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Insecticide-induced increase in the protein content of male accessory glands and its effect on the fecundity of females in the brown planthopper *Nilaparvata lugens* Stål (Hemiptera: Delphacidae)

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ABSTRACT

The brown planthopper *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) is a classical resurgent rice pest induced by insecticides. The past focus on resurgence mechanisms has been on the stimulation of the reproduction of adult females induced by insecticides. To date, the role that males play as a resurgence of *N. lugens* has not been investigated. The present study examined changes in protein levels in both male accessory glands and female ovaries induced by the insecticides triazophos and deltamethrin as well as the stimulating effect of treated males on the fecundity of adult females via mating following foliar sprays of the insecticides. For adults that had been exposed as nymphs to treated rice plants, the protein content in both the male accessory glands and in the female ovaries of *N. lugens* were significantly affected by male mating status, insecticide and insecticide concentration. There was a higher protein content in male accessory glands when males were exposed to triazophos as third instars compared to fifth instars, and there was a higher protein content before mating compared to after mating. In addition, the protein levels in male accessory glands after mating for individuals exposed to high doses of the two insecticides as 3rd and 5th instars were significantly lower than untreated control except for exposed to triazophos as 3rd instar, indicating that treated males transferred more male accessory gland protein to adult females via mating. The protein content was also affected by different combinations of treated mating pairs. Adult males (σ_t) developed from third instar nymphs treated with triazophos stimulated the fecundity of the female significantly via mating ($\sigma_t \times \varphi_{ck}$) with untreated females (φ_{ck}) (control females), increasing the reproductive rate by 43.5% as compared to the mating ($\sigma_{ck} \times \varphi_{ck}$) of untreated males and females. Also, the fecundity of the females after the mating ($\sigma_t \times \varphi_t$) of treated males and females was significantly higher than that after the mating ($\sigma_{ck} \times \varphi_t$) of untreated males with treated females. These findings indicated that the reproductive effects of insecticide on males can be transferred to females via mating. The present findings provide valuable information for understanding the potential role that males play in the pesticide-induced resurgence of *N. lugens*.

1. Introduction

The wide-spread use of chemical pesticides is known to induce the population resurgence of some pests (Reissig et al., 1982; Wang et al., 1994; Gu et al., 1996; Yin et al., 2008). The brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), is a classical one of such pest insects, recent outbreaks of which in China and other Asian countries were primarily associated with the overuse of pyrethroids and organophosphates as well as resistance to imidacloprid (Chelliah and Heinrichs, 1980; Gao et al., 1988, 2006; Liu and Liao, 2006). The pesticide-induced resurgence of *N. lugens* has been attributed to the destruction of natural enemies (Fabellar and Heinrichs, 1986; Gao et al., 1988) and the stimulation of reproduction (Gu et al., 1984; Wang et al., 1994; Zhuang et al., 1999; Yin et al., 2008). Although insecticide-induced stimulation of fecundity is regarded as one of the main causes for *N. lugens* resurgence, the role that the males play in this population process has not been well understood.

Male insects transfer not only sperms but also accessory gland products (AGPs) in mating, though the latter may be expensive to

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produce; hence with the amount or quality of these substances decreasing with increasing male mating frequency (Svard and Wiklund, 1986; Rogers et al., 2005). AGPs transferred during mating are known to affect a number of behaviors in insects, of which a reduction in female receptivity and an increase in oviposition are the most common (Cordero, 1995; Gillott, 2003). In the variable field cricket *Gryllus lineaticeps* Stål, AGPs were also found to increase the female lifespan (Wagner et al., 2001). Many studies with other insects have also demonstrated that male accessory gland peptides (mating factors) regulate the reproductive performance of females. For example, the male accessory glands of *Drosophila melanogaster* Meigen synthesize and secrete a peptide that represses female sexual receptivity and stimulates oviposition (Chen et al., 1988). Multiple matings in the female moths *Utetheisa ornatrix* (L.) bring about an increase in fecundity, but not in longevity or egg mass (Lamunyon, 1997). Lay et al. (2004) found an extension of the role of the male *Locusta migratoria* (L.) in reproduction, with peptides from white secretions of male accessory glands detected in developing eggs, while Seth et al. (2002) reported that the fecundity of adult female *Spodoptera litura* F. increases when females mate with the male moths of high quality sperms. More interestingly, Pszczolkowski et al. (2006) found that in *Heliothis virescens* (F.) the juvenile hormone (JH) stored in the male accessory glands was transferred to the females via mating and this promoted the JH synthesis and egg development of the mated females. On the other hand, the effect of insecticides on male insects and its transference effect on the reproductive performance of adult females via mating have been scarcely investigated so far.

