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Research

Ecology and Behavior

Electrophysiological, behavioral, and field responses of olive fruit fly to modified Brewer's spent yeast formulations

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Olive fruit fly, *Bactrocera oleae* (Rossi), is the primary olive pest in the Mediterranean, where management increasingly seeks sustainable alternatives to synthetic insecticides. This study evaluates recycled Brewer's spent yeast (BSY) from Croatian breweries as a potential sustainable attractant. Two BSY types (ale and lager) were processed into modified formulations at concentrations of 5%, 10%, and 15% and tested through electroantennogram (EAG) analysis, laboratory multiple-choice assays, and field trials. Adult flies were categorized into 5 groups based on age and mating status. Normalized EAG analysis revealed a significant dose-response relationship in females, with sensitivity increasing with ale-type concentration (5% to 15%), especially in young flies (0 to 4 days). In contrast, female sensitivity to lager-type BSY showed a slight age-dependent decrease. Laboratory multiple-choice assays confirmed these findings: 10% modified lager-type BSY was most attractive to young adults, while older, mated females responded consistently to 15% lager and 10% ale formulations. In field trials across 2 orchards, the 5% lager-type bait was the most effective, capturing 1.46 flies per trap per day, while higher concentrations resulted in lower captures. The research shows that olive fruit fly attraction to BSY is physiologically modulated by age and mating status. The strong performance of these low-concentration, recycled yeast baits demonstrates their potential as cost-effective, sustainable attractants that may lead to promising approaches for Integrated Pest Management in olive cultivation.

Keywords: attractant, *Bactrocera oleae*, electroantennography, Integrated Pest Management, protein-based lures, *Saccharomyces cerevisiae*, *Saccharomyces pastorianus*

Introduction

Fruit flies (Diptera: Tephritidae) are among the most harmful pests of horticultural crops worldwide (He et al. 2023). Among the most economically important species are the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann); the Caribbean fruit fly, *Anastrepha suspensa* (Loew); the cherry fruit fly, *Rhagoletis cerasi* (L.); the apple maggot fly, *Rhagoletis pomonella* (Walsh); the melon fly, *Bactrocera cucurbitae (Coquillett);* the Oriental fruit fly, *Bactrocera dorsalis* (Hendel); and the olive fruit fly, *Bactrocera oleae* (Rossi). *Bactrocera oleae* Rossi (Diptera: Tephritidae) is the primary pest of olives in Croatia and across the Mediterranean basin (Sharaf 1980, Kapatos and Fletcher 2017). Established in the region for over 2 millennia, it can cause severe, and sometimes total, annual yield losses if not controlled (Burrack et al. 2008, Martínez-Pertíñez and Vélez 2020). Global production losses are estimated at approximately 15% annually (Bueno and Jones 2002), with complete crop loss reported in certain years (Collier and van Steenwyk 2003). Damage affects both table olives and olive oil production. Infested fruits are unsuitable for the fresh market, while larval feeding and secondary pathogen infections significantly reduce oil yield and quality (Vitanović et al. 2020a, Vitanović et al. 2020b). Although *B. oleae* adults exploit a broad range of sugar- and protein-rich substrates, including nectar, honeydew, pollen, bird feces, and decaying fruits, the larvae develop exclusively within olive fruit where females oviposit, feeding solely on the mesocarp (Hendrichs et al. 1993, Balampekou et al. 2023, 2026). Recent studies have demonstrated the importa. Given the high reproductive potential of *B. oleae* and its capacity to cause severe crop losses, substantial effort has been devoted to detection and management strategies, including semiochemical lures, protein bait sprays, sterile insect technique, and synthetic insecticides.

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For decades, management of *B. oleae* has relied primarily on synthetic insecticides, raising concerns about environmental contamination (Cirio 1997, Civantos 1999) and reductions in canopy biodiversity (Vitanović et al. 2018). In line with the European Union's strategy to reduce pesticide use by 50% by 2050 (Bego et al. 2021, European Commission 2023), developing sustainable and effective alternatives is crucial. Olfactory attractants including pheromones (Carpita et al. 2012, Canale et al. 2013), ammonia-based lures (Haniotakis 2005), and proteinaceous baits (Biasazin et al. 2018) are essential tools for monitoring and improved management. Although protein-based lures demonstrated significantly greater attractiveness compared to simple ammonia lures (Prokopy and Economopoulos 1975), traditional protein hydrolysates have been criticized as monitoring tools. Specifically, they often failed to detect early-season populations and tended to overestimate the proportion of gravid females (Neuenschwander and Michelakis 1981). Despite these limitations, protein-based lures, such as torula yeast solutions, have proven superior to other attractants for *B. oleae* under many conditions (Burrack et al. 2008, Villamil et al. 2013).

Brewer's spent yeast (BSY) is a promising and underutilized resource in this context. The brewing industry generates substantial amounts of by-products, with BSY being the second largest fraction (Huige 2006), accounting for about 15% of total residues (Kerby and Vriesekoop 2017). Depending on the yeast strains used in the brewing process, beers are divided into 2 main groups: lager (bottom-fermented beers) and ale (top-fermented beers). For lager beer production, the yeast strain *Saccharomyces pastorianus* (Hansen) is commonly used, whereas ale beer production uses *S. cerevisiae* (Hansen). According to Kunze (2014), after fermentation and maturation, most of the yeast settles at the bottom of the tank or rises to the surface, allowing it to be easily separated and removed as waste (Lewis and Young 1995). This inactive, nutrient-rich biomass contains proteins, minerals, and vitamins (Karlović et al. 2020) and differs in composition from pure *S. cerevisiae* cultures. Although BSY is commonly used as livestock feed or discarded (Karlović et al. 2020, Rachwał et al. 2020, Avramia and Amariei 2021), improved management and valorization strategies could offer significant economic and environmental benefits (Rachwał et al. 2020).

Lloyd and Drew (1997) first proposed that BSY could be developed into locally produced baits. Subsequent research confirmed that modified BSY formulations can attract various tephritid pests (Ekesi and Tanga 2016, Cai et al. 2019, Patel et al. 2019, Wang et al. 2021). However, BSY derived from breweries has not been evaluated against *B. oleae*. While these previous studies on other tephritids primarily focused on general field attraction, the present work provides additional details that may better assist in the development of sustainable tools for IPM programs by comparatively evaluating how specific BSY types (ale vs. lager) and processing concentrations modulate both electrophysiological and behavioral responses, accounting for the fly's age and mating status. Although other research has investigated the responses of *B. oleae* to olive volatiles (Scarpati et al. 1993, 1996, Lo Scalzo et al. 1994, Vitanović et al. 2024) and other yeast species (Vitanović et al. 2020b), no study has characterized the antennal responses of this species to volatile emissions of modified BSY.

Volatile emissions produced by microorganisms play an important role in mediating insect-host and insect-microbe interactions (Qadri et al. 2020, Chakraborty and Roy 2021). These emissions function as olfactory cues that attract adult tephritids to food resources that are also necessary for reproductive development (Riddick 2020, Scolari et al. 2021, Antonatos et al. 2026). Therefore, characterizing the antennal sensitivity of *B. oleae* to modified BSY formulations will provide important insights into the olfactory signals involved in resource location and may contribute to the development of improved semiochemical-based monitoring and management strategies.

A more complete understanding of how BSY-based attractants affect the behavior and electrophysiological responses of *B. oleae* could lead to the use of these materials for insect control. Therefore, the objective of the present study was to evaluate the electrophysiological, behavioral, and field responses of *B. oleae* to modified ale and lager BSY formulations through electroantennogram (EAG) analysis, laboratory multiple-choice preference assays, and field trials. Currently available attractants are not sufficiently effective to control *B. oleae* in olive orchards, especially in years with high populations. We anticipate that further evaluation of modified BSY formulations will provide enhanced, sustainable tools for the monitoring and management of *B. oleae*.

