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Identification of sex pheromone chemistry, synthesis and laboratory and field male trapping in Iranian population of fall webworm, *Hyphantria cunea* (Drury) (Lepidoptera: Erebidae)

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Abstract

The fall webworm, *Hyphantria cunea* (Drury), is a globally invasive polyphagous defoliator, recognized as a destructive pest of both agricultural and non-agricultural plants. To mitigate its expanding populations, diverse control strategies have been explored across invaded regions. However, many approaches remain confined to laboratory settings, necessitating field validation to assess their practical efficacy. Given the species-specific activity and environmentally benign nature of sex pheromones, this study aimed to identify and characterize sex pheromone compounds in *H. cunea* as a potential foundation for sustainable pest management. The pheromone glands of *H. cunea* virgin females were dissected and extracted in hexane, followed by gas chromatography-mass spectrometry (GC-MS) analysis of the glandular extracts. Four compounds were identified as putative sex pheromone components: (Z,Z,Z)-9,12,15-octadecatrienoic acid, (Z,Z)-9,12-octadecadienoic acid methyl ester, (Z)-9-octadecenoic acid methyl ester, and octadecanoic acid methyl ester. The identified compounds represent preliminary candidates for the sex pheromone of the Iranian population of *H. cunea*, serving as a foundation for pheromone-mediated control strategies. Wind tunnel bioassays demonstrated that a synthetic quaternary blend of the identified compounds, formulated in a 4:2:2:3 ratio, elicited significant attraction in male *H. cunea*. Field trials corroborated these findings, with the optimized blend effectively luring males under natural conditions. The identification of this attractive sex pheromone blend enables the development of targeted strategies for monitoring and suppressing *H. cunea* populations, offering a promising tool for integrated pest management (IPM) programs.

Keywords: Field trapping, *Hyphantria cunea*, Sex pheromone, Wind tunnel bioassay.

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Introduction

The fall webworm, *Hyphantria cunea* (Lepidoptera: Erebidae), a North American native moth, is a globally invasive polyphagous pest affecting over 600 host species, including forest, fruit, and agricultural crops. Larval stages cause defoliation and silken webbing, impairing photosynthesis and reducing plant biomass (Edosa *et al*., 2019). Although chemical insecticides suppress outbreaks, excessive reliance on broad-spectrum formulations elevates risks to human health, non-target organisms, and natural enemy populations, often exacerbating resurgence through disrupted biocontrol (Alengebawy *et al*., 2021). Furthermore, *H. cunea*'s strong preference for mulberry (*Morus* spp.)—a key host plant in sericulture—introduces unique challenges. Intensive infestations in mulberry-dominant regions, coupled with chemical interventions, threaten both silk production and urban forestry ecosystems, as pesticide residues may persist in foliage used for silkworm rearing or leach into adjacent green spaces (Edosa *et al*., 2019). This dual risk underscores the need for integrated pest management (IPM) strategies that balance immediate control efficacy with long-term ecological sustainability.

Effective monitoring of highly invasive, polyphagous pests requires innovative strategies such as sex pheromone-based systems. These species-specific compounds offer an eco-friendly alternative to broad-spectrum insecticides, minimizing harm to non-target organisms. Their targeted disruption of mating behaviors avoids collateral ecological damage, while ultra-low application concentrations reduce evolutionary selection pressure, thereby delaying resistance development compared to synthetic pesticides (Klassen *et al*., 2023). Female moth pheromones are biosynthesized from modified fatty acids, which are characterized by hydrocarbon chains of varying lengths, double-bond configurations, and terminal functional groups (e.g., alcohols, aldehydes, or acetate esters) that dictate their biological activity (Groot *et al*., 2019). In IPM, these pheromones are leveraged for four core strategies: population monitoring, mating disruption, mass trapping, and push-pull systems (Rizvi *et al*., 2021).

Sex pheromone studies in *H. cunea* trace back to Hill *et al*. (1982), who identified three aldehydes—(Z,Z)-9,12-octadecadienal, (Z,Z,Z)-9,12,15-octadecatrienal, and 3,6-9,10-epoxyheneicosadiene—in U.S./USSR populations, with morph-specific ratios (1:1.2:2.6 vs. 1:8.2:1). Subsequent work revealed geographic divergence: Hungarian populations produced two epoxy-trienes (Tóth *et al.*, 1989), while New Zealand females synthesized four components, including novel aldehydes (El-Sayed *et al.*, 2005). Chinese populations exhibited optimal attraction to a quaternary blend (2:33.6:58.4:6 ratio) of aldehydes and epoxies (Su *et al.*, 2008). Despite Italian trials confirming male responsiveness to ternary blends, ratio

optimization remained unresolved (Trematerra *et al.*, 1993). Critically, regional pheromone variability undermines monitoring efficacy, as commercial lures—often calibrated to singular blends—fail to align with local male activity peaks (Yarmand *et al.*, 2009; Schowalter *et al.*, 2017). This geographic specificity underscores the imperative for regionally tailored formulations to improve invasive population control.

