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ORIGINAL CONTRIBUTION

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Methyl eugenol effects on *Bactrocera dorsalis* male total body protein, reproductive organs and ejaculate

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Abstract

Methyl eugenol (ME) and inclusion of protein into the adult diet increase the mating competitiveness of the Oriental fruit fly, *Bactrocera dorsalis* (Hendel). Exposing males to ME or protein is a promising post-teneral treatment for males being released in the sterile insect technique (SIT). However, the effect of this post-teneral treatment on male reproductive organs or the male ejaculate is unknown. During mating, males transfer sperm and accessory gland products (AGPs) to females and these compounds are reported to modulate female sexual inhibition. We studied the impact of male exposure to ME and a yeast hydrolysate (YH) diet on the protein reserves of males, male reproductive organ size, and the male ejaculate through sperm and AGPs. We show that males exposed to ME regardless of access to YH accumulated a greater amount of whole body protein. Males fed on YH also had increased total body protein and had bigger reproductive organs than YH-deprived males, but no apparent effect of ME exposure was observed on reproductive organ size. Females stored less sperm when mated with males fed on YH and ME compared to males not fed on ME. YH and ME had no effect on male AGPs. Females injected with AGPs of males fed on YH and exposed to ME were just as likely to mate as females injected with AGPs of non-treated males. However, females injected with AGPs of males exposed to ME mated faster than females injected with AGPs of non-exposed males. We conclude that while exposure to ME increases male copulatory success and protein reserves in the male body, there seem to be some potential trade-offs such as lower sperm stored by females. We discuss our results in terms of pre-release protocols that may be used for *B. dorsalis* in SIT application.

KEYWORDS
accessory glands, Diptera, protein, remating, sperm, Tephritidae

1 | INTRODUCTION

The sterile insect technique (SIT) as a component of area-wide integrated pest management (AW-IPM) is being widely used for the control of tephritid fruit flies and other pests (Hendrichs, Robinson, Cayol, & Enkerlin, 2002, Pereira 2013). For successful application of SIT, the mass-reared sterile males must be able to compete with wild males for transferring sterile sperm to wild females. However, several factors such as mass rearing or somatic damage caused by irradiation often result in the loss of natural attributes or sexual competitiveness of sterile males (Gavriel, Gazit, & Yuval, 2009; Mossinson & Yuval, 2003).

Thus, several efforts, the most effective being: (a) feeding males with protein supplements (Aluja, Jácome, & Macías-Ordóñez, 2001;

Pérez-Staples, Weldon, & Taylor, 2011; Yuval et al., 2007), (b) exposing males to essential oils (McInnis, Shelly, & Komatsu, 2002; Papadopoulos, Shelly, Niyazi, & Jang, 2006; Shelly & McInnis, 2001), (c) treating males with semiochemicals (Haq, Vreysen, Teal, & Hendrichs, 2014; Khan et al., 2017; Orankanok, Chinvinijkul, Sawatwangkhoung, Pinkaew, & Orankanok, 2013; Shelly, 2010; Shelly, Edu, & Pahio, 2005) and (d) the application of the juvenile hormone analogous, methoprene (Adnan et al., 2018; Haq, Cáceres, et al., 2010; Haq, Cáceres, Meza, Hendrichs, & Vreysen, 2018; Haq, Vreysen, Teal, et al., 2014; Teal, Gómez-Simuta, & Proveaux, 2000), have been made to enhance male mating competitiveness.

The response of males to each of these treatments can vary among different species. In particular, the incorporation of protein or yeast hydrolysate (YH) into the diet has been shown to increase the sexual competitiveness of males, increase the number of sperm stored by females and decrease the probability of female remating (Gavriel et al., 2009; Pérez-Staples, Harmer, Collins, & Taylor, 2008; Taylor & Yuval, 1999; Taylor et al., 2013; Yuval et al., 2007; Yuval, Kaspi, Field, Blay, & Taylor, 2002). However, in the Mediterranean fruit fly [*Ceratitis capitata* (Wied)] males fed protein transferred less sperm compared to the males deprived of them (Blay & Yuval, 1997). Treatments with juvenile hormone (JH) and aromatherapy are particularly promising (Caceres et al., 2007). For example, the use of JH enhances mating competitiveness in *Zeugodacus cucurbitae* (Coquillett) (Haq, Cáceres, et al., 2010) and *Anastrepha suspensa* (Loew) (Teal et al., 2000) and accelerates sexual maturation in the Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Collins, Reynolds, & Taylor, 2014). In the South American fruit fly, *Anastrepha fraterculus* (Wiedemann), methoprene, an analog of JH also accelerates sexual maturation (Segura et al., 2009) but does not increase sperm transfer and is not effective in inhibiting female remating (Abraham et al., 2013). On the other hand, aromatherapy treatments include the use of ginger oil to increase mating competitiveness in male *C. capitata* (Shelly & McInnis, 2001) and the use of methyl eugenol to increase male sexual attractiveness in *Bactrocera carambolae* (Drew & Hancock) (Haq, Vreysen, Cacéres, Shelly, & Hendrichs, 2014) and *Bactrocera dorsalis* (Hendel) (Shelly, 1994).

