Effect of Larvae Treated with Mixed Biopesticide Bacillus thuringiensis - Abamectin on Sex Pheromone Communication System in Cotton Bollworm, Helicoverpa armigera

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Abstract

Third instar larvae of the cotton bollworm (Helicoverpa armigera) were reared with artificial diet containing a Bacillus thuringiensis - abamectin (BtA) biopesticide mixture that resulted in 20% mortality (LD20). The adult male survivors from larvae treated with BtA exhibited a higher percentage of “orientation” than control males but lower percentages of “approaching” and “landing” in wind tunnel bioassays. Adult female survivors from larvae treated with BtA produced higher sex pheromone titers and displayed a lower calling percentage than control females. The ratio of Z-11-hexadecenal (Z11–16:Ald) and Z-9-hexadecenal (Z9–16:Ald) in BtA-treated females changed and coefficients of variation (CV) of Z11–16:Ald and Z9–16:Ald were expanded compared to control females. The peak circadian calling time of BtA-treated females occurred later than that of control females. In mating choice experiment, both control males and BtA-treated males preferred to mate with control females and a portion of the Bt-A treated males did not mate whereas all control males did. Our data support that treatment of larvae with BtA had an effect on the sex pheromone communication system in surviving H.armigera moths that may contribute to assortative mating.

Introduction

Pesticides provide a primary and non-substitutable method to control pests so that crop yield potential is conserved [1]. However, pesticide abuse is a serious problem all over the world and pesticide residue in fields can increase risks of threats to human health, inducing pesticide resistance, and killing of non-target species [2,3].

There are many studies that focus on the sublethal effects of pesticides on target pests and beneficial arthropods. A median lethal dose (LD50) or lethal concentration (LC50) estimate has been the classical laboratory method for assessing the side effects of pesticides on beneficial arthropods for decades but do not specifically address sublethal effects [2].

Many researchers have demonstrated that pesticides impact natural enemies directly or indirectly [4–11]. The longevity of Microplitis mediator, a parasitoid of the cotton bollworm (Helicoverpa armigera) decreased significantly after being fed with 10% honey water containing a sublethal dose of a Bacillus thuringiensis and abamectin (BtA) [10].

Studies of the sublethal effects of pesticides have also included work on agricultural pests that examined side effects on the sex pheromone and chemical communication systems of Lepidoptera. Sublethal permethrin treatment reduced the incidence of calling behavior of female pink bollworm (Pectinophora gossypiella) [12] and activation (wing fanning) of male survivors to sex pheromone [13]; similar results were obtained for cabbage loopers (Trichoplusia ni) female moths [14]. However, treatment of oriental fruit male moths (Cydia molesta) with octopamine induced hypersensitivity to olfactory signals [15], as did chloridimeform treatment of male T.ni [16]. A sublethal (LD1) dose of chloridimeform stimulated pheromone emission early in scotophase and exhibited a high percentage of calling behavior in treated T.ni moths [17]. However, female Asian corn borer (Ostrinia furnacalis) moths decreased sex pheromone titer and pheromone biosynthesis activating neuropeptide (PBAN)-like activity following treatment with deltamethrin [18].

All these studies were focused on sublethal effects of pesticides on the adult stage of lepidopteron moths. Since 2003, some researchers began to focus on adult survivors from larvae treated with a sublethal dose of pesticide and pests with high resistance to...
pesticides. Adults of O. furnacalis that survived treatment with deltamethrin during the first and third instars produced higher titers of sex pheromone, expanded coefficients of variation of the ratio of sex pheromones and displayed a lower response to sex pheromone in wind tunnel testing [19]. Tobacco cutworm moths (Spodoptera littoralis) emerging from larvae treated with a sublethal dose of deltamethrin performed similarly [20]. Male moths of H. armigera treated with Bt from the third larval instars showed higher EAG responses to sex pheromones than controls [21], as did tebufenozide and abamectin resistant diamondback (Plutella xylostella) male moths [22]. However, P. xylostella moths elicited similar EAG responses after exposure to indoxacarb compared with controls [23].