Materials and Methods

Laboratory Modification of BSY

Two types of BSY were used as starting materials: 40 L of BSY obtained from a craft brewery (Familia, Kaštel Gomilica, Croatia) after the production of ale beer (*S. cerevisiae*), and 40 L of BSY sourced from a commercial brewery (Karlovačka pivovara, Karlovac, Croatia) after the production of lager beer (*S. pastorianus*). To maintain biological stability and prevent premature fermentation or degradation, both BSY samples were transported in 50 L stainless steel containers stored on ice from the respective breweries to the Institute for Adriatic Crops (IAC). Upon arrival, the substrates were immediately subjected to the following protocols.

The 2 types of BSY were processed to obtain modified formulations following the methodology described by Ekesi and Tanga (2016), which was further adapted and optimized to enhance the attractiveness of the resulting lures for *B. oleae*. These modifications were implemented to refine the volatile emissions and nutritional quality of the substrates. For each BSY type, a 40 L volume was transferred into a 100 L electric boiling pan (Variomix, Electrolux Professional, Pordenone, Italy) and heated at 95°C for 10 to 12 h. The process used an integrated temperature sensor and an automatic water-filling system to ensure precise thermal maintenance, while the "Smart Variomix" stirring system provided continuous mechanical agitation for substrate homogenization. Thermal treatment continued until the yeast slurry was reduced to 50% of its initial volume, effectively increasing the dry matter content and ensuring that residual alcohol was evaporated as much as possible. This concentration process was essential to stabilize the substrates and ensure a standardized base for subsequent lure development.

After thermal concentration, the resulting BSY concentrates were cooled to room temperature and transferred to glass vessels for enzymatic hydrolysis. The substrates were treated with 0.4% (w/v) papain (Thermo Scientific, Geel, Belgium) to catalyze protein degradation. The solutions were incubated in a

water bath at 65°C for 96 h. This hydrolysis step was conducted to break down complex proteins into smaller peptides and amino acids, thereby enhancing the release of volatile attractants. After hydrolysis, the modified BSYs were cooled to room temperature. To prevent microbial spoilage and ensure long-term stability, the formulations were preserved with 0.4% (w/v) methyl 4-hydroxybenzoate (Thermo Scientific, Kandel, Germany). The stabilized modified BSYs were then poured into sterilized 1 L Nalgene bottles (Rochester, NY, USA) and stored at 4°C until further use in laboratory bioassays and field trials.

Laboratory Bioassays

B. oleae Collection and Rearing

The collection and laboratory rearing of *B. oleae* followed the methodology described by Vitanović et al. (2020a). Infested olive fruits were sampled from the experimental olive orchard of the IAC, located in Duilovo (43°30′N, 16°29′E). The olives were placed in open plastic boxes (40 × 40 × 8 cm) equipped with a bottom grid to allow pupating larvae to descend into foil-lined trays placed beneath. A total of 1,054 pupae were collected and transferred to Petri dishes inside insect net cages (20 × 20 × 20 cm), resulting in the successful emergence of 840 adults (398 females and 442 males). Rearing was conducted in a controlled environment room maintained at 26 ± 1°C and 65 ± 10% relative humidity (RH), under a 16:8 (L:D) photoperiod provided by fluorescent lighting. Emerged adults were collected daily and separated into new net cages based on age, sexual maturity, and mating status. From the total emerged population, a total of 510 healthy flies was successfully assigned to the experimental protocols. The adults were divided into 5 experimental groups (Table 1). Specifically, for the EAG analysis, 30 individual flies were used (5 individuals per sex and age group; $n = 5$ per group × 2 sexes × 3 age groups). For the laboratory multiple-choice preference assays, a separate group of 480 flies was distributed across the replication setups to evaluate behavioral choices across different age categories.

Flies were provided with a 1:10 (v/v) honey–water solution and water via soaked cotton dental roll, which were replaced every 48 h to ensure freshness.

EAG Analysis

Electrophysiological responses of *B. oleae* to different concentrations of modified BSY (ale 5%, 10%, and 15%; lager 5%, 10%, and 15% v/v) were recorded with a Syntech set up. Adult flies were anesthetized on ice, and their heads were carefully excised with micro-dissecting scissors. For EAG recordings, glass capillaries filled with 0.1 M KCl solution served as electrodes, with electrical connectivity to the system's preamplifier established via silver (Ag) wires. For each preparation, the excised head was used. The reference (ground) electrode was inserted into the base of the head, while the distal tip of the antenna (funiculus) was inserted into the recording electrode. Electrical signals were recorded and amplified using a Syntech IDAC-4 high-impedance amplifier (Syntech, Kirchzarten, Germany). Test stimulations were delivered as short air pulses (200 mL min^{-1}) with a 2 s duration, managed by a CS-55 stimulus controller (Syntech, Kirchzarten, Germany). The airstream was directed over the antenna through a glass delivery tube positioned 15 mm from the preparation. Stimulus cartridges were prepared by applying 10 µL of each diluted modified BSY test solution onto a filter paper strip (1 cm^2) inserted into a 15 cm Pasteur pipette. All cartridges were stored at –20°C until use to ensure formulation stability and were allowed to reach room temperature before testing. Each antenna was exposed to a series of stimuli, including water as a solvent control. Each stimulus was presented in 3 technical replicates per antenna, with a 30 s interval between replicates and a 1 min recovery period between different stimuli. To prevent sensory saturation, stimuli were applied in ascending order of concentration. Antennal responses were recorded from a total of 30 healthy flies, comprising 5 distinct individuals per sex and age group ($n = 5$ flies × 2 sexes × 3 age groups), and processed using EagPro V2.1.0 software (Syntech, Kirchzarten, Germany). The absolute amplitude of each EAG response was subtracted from the mean EAG response of the water control, to compensate for solvent and/or mechanosensory artefacts. To account for mechanosensory artifacts (the physical impact of the air puff) and baseline biological drift of the antennal preparation over time, a 2-step normalization protocol was applied to the raw electroantennographic (EAG) data. First, a stabilized baseline control value ($\bar{C}$) was established for each antenna by calculating the overall mean of the 3 solvent control (water) responses recorded at the beginning of the trial (C_{beg}) and the 3 solvent control responses recorded at the end of the trial (C_{end}):

$$\bar{C} = \frac{1}{6}\left(\sum_{i=1}^{3} C_{beg,i} + \sum_{j=1}^{3} C_{end,j}\right)$$

Second, the absolute raw amplitude deflection (mV) elicited by each test stimulus (R_{raw}) was corrected by subtracting the calculated baseline mean control response ($\bar{C}$). The resulting adjusted amplitude was then expressed as a normalized relative ratio (R_{norm}) against the mean control response using the following equation:

$$R_{norm} = \frac{R_{raw} - \bar{C}}{\bar{C}}$$

On this relative scale, a value of 0.0 represents an EAG response identical to the solvent baseline, whereas positive values represent fold increases in depolarization amplitude relative to the control. This normalization accounted for individual variations in baseline sensitivity among different insect preparations, making the relative values suitable for subsequent statistical analyses.

Table 1. Categorization of the adult flies in groups based on age, sexual maturity, and mating status

Group	Age	Sex		Mating status	Description
1	0 to 4 days	♂		×	Young, sexually immature males
2	0 to 4 days	♀		×	Young, sexually immature females
3	5 to 9 days	♂		×	Sexually mature males (unmated)
4	5 to 9 days	♀		×	Sexually mature females (unmated)
5	>10 days	♂ + ♀			Sexually mature mated adults

♂ Male; ♀ Female; × Unmated.