Methyl eugenol (ME) (1,2-dimethoxy-4-(2-propenyl) benzene) is a floral scent, phenylpropanoid compound, found in over 450 plant species (Tan & Nishida, 2012). It attracts many tephritid flies of the *Bactrocera* and *Dacus* genus (Haq, Vreysen, Cacéres, et al., 2014; Tan, Tokushima, Ono, & Nishida, 2011; Wee, Munir, & Hee, 2018; Wee, Tan, & Nishida, 2007). Its effectiveness to attract wild males is associated with the natural presence of this compound in the environment (Shelly, 1994). As a powerful attractant, it has been employed since the 1950s for purposes of monitoring and control (Steiner & Lee, 1955; Steiner et al., 1970) by attracting and trapping males (Barclay, McInnis, & Hendrichs, 2014).

The oriental fruit fly, *B. dorsalis* (Diptera: Tephritidae), is a species of quarantine importance. It is distributed in Asia, Africa and Europe. It infests several fruits and vegetables and is among the five most important pests to agriculture in Southeast Asia (White & Elson-Harris, 1992, McInnis et al., 2011). Studies have found that the copulatory success of *B. dorsalis* males is significantly improved when fed on ME and a protein-rich diet (Orankanok et al., 2013; Shelly & Dewire, 1994; Shelly & Nishida, 2004; Shelly et al., 2005). The physiological pathway of ME consumption in *B. dorsalis* showed that consumed ME is metabolized in the crop and the ME metabolites are stored in the rectal glands and then released as volatiles in the male pheromone (Tan & Nishida, 1996; Tan et al., 2011). When females were bioassayed for their attractiveness to ME metabolites, they showed higher attractiveness to rectal glands of ME-fed males (Hee & Tan, 1998; Khoo, Yuen, & Tan, 2000; Tan & Nishida, 1996). However, some studies proposed that some other mechanisms, in addition to pheromones, may also be involved in enhancing the male's copulatory success (Haq, 2018; Kumaran, Balagawi, Schutze, & Clarke, 2013). The other advantage of consuming ME is that it can increase predation evasion particularly against vertebrate predators (Wee & Tan, 2001).

Despite these benefits, we know surprisingly little about how ME affects male internal reproductive organ size or the ejaculate. In the context of energy metabolism, we know little about the costs of synthetizing ME, and how this can alter the amount of soluble protein that is stored in the tissues of treated males. The quantification of proteins stored in tissues can be used as an indicator of male quality. For example, the quantification of proteins has been used for the detection of resistance to pesticides in *Tetranychus urticae* Koch (Cerna et al., 2010), and to determine the nutritional status of *C. capitata* and *Z. cucurbitae* (Haq, Mayr, et al., 2010; Yuval, Kaspi, Shloush, & Warburg, 1998). Here, we studied if there was an increase in the amount of stored protein in *B. dorsalis* males exposed to ME and fed YH. The effects of ME on male physiology can give us a more precise understanding on how ME impacts male sexual performance.

Most of the studies on effects of post-teneral treatments have focused on pre-copulatory behaviours. However, a sterile male not only needs to mate with a wild female, it also has to inhibit the sexual receptivity of the female to prevent remating with a wild male. Males transfer sperm and accessory gland products (AGPs) in the ejaculate (Chen, 1984). AGPs can produce important changes in the post-copulatory behaviour of females, such as: a decrease in sexual receptivity, an increase in oviposition and an increase in food intake among others (Avila, Sirot, LaFlame, Rubistein, & Wolfer, 2011; Gillott, 2003). For example, in *C. capitata*, injections of AGP change the olfactory response of females from male pheromone to host fruit volatiles (Jang, 1995). AGPs have also been found to decrease the sexual receptivity of females in *A. fraterculus* and *B. tryoni* (Abraham, Cladera, Goane, & Vera, 2012; Radhakrishnan & Taylor, 2007).

Considering the important effects that AGPs can have on female behaviour and the promising use of post-teneral treatments, here we investigated in *B. dorsalis*, the effect of YH diet and exposure to ME on the efficacy of AGPs to inhibit mating behaviour by injecting AGPs into the haemocoel of virgin females. We also investigated if males fed YH and exposed to ME had a positive impact on male reproductive organ size and sperm transfer.

2 | MATERIALS AND METHODS

Bactrocera dorsalis pupae were obtained from an Indian population maintained at the Insect Pest Control Laboratory (IPCL), Joint FAO/IAEA Laboratories, Seibersdorf, Austria, and allowed to emerge. Pupae and adults were kept in a room illuminated with two white-light lamps with controlled light, temperature and humidity conditions (12:12-hr light:dark cycle, 24 ± 2°C, 54%–65% relative humidity). The natural light–dark period cycle was displaced such that dusk started at 12:00 p.m. which allowed us to obtain diurnal copulas. Upon emergence, adults were sorted by sex and 150 flies were placed in plexiglass cylindrical cages (30 × 20 cm in diameter) and maintained separately. Adults from this colony were used for protein quantification, sperm transfer and accessory gland injections. For all experiments, females were fed with sugar and yeast hydrolyzed (YH); protein diet in a ratio of 3:1. Males were fed either with the protein diet or only with sugar. The treatments were as follows: (a) YH and methyl eugenol (**+YH+ME**), (b) feeding on sugar only and no methyl eugenol (–**YH+ME**), (c) feeding on YH but no ME (**+YH**–**ME**) and (d) feeding on sugar only and no ME (–**YH**–**ME (control)**).