Biopesticide usage is increasing every year, even more with the respective that mixtures of pesticides are theoretically more effective in delaying resistance than alternating usage of pesticides [24]. An example of this strategy is the conjugation of the toxins from Bacillus thuringiensis with the toxin of abamectin, to form a new biopesticide called BAa [25]. This Ba mixture has been widely used to control agricultural pests [26–29] and the usage of Ba was in the hundreds of tons in several provinces of China in 2011. Despite this heavy use, how this biopesticide mixture affects both pests and natural enemies warrants further study. Effects of Ba on development of cotton bollworm and longevity of adult Mediator parasitoids has begun to be studied [10]. In this study, we continue to focus on effects of Ba on the sex pheromone communication system of adult survivors of H. armigera obtained from 3rd instar larvae reared on artificial diet containing sublethal doses of Ba. Although the individual effects of these two biopesticides on sex pheromone communication systems has been studied [22,29], additional work is needed to clarify the effects of the biopesticide mixture on the sex pheromone communication system. In this study, we evaluate the effects of Ba on calling behavior, production of sex pheromone in H. armigera females, the behavior responses of males to pheromone lures in a wind tunnel and mating choice in mating cages.

Methods and Materials

Insect

Larvae of H. armigera obtained from the laboratory of Zhejiang Academy of Agricultural Sciences (China) were reared at 25 ± 1°C, 75% relative humidity (r.h.), and L14:D10 photoperiod. They were separated into two experimental populations, one was reared on a standard artificial diet without any pesticides, while the other was reared on an artificial diet containing a sublethal dose of Ba applied during the 3rd larval instar. Larvae were first reared in groups in plastic casing (20 cm × 30 cm × 7 cm) with artificial diet until the 3rd instar, when they were transferred to and reared singly in plastic petri dishes (6 cm diam × 2 cm depth) where fresh diet was provided every 4 days. Pupae were separated by sex, and male and female moths were held in glass test tubes (3 cm diam × 10 cm high) with 10% honey water.

Pesticides and Sex Pheromones

The biopesticide Ba was tested, was composed of B. thuringiensis var. kurstaki (16,000 IU/mg, Fujian Pucheng Green Shell Biological Technology, Pucheng, China) and Abamectin (0.10%EC, Zhejiang Shenghua Biok Biological, Zhejiang, China) mixed by Sendedao Bioproducts (Wenzhou, China). Two sex pheromone components Z-11-hexadecenal (Z11–16:Al) and Z-9-hexadecenal (Z9–16:Al) were obtained commercially (Shin-Etsu Chemical Co. Ltd., Japan). The purity of these two pheromones was detected to be more than 95% by Gas Chromatography (GC) analysis.

Effects of Ba on Development from 3rd Instar Larvae until Eclosion in H. armigera

The method for this experiment was similar to that of Wanna et al. (2010) [10]. The sublethal Ba concentrations tested in the artificial diet were 0.5, 1, 2, 4 and 8 μg/g. The stock solutions were prepared by first dissolving Ba in distilled water and then adding it to the liquid diet at a temperature of 50°C before solidification. Neonate larvae were reared in plastic casings (20 cm × 30 cm × 7 cm) with standard artificial diet and then treatments were established by transferring single 3rd instar larvae into plastic petri dishes (6 cm diam × 2 cm high) provisioned with fresh artificial diet with different concentrations of Ba every 4 days until pupation. There were three replications for each concentration and 100 larvae per replication. Data on larval mortality, pupation rate, abnormal pupa rate, male pupa weight and female pupa weight were obtained at the end of the experiment. The surviving moths were held in glass tubes (3 cm diam × 10 cm high) with 10% honey water and used in subsequent experiments.

Calling Behavior

3-day-old virgin female moths were individually transferred into a glass test tube (3 cm diam × 10 cm high) with 10% honey water and put into a darkroom (25 ± 1°C, 75% r.h., and L14:D10 photoperiod). Calling behaviors were observed by eye with a dim incandescent red back light at 30 min intervals throughout the 10-h scotophase in entirely dark environmental conditions. Each female was scored as either calling or not calling. There were three replications for control females and Ba-treated females and 10 females per replication. Calling behavior characteristics observed were upward abdomen, wide spread wings with fanning, and ovipositor or gland visually evident protruding from the end of abdomen under red light (0.3lux).