Despite extensive global research on the sex pheromones of *H. cunea*, the pheromone profile of Iranian populations remains uncharacterized. This gap is critical, as geographic and interpopulation variations in pheromone composition—such as differences in component ratios or structural isomers—are well-documented in this species (Su *et al*., 2008). For invasive pests like *H. cunea*, region-specific identification of pheromones is essential to develop targeted monitoring and control strategies. Prioritizing the isolation and quantification of sex pheromone components in Iranian populations—including their precise ratios and synergistic interactions— will facilitate rigorous evaluation of their behavioral activity through laboratory bioassays and field trials. Such data are foundational for designing effective, population-specific lures to mitigate this pest's ecological and agricultural impact in Iran.

Materials and Methods

Insect Collection

First-generation *H. cunea* larvae were collected from mulberry trees on the University of Guilan campus in Rasht, Iran (37.2682°N, 49.5891°E). Larvae were reared in mesh cages (30 × 30 × 40 cm) under 25 ± 2 °C, 60 ± 5% RH, and a 16:8 h (L:D) photoperiod, fed daily with fresh mulberry leaves. Pupae were sexed morphologically (Tuncer and Aker, 2017) and adults were isolated to ensure virginity.

Sex Pheromone Extraction

Sex pheromones were extracted from 1–3-day-old virgin *H. cunea* females using a modified El-Sayed *et al*. (2005) protocol, prioritizing gland isolation efficiency. Females were immobilized on ice, and the intersegmental membrane between abdominal segments 8–9 was surgically exposed via gentle ovipositor extrusion. Pheromone glands from actively calling moths were dissected under aseptic conditions using ethanol-sterilized scalpels and immediately submerged in 20 µL hexane (1 gland/tube) to preserve compound integrity. After 60 min incubation at 20°C, tissues were removed, and pheromone-laden hexane extracts were stored at −20°C prior to GC-MS analysis. Sterile handling and rapid processing minimized degradation, ensuring robust chemical characterization.

GC-MS Analysis

Pheromone extracts were analyzed via gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890B GC coupled to a 5977A MS (HP-5MS column: 30 m × 0.25 mm ID, 0.25 µm film). High-purity helium carrier gas flowed at 3 mL min^{-1}. The oven program initiated at 50°C (2 min hold), ramping to 280°C at 5°C min^{-1}. Samples (2 µL) were injected splitless at 250°C, with MS operating in electron ionization (EI) mode (70 eV). Tentative identification of pheromone components was achieved by comparing retention indices and mass spectral fragmentation patterns to reference standards in the NIST and Wiley libraries.

Chemicals

Identified compounds were cross-referenced with Pherobase (http://www.pherobase.net) to confirm their classification as sex pheromones. Four Sigma-Aldrich-sourced candidates were selected for evaluation: (Z,Z,Z)-9,12,15-octadecatrienoic acid (>99%; CAS 463-40-1), (Z,Z)-9,12-octadecadienoic acid methyl ester (≥98%; CAS 112-63-0), (Z)-9-octadecenoic acid methyl ester (99%; CAS 112-62-9), and Octadecanoic acid methyl ester (99%; CAS 112-61-8).

Wind Tunnel Bioassays

The behavioral responses of unmated *H. cunea* males to synthetic sex pheromone blends were evaluated under controlled laboratory conditions using a custom-designed wind tunnel, adapted from the Miller and Roelofs (1978) model. The tunnel was constructed from transparent polymethyl methacrylate (Plexiglas) and measured 120 × 50 × 50 cm (L × W × H). A unidirectional laminar airflow was generated using two 12 × 12 cm axial fans (SUNON DP201A, Taiwan) mounted at opposite ends, with the front fan supplying airflow and the rear fan acting as an exhaust. Fan speed was maintained at 3150/2850 rpm, generating an airflow velocity of 0.3 ± 0.05 m/s at the midsection of the tunnel.