Males were treated with ME following the protocol described by Shelly et al. (2005). Sexually mature males (12–14 days) were moved to a separate room and supplied with 100 µl ME in a cotton wick. Males were exposed to ME for one hour during 10:00–11:00 hr, and then, males were again moved to an independent room where trials were conducted, 3 days after treatment.

Reproductive organ measurements were carried out with a colony established at the Division of Entomology and Nematology, Indian Institute of Horticultural Research, Bangalore, India. Adults were sorted by sex into plastic containers (120 × 80 mm Plastic, SPL Life sciences, Korea) with 10 individual flies per treatment. All insects were maintained at room temperature (24–28°C) with 60%–90% RH and photoperiod of 12:12-hr light–dark cycle.

2.1 | Protein quantification of males exposed to YH and ME

When males were 14 days of age, 30 males from each treatment were preserved in a Cell Lysis Buffer (50 ml PBS with protease inhibitor, Complete Mini, free of EDTA, Roche® Complete Protease inhibitor cocktail, Sigma-Aldrich, Darmstadt, Germany) and were placed in a liquid nitrogen container for shipment to INBIOTECA, Xalapa, Mexico, where experiments were conducted. Three males from each treatment were used for whole body protein content quantification. The biological material was thawed in ice, and each male was individually placed in a 1.5-ml Eppendorf tube with phosphate-buffered saline (PBS) with a protease inhibitor (Complete Mini, libre de EDTA, Roche®) and was gently homogenized with a plastic micro homogenizer for 1 min. Males from each treatment were placed in a 50 µl solution. The homogenized sample was centrifuged at 10,000 *g* at 4°C for 3 min (Hermle Z 300 K centrifuge, Franklin, WI, USA). The supernatant was transferred to a new tube on crushed ice, and the pellet was discarded. Protein quantification was carried out with the Bradford method (Bradford, 1976), using the Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). To obtain the standard curve, bovine serum albumin (BSA) was used as a reference protein in a set of dilutions (0, 0.5, 1, 2, 4, 8 and 16 mg/ml) with PBS and protease inhibitors as a diluent. After five minutes of incubation at room temperature, the absorbance of each sample was measured at a wavelength of 595 nm in an ELISA spectrophotometer (GENESYS, model Genesys 10, Rochester, NY, USA). Protein concentration was determined by contrasting against the standard curve obtained from the absorbance values with the reference BSA. This procedure was repeated three times for each treatment.

2.2 | Male reproductive organ size

At 17 days of age, (3 days after ME treatment), males from each treatment were anesthetized with ethyl acetate and dissected in distilled water under a stereomicroscope (Leica MZ7.5; Leica Microsystems Ltd, Switzerland). The male accessory glands, testes, apodeme and ejaculatory duct were photographed with a camera mounted on a stereomicroscope (Leica ME120HD). The area of both testes, the long arms of accessory gland, the area of the ejaculatory duct and the area of the apodeme were measured using ImageJ software, version 1.50i (National Institutes of Health, Bethesda, MD, USA). The standard scale was set using the scale bar on photographs, and global scale parameters were applied for all measurements. Testes areas were measured using the colour threshold tools with threshold colour red and colour space of HSB and RGB. The accessory glands, apodeme and ejaculatory duct were traced carefully along the boundaries with freehand selections tools and measured on set scale. As a proxy for size, thorax length was also measured for each individual. A total of 54 virgin males from the different treatments were used for reproductive organ measurement (+YH +ME: $N = 16$; +YH −ME: $N = 16$; −YH +ME: $N = 11$; −YH −ME: $N = 11$).

2.3 | Effect of YH and ME on sperm transfer

Flies at the age of 14 days (3 days after males fed on ME) were used for this experiment. One hundred sixty males from the four treatments and 160 females (only fed on YH based diet) were used. Males (40) and (40) females were placed in rectangular cages (32 × 20 × 15 cm). As soon as the flies started mating, the pairs were carefully taken out with a vial and placed in a 250-ml plastic cup with diet (sugar and hydrolysed yeast diet in a ratio of 3:1) and water. The next morning all males were removed, and only the mated females were maintained in the cups.

Females were anesthetized in an ethyl acetate chamber, and the pair of spermathecae was removed and dissected in a drop of 10 µl of saline solution with 0.1% of Triton, by observing under a stereomicroscope (Carl Zeiss, model Stemi 2000, with external lamp CL 6000 LED, USA). Once the spermathecae had been broken down, the content of the drop was vigorously shaken for 1 min, covered with a cover slip (22 × 22 mm) and sealed with a few drops of transparent nail polish. The slides were stored in a microscope slide

storage box for transfer to INBIOTECA where sperm was counted with a phase-contrast microscope (Iroscope series 300, Model MG-11, Irapuato, Mexico), following methodology of Pérez-Staples and Aluja (2006). The microscope slides were scanned at 100× to locate 30 fields, equivalent to 17% of the cover of the slide. When counts were finalized, observed sperm were multiplied by the conversion factor of 5.81. If no sperm were observed within the 30 fields, a total count of the sample was made. A total of 140 females were dissected (n = 67 females mated to males fed YH −ME males, n = 73 for females mated to males fed YH +ME).