The objective of the present investigation was to examine the effects of sub-lethal doses of two insecticides on protein contents in the male accessory glands and the female ovaries of *N. lugens*, as well as the stimulating effect of insecticide-treated males on the fecundity of the females via mating. The two insecticides used in this study were triazophos and deltamethrin that had been applied to control rice stem borers and rice leafroller for many years, and were found to induce the population resurgence of *N. lugens*.

2. Materials and methods

2.1. Rice variety, insects and insecticides

The rice (*Oryza sativa* L.) variety Shengyou 1 (japonica rice) was used in the trials. This variety of rice was selected because it is commonly planted in Jiangsu province, China. Seeds were sown outdoors in a standard rice-growing soil in cement tanks (height 60 cm, width 100 cm and length 200 cm). When seedlings reached the 6-leaf stage, they were transplanted into 16 cm diameter plastic pots, with four hills per pot, and two plants per hill. Rice plants used in the experiments were at the tillering stage.

A laboratory strain of *N. lugens*, originally obtained from China National Rice Research Institute (CNRRI; Hangzhou, China), was reared in a greenhouse at Yangzhou University.

Two insecticides were used in the trials: the pyrethroid 2.5% deltamethrin EC (Yangnon Chemical Co. Ltd., Yangzhou, Jiangsu, China) and the organophosphate 20% triazophos EC (Changqin Pesticide Co. Ltd., Jiangdu, Jiangsu, China).

2.2. Experiments

In 2008, two concentrations of each insecticide, i.e. 15 and 30 ppm of deltamethrin and 25 and 50 ppm of triazophos, were selected based on previous results of a sub-lethal test (Samer et al., 2009). A total of 80 third instar nymphs and 80 fifth instar nymphs were released onto each hill, with separate pots for each instar. Twenty-four hours after being infested, foliar sprays were applied to the rice plants at the tillering stage using a Jacto sprayer (Maquinas Agricolas Jacto S.A., Brazil) equipped with a cone nozzle (1-mm diameter orifice, pressure 45 psi, flow rate 300 ml/min). Control plants at the same stage were sprayed with tap water. Each treatment and control was replicated three times. The treated and control plants were covered with cages (screen size: 80-mesh). The nymphs on the treated and control plants were collected at 36 h following foliar sprays and a single nymph was placed in a glass jar (diameter 10 cm, height 12 cm) with untreated rice plants (26 ± 1 °C and 16L:8D). After adult emergence, males and females were separated, with 20 males per replicate used to measure total protein content in male accessory glands (MAGs). The females and the other males were used to evaluate changes in protein content in the MAGs with different mating combinations of treated and untreated adults. The four mating combinations included: 1) treated males ($♂_t$) × treated females ($♀_t$), 2) $♂_t$ × untreated females ($♀_{ck}$), 3) untreated males ($♂_{ck}$) × $♀_t$, and 4) $♂_{ck}$ × $♀_{ck}$. For each of these four combinations, there were three replicates, each of 15 males that were used in determining protein content in the MAGs prior to mating and 48 h after mating.

2.3. The transference effect of triazophos-treated males on the fecundity of females via mating

In 2009, the experiment of different mating pairs was conducted to evaluate the stimulating effect of insecticide-treated males on the fecundity of females. The third instars were exposed to 42 ppm (LC_{50}) triazophos in the same manner described above. Two adults (female × male) after emergence were placed in a cage (60-mesh) covering a pot (height 50 cm, diameter 30 cm) containing rice plants untreated at the booting stage for egg-laying under natural conditions. Insect mortalities were checked at 24 h after the release of adults, and dead adults (if any) were replaced with live ones of the same age. The numbers of eggs laid per female were counted under a microscope. Eggs were scraped from the leaf sheaths and leaf blades using a pin. Each treatment and control was replicated twenty times (20 mating pairs). Mating pairs were designed as follows: 1) $♂_{ck} \times ♀_{ck}$, 2) $♂_{ck} \times ♀_t$, 3) $♂_t \times ♀_{ck}$ and 4) $♂_t \times ♀_t$.

