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Research

Effect of Pheromone Blend Components, Sex Ratio, and Population Size on the Mating of *Cadra cautella* (Lepidoptera: Pyralidae)

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Abstract

The almond moth *Cadra cautella* (Walker), a key pest of storage facilities, is difficult to manage using synthetic chemicals. Pheromone-based management methods remain a high priority due to advantages over conventional management practices, which typically use insecticides. *Cadra cautella* females release a blend of pheromone including (*Z, E*)-9,12-tetradecadienyl acetate (ZETA) and (*Z*)-9-tetradecadien-1-yl acetate (ZTA). The effect of these components on mating of *C. cautella* and how response varies with the population density and sex ratio remain unknown. In this study, the mating status of *C. cautella* was studied inside mating cages under different ratios of ZETA and ZTA diluted in hexane and at different population sizes either with equal or unequal sex ratio. The lowest percentage of mated females (highest mating disruption [MD] effects), corresponding to roughly 12.5%, was produced by a 5:1 and 3.3:1 ratio of ZETA:ZTA. Populations with equal sex ratio showed the lowest percentage of mated females, at 20% and 12.5% under lower and higher density, respectively. The next lowest percentage of mated females was produced when the sex ratio was set to 1: 2 and 2:1 male:female, with just 25% and 22.5% of moths mated, respectively. This study shows that mating status of *C. cautella* is influenced by ZETA:ZTA ratio, sex ratio, and population size. This current knowledge would have useful implications for mating disruption programs.

Key words: *Cadra cautella*, sex pheromone, mating disruption, ZETA, ZTA

The damage caused by insects to the raw and processed food in storage is quite high and of numerous ways (Kumar and Kalita 2017, Dissanayaka et al. 2018c, Sajeewani et al. 2018, Wijayaratne et al. 2018, Kumari et al. 2020, Sajeewani et al. 2020). The most common management practices for insects infesting stored products include the use of contact insecticides (Arthur et al. 2019), fumigation (Ridley et al. 2011, Fields 2012, Hwaidi et al. 2017), exposure to extreme temperatures (Beckett 2011), and grain aeration (Arthur et al. 2015). However, there are several challenges for continued chemical management of stored-product insects in the postharvest supply chain, including the phase-out of fumigants such as methyl bromide (Andersen 2018), development of resistance to available insecticides (Arthur et al. 1988, Opit et al. 2012), and nontarget impacts of control measures on other species, workers, and the environment (Fields 1992, Arthur 1996, Phillips and Throne 2010, Wijayaratne et al. 2018). Ongoing and recent advances in developing alternatives to combat these issues include sanitation (Morrison et al. 2019a), airtight storage (Sanon et al. 2011, Hasaranga et al. 2018), controlled or modified atmospheres (Wijayaratne et al. 2009, Wijayaratne et al. 2019), use of natural products such as diatomaceous earth (Korunic et al. 2020) or botanical compounds (Dissanayaka et al. 2018b), and use of safer insecticides (Wijayaratne and Fields 2010; Wijayaratne et al., 2012a,b, Wijayaratne and Rajapakse 2018, Wijayaratne et al. 2018, Morrison et al., 2019b, Dissanayaka et al., 2020a,c, Sammani et al., 2020).

The almond moth, *Cadra cautella* (Walker) (Lepidoptera: Pyralidae), is a serious pest of stored food commodities including rice, other grains, flour, nuts, dried fruits, and seeds (Cox 1975, Hill 1990, Arbogast et al. 2005). It is a cosmopolitan species (Sinha and Watters 1985), including occurring in Sri Lanka (Sajeewani et al. 2018), where it has been reported as a pest infesting stored paddy rice, which is the staple commodity of Sri Lankans. Mating disruption (MD) is a technique that has been developed to combat this species. Mating disruption involves the utilization of synthetic pheromone mimic in higher concentrations that overwhelm those produced naturally by individuals of the target species in higher concentrations than those released by individuals naturally, resulting in the suppression of mating followed by reduced laying of