Gas Chromatography Analysis

The ovipositors and associated sex pheromone glands were excised from 3-day-old virgin female moths during their peak calling period (7 h–8 h after the initiation of scotophase). Glands were forced out by squeezing the abdomen and excising the tip at the 8–9th abdominal segment with ophthalmic scissors (Figure 1). Each gland was soaked in 10 μL redistilled hexane for 30 min to extract pheromone components. Then, the ovipositors and associated sex pheromone glands were removed and the extracts were maintained at 0°C until GC analysis by using standard methods. The GC-standards (GCS) of Z11–16:Al and Z9–16:Al in hexane were detected by GC first with concentrations of 25, 50, 100, 200 and 400 ng/μL. The injection volume was set at 1 μL. Standard curves were drawn by calculating the peak areas of GCS. The extracts were concentrated to 2 μL, and then the concentrated extracts were injected into a GC (GC-2010, Shimadzu, Japan) equipped with a flame ionization detector (FID), a split/splitless injector, and a fused silica capillary column (RTX-5, 30 m × 0.25 mm ID, 0.25 μm film thickness, SGE, Australia) in splitless mode. Nitrogen was used as the carrier gas. The oven temperature was maintained at 100°C for 2 min, and programmed at 10°C /min to 200°C. The injector and FID temperature were set at 220°C and 250°C. The quantity of each component was calculated based on the peak area, and calibrated by comparing it with standard curves of Z11–16:Al and Z9–
16:Ald. 22 glands of both control females and BtA-treated females were extracted and tested.

**Wind Tunnel Bioassay**

The methods for wind tunnel tests were similar to Deng et al. (2004) [31]. The tests were performed in a Plexiglas wind tunnel, 230 cm x 90 cm x 90 cm under conditions of 25±1°C, 75% r.h., 0.3 lux (red light), and air speed of 0.3 m/s. Naïve male moths were tested at the 3rd scotophase by exposure to sex pheromone. Before the onset of scotophase, both control male moths and adult male survivors from larvae treated with BtA were transferred individually into glass test tubes (3 cm diam x 10 cm high). All tubes were held until 7th hr into scotophase. Moths were allowed to acclimate to tunnel conditions for 60 min, and then introduced into the tunnel individually. Lure (filter paper) containing 300 ng of the 97:3 blend of Z11–16:Ald and Z9–16:Ald was pinned on a 25 cm high iron shelf placed on the midline of the wind tunnel and 25 cm away from the up-wind end. The iron shelf with lure was packaged with a cylindrical wire netting cage (10 cm diam x 25 cm high). Male moths were introduced into the wind tunnel by using an open-ended release cage (5 cm diam x 10 cm high) placed 17 cm high and 200 cm from the lure. Each male was allowed 2 min to respond and scored for the following behaviors: taking flight (TF), orientation flight (OR), half up-wind to lure (HW), approaching the cage containing the lure (within 10 cm) (APP), landing on the cage containing the lure (LA) (Figure 2). 69 control males and 81 BtA-treated males were tested. Each male was tested only once.

**Mating Choice Experiment**

3-day-old virgin moths were used in this experiment. One group consisted of 10 control males and 10 BtA-treated and control females. The other group was 10 BtA-treated males and 10 BtA-treated and control females. These moths were introduced into mating cages (120 cm x 80 cm x 80 cm) before scotophase. Both BtA-treated males and females were marked on their wings with carbon black ink (this does not interfere with males' mating behavior). Three replicates were performed for each group (Figure 3). Observations were made at 1 h intervals throughout the 10 h scotophase by using red light (0.3 lux). Male moths were removed if mated. Experiments were tested in darkroom under conditions of 25±1°C, 75% r.h. and 10 h scotophase in entirely dark environmental conditions.
Statistics

Data analysis for development of *H. armigera* was performed using LSD test (*P* < 0.05). Statistical comparisons of percentage of calling behaviors and female mating percentages between control moths and moth survivors from the larval treatment with BtA used the student’s t test. The distribution of male moth responses to lures in the wind tunnel bioassay used a $\chi^2$ test. Mean titers of sex pheromones between control females and BtA-treated females were analyzed using a nonparametric test (Mann-Whitney U test). Percentage data were arcsine square root transformed before analysis. All data were analyzed by IBM Statistics SPSS 19.0 and DPS 9.5 [32].