2.4. Extraction of total protein

Protein content was measured using the method described by Li and Yu (1997). The MAGs from each of the males and the ovaries from each of the females within a replicate were removed, placed in ice-cold physiological saline solution under a microscope, and washed with phosphate buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 were dissolved in 800 ml distilled water, adjusted with HCl to pH 7.4 and fixed with distill water to 1000 ml). The MAGs and ovaries were homogenized on ice, placed on ice centrifugal tubes and centrifuged at 12,000 rpm at 4 °C for 25 min. The supernatant was collected after removing the upper fat layer. Sediment in the tubes was washed with distilled water three times and centrifuged again, and the supernatant was put into the tubes.

2.5. Measurement of protein content

The procedure described in Li and Yu (1997) was followed to measure protein content with Coomassie Brilliant Blue R 250 (Shanghai Chemical agent Co., Ltd., Shanghai, China). A standard curve was established based on a standard protein (bovine serum albumin, made in Shanghai Biochemistry Research Institute, Shanghai, China). The absorbance at 670 nm was determined in a UV755 B spectrometer (Shanghai Precision Instrument Co., Ltd,

Shanghai, China). Protein content in the sample solution was calculated according to the standard curve.

2.6. Statistical analysis

Data were tested for normality and homogeneity of variance prior to use for statistical analysis. The data of protein contents in the male accessory glands were analyzed using 3-way Analysis of Variance (ANOVA) (insecticide, concentration and mating status). The data for the numbers of eggs laid were analyzed using one-way ANOVA. Multiple comparisons of means were conducted for each exposure age for each insecticide, using Fisher's Protected Least Significant Difference (PLSD) test. All analyses were conducted using the GLM procedure (SPSS Inc, 2002).

3. Results

3.1. Protein contents from before and after mating

When the insects were exposed to insecticides as third instars, protein content in the MAGs was found to be significantly affected by mating status (A) ($F = 126.6$, df = 1, 24, $P < 0.001$), insecticide type (B) ($F = 322.2$, df = 1, 24, $P < 0.001$), insecticide concentration (C) ($F = 85.4$, df = 2, 24, $P < 0.001$), and all interactions between these factors ($F = 21.0$, df = 1, 24, $P < 0.001$ for A × B; $F = 38.3$, df = 2, 24, $P < 0.001$ for A × C; $F = 83.3$, df = 2, 24, $P < 0.001$ for B × C; $F = 16.0$, df = 2, 24, $P < 0.001$ for A × B × C). As shown in Table 1, exposure to triazophos and deltamethrin resulted in a 41 and 40% decrease, respectively, in protein content in the MAGs after mating, whereas the untreated control had a 5% increase in the amount of protein after mating, compared to before mating. For triazophos, the highest levels of protein contents were found for both treatment concentrations before mating and were significantly higher than the protein contents after mating; the low treatment concentration after mating was not significantly different from either the untreated control either before or after mating. For deltamethrin, the highest rate of protein was found with the high dose before mating, followed by the low dose before mating, which was not significantly different from the untreated control before mating. However, the protein content in MAGs after mating for 15 ppm deltamethrin was significantly lower than the untreated control.

When the insects were exposed to insecticides as fifth instars, the amount of protein in the MAGs was found to be significantly affected by mating status ($F = 15.1$, df = 1, 24, $P < 0.001$), insecticide type ($F = 27.8$, df = 1, 24, $P < 0.001$), and the interactions mating status × insecticide concentration ($F = 61.4$, df = 2, 24, $P < 0.001$) and insecticide type × insecticide concentration ($F = 7.2$, df = 2, 24, $P < 0.01$). Protein content after mating was reduced by 51 and 45% when there was exposure to triazophos and deltamethrin, respectively, whereas the untreated control resulted in an 85% increase in protein content after mating. For triazophos, the highest protein content occurred with the high dose before mating, which was significantly higher than the untreated control. However, the lowest protein content occurred with the high dose after mating, and it was significantly lower than all the other treatments. For deltamethrin, both doses before mating resulted in significantly higher protein content. Similarly, the protein content after mating for high dose treatment was significantly lower than untreated control. These findings indicated that treated males transferred more MAG protein to adult females via mating in comparison with before mating and after mating.