2.4 | Effect of AGPs on female sexual receptivity

Accessory glands of males (14–16 days old) from the four treatments were dissected between 8:30 and 10:30 hr, 2 days after ME treatment. Ten accessory glands (from each of the four treatments) were placed in a 50 µl saline solution, then homogenized and centrifuged following Abraham et al. (2012), Abraham, Nuñez-Beverido, Contreras-Navarro, and Pérez-Staples (2014). A Hamilton syringe was used to inject virgin sexually mature females with either 1 µl of male accessory gland water extract or saline solution (NaCl 0.9%, Praxisdienst, Germany) as a control.

Twenty-four hours after injections, female sexual receptivity was recorded. One hour before dusk started, each female (virgin or injected) was offered a virgin sexually mature male (14–17 days old). Time of mating initiation was recorded to calculate mating latency (time between the start of the experiment and mating initiation).

2.5 | Statistical analyses

For all variables, Levene's test was used to check variance homogeneity, while normality was tested with a Shapiro–Wilk test. Protein quantification in males fed with YH and ME was analysed with a two-way ANOVA. Each reproductive organ was analyzed by ANCOVA with male thorax length, replicate and dietary treatment (ME and YH combinations) as independent variables. These variables were analysed in JMP version 9. To determine the effect of the diet on the AGPs, a generalized linear model (GLM) with binomial distribution and a logit link function were performed. Mating latency according to treatment was analysed using GLM with a Poisson distribution and logit link function. The data on the effect of YH and ME on number of sperm stored by females after copulation were non-normally distributed; therefore, a Wilcoxon test was used. Probability of sperm storage (females with no sperm vs. females that did store sperm) was analysed by chi-square. Analyses were performed in *R studio* (version 0.98.953).

3 | RESULTS

3.1 | Quantification of total soluble protein in males exposed to YH and ME

For this experiment, a total of 36 males (three for each of the four treatment × three replicates) were analysed. There was a significant effect of YH diet (F = 98.03; df = 1; p < 0.0001), exposure to ME (F = 11.90; df = 1; p = 0.001) and replicate (F = 18.99; df = 1; p = 0.0001) on whole body protein content in *B. dorsalis*. However, there was no significant effect of the interaction between diet and ME (F = 0.01; df = 1; p = 0.901). Whole body protein content in males was higher in males fed with YH compared to males deprived YH (fed sugar only). It was also higher in males with access to ME compared to males without ME access despite of having been deprived of YH (Figure 1).

3.2 | Reproductive male organ size

3.2.1 | Testes

Male treatment (F = 5.105; df = 3; p < 0.003), size (F = 10.761; df = 1; p = 0.002) and replicate (F = 17.577; df = 1; p = 0.0001) had a significant effect on testes size (N = 52). Tukey's HSD test showed that males fed on YH only (+YH−ME), had the biggest testes ($\bar{x}$ = 0.326 mm, SD = 0.07) compared to YH-deprived males and exposed to ME (−YH+ME, $\bar{x}$ = 0.256 mm, SD = 0.03) and the control (−YH−ME, $\bar{x}$ = 0.261 mm, SD = 0.07), with no significant difference for males fed YH and exposed to ME. There were no significant differences between any of the other male dietary treatments.

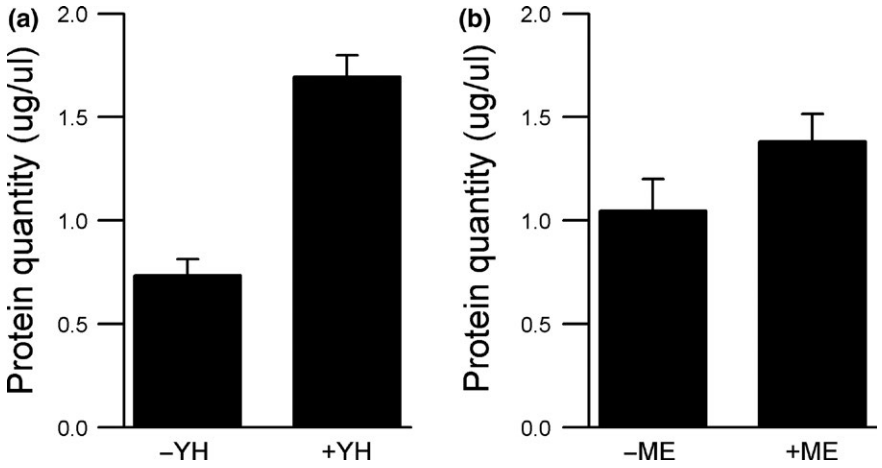

FIGURE 1 Mean ± *SE* whole body protein content of *Bactrocera dorsalis* males. (a) Males fed on yeast hydrolysate (+YH) or YH-deprived (−YH) and (b) males exposed to Methyl Eugenol (+ME) or not (−ME)