Results

Effects of BtA on Development of *H. armigera* from 3rd Instar Larva to Eclosion

Treatment with BtA significantly affected the development of *H. armigera* (Table 1). The mortality of 3rd instars after feeding with BtA diet was significantly higher (*P* < 0.05) than the control. In our assays, a BtA concentration of 2 µg/g resulted in 20% mortality and subsequent experimentation was done with this concentration compared to control. The means of pupation rate, normal pupa rate and male pupal weight of larvae treated with treatment from 0.5 µg/g to 8 µg/g of BtA were significantly lower (*P* < 0.05) in controls, but the mean of female pupal weight was significantly higher (*P* < 0.05) in BtA-treated compared to controls. Also, male and female mean pupal stages were delayed with larval treatment from 0.5 µg/g to 8 µg/g and most pupae were in a diapause condition (four eye spots in line observed on the compound eye).

Calling Behavior of Females

The periodicity of calling behaviors by females that survived treatment as 3rd instar larvae with BtA was similar to that of the control (Figure 4). Control females started calling after 1 h into the 10-h scotophase, but BtA-treated females started 0.5 h later (Figure 4). From 0.5 h to 2.5 h and 8 h to 9 h, there were no significant differences in percentages of calling between control females and female survivors (Figure 4). From 3 h to 6 h and 9.5 h to 10 h, calling percentages of control females were significantly higher than female survivors (*P* < 0.05) except at 4 h (Figure 4). Compared with BtA-treated females, calling percentages of control females were significantly higher (*P* < 0.01) during the calling peak period (6.5 h to 8 h) (Figure 4). Calling peak time of BtA-treated females was at 8 h, but calling percentage of control females was decreased from their peak at this time. After calling peak periods, both calling percentages had decreased sharply but there were still some females that kept calling after scotophase ended.

GC Analysis of Female Sex Pheromones

The mean titers of sex pheromones from control females conformed to a normal distribution but BtA-treated females did not. These data were analyzed using a nonparametric test (Mann-Whitney U test). The BtA-treated females produced significantly higher (*P* < 0.01) amounts of Z11–16:Ald and Z9–16:Ald compared to control females (Table 2). The mean blend ratio of Z11–16:Ald and Z9–16:Ald in BtA-treated females was 5.80 ± 0.28 and significantly different (*P* < 0.01) from that of control females (9.76 ± 0.16) (Table 2). In addition, coefficient of variations (CV) in Z11–16:Ald, Z9–16:Ald and the sum of BtA-treated females were 52.57%, 44.74% and 50.77%, significantly higher (*P* < 0.01) than that of control females (10.62%, 12.55% and 10.54%) (Table 2).

Wind Tunnel Bioassay

Compared with control males, the behavioral responses of male survivors from 3rd instar larvae treated with BtA were similar in “TF” and “HW” behavior, however, the percentage of BtA-treated males was significantly higher (*P* < 0.05) than control males in “OR” behavior (Figure 5). There were significant differences (*P* < 0.01) between BtA-treated males and control males in behaviors of “APP” and “LA” and percentages of BtA-treated males approaching to the cage and landing on the cage containing the lure were significantly lower (*P* < 0.01) than in controls (Figure 5).
Mating Choice

Results from two non-random mating choice experiments showed that control and BtA-treated males preferred to mate with control females. Percentages of mating control females with control males and BtA-treated males were both significantly higher (P < 0.01) than for BtA-treated females (Figure 6). The percentage of unmated BtA-treated males was 16.67%.

Discussion

Chemical communication systems in moths may change due to selection factors operating in the natural and the managed environment. Female *P. gossypiella* moths emitted significantly more sex pheromone when subjected to mating disruption for population control [33]. In this study, we found changes in the sex pheromone communication system in *H. armigera* under treatment by BtA.

A significant reduction in male pupal weight was observed after feeding with artificial diet containing different concentrations (from 0.5 μg/g to 2 μg/g) of BtA especially at the concentration of 2 μg/g (Table 1). Wanna et al. (2010) showed that mean pupal weight decreased significantly as BtA concentration increased [10]. We speculate that larvae strong enough to survive with BtA treatment and display an increased mean pupal weight may have improved fitness to contribute to subsequent generations. However, there was no evidence of correlation between pupal weight and sex pheromone titer. Similar results were achieved by Miller and Roelofs (1980), Charlton and Cardé (1982), Schal and Cardé (1987), and Löfstedt et al. (1985) [34–37].