viable eggs (Jones 1998). MD has several advantages, such as lower impact on nontarget and beneficial insects (Mori and Evenden 2015) as well as humans (Soopaya et al. 2015). Young *C. cautella* females release a sex pheromone consisting principally of (*Z*, *E*)-9,12-tetradecadienyl acetate (ZETA) and (*Z*)-9-tetradecadien-1-yl acetate (ZTA) in a 14:1 ratio, though actual ratio may vary based on the age of the individual (Allison and Cardé 2006, 2007). ZETA is the major component for the male attraction whereas ZTA is a synergist of ZETA (Read and Haines 1976). MD for this species has used both of these compounds to disrupt the life cycle of the pest in food storage environments.

Mating disruption in stored-product moths has been studied for *C. cautella*, *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) and *Plodia interpunctella* (Hubner) (Lepidoptera: Pyralidae) (Cardé 2007, Burks et al. 2015, Sammani et al. 2018; Wijayaratne and Burks 2020). These studies mostly tested the effects of high concentrations of pheromones for suppressing progeny population levels (Mafra-Neto and Baker 1996, Phillips 1997, Plarre 1998, Süss et al. 1999, Shani and Clearwater 2001, Ryne et al. 2001, Cox 2004, Ryne et al. 2006, Anderbrant et al. 2007, Ryne et al. 2007, Mueller 2010), MD and male trap catch (Burks and Kuenen 2012). However, there has been little research that has investigated the success of MD in *C. cautella* under different pheromone component ratios, sex ratios, and population sizes. Therefore, the objectives of this study were to determine the effect of optimum blend of the pheromone components ZETA and ZTA on mating disruption and to determine whether MD breaks down under larger population sizes.

Materials and Methods

Rearing of *Cadra cautella*

Adult *C. cautella* were captured from a rice milling center at Puliyankulama, Anuradhapura, Sri Lanka, using an aspirator connected to a vacuum pump (Rocker 300, Rocker Scientific Co. Ltd., New Taipei City). Fifty adults were introduced to 250 g of rice flour in plastic bottles, covered with a piece of cloth and maintained inside an incubator (FH-1200 LED T8, HiPoint Laboratory, Taiwan) at 33 ± 1°C and 60 ± 5% relative humidity (RH) until larvae eclosed and pupae emerged. At the pupal stage, culture medium was sieved through a 850 μm^2 and 2 mm^2 mesh (ASTM E11, W.S. Tyler Industrial Group, USA) to separate life stages from growth media. Male and female pupae were separated under a microscope (OPTIKA, Triace, Italy) using the two nodes located close to the genital scar on the ventral side of the eighth segment (Zhu et al. 1999). Each pupa was introduced into a separate vial (3.6 cm diameter and 6.2 cm height) with 5 g of rice flour and maintained inside an incubator (33 ± 1°C and 60 ± 5% R.H.) until the adults emerged. The rice flour was added to each vial having the pupa to avoid its desiccation and maintain in fresh/healthy condition.

Mating Cage Construction

Four cages (each 1.5 m × 1.5 m × 1.5 m) with metal frames were constructed. The top side, bottom side, and two opposite vertical sides of each cage were covered using transparent polythene (25 mm thickness). The remaining two vertical sides were covered using insect-proof net (approximately 300 holes/cm^2) to facilitate air circulation. The polythene and netting material were attached to the cage by using Velcro (Garment Accessories.lk, Rajagiriya, Sri Lanka) along the sides.

Experiment 1: Determination of the Optimum Ratio of Pheromone Components (Z, E)-9, 12-Tetradecadienyl Acetate and (Z)-9-Tetradecadien-1-yl Acetate on Mating Disruption of *C. cautella*

Preparation of different pheromone blends

Commercially available stock solutions of the pheromone components ZETA (100%) and ZTA (100%) (Insects Ltd. Inc., Westfield) were used in the study. These pheromone solutions were maintained at 5°C inside a refrigerator until used. Different volumes of ZETA and ZTA were measured by using a micropipette (Labnet International Inc., Poland) into a test tube to prepare different pheromone blends (Table 1). The pheromone components were diluted using hexane as the solvent (Zhu et al. 1999). First, the relevant volume of hexane was measured using the micropipette into the test tube and then the pheromone components were added.