Multiple-Choice Preference Assay

To quantitatively assess the attraction of the *B. oleae*, a rigorous multiple-choice preference assay based on the protocol developed by Zalom's laboratory was used. For each test, an 8 mL aliquot of the respective BSY solution was placed in a plastic cup (Solo 10). Each cup was covered with an inverted fiberglass window screen mesh cone (1 cm central aperture) and secured with a rubber band to serve as a trap. The traps were placed inside an insect net cage (20 × 20 × 20 cm). The experimental design used 2 independent duplicate cages evaluated simultaneously for each of the 3 age groups, for a total of 6 experimental cages. For the first age category, a total of 160 flies (0 to 4 days old) were deployed, with 40 males and 40 females introduced into each of 2 duplicate cages. The assay was conducted in a controlled environment room (26 ± 1°C and 65 ± 10% RH). Seven samples were assayed simultaneously: 2 modified BSYs at 3 concentrations (5%, 10%, and 15% v/v) and water as a control. The samples were randomly positioned within 2 duplicate cages. The number of flies captured in each trap was recorded hourly for 8 h. To eliminate potential bias from trap positioning within the cage, the traps were rotated 1 position forward after each hourly count. The same experimental procedure was repeated twice for the other age groups, using a group of 160 sexually mature, unmated flies (5 to 9 days old; 40 males and 40 females per cage, across 2 replicates) and 160 sexually mature, mated flies (≥10 days old; 40 males and 40 females per cage, across 2 replicates), bringing the total number of flies used in the behavioral assays to 480. Upon completion of the assay, the final number of males and females in each trap was recorded for subsequent statistical analysis. Prior to the assay, the flies were starved for 24 h, and each individual was used only once in the experiments.

Field Trials

The attractiveness of both modified BSY to *B. oleae* was evaluated at 3 concentrations (5%, 10%, and 15% v/v) in 2 different olive orchards in Dalmatia, Croatia. The first site was an IPM-managed experimental multivarietal orchard at the IAC in Kaštel Stari (43°33′N, 16°20′E), characterized by high varietal diversity. The second site was an organic monovarietal orchard (cv. Oblica) in Kaštel Lukšić (43°33′N, 16°21′E). Field trials were conducted from August to November 2025 to assess the dose-response relationship of the modified substrates under varying environmental and agronomic conditions. The field trials lasted 98 days (14 consecutive weeks). Across both orchards, the cumulative sampling effort was 4,116 trap-days, with an overall average capture rate of 0.45 *B. oleae* individuals per trap per day.

The experimental setup followed a randomized complete block design with 3 replicates per olive orchard. Each experimental unit consisted of a McPhail trap filled with 200 mL of a specific modified BSY concentration. Traps containing 200 mL of water were included in each block as a negative control. McPhail traps were suspended within the olive canopy at a height of 1.5 to 2.0 m. To minimize interference between treatments and minimize plume overlap, a minimum distance of 15 m was maintained between adjacent traps within a block, while blocks were separated by at least 25 m. Traps were inspected weekly, and the number of male and female *B. oleae* was recorded. The lure solutions were replaced in all traps during each inspection. To eliminate potential positional bias, traps were rotated 1 position forward within each block at every 7-d interval.

Statistical Analysis

Normalized EAG responses were evaluated using a Linear Mixed Model (LMM) with lme4 package in R (Bates et al. 2015, R Core Team 2025). The model included "stimulus," "sex," "age group," and their interactions as fixed effects, and "antenna ID" as a random effect to account for physiological variability. Data from field and laboratory bioassays were analyzed using Generalized LMMs. These models use ANOVA to directly accommodate the non-homogeneous variance and discrete nature of tephritid fruit fly capture data without relying on data transformation (Biasazin et al. 2022, 2025). Field count data were primarily fitted using a Poisson distribution with a log link function. To account for potential overdispersion, models were adjusted to a Negative Binomial distribution when variance significantly exceeded the mean (Biasazin et al. 2022, 2025). The field model included "BSY solution," "sex," and "time sampling" as fixed factors, and "block" as random effect to compensate for spatial variations in olive orchards. For laboratory assays, a Binomial distribution was employed to model the probability of capture (Daher et al. 2022). This model included "BSY solution," "sex," and "age group" and their interactions as fixed effects, and "cage ID" as random effect to capture inter-cage variability and ensure that the estimated fixed effects are robust across different experimental setups. Both models were implemented using the glmmTMB package in R (Brooks et al. 2017). The significance of main effects and their interactions for all models was assessed using 3-way ANOVA (Wald x^2 tests) via the car package (Fox and Weisberg 2019). Following ANOVA, post-hoc comparisons were performed to determine specific differences between groups using the emmeans package (Lenth and Piaskowski 2025). Tukey's Honestly Significant Difference test was applied for EAG and field trap data, while the Least Significant Difference (LSD) test was used for laboratory assays. All statistical analyses were conducted in R version 4.5.2. Model diagnostics, including assessments of homoscedasticity, residual distributions (Q–Q plots), overdispersion, and zero-inflation, were verified using the DHARMa, performance, and see packages (Hartig 2024, Lüdecke et al. 2021a, 2021b). Additionally, multicollinearity was assessed by calculating Variance Inflation Factors on models excluding interaction terms to ensure the stability of the main effects. Data manipulation and organization were performed using the tidyverse package (Wickham et al. 2019), while all figures and graphical representations were generated using the ggplot2 package (Wickham 2016).

Results

Electroantennographic Responses of *B. oleae* to Modified BSYs

Electroantennographic analysis identified specific trends in the olfactory sensitivity of *B. oleae*, which were significantly influenced by the type of stimulus ($x^2 = 203.93$, df = 6, $P < 0.001$). While *B. oleae* exhibited the non-significant result in their response for sex ($x^2 = 2.204$, df = 1, $P = 0.137$) and age ($x^2 = 5.509$, df = 2 $P = 0.063$) group as single factors. However, their interactions stimulus × sex ($x^2 = 20.29$, df = 6, $P = 0.002$)