The female survivors from the larvae treated with a lethal dose (LD20) of BtA reduced the percentage of calling while the periodicity of female survivors was similar to control females (Figure 4). The start calling time and peak calling timing of BtA-treated females was delayed slightly compared with control females (Figure 4). Female *P. xylostella* moth resistance to tebufenozide and abamectin exhibited a similar trend [22], as did *S. littura* [20] and *O. furnacalis* female survivors with larvae treated with deltamethrin [19]. Our results were also consistent with those in adult moths treated with pesticides such as *O. furnacalis* dosed with deltamethrin [18], *P. gossypiella* dosed with permethrin [12]...
Effects of BtA on development of Helicoverpa armigera from 3rd larvae to eclosion (Mean ± SEM) N = 100.

| No. of normal male pupa weight (mg) | No. of normal male pupa stage (day) | Female pupal stage weight (mg) | Female pupal stage (day) | Female pupal weight (mg) | No. of female normal pupa rate (%) | Pupation rate normal pupa rate(%) | Mortality (%) |
|-------------------------------------|-------------------------------------|-------------------------------|--------------------------|--------------------------|----------------------------------|---------------------------------|---------------|
| 3.6                                | 3                                   | 0.5                           | 98.96 ± 0.39             | 91.12 ± 0.92             | 82.61 ± 2.13                  | 128.71 ± 2.84                  | 99.33 ± 1.15 |
| 3.1                                | 3.5                                 | 0.6                           | 93.93 ± 1.15             | 97.81 ± 2.93             | 87.23 ± 2.64                  | 133.74 ± 3.58                  | 93.31 ± 1.64 |
| 2.8                                | 3.6                                 | 0.7                           | 94.33 ± 1.56             | 99.33 ± 2.96             | 87.23 ± 2.64                  | 134.74 ± 3.58                  | 93.31 ± 1.64 |
| 2.5                                | 3.7                                 | 0.8                           | 94.33 ± 1.56             | 99.33 ± 2.96             | 87.23 ± 2.64                  | 134.74 ± 3.58                  | 93.31 ± 1.64 |
| 2.2                                | 3.8                                 | 0.9                           | 94.33 ± 1.56             | 99.33 ± 2.96             | 87.23 ± 2.64                  | 134.74 ± 3.58                  | 93.31 ± 1.64 |
| 2.0                                | 3.9                                 | 1.0                           | 94.33 ± 1.56             | 99.33 ± 2.96             | 87.23 ± 2.64                  | 134.74 ± 3.58                  | 93.31 ± 1.64 |
| 1.8                                | 4.0                                 | 1.1                           | 94.33 ± 1.56             | 99.33 ± 2.96             | 87.23 ± 2.64                  | 134.74 ± 3.58                  | 93.31 ± 1.64 |
| 1.6                                | 4.1                                 | 1.2                           | 94.33 ± 1.56             | 99.33 ± 2.96             | 87.23 ± 2.64                  | 134.74 ± 3.58                  | 93.31 ± 1.64 |
| 1.4                                | 4.2                                 | 1.3                           | 94.33 ± 1.56             | 99.33 ± 2.96             | 87.23 ± 2.64                  | 134.74 ± 3.58                  | 93.31 ± 1.64 |
| 1.2                                | 4.3                                 | 1.4                           | 94.33 ± 1.56             | 99.33 ± 2.96             | 87.23 ± 2.64                  | 134.74 ± 3.58                  | 93.31 ± 1.64 |
| 1.0                                | 4.4                                 | 1.5                           | 94.33 ± 1.56             | 99.33 ± 2.96             | 87.23 ± 2.64                  | 134.74 ± 3.58                  | 93.31 ± 1.64 |
| 0.8                                | 4.5                                 | 1.6                           | 94.33 ± 1.56             | 99.33 ± 2.96             | 87.23 ± 2.64                  | 134.74 ± 3.58                  | 93.31 ± 1.64 |
| 0.6                                | 4.6                                 | 1.7                           | 94.33 ± 1.56             | 99.33 ± 2.96             | 87.23 ± 2.64                  | 134.74 ± 3.58                  | 93.31 ± 1.64 |
| 0.4                                | 4.7                                 | 1.8                           | 94.33 ± 1.56             | 99.33 ± 2.96             | 87.23 ± 2.64                  | 134.74 ± 3.58                  | 93.31 ± 1.64 |
| 0.2                                | 4.8                                 | 1.9                           | 94.33 ± 1.56             | 99.33 ± 2.96             | 87.23 ± 2.64                  | 134.74 ± 3.58                  | 93.31 ± 1.64 |
| 0.0                                | 4.9                                 | 2.0                           | 94.33 ± 1.56             | 99.33 ± 2.96             | 87.23 ± 2.64                  | 134.74 ± 3.58                  | 93.31 ± 1.64 |