3.2. Protein content with different mating combinations

The protein content was affected by different combinations of mating pairs ($♂_t \times ♀_t$, $♂_t \times ♀_{ck}$, $♂_{ck} \times ♀_t$, and $♂_{ck} \times ♀_{ck}$), but the response was not consistent for different treatments (Table 2). For triazophos, the adult male accessory glands protein content was significantly affected by insecticide concentration ($F = 5.4$, df = 1, 14, $P < 0.05$ for exposed as third instar; $F = 33.2$, df = 1, 14, $P < 0.001$ for exposed as fifth instar), mating combination ($F = 10.2$, df = 3, 14, $P < 0.001$ for exposed as third instar; $F = 98.8$, df = 3, 14, $P < 0.001$ for exposed as fifth instar), and the interaction between these two variables ($F = 3.2$, df = 3, 14, $P < 0.05$ for exposed as third instar; $F = 5.1$, df = 3, 14, $P < 0.01$ for exposed as fifth instar). For deltamethrin, the adult male accessory gland protein content was significantly affected by mating combination ($F = 12.3$, df = 3, 14, $P < 0.001$ for exposed as third instar; $F = 8.9$, df = 3, 14, $P < 0.001$ for exposed as fifth instar), but not insecticide concentration, regardless of whether the adult had been exposed as a third or fifth instar.

The exposure of third instars resulted in higher protein content than the exposure to fifth instars for triazophos, whereas the reverse was true with deltamethrin. Differences between the two insecticides were also apparent in that the protein content of *N. lugens* exposed as third instars was 2.8 times higher for triazophos compared to deltamethrin, and the protein content of MAGs exposed as fifth instars was 1.8 times higher for deltamethrin compared to triazophos. The overall trend between the

Table 1
Protein content in the accessory glands of males developed from *N. lugens* third and fifth instar nymphs treated with insecticides.

Insecticide	Mating status	Concentration (ppm)	Protein content (μg/male) in adults exposed as third instars		Protein content (μg/male) in adults exposed as fifth instars	
			Mean	SE	Mean	SE
Triazophos	Before mating	0	36.4c	2.7	20.8c	0.8
		25	104.7a	7.3	32.7b	3.0
		50	109.8a	6.5	34.1ab	0.4
	After mating	0	38.4c	2.6	38.4a	2.6
		25	47.4c	6.7	21.4c	0.9
		50	78.1b	4.1	11.5d	1.9
Deltamethrin	Before mating	0	36.4bc	2.7	20.8c	0.8
		15	37.0b	1.4	47.4a	4.6
		30	49.6a	3.4	53.5a	3.2
	After mating	0	38.4b	2.6	38.4b	2.6
		15	26.4cd	5.4	31.9b	2.1
		30	25.7d	3.5	23.3c	3.2

Within each insecticide and column, means followed by the same letter do not differ significantly ($\alpha = 0.05$, ANOVA followed by Fisher's protected least significant difference test).

Table 2
Protein content in the male accessory glands after mating, with different mating combinations of treated and untreated males and females that had developed from third and fifth instars exposed to insecticides.

Insecticide	Insecticide Concentration (ppm)	Mating combination	Protein content (μg/male) in adults exposed as third instars		Protein content (μg/male) in adults exposed as fifth instars	
			Mean	SE	Mean	SE
Triazophos	0	$♂_{ck} \times ♀_{ck}$	38.4c	2.6	38.4a	2.6
	25	$♂_{t} \times ♀_{t}$	47.4bc	6.7	21.4c	0.9
		$♂_{t} \times ♀_{ck}$	54.4b	4.0	26.9b	1.2
		$♂_{ck} \times ♀_{t}$	85.9a	6.3	29.7b	1.2
	50	$♂_{t} \times ♀_{t}$	78.1a	4.1	11.5d	1.9
		$♂_{t} \times ♀_{ck}$	85.8a	6.5	20.9c	1.1
		$♂_{ck} \times ♀_{t}$	80.9a	5.9	25.4c	1.8
Deltamethrin	0	$♂_{ck} \times ♀_{ck}$	38.4a	2.6	38.4bc	2.6
	15	$♂_{t} \times ♀_{t}$	26.4b	5.4	31.9c	2.1
		$♂_{t} \times ♀_{ck}$	22.1b	4.2	53.0a	2.2
		$♂_{ck} \times ♀_{t}$	32.4ab	3.0	53.1a	2.2
	30	$♂_{t} \times ♀_{t}$	25.7b	3.5	23.3d	3.2
		$♂_{t} \times ♀_{ck}$	21.2b	4.1	40.6b	4.5
		$♂_{ck} \times ♀_{t}$	29.1b	3.0	42.3b	3.1