Introduction of pheromone blends into the mating cage

In the control experiment, 100 µl hexane was added to a triangular filter paper (8 cm^2) attached to a piece of rigiform by a needle, and placed on the bottom portion of a Petri dish (Zhu et al. 1999). The same-sized triangular filter papers (4 cm × 4 cm, base × height) were used similarly throughout all the following experiments to equalize the pheromone release rate. The Petri dish was placed inside a monitoring trap (Storgard kit insect monitoring system, Trece Inc., Adair, OK) and hung at the center of the mating cage (75 cm distance from bottom) 3 h before introducing the moths (Ryne et al. 2001). Following the control experiment, different ratios of the pheromone components ZETA, ZTA, or the hexane solvent were used in experiments as mentioned in Table 1. These solutions were added to filter paper as above and hung in the center of mating cages 3 h before introducing the moths.

Exposure of *C. cautella* adults to pheromone inside the mating cage

Ten male and ten female *C. cautella* adults (population size of 20 individuals) that each emerged in individual vials (3.6 cm diameter and 6.2 cm height) were introduced individually into the mating cages 2–4 d following emergence, and maintained in the mating cage for 24 h.

Table 1. Different ratios of pheromone blends of ZETA, ZTA, and Hexane tested in experiment 1

Blend number	ZETA:ZTA:Hexane ratio	ZETA (µl)	ZTA (µl)	Hexane (µl)	Total amount (µl)
1	20: 1: 20	100	5	100	205
2	10: 1: 10	100	10	100	210
3	5: 1: 5	100	20	100	220
4	3.3: 1: 3.3	100	30	100	230
5	1: 0: 1	100	-	100	200
6	0: 1: 3.3	-	30	100	130
7	0: 1: 20	-	5	100	105
8	0: 0: 1	-	-	100	100

Following exposure to pheromone (or hexane), the adult moths in each cage were aspirated into separate conical flasks (250 ml), frozen at –10°C for 1 h and dissected using dissecting pins under a microscope (OPTIKA, Triace, Italy) to determine the presence/absence of spermatophores, which is indicative of mating (Mafra-Neto and Baker 1996). As the spermatophores are preserved best if not frozen for longer than 2 h (Ryne et al. 2001), the females were dissected within that period following removal from the mating cage to avoid deterioration (Drummond 1984). The number of mated females were determined using the above criterion for each pheromone ratio (or control) used. Furthermore, the number of males attracted to the Petri dish with a particular pheromone ratio were also counted. For each pheromone ratio (or control), four replicates were tested.

Recording temperature and relative humidity

Dataloggers (TM-305U, Tenmars Electronics Co., Ltd., Taiwan) were placed both inside and outside of the mating cages to record the temperature and relative humidity. The average temperature and relative humidity inside and outside of the mating cages were 30 ± 1°C, 63 ± 1% and 32 ± 1°C, 64 ± 2% respectively.

Experiment 2: Determining the Effective Population Size of *C. cautella* That Maximizes MD Under Optimum Pheromone Blend

The pheromone blend with the highest MD from experiment 1 (e.g., 5:1 ZETA:ZTA) was placed at the center of the mating cage, approximately 75 cm from the bottom using a monitoring trap, at least 3 h before the introduction of moths (Ryne et al. 2001). The 2–4-d-old virgin male and female *C. cautella* adults each emerged in separate vials, and were introduced individually into the mating cages at different population densities (10 or 40 insects) either at an equal (=1:1 male:female) or unequal (≠1:1 male:female) sex ratio (Table 2). The male and female moths were allowed to mate for 24 h and then were recovered by aspirating into individual conical flasks. Moths were kept in a freezer at –10°C for 2 h and dissected under microscope to determine their mating status as did in the previous experiment. In the control treatment, hexane alone was used instead of pheromones. For each population size and sex ratio, four replicates were tested. Thus, the population size that demonstrates the maximum mating disruption (minimum mating response) was determined. Following each experimental step, the entire facility including the mating cages were all thoroughly washed using biodegradable detergent (Britol Disinfectant Pine, Antler Industries Pvt. Ltd., Piliyandala, Sri Lanka) to remove any pheromone residues.

