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Research Paper

A Promising Male Phenylbutanoid Attractant for Enhancing Mating Performance in *Bactrocera dorsalis* (Hendel)

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Abstract

***Bactrocera dorsalis*, a devastating pest worldwide, has a strong affinity for methyl eugenol (ME), which is used in its management strategies. ME is a male lure, a species-specific compound that enhances the mating behaviour of *B. dorsalis*. However, using ME in combination with the sterile insect technique (SIT) and male annihilation techniques (MAT) presents challenges, as it reduces the efficacy of SIT. Additionally, some insensitivity to ME has been reported, further decreasing the effectiveness of MAT. Therefore, it is essential to identify alternative male lures for *B. dorsalis*, including those that can attract non-ME-responding (NR) males. The potential of using phenylbutanoid compound, zingerone (ZN) to enhance male mating performance in NR and normal *B. dorsalis* males was studied. Initially, NR males showed comparable attraction to ZN compared to normal males. Subsequently, 5 days after ZN feeding, NR-males exhibited significantly higher copulation rates ($p < 0.001$) than ZN-deprived males, with ZN-fed males mating earlier than their non-fed counterparts. Semi-field cage assays further supported these findings, showing significantly higher copulation rates in ZN-fed males than those deprived of ZN. Additionally, wind tunnel assays revealed that virgin females have significantly greater attraction ($p < 0.05$) to ZN-fed males compared to ZN-deprived males. These results suggest that ZN is a potential candidate for enhancing male mating performance in *B. dorsalis* males, offering an alternative to ME-based strategies.**

Keywords: *B. dorsalis*, Mating behaviour, Non-ME-responders, Zingerone

Introduction

Fruit fly males are attracted to certain plant secondary metabolites or their synthetic analogues, known as "male lures," such as phenylpropanoids, phenylbutanoids, and sesquiterpenes (Metcalf & Metcalf, 1992; Tan *et al*., 2014). These substances play a crucial role in stimulating male mating

behaviour of Tephritid flies (Nishida & Tan, 2016). Methyl eugenol (ME) is a well-studied male lure that enhances male mating success by influencing behavioural, physiological changes and attractiveness (Shelly, 2001; Tan & Nishida, 2012; Segura *et al*., 2018). Males are instinctively drawn to specific male lures, a behaviour observed by Shelly (2010) and further supported by Tan & Nishida (2012), who noted that feeding often follows this attraction. After consumption, the lure passes through the crop (an enlarged area of an insects' gut) and it is either metabolized into analogues (Nishida *et al*., 1988) or sequestered unchanged (Nishida *et al*., 1993) and transported through the hemolymph to be stored in the male rectal glands (Hee & Tan, 2004). These stored compounds blended with endogenous pheromones of males leading to enhanced: (1) male behavioural sexual signalling (Wee *et al*., 2007; Kumaran *et al*., 2014a; Rabiatul & Wee, 2019); (2) attractiveness of chemical sexual signals for the female attraction (Shelly 2001; Kumaran *et al*., 2014b; Rabiatul & Wee, 2019); and (3). physiological changes in bodies (Kumaran et al., 2014a; Kumaran & Clarke, 2014) which finally enhance male mating success.

Despite the extensive research on ME, limited studies have explored the biological mechanisms underlying other lures like beta-caryophyllene, zingerone, and ME fluorine analogues (Tan & Nishida, 2000; Khrimian *et al*., 2009). Interestingly, instances of males displaying insensitivity to ME have been observed (Liu *et al*., 2017; Chen *et al*., 2019; Mandanayake & Hee, 2023), suggesting the potential for non-ME-responding (NR) males to be utilized in fruit fly management strategies.

Zingerone, a phenylbutanoid with structural similarities to both ME and raspberry ketone (RK), has emerged as a promising lure for various fruit fly species (Tan & Nishida, 2000, 2012; Nakahira *et al*., 2018; Leblanc *et al*., 2019). It has shown potential for attracting diverse fruit fly species, including those unresponsive to ME or RK (Leblanc *et al*., 2018a, b, 2019; Royer *et al*., 2018, 2019). Additionally, zingerone has been identified as a floral synomone for several Bactrocera fruit fly species, attracting males to specific orchid species where it is found (Tan & Nishida, 2000, 2012; Nakahira *et al*., 2018; Katte *et al*., 2020).

