late population", therefore pymetrozine is still recommended for the control of migrating-generation *N. lugens* in order to control the offspring population. Nevertheless, the inhibitory effect of pymetrozine on the fecundity of *N. lugens* requires further attention. Once strong resistance is observed, the fecundity of *N. lugens* cannot be significantly inhibited in this manner. In these cases, the use of pymetrozine should be abandoned and other types of insecticides should be used.

5. Conclusions

In summary, we established two fecundity assay bioassay methods to determine the sensitivity of the *N. lugens* pymetrozine-resistant strain and field populations to pymetrozine. Our findings demonstrate that all *N. lugens* strains tested using the traditional rice-stem dipping method were highly resistant to pymetrozine. The results of the fecundity assay methods showed that pymetrozine could also effectively inhibit the next-generation population of brown planthopper, and the resistance was low to moderate. Further studies revealed that pymetrozine interferes with the courtship behavior of males and the receptivity behavior and oviposition behavior of females, thus significantly inhibiting the fecundity of *N. lugens*.

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