Experimental design and data analysis

Due to the uniformity of the ambient environmental conditions prevailed in the rooms in which the experiments were conducted, the first and second experiments were designed as completely randomized design (CRD) with four replicates. In experiment 1, the percentages of mated females and males attracted to a given pheromone ratio were transformed using the square root of the arcsine value and analyzed using ANOVA procedures of Statistical Analysis System (SAS) (SAS Institute 2002–2008) (Zar 1999, Toews et al. 2010, Burks and Kuenen 2012, Dissanayaka et al. 2018a,b, Wijayaratne and Rajapakse 2018, Dissanayaka et al., 2020a,b,c). The means were separated by Tukey's test upon a significant result of the model, with significance tested at $\alpha = 0.05$.

In experiment 2, the percentage of mated females for each population size was transformed using the square root of the arcsine value and analyzed using ANOVA procedures of SAS (SAS Institute 2002–2008). Tukey's test was used to separate the means of treatments at the significance level $\alpha = 0.05$.

Results

Experiment 1: Determination of the Optimum Ratio of Different Pheromone Components (Z, E)-9,12-Tetradecadienyl Acetate and (Z)-9-Tetradecadien-1-yl Acetate on Mating Disruption of *C. cautella*

When exposed to the pheromone ratios, the percentage of mated females was significantly lower than the hexane control ($F_{7,24} = 48.53$, $P < 0.001$). The lowest percentage of mated female moths (12.5%) (the highest MD) was obtained when individuals were exposed to 5:1 and 3.3:1 ZETA:ZTA ratios (Fig. 1). The next highest levels of mating were obtained when moths were exposed to 20:1, 10:1, or 1:0 ZETA:ZTA ratios. Mating was further increased with the ZETA:ZTA ratios 0:0.05 and 0:0.3. The maximum percentage of mated female moths was obtained in the hexane only control (ZETA:ZTA at 0:0), while the percentage of mated females was not significantly different from the control where moths were exposed to a ZETA:ZTA ratio of 0:0.05. There were no significant differences among male attraction to traps with different pheromone ratios compared with the hexane control ($F_{7,24} = 1.14$, $P = 0.3705$; Fig. 2).

The addition of ZETA and ZTA to hexane in various ratios reduced mating compared with using hexane alone; in controls, 95% of moths were able to mate. The importance of ZTA as a synergist is evident, for example in the 1:0 ZETA:ZTA ratio where ZTA was not present, 42.5% of females were mated (Fig. 1). Furthermore, the combined presence of both ZETA and ZTA in the same solution reduced mating by 77.5% compared with ratios where ZTA was the only component present (comparison of pheromone blends 5:1 and 0:1 ZETA:ZTA ratios; Fig. 1). While the presence of ZTA alone was not sufficient to cause a marked decrease in mating, the use of ZETA alone (e.g., 1:0) was able to suppress mating equally to some ratios with both ZETA and ZTA (e.g., 20:1 and 10:1 ZETA:ZTA ratios). However, the optimum pheromone ratios for mating disruption of *C. cautella* were 5:1 and 3.3:1 ZETA:ZTA, which resulted both in

Table 2. Different population sizes of *Cadra cautella* tested in experiment 2

	Treatment No.	Male: Female	Population size/replicate	Population density (moths/m^2)
Similar male and female combination	1	1:1	10	4.44
	2	2:2	20	8.88
	3	3:3	30	13.33
	4	4:4	40	17.77
Different male and female combination	1	1:2	15	6.66
	2	2:1	15	6.66
	3	2:5	35	15.55
	4	5:2	35	15.55