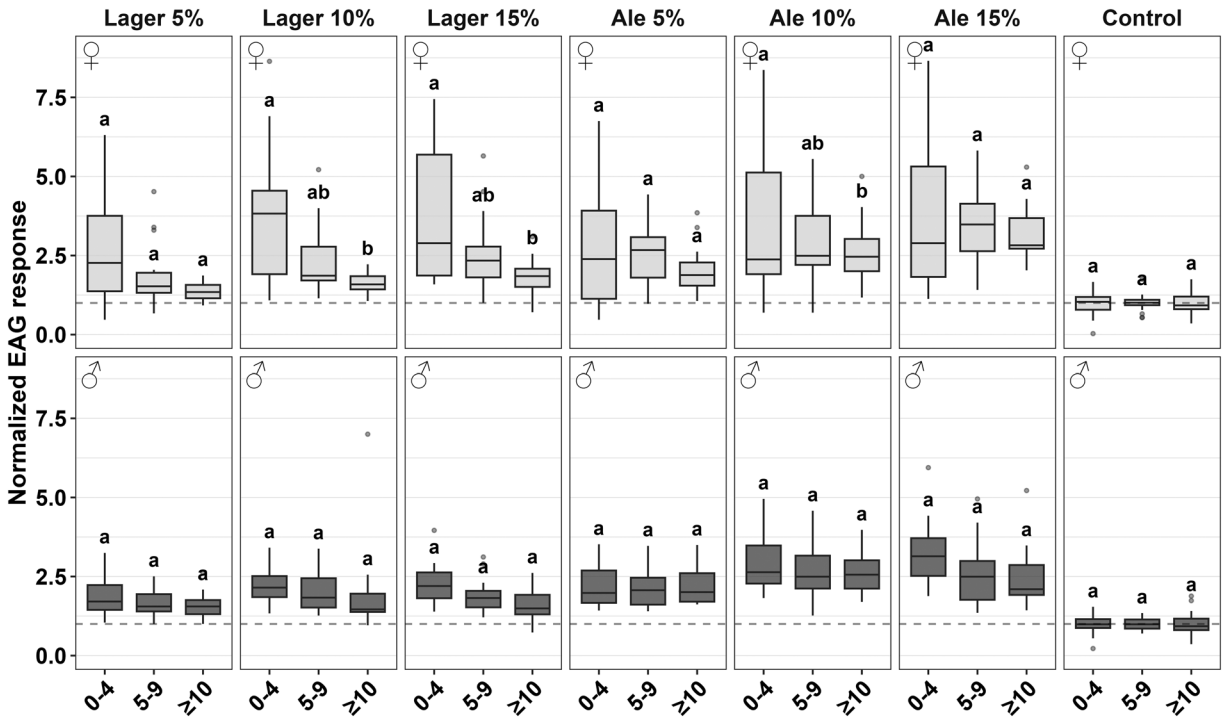

Fig. 1. Normalized electroantennographic (EAG) responses of (upper panels) female and (lower panels) male *Bactrocera oleae* to various stimuli and the water control across three age groups (0 to 4 days old, 5 to 9 days old, and ≥10 days old). The dashed horizontal line at $y = 1$ represents the baseline response to the control. Within each stimulus, different letters indicate significant differences between age groups (Tukey's HSD test, $P < 0.05$). Individual points beyond the whiskers represent outliers.

and stimulus × age ($x^2 = 40.42$, df = 12, $P < 0.001$) were highly significant, justifying a detailed comparison of these groups (Fig. 1).

All tested stimuli elicited significant EAG responses compared to the control ($t = 4.13023$ to 10.865, df = 654, $P < 0.001$; Fig. 1). Females exhibited substantially higher normalized EAG responses and greater physiological variability than males (Fig. 2), among all ages group, particularly when stimulated with ale-type modified BSY at 15% (Fig. 1).

Within the female groups, significant differences in normalized EAG amplitudes were recorded across age groups, particularly between young and >10 days old flies when stimulated with lager-type BSY at 10% ($t = 3.61$, df = 50.06, $P = 0.002$) and 15% ($t = 3.39$, df = 50.06, $P = 0.004$; Fig. 1). In contrast, female responses to ale-type modified BSY, notably at 5% and 15%, did not differ significantly across age groups, with no significant differences ($t = 0.368$ to 2.559, df = 50.06, $P = 0.134$ to 0.929) found between them (Fig. 1).

Normalized EAG ratios for males lower and remained consistent across all age groups, with no significant ($t = 0.094$ to 1.256, df = 50.06, $P = 0.426$ to 0.999) differences compared to control group (Fig. 1). In contrast to the significant differences observed in female flies (>10 days old) at specific concentrations, male sensitivity remained relatively stable, with no significant differences ($P > 0.05$) observed between the 0 to 4, 5 to 9, and ≥10 days old groups for all tested modified BSYs (Fig. 1).

Significant differences between sexes were recorded in the youngest flies, with females showing greater sensitivity to lager-type BSY at 10% ($t = 2.562$, df = 50.06, $P = 0.013$) and 15% ($t = 2.558$, df = 50.06, $P = 0.014$) compared to males. A similar but marginal trend was observed for the 10% ale-types BSY ($t = 2.00$, df = 50.06, $P = 0.051$), while no significant differences between sexes were detected in older age groups ($t = 0.465$ to 1.239, df = 50.06, $P = 0.085$ to 0.999). In both sexes and all age groups, ale 10% and 15% consistently elicited the highest response amplitudes, while the water control elicited baseline responses (Fig. 3).

Dose-response results indicate that the olive fruit fly antennal sensitivity is characterized by a significant interaction of sex, age, and stimulus concentration, ie 5%, 10%, and 15% (Fig. 4).

Specifically, the normalized EAG analysis showed significant main effects and interactions for sex, age, and stimulus concentration influence the magnitude of the physiological response to modified BSY formulations. In all tested groups, the ale-type modified BSY consistently elicited stronger physiological responses in both males and females than the lager-type. Significant differences were recorded when comparing ale-type BSY 15% with lager-type BSY 5% which significantly affected responses in young females ($t = 4.441$, df = 654, $P = 0.0002$) and in >10 days old females ($t = 5.578$, df = 654, $P < 0.0001$; Fig. 4).

A significant effect of concentration was observed in females, with sensitivity increasing ($t = 2.991$ to 4.150, df = 654, $P = 0.0001$ to 0.003) with concentration from 5% to 15%, particularly in the youngest flies (0 to 4 days old). In this group (0 to 4 days old females), ale-type stimuli at 10% and 15% reached peak normalized ratios above 4.0, which was significantly higher compared to 5% concentration ($t = 3.797$, df = 654, $P < 0.001$; $t = 4.150$, df = 654, $P < 0.001$, respectively). While female sensitivity to lager-type modified BSY showed a slight age-dependent decline compared to the female sensitivity to ale-type modified BSY (Fig. 4). The oldest mated flies (≥10 days old), at 15% ale elicited higher responses than the

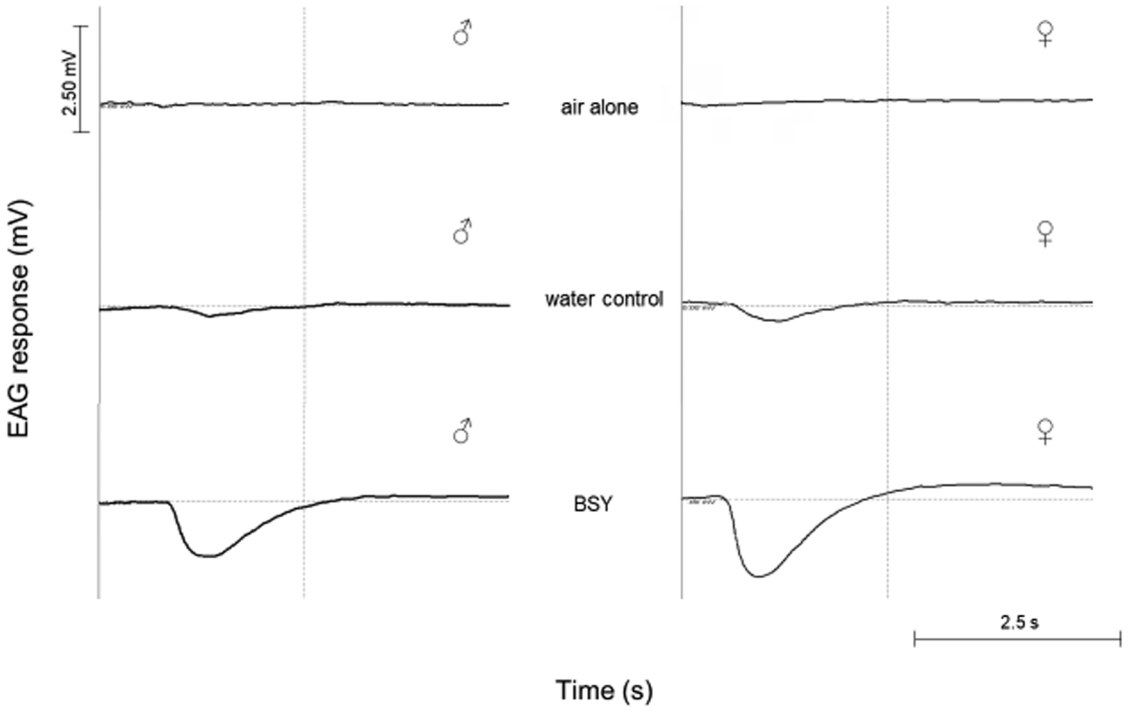

Fig. 2. Representative EAG responses of male (♂) and female (♀) *Bactrocera oleae* to volatile stimuli. Responses are shown for the blank (air alone), the control (water) and BSY (Brewer's spent yeast) stimuli.