Means within the same column followed by the same letter are no significantly different (LSD test: P<0.05).

Table 1. Effects of BtA on development of Helicoverpa armigera from 3rd larvae to eclosion (Mean ± SEM) N = 100.

Table 2. High sex pheromone titers did not directly translate to an increase in release rate as shown in Figure 4 and Table 2. High emitting rate would increase the distance of communication [37], but the ratio of components is a more important factor [22,54]. The mean ratio of Z11–16:Ald and Z9–16:Ald produced by BtA-treated females was shifted from 9.76 to 5.80 (Table 2), similar to that found in abamectin-resistant P. xylostella [22]. This shift of blend ratio may also lead to a lower mating rate of female survivor.
Figure 4. Calling percentages of female moths. The moths were recorded as calling or not calling during a 10-h scotophase after treating 3rd instar larvae with BtA artificial diet (N = 30) or standard artificial diet (N = 30). The data (Mean ± SEM) marked with ** and *** are significantly different at P < 0.05 and P < 0.01 based on student’s t test after mean percentages were arcsine square-root transformed.
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Table 2. Mean pheromone titer(ng)±SEM (CV%) and blend ratio in BtA and control females of *H. armigera* (N = 22).

| Treatment | Mean Titer(ng) ± SEM (CV%) | Z11–16:Ald | Z9–16:Ald | Sum | ratio\(^b\) |
|-----------|-----------------------------|------------|------------|-----|------------|
| Control   | 47.73 ± 1.08(10.62)         | 4.92 ± 0.23(12.55) | 52.64 ± 1.18(10.54) | 9.76 ± 0.16** |
| BtA       | 166.45 ± 19.10(52.57)**     | 28.76 ± 2.81(44.74)** | 195.21 ± 21.62(50.77)** | 5.80 ± 0.28 |

*The data marked with ** are significantly different at P < 0.01 by using a nonparametric test (Mann-Whitney U test). CV = SD/Mean.

\(^b\)The blend ratio is Z11–16:Ald/Z9–16:Ald.
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Biocide on Moth Sex Pheromone Communication System
We initiated using *H. armigera* as a model organism to evaluate the effect of mixed BtA biopesticide on the sex pheromone communication system and other reproductive behaviors.

The specific mechanisms for why the sex pheromone communication system changed after larvae were treated with BtA remains unclear; a better understanding of how BtA affects *H. armigera* could point us to a course of action that may include using a new ratio of lure, a more suitable dose of pesticide in the field, etc. to accommodate these changed reproductive behaviors.

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**Figure 5. Behavioral responses of male moths to a pheromone lure in the wind tunnel bioassays.** In lure (filter paper), the dosage of sex pheromones was 300 ng with the 97:3 blend of Z11-16:Ald and Z9-16:Ald. Five parameters taken in the wind tunnel: TF = taking flight, OR = orientation flight, HW = half up-wind to lure, APP = approaching the cage containing the lure (within 10 cm), LA = landing on the cage containing the lure. The data marked with ‘*’ and ‘**’ are significantly different at *P* < 0.05 and *P* < 0.01 as shown by a χ² test.

**Figure 6. Percentages of females that mated with control males and BtA females.** The bars (Mean ± SEM) marked with ‘***’ are significantly different at *P* < 0.01 by using Student’s *t* test after percentages were arcsine root square transformed.

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Author Contributions

Conceived and designed the experiments: LZS ZHX JYD. Performed the experiments: LZS PZC FMW. Analyzed the data: LZS JYD GXZ. Contributed reagents/materials/analysis tools: LZS JYD MKH RW FMW. Wrote the paper: LZS ZHX MKH.

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