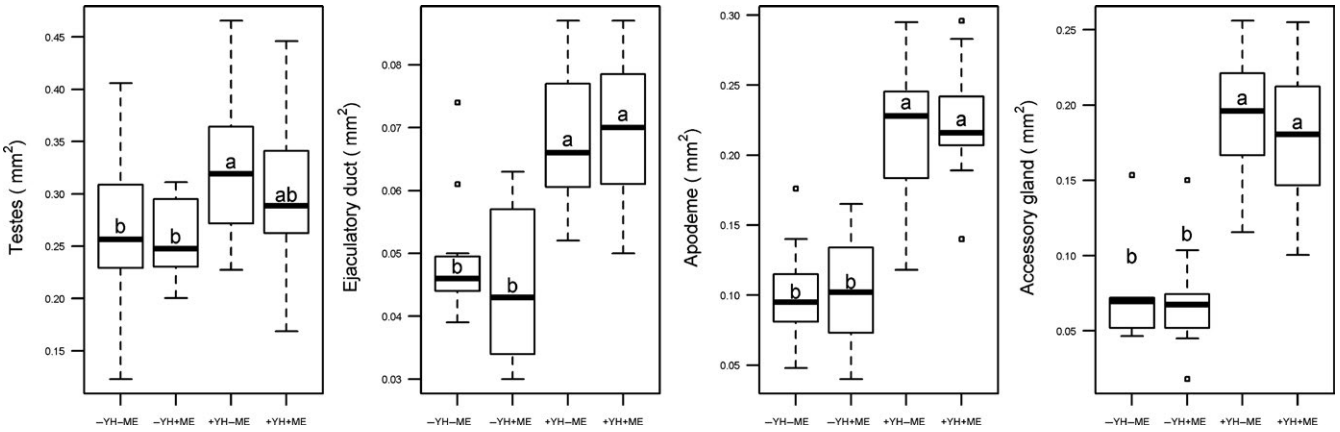

FIGURE 2 Reproductive organ size (mm^2) of *Bactrocera dorsalis* males fed on YH (+YH) or YH-deprived (–YH) and exposed to methyl eugenol (+ME) or not (–ME). Horizontal lines inside boxes represent the median; the box represents the percentile 25–75. Whiskers represent 1.5 times the interquartile range. Circles outside whiskers represent the outliers

3.2.2 | Ejaculatory duct

There was a significant effect of male treatment on the ejaculatory duct ($F = 18.427$; $df = 3$; $p < 0.0001$). Tukey's HDS test showed significant differences between the size of the ejaculatory duct of males fed YH (+YH+ME and +YH−ME) and those deprived of YH regardless of access to ME. There was no significant effect of male size ($F = 1.454$; $df = 1$, $p = 0.233$), or replicate ($F = 2.910$; $df = 1$; $p = 0.094$), ($N = 54$).

3.2.3 | Apodeme

There was a significant effect of treatment on the size of the apodeme ($F = 36.124$; $df = 3$; $p < 0.0001$); male size also had a significant effect ($F = 8.136$; $df = 1$; $p = 0.006$), but not replicate ($F = 1.824$; $df = 1$; $p = 0.183$), ($N = 52$). Tukey's HSD test showed significantly bigger apodemes for males fed on YH diet, regardless of whether they were exposed to ME or not (+YH+ME and +YH−ME), and compared to YH-deprived males.

3.2.4 | Male accessory glands

Male treatment also had a significant effect on the size of male accessory glands ($F = 32.983$; $df = 3$; $p < 0.0001$), ($N = 46$). Tukey´s HSD test showed that males fed on YH had significantly bigger accessory glands regardless of whether they were exposed to ME or not (+YH+ME and +YH−ME), and compared to YH-deprived males (Figure 2).

3.3 | Effect of YH and ME on number of sperm

Males fed with a sugar and YH diet, either exposed to ME or not, were able to mate with females, while YH-deprived males did not mate (Table 1). Females mated with males fed on YH but not exposed to ME (+YH−ME) were found to store more sperm than females mated to males fed with YH and exposed to ME (+YH+ME) ($W = 3{,}481.5$, $p < 0.0001$, $N = 140$) (Figure 3). Only nine females (6%) did not store any sperm, out of these seven were females mated to males fed YH and exposed to ME (+YH+ME). However, there was no significant difference in the likelihood to store sperm according to male treatment ($\chi^2 = 2.692$, $p > 0.100$).

TABLE 1 Number of matings by *Bactrocera dorsalis* males fed with sugar only (–YH) or sugar and yeast hydrolysate (YH) andstored a lower number of sperm in the exposed or not to methyl eugenol (±ME). Males fed YH and exposed to ME had higher mating success ($\chi^2 = 27.96$, $df = 3$, $p < 0.001$)

Male treatment	No. of pairs	No. of mated	Per cent mated
+YH +ME	53	14	26.41
–YH +ME	55	1	1.81
+YH –ME	60	12	20
–YH –ME	60	0	0

3.4 | Effect of AGPs on female sexual receptivity

A total of 244 females were injected, and twenty-four hours after being injected, 182 females were placed with virgin males for evaluation of female receptivity for mating (+YH +ME: $N = 37$; +YH −ME: $N = 34$; –YH +ME: $N = 36$; –YH −ME: $N = 37$; saline solution (control): $N = 38$).