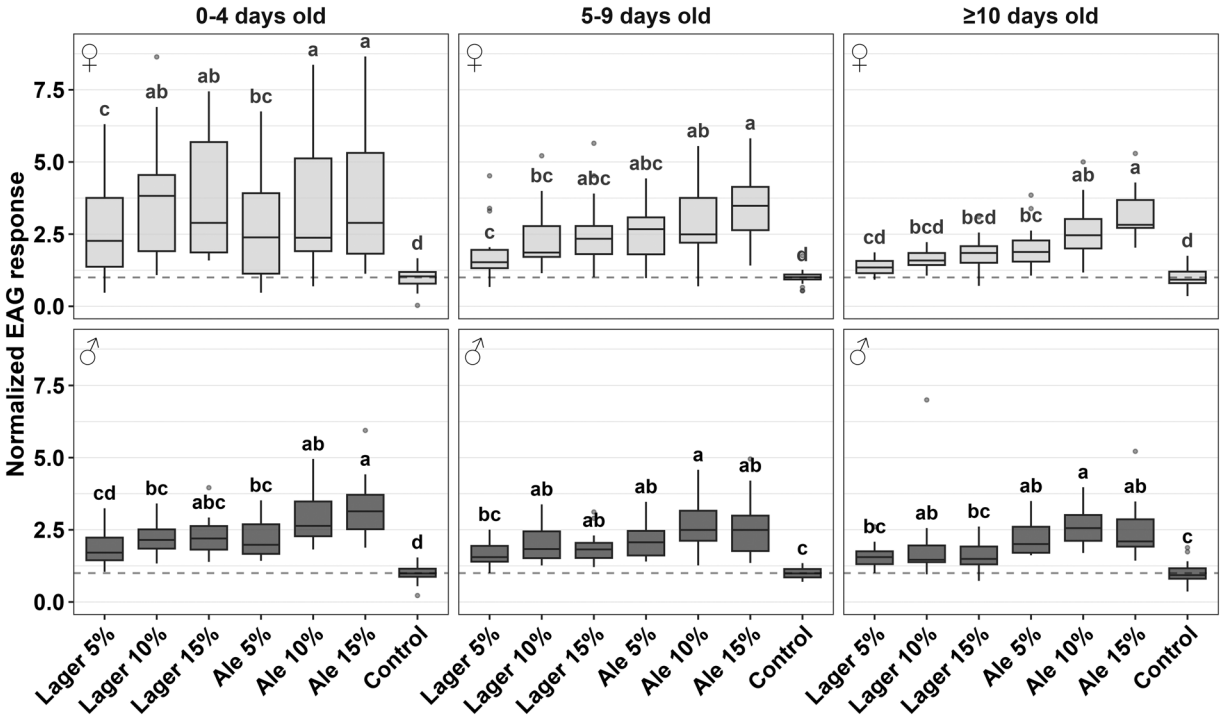

Fig. 3. Comparison of normalized electroantennographic (EAG) responses of (upper panels) female and (lower panels) male *Bactrocera oleae* to different stimuli and water control, categorized by age group (0 to 4 days old, 5 to 9 days old, and ≥ 10 days old). The dashed horizontal line at $y = 1$ indicates the baseline response to the control. Different letters above the boxes denote significant differences in responses to different stimuli within each age group (Tukey's HSD test, $P < 0.05$). Individual points beyond the whiskers represent outliers.

5% concentration ($t = 3.503$, df = 654, $P = 0.001$), maintaining a mean ratio above 3.0. Young males showed significantly greater EAG response only for 15% ale-type BSY then 5% ($t = 3.086$, df = 654, $P = 0.005$). Differences in EAG responses were not found across other male age groups between the 5% and 15% concentrations for either BSY ($t = 0.028$ to 2.122, df = 654, $P = 0.086$ to 0.999), with normalized ratios remaining below 3.0 at all tested concentrations (Fig. 4).

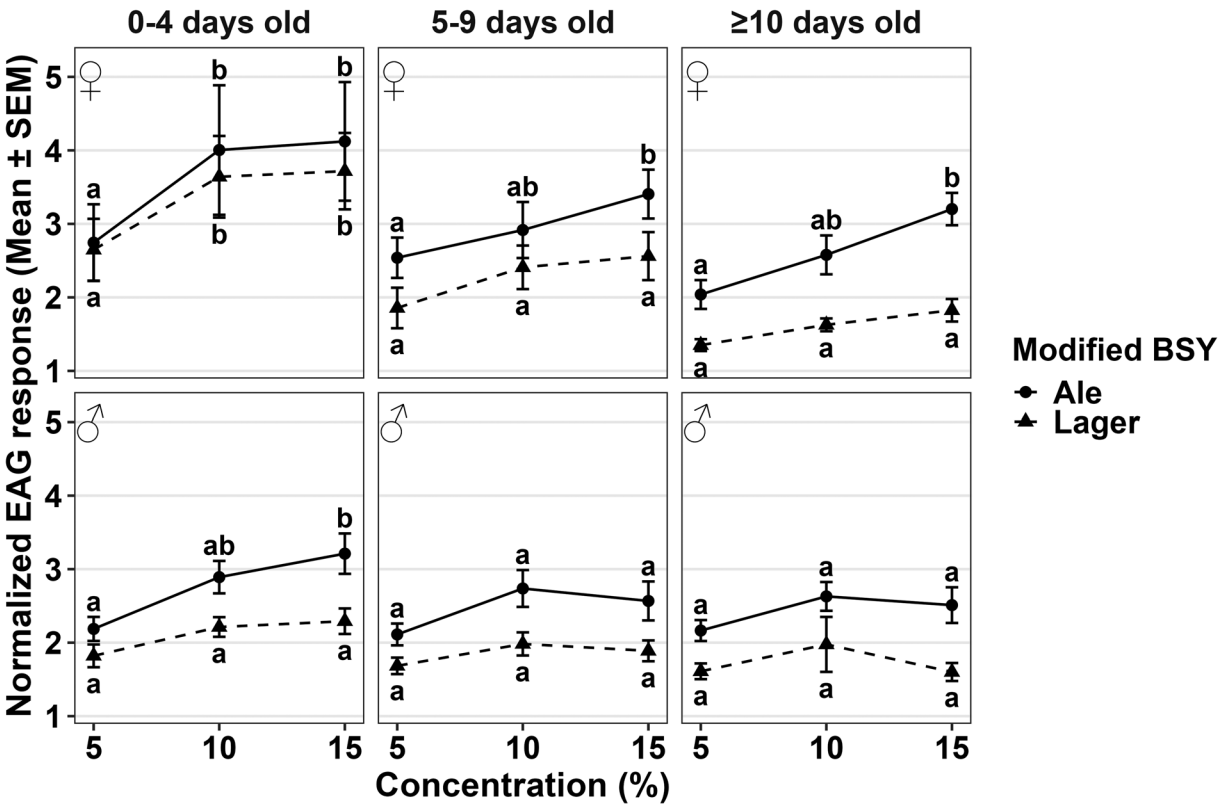

Fig. 4. Dose-response curves of normalized electroantennographic (EAG) responses in (upper panels) female and (lower panels) male *Bactrocera oleae* to modified ale and lager Brewer's spent yeast (BSY) stimuli at 5%, 10%, and 15% concentrations. Panels are categorized by fly age groups (0 to 4 days old, 5 to 9 days old, and ≥ 10 days old). Different letters indicate significant differences between concentrations within each stimulus type (Tukey's HSD, $P < 0.05$) with letters for ale placed above and letters for lager placed below the respective data points.

Behavioral Responses of *B. oleae* to Modified BSYs in Multiple-Choice Assays

Results of the laboratory preference assay (Fig. 5) showed that both modified BSY formulations (lager and ale) were more attractive to *B. oleae* than the control ($x^2 = 13.592$, df = 6, $P = 0.035$) which is generally consistent with the trends observed in the EAG assays.