Within each insecticide and column, means followed by the same letter do not differ significantly at the 5% level ($\alpha = 0.05$ ANOVA followed by Fisher's protected least significant difference test).

high and low doses also differed for each insecticide. On average the high dose resulted in 13% higher protein content for triazophos compared to the low dose, whereas for deltamethrin the high dose resulted in 17% lower protein content compared to the low dose. There was also variability among the different combinations of mating pairs, although for both the third and fifth instars exposed to triazophos, the pairs with both sexes treated ($♂_t \times ♀_t$) always had the lowest protein content; this was also found for pairs exposed as fifth instars to deltamethrin, but not for third instars exposed to deltamethrin.

3.3. Effect of triazophos-treated males on the fecundity of females via mating

ANOVA of the numbers of eggs laid for different mating pairs showed that treated males or females significantly influenced the fecundity of the females ($F = 13.6$, df = 3, 72, $P = 0.0001$). The adult males ($♂_t$) developed from the third instar nymphs treated with triazophos significantly stimulated the fecundity of the females via mating ($♂_t \times ♀_{ck}$) with untreated females ($♀_{ck}$) (control females), increasing the reproductive rate by 43.5% as compared to the mating ($♂_{ck} \times ♀_{ck}$) of untreated males with females (Fig. 1). Also, the fecundity of the females after the mating ($♂_t \times ♀_t$) of treated males and females was significantly higher than that after the mating ($♂_{ck} \times ♀_t$) of untreated males with treated females, with an increase of 20.5%. No significant fecundity difference between $♂_t \times ♀_t$ and $♂_t \times ♀_{ck}$ was found, indicating that the insecticide-induced reproductive effects of the males were transferred to females via mating and stimulated the fecundity of the females.

4. Discussion

Exposure to sub-lethal doses of triazophos and deltamethrin affected protein content in the male accessory glands (MAGs) via mating, thus demonstrating a potential role that males play in the pesticide-induced resurgence of *N. lugens*. In general, there was higher protein content in the MAGs when males were exposed to insecticides as third instars compared to fifth instars, and there was higher protein content before mating compared to after mating. The protein content was also affected by different combinations of mating pairs ($♂_t \times ♀_t$, $♂_t \times ♀_{ck}$, $♂_{ck} \times ♀_t$, and $♂_{ck} \times ♀_{ck}$), but the response was not consistent for different treatments, as exposure to deltamethrin and triazophos often had opposite trends (e.g., the higher protein content was associated with fifth instars for triazophos vs. third instars for deltamethrin exposure, and the higher dose resulted in a higher protein content for triazophos-exposed instars and a lower protein content for deltamethrin-exposed instars). Total proteins in MAGs and in female ovaries may also include cellular components due to the insect being too small to obtain the protein, except cellular components. The current findings showed that the protein content in insecticide-treated MAGs after mating was significantly lower than that in control MAGs except for individual exposed to triazophos as 3rd instars. This demonstrates that insecticide-treated males transmitted more accessory gland products into females via mating relative to control males. In insects, secretions from the MAGs are known to improve a male's chances of siring offspring by a variety of means, including sperm mobility, sperm storage, stimulation of ovulation/oviposition, and egg protection (Gillott, 2003). For example, Garcia-Gonzalez and Simmons (2005) demonstrated that greater sperm viability was positively correlated with the proportion of offspring sired for the cricket