While zingerone's role in male mating behaviour has been studied in some species such as *B. tryoni* (Kumaran *et al*., 2013, 2014a) and *Zeugodacus cucurbitae* (Khoo & Tan, 2000), its relationship with NR males remains unexplored. This study aimed to investigate the potential of zingerone to enhance the mating performance of NR males, with implications for integrated pest management strategies like the sterile insect technique (SIT) and male annihilation technique (MAT). This approach could bolster the effectiveness of both SIT and MAT by attracting NR males and improving their mating success. The study included cage assays to attract males to zingerone under laboratory conditions and assessed female attraction to post-fed males in a wind tunnel, along with mating success in semi-natural conditions.

Materials and Methods

Insects

Two non-ME responder line progenies (herein mentioned as NR lines) were developed (Mandanayake *et al*., 2023) and were maintained for eight generations/ year at the Insectary in the Laboratory of Entomology and Chemical Ecology in the Department of Biology, Faculty of Science, University Putra Malaysia (UPM). Correspondingly, laboratory strain (reared since 2009 with nine generations per year with irregular introgression) was kept up at the same place to utilize as a control line for all experiments.

Chemicals

Zingerone (ZN) (96% purity as crystals, Sigma-Aldrich, USA) was used for experiments, and Ethyl alcohol (99.8% purity, Merck®, Germany) was used to prepare solutions of ZN.

Assessing male attraction to ZN

Sexually matured 16-17 days old virgin males from NR lines and the laboratory strain (16 days after emerging -DAE) were compared in their response to ZN within a short distance of ≤40 cm. Based on availability, treatments included 100 males from the laboratory strain and 50-60 from NR lines. Flies were exposed to two concentrations of ZN (10 mg mL^{-1} and 100 mg mL^{-1}) on filter paper placed in the center of a plastic Petri dish arena.

A positive response was recorded when a male landed and fed on the attractant source for 30 minutes. After 30 minutes, the source with attracted males was covered and stored in the refrigerator for later counting. The experiment was replicated seven times on different days with different cohorts for each selected concentration. All experiments were conducted between 09:30-11:00, as preliminary studies indicated higher attraction within this timeframe.

The percentage (%) of attracted males was calculated as follows.

$$\text{Percentage of attracted males} = \frac{\text{Males feeding or landing on the source}}{\text{Total number of males exposed}} \times 100 \quad \text{..... (1)}$$

Investigating the effects of post-feeding ZN on mating success

Cage bioassays were conducted with 16-17 days old virgin males from NR flies and the laboratory strain, simulating semi-natural conditions. Virgin males were exposed to ZN spots (100 µL $spot^{-1}$) for 30 minutes before the study. Treatment groups consisted of 50 pairs of insects. Females, deprived of providing water and food for 30 minutes prior to the experiment were introduced to male cages 2-3 hours before the experiment.

Two control treatments were included: laboratory strain males fed with ZN (control 1) and laboratory strain males without ZN feeding (control 2), both were compared against two NR lines. Observations were made between 17:30-20:00, aligning with *B. dorsalis* mating habits. Mating success was assessed by observing pairs remaining in copula for at least 30 minutes. The number of mated pairs was recorded at 1-, 3-, 5- and 7-days post-ZN feeding with nine replications.

The percentage of mated pairs was calculated as follows.

$$\text{Percentage of mated pairs} = \frac{\text{Number of mated pairs}}{\text{Total number of pairs introduced}} \times 100 \quad \text{..... (2)}$$

Assessment of the mating success of ZN-fed and deprived males

A cage bioassay was conducted to assess the mating success of ZN-fed and ZN-deprived males. Sixteen to seventeen-day-old virgin males from each line were selected, and same-aged virgin females were used for the experiments. The treatment groups consisted of 50 pairs of insects. Males were fed with ZN (100 µL $spot^{-1}$ for 30 minutes) five days before the experiments. In the morning of the experiment, females were introduced into the cages. The female flies were deprived of food and water until 30 minutes before the experiment. Observations were begun at 17:30 hour and continued at 15-minute intervals thereafter. Mated pairs were carefully collected using clean glass specimen vials to record their numbers. The experiment was replicated five times, with each NR line and laboratory strain tested on different days.

Wind tunnel assay for the attraction of conspecific females to the ZN-fed males

Males were pre-fed with ZN five days before testing in a wind tunnel during the late afternoon and dusk (17:30-19:30 h). Four treatment groups were examined: males from two NR lines fed with ZN; laboratory strain males fed with ZN; and laboratory strain males without ZN feeding (control), with 15 males in each group.

Thirty minutes before experiments, ZN-fed males were acclimatized in the upwind area of the wind tunnel. Then, 12 sexually matured virgin females were released at the downwind end, and a positive response was recorded when a fly performed a zig-zag flight towards an active upwind section of the tunnel.