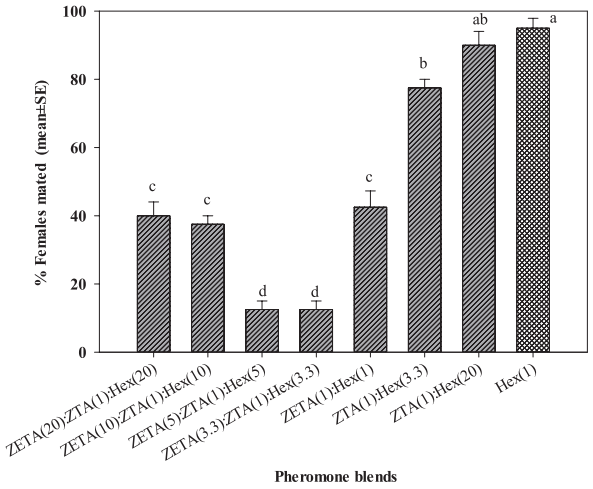

Fig. 1. Percentage (mean ± SE) of mated *C. cautella* females following exposure to different pheromone ZETA:ZTA ratios (n = 4). Means followed by the same letter are not significantly different according to Tukey's test following ANOVA (α = 0.05). Abbreviations: ZETA = (*Z, E*)-9,12-tetradecadienyl acetate; ZTA = (*Z*)-9-tetradecadien-1-yl acetate; and Hex = hexane solvent.

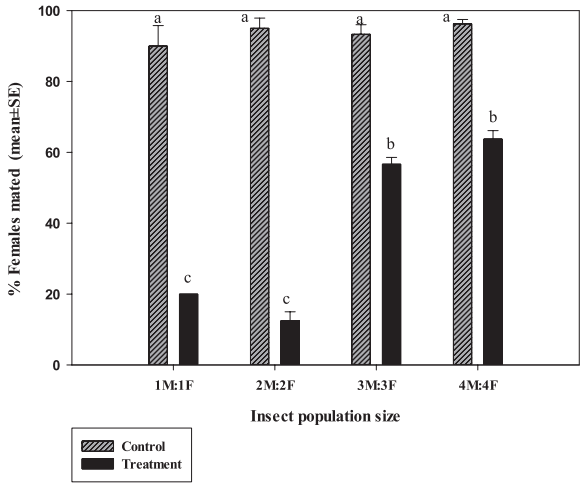

Fig. 3. Percentage (mean ± SE) *C. cautella* adult females mated following exposure to ZETA (5): ZTA (1): Hexane (5) when used in different population sizes (male: female = 1:1). Means followed by the same letter are not significantly different according to Tukey's test following ANOVA (α = 0.05).

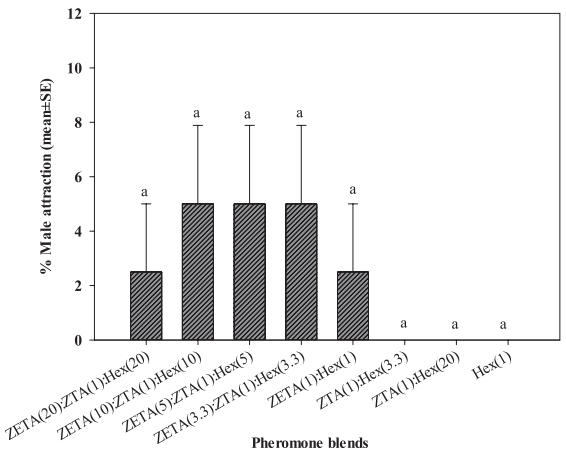

Fig. 2. Percentage (mean ± SE) of *C. cautella* males attracted to pheromone ratios (n = 4). Means followed by the same letter are not significantly different according to Tukey's test following ANOVA (α = 0.05). ZETA = (*Z, E*)-9,12-tetradecadienyl acetate; ZTA = (*Z*)-9-tetradecadien-1-yl acetate; and Hex = hexane solvent.

only 12.5% of females mating (Fig. 1). This represents a reduction in the number of mated females by 88–90% compared with the hexane only controls.