There were no significant differences in sexual receptivity between females injected with AGPs of males fed with YH ($\chi^2 = 1.863$; $df = 1$; $p = 0.172$), or those injected with AGPs of males fed with ME ($\chi^2 = 1.576i$; $df = 1$; $p = 0.2101$). The interaction between YH and ME was not statistically significant ($\chi^2 = 0.3261$; $df = 1$; $p = 0.568$). The highest female receptivity (51.35%) was observed for females injected with AGPs from males fed with sugar (–YH −ME). The lowest receptivity (25%) was found in females injected with AGPs from males fed with YH and without exposure to ME (Figure 4).

Exposure to ME had a significant effect on mating latency ($\chi i^2 = 0.182$; $df = 1$; $p = 0.01$) (Figure 5). Females that were injected with AGPs of males exposed to ME mated faster (average of 56.96 min) compared to females injected with AGPs from males

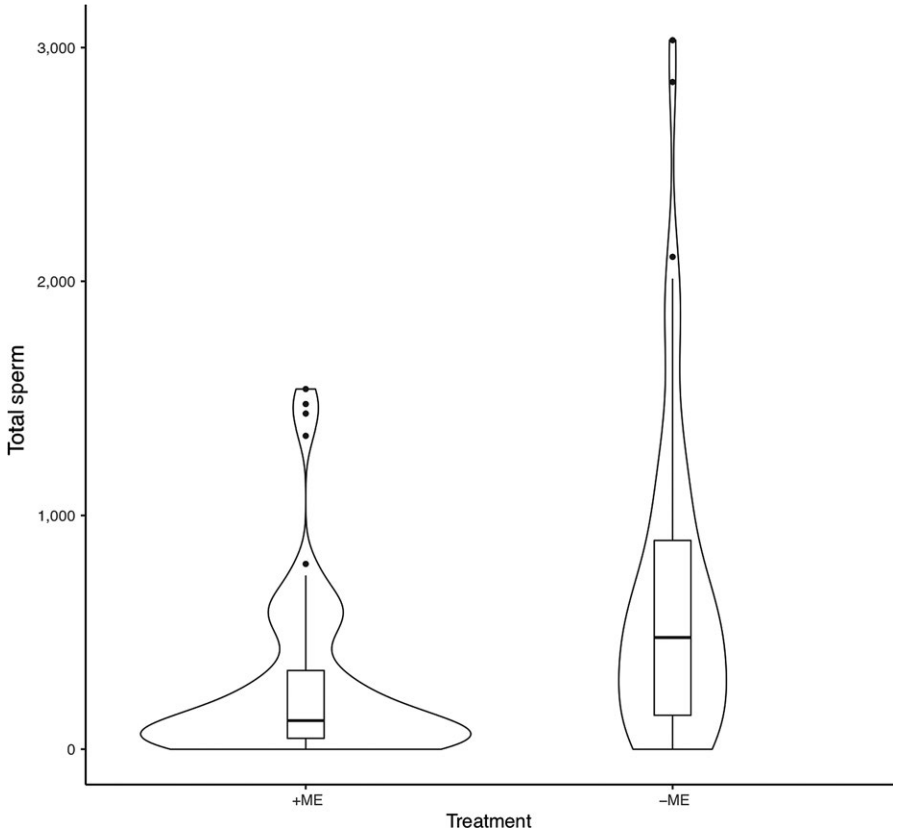

FIGURE 3 Violin plot of number of sperm stored by females, mated with *Bactrocera dorsalis* males fed on yeast hydrolysate (YH) and not exposed to ME (−ME) or fed on YH and exposed to methyl eugenol (+ME). Females were fed YH. Horizontal lines inside boxes represent the median; the box represents the percentile 25–75. Whiskers represent 1.5 times the interquartile range. The distribution of the variable is illustrated as density curves