Preliminary Trials to Optimize Wind Tunnel Conditions

To refine experimental parameters mimicking natural mating behavior, initial tests were conducted in a wind tunnel. Unmated *H. cunea* females (1–3 days old) were housed in a dark, temperature- and humidity-controlled cubic mesh cage (60 × 60 × 60 cm; 25 ± 1°C, 60 ± 1% RH). Male moths were released to evaluate responsiveness; both sexes were acclimated to darkness for 1–6 hours prior to trials to align with their nocturnal pheromone release cycle. Observations revealed that males exposed to 2–4 hours of darkness displayed peak activity

(e.g., sustained flight and orientation), whereas attraction to live females occurred within 10–20 minutes of release. Consequently, this 10–20-minute interval was standardized as the habituation period for assessing male attraction to artificial pheromone sources in subsequent experiments.

Final Test

Male *H. cunea* moths were acclimated in cylindrical containers for one hour inside the wind tunnel prior to testing. Synthetic pheromone compounds (individual components, binary to quaternary mixtures) and a hexane solvent control (500 µL each) were loaded into cotton-plugged tubes and placed 10 cm above the tunnel floor. Males were released 100 cm downwind under red light illumination (10 lux), with airflow maintained at 0.3 m/s. Behavioral responses were categorized as follows: orientation (upwind flight), proximity approach (movement within 40 cm of the release point), or source contact (landing on the pheromone tube). Trials lasted 10–20 minutes in darkness, with ten males tested per treatment (single exposure only); non-responsive individuals were excluded. Data (arcsine-transformed contact percentages) were analyzed using one-way ANOVA and Tukey's HSD tests ($\alpha = 0.05$) in SAS 9.4, with treatments as fixed factors and individual moths as replicates. Statistical significance was set at $p \leq 0.05$.

Field Trapping Experiments

Field trials were designed based on preliminary wind tunnel bioassays and conducted in a pesticide-free mulberry orchard at the University of Guilan, Rasht, Guilan Province, Iran (37°11'53.6" N, 49°39'06.7" E), between 21 August and 24 September 2023. A randomized complete block design with four replicates and three treatments was employed: (A) Synthetic pheromone: mix-7 (Comp. 1–4, 4:2:2:3). (B) Control: hexane solvent only. (C) Natural pheromone: unmated adult *H. cunea* females. Pheromone loading protocol: To optimize slow-release efficiency, 500 µL of hexane was mixed with 500 mg of zeolite, vigorously stirred, and air-dried at room temperature for 30 minutes until the solvent evaporated completely, restoring the zeolite to its original mass. For synthetic pheromone treatments, the quaternary blend was incorporated into zeolite-filled cryovials, while controls received hexane-only mixtures. Although direct quantification of the release rate was not conducted during the field trials, previous research indicates that the zeolite-based carrier exhibits a controlled slow-release profile under ambient conditions similar to those of this study (Muñoz-Pallares *et al*., 2001; Kim and Park, 2013). Trap deployment: Yellow delta traps baited with treatments were

positioned on trees at 1.5 m height, spaced 3 m apart within rows. Trap positions were randomized weekly using a random number table, with installation order reassigned per block while retaining fixed spatial coordinates. Sticky liners were replaced weekly, and trapped males were counted and removed during each monitoring interval. Species captured in the traps were identified under a stereomicroscope by trained entomological personnel using established morphological keys. Features such as wing patterns and genitalia structures were examined to confirm the presence of target species (*H. cunea*) and to exclude non-target captures.

Statistical Analysis

All experiments were analyzed separately. One-way ANOVA was used to test for treatment effects, followed by Tukey's Honestly Significant Difference (HSD) test for multiple comparisons when the ANOVA indicated significant differences ($\alpha = 0.05$). All analyses were performed using SAS software (version 9.4, TS Level 1M6; SAS Institute Inc., USA). Results were considered statistically significant at $p \leq 0.05$.

Results

Identification of Pheromone Compounds

GC-MS analysis of *H. cunea* female pheromone gland extracts revealed a complex chemical profile (Figure 1). Four candidate pheromone compounds were identified by retention time and spectral matching against the Pheromone Database (www.pherobase.net): Compound a: (Z,Z,Z)-9,12,15-Octadecatrienoic acid (13.30 min). Compound b: (Z,Z)-9,12-Octadecadienoic acid methyl ester (28.30 min). Compound c: (Z)-9-Octadecenoic acid methyl ester (28.41 min). Compound d: Octadecanoic acid methyl ester (28.73 min). Compounds eluted in order of increasing polarity, consistent with their structural complexity. Relative peak intensities provided approximate abundance estimates, though ionization efficiency biases preclude direct quantitative comparisons.

Mass Spectrometric Validation: Compound a (Figure 2a): Dominant fragment at m/z 80; molecular ion [M^+] at m/z 278. Compound b (Figure 2b): Base peak at m/z 67; molecular ion [M^+] at m/z 294. Compound c (Figure 2c): Base peak at m/z 55; molecular ion [M^+] at m/z 296. Compound d (Figure 2d): Base peak at m/z 74; molecular ion [M^+] at m/z 298. Spectral fragmentation patterns and molecular ions aligned with reference data for known lepidopteran pheromones.