Females were released at 17:30 h, and responses were recorded after 10 minutes in the first trial. A 5-minute interval was allowed for fly collection using clean specimen vials to prevent habituation. Light intensities were measured in the upwind and downwind areas using a light meter CA-813 (AEMC® Instruments., Taiwan). The control experiment was conducted separately on different days, and the entire process was repeated 24 times across various days.

Statistical Analysis

Data from cage bioassays investigating male attraction to ZN, mating success of post-ZN consumption, and wind tunnel assays involving ZN-fed males was subjected to Poisson regression analysis. Post-hoc tests with Tukey-Kramer adjustment were conducted to identify significant pairwise differences between treatments. Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4).

Results & Discussion

Assessing male attraction to ZN

Table 1 indicates the mean percentage of flies attracted and subsequently fed on the source. There was a significant difference (p=0.000) two concentrations of ZN.

Table 1. Number of different strains of males attracted to ZN

Treatment	Males respond to ZN (mean %)	
	10 mg mL^{-1}	**100 mg mL^{-1}**
NR line 1	23.82±5.1	53.70±3.2
NR line 2	21.10±2.7	49.46±1.4
Laboratory strain	24±4.1	46.6±2.8

Note: Mean separation was done using Tukey-Kramer adjustment with pair-wise comparison and statistical significance at p<0.05 was not observed across the columns

However, the difference was not significant across treatments at the 10 mg mL^{-1} (p=0.785) and 100 mg mL^{-1} (p=0.414) concentrations of ZN separately. However, the percentage of males attracted was notably higher at 100 mg mL^{-1} (54-46%) compared to the 10 mg mL^{-1} (21-24%) concentration of ZN. Additionally, it was observed that male flies attracted to the source exhibited strong congregation and feeding behaviour, similar to the response observed with ME. These findings emphasize the potential of ZN as a compound for evaluating its effect on male mating success, particularly for NR flies, as it demonstrates a comparable level of attraction to that observed in normal males.

Research conducted by Tan and Nishida (2000, 2012) and Nishida & Tan (2016) highlighted the attraction of *B. dorsalis*, a highly ME-responsive species, to ZN, characterized by a methoxy-moiety attached to the benzene ring. Although *B. dorsalis* males were observed visiting ZN-containing orchid species like *Bulbophyllum patens*, *B. baileyi*, and *B. macranthum*, the occurrence was infrequent (Tan & Nishida, 2000, 2012; Nakahira *et al*., 2018).

Numerous dacini species, irrespective of their ME/RK responsiveness, have been reported to be attracted to ZN in field traps across various regions worldwide (Royer *et al*., 2018; Leblanc *et al*., 2018a, 2018b, 2019; Wee *et al*., 2020). Notably, *B. jarvisi* initially considered non-lure responsive or weakly responsive to Cue-lure (CL), demonstrated a strong attraction to ZN (Fay, 2012; Royer, 2015). Wee *et al*. (2018) found that *B. jarvisi* has a lower olfactory threshold and faster response to ZN compared to Raspberry-Ketone (the natural form of CL), with immature males also displaying attraction to ZN.

Investigating the effects of post-feeding ZN on mating success

Table 2 presents the impact of ZN feeding on the mating behaviour of NR and laboratory-strain males. The results revealed significant effects of post-feeding days (Chi-Square=25.65, p<0.0001), line (Chi-Square=27.14, p<0.0001), and their interaction (Chi-Square=78.47, p<0.0001) on mating pairs. One day post-ZN feeding did not show a significant difference (Chi-Square=0.29, p=0.592) in the number of mated pairs between ZN-fed males and deprived groups. However, by the third day post-feeding, males fed with ZN exhibited a significantly higher number of mating pairs (Chi-Square=17.68, p<0.0001) compared to the untreated control.

Table 2. The mean number of mated pairs occurred at post-feeding of ZN

Treatment	Number of mated pairs (mean %)			
	1-day post feeding	3-day post feeding	5-day post feeding	7-day post feeding
NR line 1	64.58 ±1.3	64.98± 3.7*	62.19±4.1*	57.27±4.1
NR line 2	64.37±1.4	67.05 ± 2.8*	64.48±3.9*	56.94 ±1.9
Control 1	67.33±1.5	70.66±2.7*	62.57 ± 1.9*	59.58 ±2.3
Control 2	60.62±1.3	54.61±1.5*	46.5± 2.7*	49.87± 3.1

Note: ± Asterisks (*) designate the level of statistical significance among the treatment across the column at the p<0.05 compared to the control 2 (ZN-deprived treatment)

Further five days after ZN treatment showed a significant difference (Chi-Square=13.25, p = 0.0003) compared to the control group. There was no significant difference (p>0.05) among treatment groups at 7 days post-feeding.