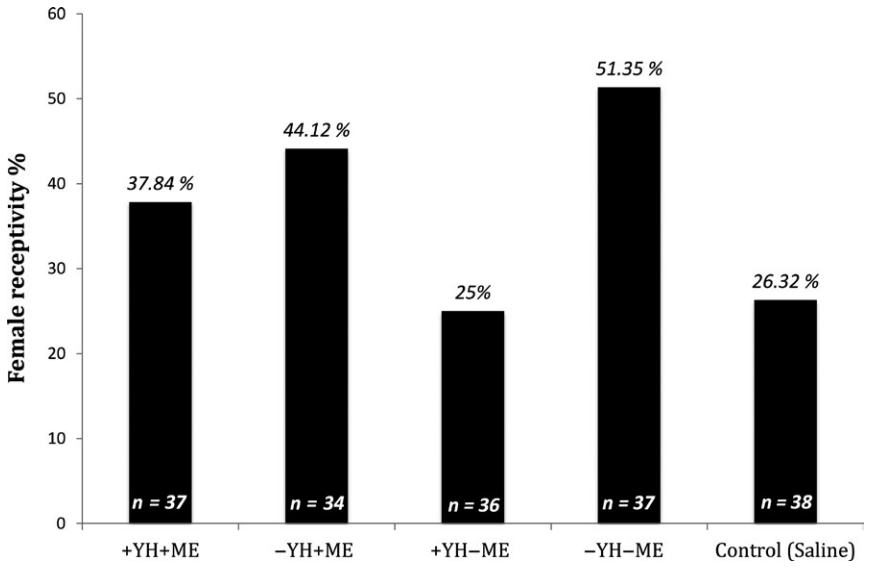

FIGURE 4 Sexual receptivity of *Bactrocera dorsalis* females that mated with virgin males 24 hr after being injected with saline solution (Control [Saline]), Accessory gland products (AGPs) of males fed on YH or without YH, and exposed or unexposed to methyl eugenol (ME). Numbers within bars (*N*) show sample size of injected females. Females were fed YH

without contact with ME (average of 72.39 min). There was no significant effect of either YH ($\chi i^2 = 0.182$; $df = 1$; $p = 0.667$) or the interaction between YH and ME ($\chi i^2 = 0.810$; $df = 1$; $p = 0.368$) on mating latency (Figure 5).

4 | DISCUSSION

Methyl eugenol has been used for the detection, monitoring and control of pest tephritid fruit flies (Fitt, 1981). Males treated with ME have higher copulatory success, as ME serves as a precursor of male sexual pheromone (Nishida et al., 1988; Shelly & Dewire, 1994; Tan & Nishida, 1996). Here, we tested if a protein-rich diet and the addition of ME affected male quality in terms of body protein content, reproductive organ size, male ejaculate attributes and the post-copulatory response of females. The mating success of males fed with YH was improved with the addition of ME, similar to results by Orankanok et al. (2013). Males fed only with ME and without YH have a low mating success similar to YH-deprived males. These results are in accordance with those obtained by Shelly et al. (2005).

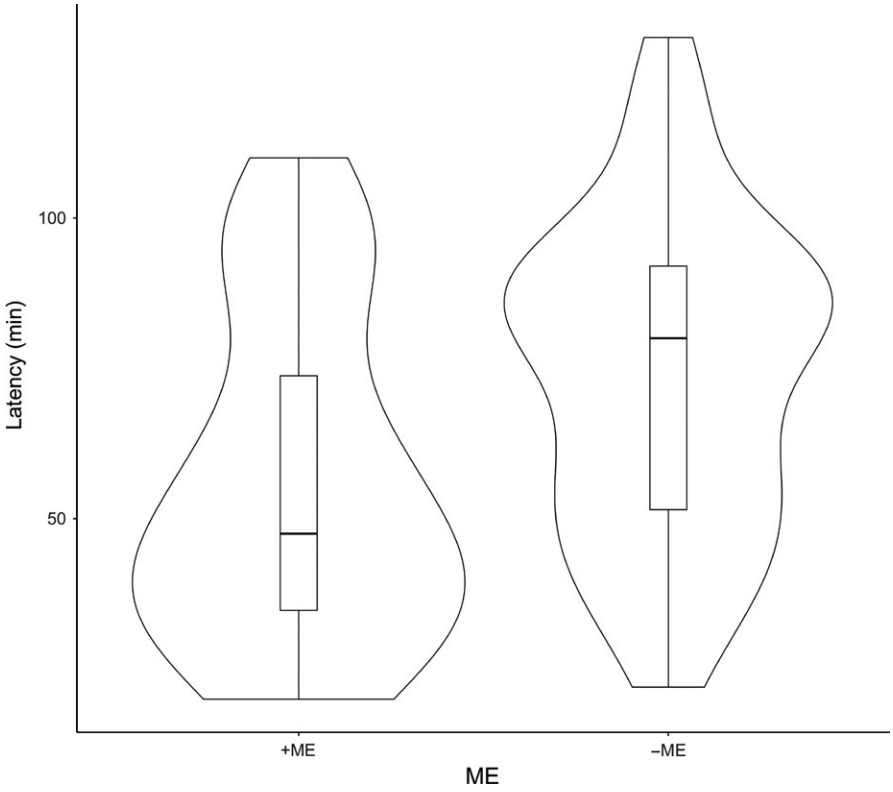

FIGURE 5 Violin plot of latency (min) to mate of *Bactrocera dorsalis* females that were injected with accessory gland products of males fed on yeast hydrolysate and not exposed to ME (–ME) or fed on yeast hydrolysate and exposed to methyl eugenol (+ME). Females were fed YH. Horizontal lines inside boxes represent the median; the box represents the percentile 25–75. Whiskers represent 1.5 times the interquartile range. The distribution of the variable is illustrated as density curves

Males exposed to ME contained higher amounts of protein in their adult body compared to ME-deprived males. This suggests that regardless of access to YH, ME is affecting protein metabolism or protein synthesis. Proteins are synthesized as structural and catalytic components in most cellular reactions (Torres-Cabra, Hernández-Fernández, & Pérez-Rubiano, 2015); thus, whole body protein content can be an important indicator of overall male quality. Protein content may impact male pheromone production and energy expenditure in courtship resulting in overall higher copulatory success for ME-fed males (Haq et al., 2018; Kumaran et al., 2013; Shelly & Dewire, 1994). Improved male copulatory success could be due to increased female attraction to a higher quality male and/or increased success through intrasexual competition. The increase in protein accumulation manifested itself after only 3 days from exposure to ME, when males were already sexually mature. From an applied perspective, this could imply that males treated with ME and then released a few days later could have a higher energy metabolism. This could have positive impacts on male mobility and sexual behaviour as outlined above. The effects of ME on protein synthesis need to be further studied when males are immature, and when there is a longer period (e.g., 10 days) between exposure to ME and protein analysis.