The total number of captured flies was also significantly influenced by the interaction between sex and age group ($x^2 = 6.245$, df = 2, $P = 0.044$; Fig. 5). The 10% modified lager BSY elicited the greatest overall attraction in the youngest flies (0 to 4 days old for both sexes), with strong responses from both males ($z = 2.849$, $P = 0.004$) and females in this age group ($z = 2.357$, $P = 0.018$). In contrast, an opposing pattern was observed for the 10% ale-type BSY, which was significantly less attractive to young flies but showed a progressive increase in attractiveness as the flies aged, peaking in the ≥10-day group (Fig. 6). Direct comparison of treatments within age groups further clarified these preferences. For young flies (0 to 4 days), 10% lager-type BSY consistently showed greater attractiveness to both sexes compared to all other treatments, including the 10% ale-type, which recorded the lowest attraction among the BSY concentrations for this age group (0 to 4 days old for both sexes, Fig. 6).

However, this preference shifted in the older, mated group (≥ 10 days; Fig. 6). In this group, 15% lager-type captured the greatest number of females while 10% ale-type BSY captured the greatest number of males, compared to the control. In the 5 to 9 days old group, unmuted female responses showed no differences between all treatments tested ($P > 0.05$), including the control. In contrast, male captures were significantly greater in the 5% lager-type and 15% ale-type BSY treatments compared to the control and other tested concentrations (Fig. 6). Across all age groups and both sexes, the control treatment consistently attracted the lowest number of *B. oleae*, confirming that the observed attraction was specifically triggered by the BSY formulations (Figs 5 and 6).

Field Efficacy of Modified BSYs as Attractants for *B. oleae*

Greater numbers of both sexes of *B. oleae* were attracted to every modified BSY treatment and concentration than the control in both olive orchards (Location A: $x^2 = 51.082$, df = 6, $P < 0.001$; Location B: $x^2 = 22.122$, df = 6, $P = 0.001$; Fig. 7).

More *B. oleae* were captured in all traps combined during the course of the field study in the multi-variety olive grove (1,569 flies) then in the mono-variety olive grove (669 flies), with the 5% lager-type bait being the most attractive for both females ($z = 6.594$, $P < 0.001$) and males ($z = 7.488$, $P < 0.001$; Fig. 7A). Figure 7 also shows a general decline in trap captures with increasing concentration for the modified lager-type BSY that was significant for males ($z = 4.383$, $P < 0.001$). However, a similar trend of declining trap captures corresponding to higher concentrations was not observed for the ale-type modified BSY which showed similar efficacy in attracting both male ($z = 4.798$ to 5.908, $P < 0.001$) and females flies ($z = 3.718$ to

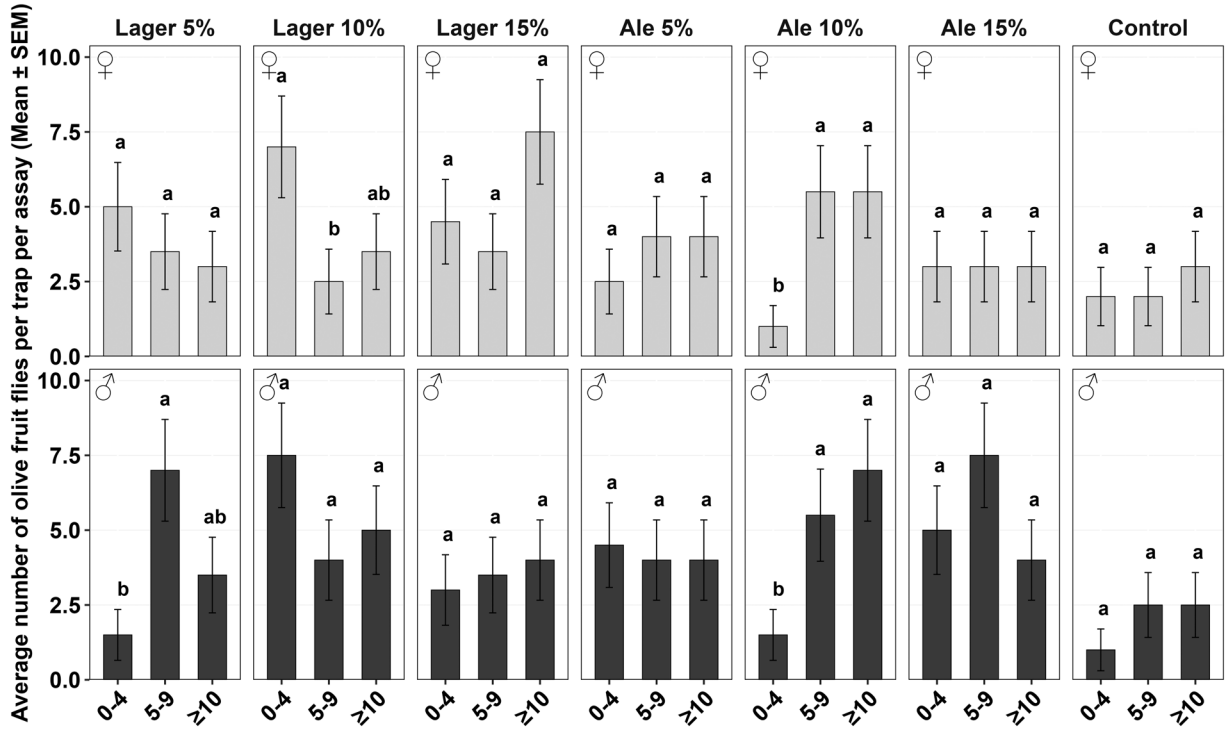

Fig. 5. Results of the multiple-choice preference assay across different concentrations of modified ale and lager Brewer's spent yeast (BSY) on responses of female (upper panels) and male (lower panels) *Bactrocera oleae*. Bars labeled with different lowercase letters indicate significant ($P < 0.05$) differences between flies age groups within a single treatment.