The study revealed that ZN post-feeding significantly affected the male mating success of *B. dorsalis*, particularly in the short term. Also, laboratory-strain males exhibited greater responsiveness to ZN compared to NR males. While no previous records existed regarding the impact of ZN post-feeding on *B. dorsalis* male mating success, the effects of ZN on pre- and post-mating behaviour have been documented in other fruit fly species. For instance, ZN has been shown to enhance male signalling in *B. tryoni* (Kumaran *et al*., 2014 a), and *Zeugodacus tau* (Rabiatul & Wee, 2019; Shamshir & Wee, 2019), while also strengthening female attraction in *Z. cucurbitae* (Khoo & Tan, 2000) and *Z. tau* (Rabiatul & Wee, 2019). Additionally, ZN has been linked to increased female fecundity in *B. tryoni*, albeit with no effect on female fertility or remating propensity (Kumaran *et al*., 2013). The physiological mechanisms underlying these effects include weight loss, increased locomotion activity, and changes in pheromonal composition (Kumaran *et al*., 2014a).

The accumulation and biotransformation of ZN in rectal glands vary between species. In *B. papayae* (now synonymous with *B. dorsalis*), ZN is accumulated and converted to zingerol after consumption, suggesting its role as a sex pheromonal component (Tan & Nishida, 2000).

In *B. tryoni*, ZN is mainly stored unchanged, while some are converted to raspberry ketoneandb-(4-hydroxy-3-methoxyphenyl) -propionic acid. Furthermore, ZN feeding increases the content of endogenous pheromone compounds in males, and in *B. frauenfeldi.* ZN is sequestered unchanged in rectal glands for up to five days post-feeding (Wee *et al*., 2020).

However, the effect of ZN post-feeding differed between laboratory and NR flies. While NR and laboratory strain males displayed phenotypic effects five days after feeding, while laboratory strain males exhibited these effects earlier, at three days post-feeding. Thus, rectal gland analysis of NR flies is necessary to elucidate the role of ZN in male mating success in *B. dorsalis*.

Assessing the mating success of ZN-fed and deprived males

Table 3 illustrates the mean number of mated pairs for ZN-fed and ZN-deprived males in NR lines and a laboratory strain. ZN-fed males in NR lines 1 and 2 mated earlier than ZN-deprived ones, while no difference was observed in the laboratory strain. Mating patterns varied across time intervals. ZN-fed NR line 1 male initiated mating around 17:45, peaked at 18:30, and gradually declined. Conversely, ZN-deprived males started mating around 18.00 and peaked at 18:45. Similar trends were recorded in NR line 2 and the laboratory strain. Compared with the other 2 lines, Line 1 showed clear and significantly higher mating in various time points such as 18.00 (Chi-Square =14.11, p=0.005), 18.15 (Chi-Square=10.11, p=0.0015), and 18.30 (Chi-Square=15.5, p<0.0001) between ZN-fed and ZN-deprived males. This study marks the first report on mating initiation differences between ZN-fed and deprived *B. dorsalis* males. Kumaran *et al*. (2013) also noted that *B. tryoni* males fed on ZN and Cue-lure initiated mating earlier than ZN-deprived males, further supporting these findings.

Wind tunnel assay with ZN-fed males

Figure 1 shows a significant difference in female attraction (p=0.001) between ZN-fed and ZN-deprived males of the laboratory strain and NR lines. Significant differences in female attraction among the treatments were observed at the time points of 18.15 (Chi-Square=5.77, p=0.004), 18.30 (Chi-Square=12.59, p=0.009), and 18.45 (Chi-Square=15.39, p=0.0001). Peak attraction varied among different lines, with NR line 1 reaching 50% attraction at 18:45 (approximately 45 minutes before sunset) under an average light intensity of 80 lux, NR line 2 reaching 39.1% at 19:00 hr (approximately 30 minutes before sunset) under 36 lux, and the laboratory strain reaching at 47.1% at 18:30 under 134 lux. ZN-deprived males also elicited attraction, with peak attraction (32.5%) occurring around 19:00 hr under a light intensity of 45 lux. However, NR line 1 attracted more females than the laboratory strain and NR line 2. Observations on caged ZN-fed and ZN-deprived males acting as pheromone sources revealed intriguing behaviour. Female responses correlated with male calling activity, with at least one-third of caged males exhibiting increased locomotion, head-on buttings, 'boxing' behaviour, wing fanning, and attempts at mounting and copulating with other males. Females responded with zig-zagging anemotaxis, landing on the male cage, mesh of the upwind grill, or sides of the poly acetate tube. Female movement increased as light intensity decreased, with some females exhibiting ovipositor extrusion upon landing. However, no copulation occurred between the caged males and released conspecific females. Light intensity played a crucial role in fruit fly mating behaviour, with female movement increasing as light intensity decreased. Peak attraction for each line occurred around an average light intensity of 135-36 lux, consistent with previous studies (Hee & Tan, 1998).