Experiment 2: Determining the Effective Population Size of *C. cautella* That Maximizes MD Under Optimum Pheromone Blend

When adult C. *cautella* moths were present under an equal sex ratio, the percentage of mated females was significantly lower than their respective controls at every population size tested ($F_{7,24}$ = 38.57, P < 0.0001; Fig. 3). The low population sizes (1:1 and 2:2 M:F) showed lower percentages of mated females (e.g., higher MD), but between the two lower population sizes, there was no significant difference. The two higher population sizes (3:3 and 4:4 M:F) showed significantly larger percentages of mated females than the lower population sizes (1:1 and 2:2 M:F); however, there was no statistical difference between the two highest population sizes.

When adult C. *cautella* moths were used in a sex ratio that was not 1:1, the percentage of mated females was significantly lower than their respective controls at all the population sizes tested ($F_{7,24}$ = 37.39, P < 0.0001; Fig. 4). At low population sizes, both male- and female-biased populations (2:1 and 1:2 M:F) showed lower percentages of mated females (higher MD) than at the two high population sizes (2:5 and 5:2 M:F ratios). Furthermore, there were no significant differences in the percentages of mated females between the two low population sizes (2:1 and 1:2 M:F). Similarly, no significant differences in the percentage of mated females was observed between the two high population sizes (2:5 and 5:2 M:F ratios).

Discussion

According to Mafra-Neto and Baker (1996), the mating of *C. cautella* is suppressed by up to 100% using ZETA at low population densities (1.27 moths/m^2) (experiments conducted inside 3 × 3 × 3 m rooms). Similarly, in the current research, ZETA used at 100 µl in combination with ZTA (5 µl) and/or hexane (100 µl) did not allow mating success to reach higher than 40% of individuals in a situation where the population size was 20 at a 1:1 sex ratio (10 females + 10 males). It is known that ZTA stimulates increased mating attempts by moths (Sower and Whitmer 1977, Hodges et al. 1984), which is also in agreement with the current findings as the use of ZTA (dissolved in hexane) facilitated increased mating by as much as 77.5% and 90% compared to ZETA alone (42.5%). Previous studies reported that the mating status of female moths can be confirmed with the presence of spermatophores in bursa copulatrix of females (Doane and Brooks 1981, Ryne et al. 2001), and this was the indicator used in the current research to verify mating success. Another factor that may affect mating disruption is the amount of pheromone production by *C. cautella*, which varies with their age. The highest ZETA production typically occurs 1 d after adult emergence. The highest ZTA production, by contrast, is 2 or 3 d after adult emergence

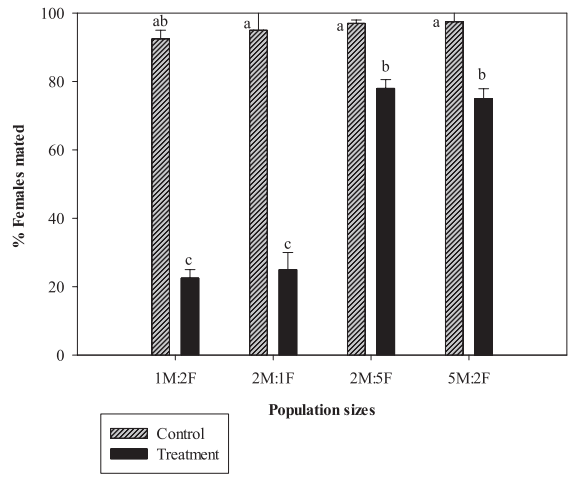

Fig. 4. Percentage (mean ± SE) *C. cautella* adult females mated following exposure to ZETA (5): ZTA (1): Hexane (5) when used in different population sizes (male: female≠1:1). Means followed by the same letter are not significantly different according to Tukey's test following ANOVA ($\alpha = 0.05$).