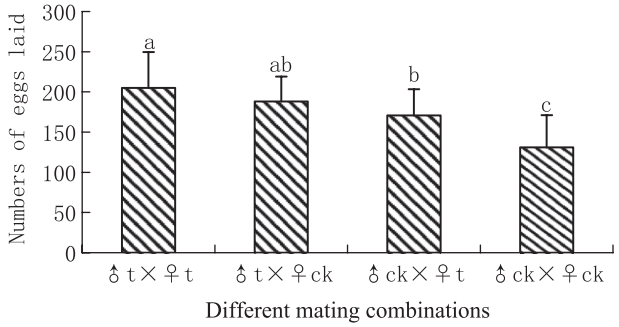

Fig. 1. Effect of different mating pairs of triazophos-treated males and/or females on the fecundity of the females. $♂_t \times ♀_t$, $♂_t \times ♀_{ck}$, $♂_{ck} \times ♀_t$ and $♂_{ck} \times ♀_{ck}$ are mating pairs of treated males with females, treated males with untreated females, untreated males with treated females and untreated males with females, respectively. Bars with different letters indicate that means differ significantly at the $P = 0.05$ level.

Teleogryllus oceanicus (Le Guillou). The identification of the types of proteins that are elevated after exposure to insecticides could also assist in the determination of the physiological processes involved. In a review article, Gillott (2003) placed accessory gland secretions into three groups: small peptides (e.g. sex peptide in *D. melanogaster*), molecules of 200–400 amino acids that are commonly glycosylated and large proteins (e.g., structural proteins such as sperm-storage protein Acp36DE of *D. melanogaster* (Bertram et al., 1996)). However, this study was not able to characterize proteins in the MAGs of *N. lugens*.

Differences in the protein content of the MAGs of different mating pair combinations indicated that, in general, the reproductive effect of insecticide-treated males on ovary proteins and fecundity of adult females via mating corresponded to the effect of the treated females. Therefore, we considered that insecticide-treated insects have a strong resistant stress. Luckey's hormoligosis hypothesis predicts that sub-harmful quantities of any stressing agent will be stimulatory to the organism by providing it increased sensitivity to respond to changes in its environment and increased efficiency to develop new or better systems to fit a sub-optimum environment (Luckey, 1968). Wang et al. (2009) reported that the imidacloprid-resistant population of *N. lugens* following exposure to sub-lethal doses of triazophos and deltamethrin sprays appears to have reproductive advantages over the susceptible population. The mechanism of increase of insecticide-induced protein content in the MAGs or female ovaries of *N. lugens* may be associated with the accumulation of more energy in the insect developed from feeding on rice plants treated with triazophos and deltamethrin (Yin et al., 2008). Investigation by Yin et al. (2008) demonstrated that there were increases in both the soluble sugar and the crude fat content in adults, as well as in the numbers of eggs laid as adults, after exposure as third instar nymphs to rice plants treated with either deltamethrin, triazophos, or imidacloprid. The current study and the one by Yin et al. (2008) both provide support that exposures to rice plants treated with sub-lethal doses of insecticides can be beneficial to *N. lugens* through the accumulation of biochemical substances in the insect body. In addition, many studies have characterized the sub-lethal effects of pesticides, which result in stimulating reproductive effects in adult *N. lugens* females (Chelliah and Heinrichs, 1980; Gu et al., 1984, 1996; Wang et al., 1994; Zhuang et al., 1999; Wu et al., 2001a,b; Yin et al., 2008).

Thus, mating pairs of treated males and females resulted in the promotion of the reproduction of the females. The amount of sucking sap of *N. lugens* on rice plants treated with pesticides is significantly higher than that on control plants (Wu et al., 2001b), indicating that treated plants are beneficial to *N. lugens* feeding. However, the amount and quality of food ingested by a male affect the quality and quantity of its ejaculate (Simmons, 2001). Therefore, host quality has been shown to affect sperm numbers in the Indian meal moths *Plodia interpunctella* Hubner (Gage and Cook, 1994). Male diet and larval host have also influenced other aspects of the reproductive performance of insects (Aluja and Sivinski, 2008; Aluja et al., 2009).

To further substantiate the role of *N. lugens* males in pesticide-induced resurgence, it needs to be demonstrated that when males having higher amounts of protein content in the MAGs mate, resulting in a higher numbers of eggs laid, on top of the changes in ovary proteins. In addition to protein in the MAGs there are other compounds that are influenced by pesticide exposure. The economic impact of this significant pest of rice will result in continued studies to further elucidate the roles that both males and females have in the population dynamics of pesticide-induced resurgence.

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