Table 3. Mean number of mated pairs occurred ZN-fed (5-PDF) and ZN-deprived males in each treatment

Time (hr)	The mean number of mated pairs					
	NR line 1		NR line 2		Laboratory strain	
	ZN-fed	ZN-deprived	ZN-fed	ZN-deprived	ZN-fed	ZN-deprived
17.45	1.2±0.09	-	1±0.4	-	-	-
18.00	4.6± 1.3*	2.4±0.4*	2.4±0.7	1±0.4	1.1±0.2	1±0.6
18.15	7.8±1.9*	3.8±1.2*	3.2±0.8	3.6±0.4	2.8±1.2	1.2±1.2
18.30	8±1*	4.8±1.5*	5.4±0.7	5.2±0.3	5.6±1.3*	3.8±1.5*
18.45	7.6±1	6.8±1.2	12±0.9*	6.6±0.8*	9.4±1.1*	5.2±1.2*
19.00	3±0.5	2.8±0.7	8.2±1.2	5.6±0.8	8±1.2*	5.2±0.4*
19.15	2.4±0.8	2.4±0.4	4±1.1	4±0.8	2.4±0.5	6.4±0.6
19.30	1.6±0.4	1±0.2	2.2±0.6	2.4±0.8	2.6±0.2	2.2±0.2
19.45	1±0.3	1±0.2	1±0.2	1±0.2	1.2±0.1	1±0.2

Note: Notes: Asterisks (*) designate the level of statistical significance between ZN-fed and ZN-deprived treatment of a particular time interval at p< 0.05

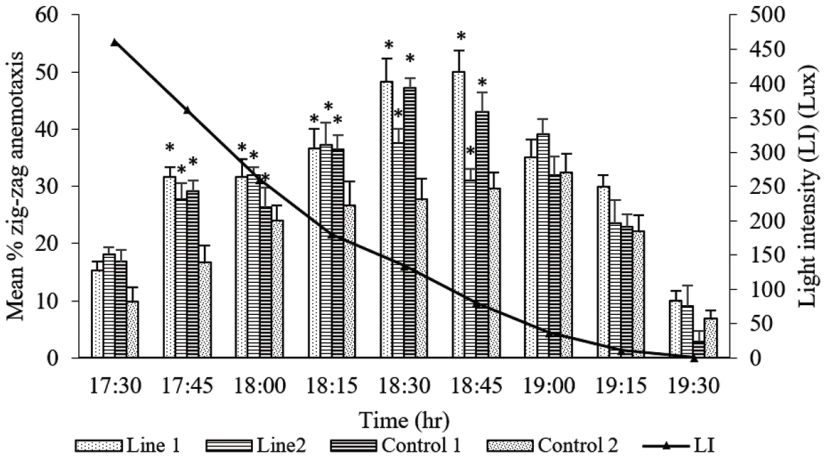

Figure 1. Mean number of zig-zag anemotaxis virgin females to both ZN-fed and ZN-deprived males of *B. dorsalis*

Note: Asterisks (*) designate the level of statistical significance among the treatment within the time intervals at the p<0.05 compared to the control 2

Decreasing light intensity at dusk appears to be a key stimulus for initiating mating behaviour in fruit flies (Hee & Tan 1998). Arakaki *et al*. (1984) also noted increased locomotion activity in females and males when light intensity decreased to less than 1000 lux, with female activity declining rapidly at light intensities below 10 lux, while caged-males became inactive and exhibited random behaviour.

Conclusions

This study revealed that NR-males attract to ZN after feeding, similar to the laboratory strain (ca. 35%). Feeding ZN enhanced male mating success in both NR lines and the laboratory strain, by initiating the mating activities earlier (5 days and 3 days post-feeding, respectively). These findings were consistent under semi-natural conditions. Additionally, ZN-fed males initiated copulation earlier than ZN-deprived males. Results from wind tunnel assay, showed significantly higher attraction of females to ZN-fed males than control males. These findings suggest that ZN is an efficacious pheromone precursor for male *B. dorsalis*. Moreover, ZN could be a promising compound to enhance mating performance in NR-males, potentially overcoming barriers associated with the concurrent use of SIT and MAT strategies.

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