(Allison and Cardé 2006). Therefore, the response of *C. cautella* adults to pheromone under natural conditions mainly correlates with temporal and physiological changes in the biosynthesis of ZETA and ZTA. In the current study, 2–4-d-old moths were used and they showed highest mating disruption at 5:1 and 3.3:1 ZETA:ZTA ratios.

In this study, the mating success of female *C. cautella* was reduced to 12.5% (highest MD) using two pheromone blends, namely 5:1 and 3.3:1 ZETA:ZTA ratios. Ryne et al. (2001) reported that the mating disruption of *P. interpunctella* in small plastic tents in a greenhouse was equally effective when using only the major component (ZETA) as when using the complete four-component blend (ZETA; ZTA; (*Z*, *E*)-9, 12-tetradecadienol (ZETOH); (*Z*, *E*)-9, 12-tetradecadienal). By contrast, our work has demonstrated that the mating disruption of *C. cautella* may be significantly affected by different ratios of ZETA and ZTA (Fig. 1). This is also supported by prior preliminary work by Hodges et al. (1984).

Mating disruption of *C. cautella* may be increased by the combination of ZETA and ZTA (Brady and Daley 1975, Sower and Whitmer 1977). We have resolved this knowledge further by determining that the optimal ratios of ZETA and ZTA (among those tested here) for mating disruption was 5:1 or 3.3:1. We also found evidence of a synergistic effect of ZTA on ZETA's ability to disrupt mating. By contrast, prior field trials did not find an effect of attractant or attractant-synergist mixture (Brady 1973). Hodges et al. (1984) reported that lower concentrations of ZETA in the mixtures resulted in poor mating. This needs to be systematically tested in future research. While Brady (1969) reported that more males are attracted by large quantities of ZTA, we did not find evidence to support this hypothesis, as the pheromone ratio appeared to have no effect on male attraction by varying levels of ZTA. Doane and Brooks (1981) reported that determination of mating disruption using spermatophores is more accurate than male trap catches. Our findings support this, as we found interesting and useful patterns in mating success through dissection that would have been overlooked if only male captures on traps were considered.

Low population densities of insects may be especially amenable to control through behavioral manipulation, as artificial pheromone sources compete with those naturally produced by females (Beroza and Knipling 1972). Thus, reproduction of *C. cautella* can be suppressed by ZETA at low population densities (Sower and Whitmer 1977, Sammani et al. 2018). Under higher densities, behaviorally based management techniques such as mating disruption may break down because mate-finding is relatively easier with increased chance encounters (Sarfraz et al. 2006, Miller et al. 2015). In this kind of situation, moths do not require long-distance, pheromone-mediated orientation (Phelan and Baker 1990, Mafra-Neto and Baker 1996). At close range, males may find their mate with the help of visual observation and tactile cues, which may take priority over chemical communication (Charlton and Carde 1990). These prior findings reasonably describe the reduced mating facilitated by ZETA at low population densities under the controlled conditions in the current research.

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Author Contributions

AMP and LKW conceptualized the research. AMP conducted experiments. DMSK assisted in data collection. AMP did major part of the data analysis. DMSK helped in data analysis. AMP wrote the first draft of the manuscript and attended revisions. LKW supervised throughout including initial research concept, designing the experimentation, methodology, conduct of experiments, data analysis and interpretation of results, writing of the manuscript, and all the revisions to the manuscript. LKW did validation of experiments and supply of resources. LKW corrected several versions of revised manuscript including final editing. WRM revised the manuscript and provided valuable comments to improve it. The funding for this study was through the research grant in which LKW is the Principal Investigator. LKW did the entire project administration. LKW did coordination among all the authors. All authors read and approved the manuscript.

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