While feeding on YH also significantly increased total body protein, the interaction between access to YH and ME did not increase protein content. Although ME was not studied, similar results were found in the total body nitrogen (a proxy for protein) of *Z. cucurbitae*. Males fed YH had twice as much nitrogen than YH-deprived males (Haq, Mayr, et al., 2010). In contrast, exposure to methoprene, a juvenile hormone analog, had no effect on nitrogen acquisition. Here, the testes, ejaculatory duct, apodeme and male accessory glands were all bigger for males fed YH regardless of access to ME. Thus, the increase in male mating success by exposure to ME is not translated into greater allocation of resources to male reproductive organs. On the other hand, protein is necessary for spermatogenesis and other mechanisms of cellular synthesis. Feeding on YH in many tephritids translates into faster development, higher male mating success, increased sperm storage by females, higher female remating inhibition and bigger reproductive male organ size (reviewed in Pereira et al., 2013; Taylor et al., 2013).

A surprising result was that females mated with males fed with ME stored a lower number of sperm in the spermathecae compared to females mated with males not exposed to ME. It appears that ME increases the sexual performance of males, but it also could compromise their capacity to transfer sperm. A possible explanation for lower sperm transfer for ME exposed males could be related to costs of metabolizing ME due to its toxicity. Even though *B. dorsalis* males are highly attracted to ME, concentrations of 10%–100% induced mortality or knockdown of 35%–53% (Chang, Cho, & Li, 2009). For *C. capitata* and *Z. cucurbitae*, ME is lethal even after only a 2-hr exposure. Methyl eugenol and it´s derivates have been found to cause abnormalities in livers of geckos fed on *Bactrocera papayae* (Drew & Hancock) ME-fed males (Chang et al., 2009; Wee & Tan, 2001). The toxicity of ME could perhaps be diminished if males do not ingest it. This could be solved with the application of ME through aromatherapy (without direct contact). For example, *B. carambolae* males either exposed to ME (through aromatherapy) or fed with ME, increase their mating success as compared to untreated males (Haq, Vreysen, Cacéres, et al., 2014). Recently, it has been shown that exposure to ME either through feeding, aromatherapy or using an

air-blown system, has similar positive results on male mating success (Haq et al., 2018). In future studies, ME aromatherapy could be used to determine whether the negative effect of ME on sperm transfer is offset when it is not ingested by males.

Exposure to ME had no clear effects on male accessory glands, in terms of size or their effect on female sexual receptivity. However, there was an effect of ME on female mating latency, as we found that females injected with the AGPs of males exposed to ME mated faster than females injected with non-exposed AGPs. Thus, a disadvantage of the use of ME would be that females mating with ME exposed males may store less sperm and remate sooner. However, there was no effect on overall mating inhibition. Likewise, Shelly and Edu (2008) found that females were just as likely to remate regardless of having mated with an ME-fed male. Increased attractiveness to ME-fed males has also been found for mated females. When females first mated with a semi wild male, and 5 days later, females were exposed to ME-fed or ME-deprived males as a second mate, there was no effect on female propensity to remate. However, by 10–15 days after a first mating with a semi wild male, females were more attracted to and remated more frequently with ME-fed males (Ji, Chen, McInnis, & Guo, 2013). Thus, an added advantage of utilizing ME as a pre-release supplement would be that males would be more attractive to both virgin and mated females. Our results also indicate that AGPs might not be directly involved in the inhibition/modulation of sexual receptivity of *B. dorsalis*. This would need to be corroborated across longer female sexual refractory periods.

Overall, the advantages of using ME as a pre-release supplement probably outweigh the costs. Precursors of pheromone are provided by a host plant or molecules incorporated into the diet (Yew & Chung, 2015). The energetic cost of sexual signalling and producing pheromone during courtship is metabolically high; thus, males obtain direct benefits from the consumption of attractants (Kumaran, Prentis, Mangalam, Schutze, & Clarke, 2014). Here, we observed that incorporation of ME in the diet increased the total body protein of males, which together with a more attractive pheromone, probably increases male copulatory success. A disadvantage will be that ME-fed males will transfer very little sperm; however, this does not seem to result in increased rematings for females. Nevertheless, a faster mating by females injected with the AGPs of ME-fed males suggests that the effect of ME on female remating inhibition warrants further research.

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AUTHOR CONTRIBUTION

DPS, SA, MRH conceived research. MRH, RT, DPS conducted experiments. PDKJ, SA and DPS contributed material and secured funding. MRH analysed the data, and DPS and MRH wrote the manuscript. All authors read and approved the manuscript.

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