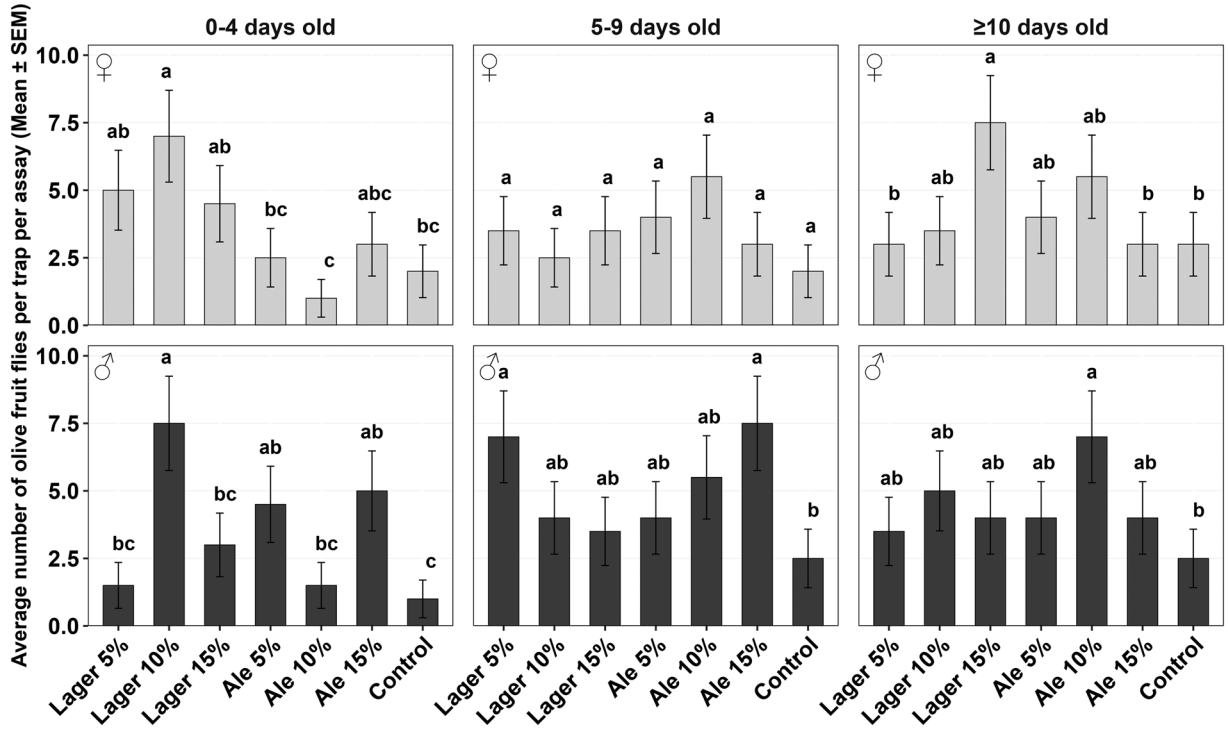

Fig. 6. Results of the multiple-choice assay on responses of female (upper panels) and male (lower panels) *Bactrocera oleae* characterized by age groups: 0 to 4 days old olive fruit flies–young; 5 to 9 days old flies–old, unmated; and ≥10 days old flies–old, mated. Bars labeled with different lowercase letters indicate significant differences among traps within each age group (LSD test, $P < 0.05$).

5.343, $P < 0.004$). *Bactrocera oleae* were significantly less attractive to 15% ale-type bait than traps with the 5% lager-type bait for both males ($z = 3.581$, $P = 0.006$) and females ($z = 3.989$, $P = 0.001$), while males were also significantly less attractive to 5% ale-type ($z = 3.798$, $P = 0.003$) and lager-type 15% ($z = 4.048$, $P = 0.001$) than to 5% lager-type. Additionally,

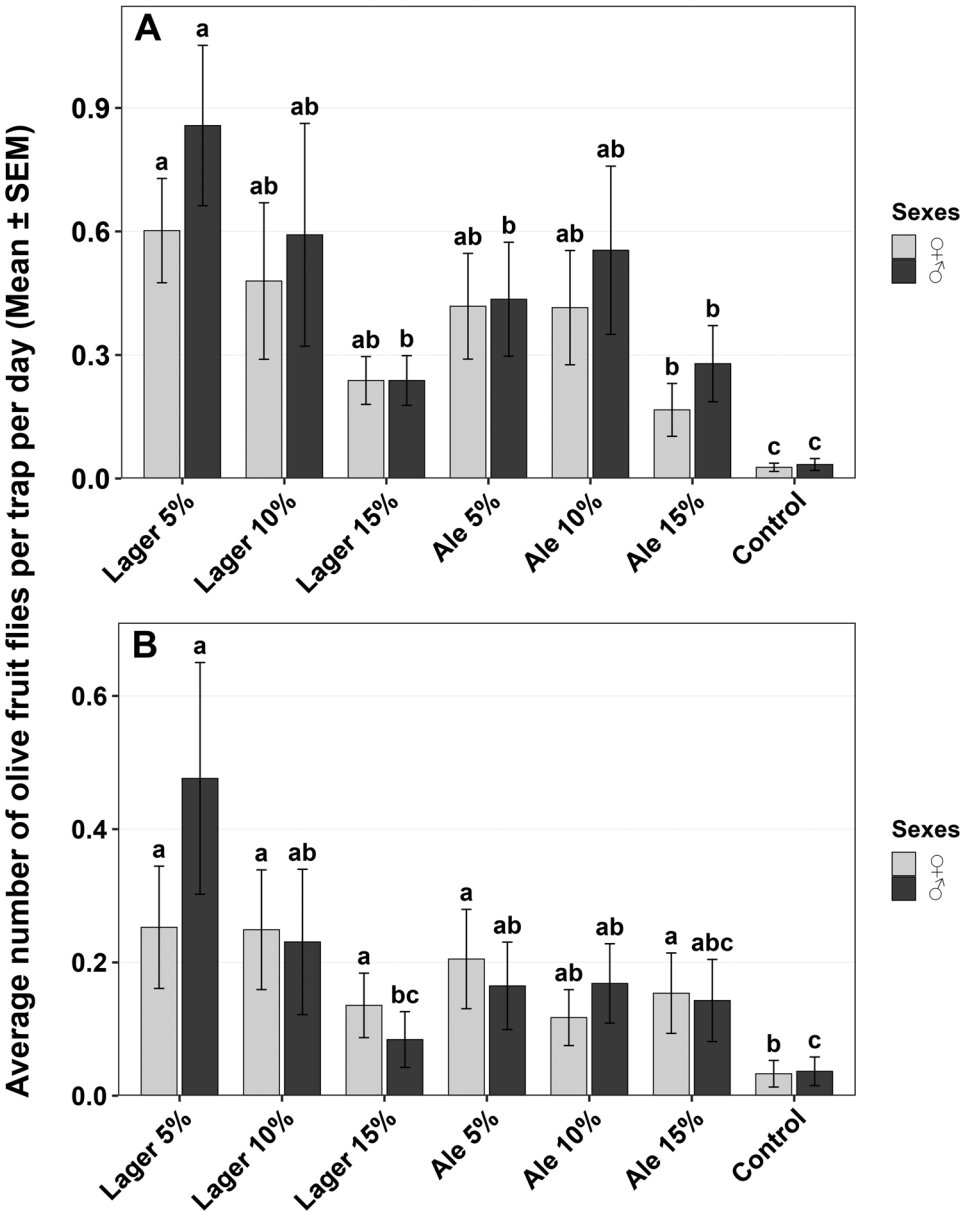

Fig. 7. Average number of male and female *Bactrocera oleae* captured per trap per day during the 14-week study period in traps baited with different modified lager and ale brewer's spent yeast (BSY) and a water (control) in (A) IPM-managed multi-variety olive grove and (B) organic mono-variety olive grove (cv. Oblica). Different letters above the bars indicate significant differences in the average number of flies caught between traps (Tukey's HSD test, $P < 0.05$).

there were no significant differences observed between male and female captures in the tested traps ($z = 0.124$ to 1.879, $P = 0.901$ to 0.060), a slight male prevalence was observed in the traps with the most attractive bait (5% lager-type modified BSY; Fig. 7A).

Figure 7B showed that in the mono-variety olive grove, population density was significantly lower than in the multi-variety grove, with peak captures reaching approximately 200 flies. Despite differences in management and lack of cultivar diversity in the mono-variety olive grove, traps containing 5% lager-type bait were the most attractive to both *B. oleae* males ($z = 5.385$, $P < 0.001$) and females ($z = 4.131$, $P = 0.001$) as was the case for the multi-variety orchard. However, differences between treatments at this site were less pronounced. As in the first olive grove, the 15% lager-type bait showed the lowest attraction efficiency, supporting that higher concentrations correspond to reduced trap captures (Fig. 7A). Notably, there was no difference between the 15% lager-type BSY and the control ($z = 1.511$, $P = 0.738$) in males. Trap captures of male and female flies were relatively similar among the remaining BSY treatments in the mono-variety olive grove, with no significant differences ($z = 0.132$ to 1.798, $P = 0.072$ to 0.894) between them (Fig. 7B).

Discussion

Physiological and behavioral assessments confirm that modified BSY formulations derived from both ale and lager production serve as potent attractants for the *B. oleae*. Rather than a non-specific response driven by physical or moisture-related

factors, the significantly higher EAG amplitudes elicited by all tested modified BSY stimuli compared to the water control validate the specificity and functionality of the olive fruit fly's antennal olfactory receptors and suggests a genuine macro-olfactory perception of specific yeast volatile organic compounds (VOCs). This efficacy is notably influenced by the fly's reproductive status, age, and lure concentration. This enhanced response is biologically plausible and likely reflects a sensory sensitivity triggered by the urgent requirement for proteins during early adulthood, a critical period for ovarian development and vitellogenesis. As noted by Clarke et al. (2011), the attractiveness of protein resources to females varies significantly with their physiological status, as protein-fed, gravid individuals exhibit reduced foraging activity compared to immature, protein-hungry females (Prokopy et al. 1991). Our findings align also with those of Piñero et al. (2017) and Pogue et al. (2024) by confirming that young, sexually immature tephritid females are highly motivated to seek nitrogen sources to develop their eggs, making them exceptionally responsive to protein baits during the first week of adulthood.

Our observation of a clear dose-response relationship further suggests that young females in particular are physiologically optimized to detect and locate high-quality nitrogen sources that are typically scarce in the olive orchard ecosystem. This is consistent with studies indicating that protein-deprived individuals exhibit significantly lower thresholds for odors associated with ammonia and proteins compared to protein-fed individuals, with female responses often being even more intense due to their reproductive requirements (Robacker 1991, Piñero et al. 2011). Notably, sexual dimorphism in olfactory perception was evident within all age groups, with females consistently displaying higher antennal sensitivity than males to both modified BSY types. This is ecologically expected as males generally prioritize chemical cues linked to mate location and courtship, resulting in comparatively lower sensitivity to food-based odors.

As the flies age, their preferences change which would suggest a dynamic transition in metabolic or reproductive priorities. The significant age-dependent decline in sensitivity to lager-type BSY observed exclusively in females highlights this shift in physiological priorities as the flies mature. Younger females require intensive protein foraging to reach sexual maturity, which correlates with their peak antennal sensitivity to specific yeast types. As females age (≥10 days old) and shift their behavioral focus from protein foraging to mating and oviposition site selection, their peripheral sensitivity to certain foraging cues such as lager-type BSY significantly decreases. Another possible reason is that sensitivity behavior changes with age, and it can influence behavioral activity. For example, Tasnin et al. (2020) noticed that the probability of behavioral responses to volatile emissions declines with increasing age in *Bactrocera tryoni* (Froggatt), indicating that aging affects both sensory perception and exploratory activity. Additionally, insect sensitivity behavior is plastic and can be modified by physiological state, including age, nutritional condition, and reproductive status (Xu et al. 2024).

We noted a difference between physiological and behavioral data. While EAG sensitivity showed an age-dependent decline in certain female groups (Fig. 1), behavioral responses in older flies (≥10 days old) remained stable (Fig. 6). In this context, it is worth noting that the strongest EAG responses were not always directly associated with the highest field captures, and this discrepancy deserves careful consideration. This lack of strict linearity between peripheral olfactory reception and behavioral output is a well-documented phenomenon in tephritid fruit flies (Canale et al. 2013, Jang et al. 2020, Tasnin et al. 2020). While EAG measures the overall electrical activity of the antennae, demonstrating the peripheral capacity to detect specific compounds (Biasazin et al. 2018), field attraction depends on complex central nervous system integration influenced by the insect's physiological status and environmental conditions (Pogue et al. 2024, Xu et al. 2024). Factors such as volatile concentration, field volatilization dynamics (Vargas et al. 2010), and the requirement for precise synergistic ratios within the modified BSY profile play a critical role (Bego et al. 2021, Vitanović et al. 2020b). Therefore, a robust antennal response indicates high sensitivity to a specific volatile component but does not inherently guarantee a corresponding level of behavioral attraction in a complex field environment (Biasazin et al. 2022). This indicates that even as antennal sensitivity decreases, behavioral responses in older flies remain stable, which is hypothesized to be driven by a complex profile of fermentation-derived volatiles.

A key limitation of this study is that the precise chemical composition of the VOCs and specific synergistic ratios emitted by our specific modified BSY formulations were not qualitatively or quantitatively characterized. In tephritid fruit flies, foraging and host-seeking behaviors are widely recognized to be mediated not by single isolated compounds but by putative synergistic interactions within multicomponent chemical mixtures. Such synergistic effects among fermentation-derived chemical groups are well documented in other *Bactrocera* species and tephritids (Vargas et al. 2010, Epsky et al. 2014, Scolari et al. 2021, Delgado et al. 2022, He et al. 2023, Xu et al. 2024, Ramniwas et al. 2025), and a similar multicomponent attraction mechanism may be operating here. However, further chemical characterization of these specific BSY formulation is required to confirm this.

In field conditions, modified BSYs, particularly the 5% lager-type, proved statistically more effective in attracting *B. oleae* than the control (Fig. 7). Historically, while protein-based lures have shown greater attractiveness than simple ammonia baits (Prokopy and Economopoulos 1975), traditional hydrolysates often faced criticism for failing to detect early-season populations or overestimating the abundance of gravid females (Neuenschwander and Michelakis 1981). However, recent studies demonstrated the importance of food-based attractants as tools for monitoring and capturing *B. oleae* populations through the modification of biomass-derived substrates. For example, Varikou et al. (2024) produced a sticky panel to identify the response of *B. oleae* to hydrolyzed protein baits and ammonium salt baits in combination with various insecticides. They reported that a modified formulation of protein-based bait could significantly increase the performance of traps developed to capture *B. oleae*. Attractants obtained from mealworm frass provided greater attraction to *B. oleae* adults when compared to widely used attractants such as hydrolyzed protein and ammonium sulfate in a study by Koufakis et al. (2025). Moreno-Alcaide et al. (2025) used a trap-optimization approach involving trap model, color, size, and density for integrated pest-management of *B. oleae* that resulted in improved detection accuracy, reduced environmental impact, and lower cost when compared with traditional monitoring methods.

In this regard, our modified BSY results are promising, as they suggest that these waste-derived formulations could serve as effective alternatives to traditional protein-based lures by potentially mimicking natural fermentation processes, although further evaluation of their longevity under field conditions is warranted. Furthermore, the attraction of both sexes observed in the laboratory was consistent with field captures, as the modified BSY solutions consistently attracted substantial numbers of both sexes. Further investigation is necessary to assess their potential application in "attract-and-kill" or mass-trapping strategies. The field observation that higher concentrations were associated with reduced field captures suggests a "saturation effect" or a potential repellent response to over-intense aromatic profiles that may mimic over-fermented or unsuitable resources (Foster and Harris 1997, Epsky et al. 2014). This bell-shaped dose-response curve is a well-documented phenomenon in insect chemical ecology, where excessive concentrations of an attractant can become inhibitory (Foster and Harris 1997).

The successful field validation of these low-concentration, waste-derived baits highlights their potential as sustainable candidates for olive fruit fly monitoring in the framework of IPM that aligns with circular economy principles, though future studies are required to provide direct comparisons to commercial standards in efficacy, long term stability, and relative costs. While modified BSY formulations show great potential as olfactory attractants, future research should prioritize characterizing their volatile chemical profiles and evaluating their effectiveness as attractants under both laboratory and field conditions.

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Author Contributions

Elda Vitanović (Conceptualization [lead], Data curation [equal], Formal analysis [lead], Funding acquisition [lead], Investigation [lead], Methodology [equal], Project administration [lead], Supervision [equal], Visualization [equal], Writing—original draft [lead]), Luka Čotić (Data curation [equal], Formal analysis [supporting], Writing—original draft [equal], Writing—review & editing [supporting]), Walter S. Leal (Data curation [equal], Investigation [supporting], Methodology [equal], Supervision [equal], Visualization [supporting], Writing—original draft [supporting], Writing—review & editing [equal]), Qaiser Javed (Data curation [equal], Writing—original draft [equal], Writing—review & editing [supporting]), and Frank G. Zalom (Data curation [equal], Investigation [equal], Methodology [equal], Supervision [equal], Visualization [equal], Writing—original draft [equal], Writing—review & editing [equal])

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Conflicts of Interest

The authors declare no conflicts of interest.

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