Evaluation of *H. cunea* Sex Pheromone Efficacy Under Laboratory Conditions

Figure 3 displays male moth contact responses (mean ± SD, 95% CIs, Tukey's HSD groupings) for a hexane control, virgin females, and four pheromone components (Comp.1–4) at 10–50 mg doses. One-way ANOVA confirmed significant treatment differences ($F_{21,44} = 45.74$, $p < 0.0001$), with strong model fit ($R^2 = 0.956$, adj. $R^2 = 0.935$, pred. $R^2 = 0.901$) and low variance (pooled $SD = 4.92$), underscoring dose-dependent pheromone effects. Virgin females elicited the highest response (90.0 ± 10.0 %; CI [84.3 – 95.7], group a), significantly greater than all single compounds ($p < 0.05$). Comp.1 (Z,Z,Z-9,12,15-octadecatrienoic acid) reached maximal attraction at 40 mg (36.7 ± 5.8 %; CI [30.9 – 42.4], group b). Lower (10, 20, 30 mg) and higher (50 mg) doses of Comp.1 failed to induce more than 13.3 % contact (groups c–e). Comp.2 ((Z,Z)-9,12-octadecadienoic acid methyl ester) peaked at 20 mg (16.7 ± 5.8 %; CI [10.9 – 22.4], groups c–d), with negligible responses (< 6.7 %) at other doses (groups d–e). Comp.3 ((Z)-9-octadecenoic acid methyl ester) showed a similar pattern, with a maximum of 16.7 ± 5.8 % at 20 mg (CI [10.9 – 22.4], groups c–d) and low responses elsewhere (groups c–e). Comp.4 (octadecanoic acid methyl ester) induced its highest attraction at 30 mg (20.0 ± 0.0 %; CI [14.3 – 25.7], group c), with moderate responses at 20 mg (13.3 ± 5.8 %; groups c–e) and minimal contact at other concentrations (groups d–e). The hexane control, Comp.1 at 10 mg, Comp.2 at 50 mg, and Comp.3 at 50 mg produced no contact (0 %; group e). Dose–response curves for each component were non-monotonic, each exhibiting a narrow optimal window (40 mg for Comp.1; 20 mg for Comps.2 and 3; 30 mg for Comp.4), beyond which attraction declined. None of the single compounds, even at their optimal dose, approached the attractiveness of a calling female, confirming that a single semiochemical is insufficient to elicit full male behavioral activation and supporting the necessity of multicomponent blends.

Figure 4 shows mean male contact percentages ±SD (95% CI) for virgin females and eight synthetic pheromone blends in wind tunnel tests. One-way ANOVA revealed significant treatment differences ($F_{8,18} = 28.96$, $p < 0.0001$, $R^2 = 0.9279$), with Tukey's HSD grouping blends by efficacy. Virgin females elicited the highest response (96.7 ± 5.8 %; CI [85.5 – 107.9], group a), significantly exceeding all synthetic treatments ($p < 0.05$). Among quaternary blends, only Mix-7 (4:2:2:3) approached the natural standard, with 73.3 ± 11.6 % (CI [62.1 – 84.5]) and no significant difference from virgin females ($p > 0.05$, group a). The other quaternary mixtures—Mix-6 (1:1:1:1) at 40.0 ± 10.0 % (group b) and Mix-8 (2:2:2:2) at 30.0 ± 10.0 % (groups b and c)—performed significantly worse than Mix-7 and virgin females

($p < 0.05$). Binary (Mix-1, Mix-2) and ternary (Mix-3–Mix-5) formulations elicited only 10.0 – 30.0 % contact (± 10.0 for Mix-1/Mix-2; ± 10.0 for Mix-3/Mix-5; ± 0.0 for Mix-4; groups b – c), highlighting insufficient synergistic activity at lower component ratios. The distinct CIs further support these distinctions: Mix-7's interval overlaps meaningfully with that of virgin females, whereas other blends show minimal or no overlap. Collectively, these data demonstrate that both the presence of all four pheromone compounds and their precise 4:2:2:3 ratio are critical to reproducing the natural pheromone profile and achieving high male contact rates, supporting the selection of Mix-7 for subsequent field trials and behavioral monitoring programs.

Field Evaluation of the Synthetic Sex Pheromone

Figure 5 presents the number of male moths captured in field traps over four weeks. Week 1: One-way ANOVA revealed a significant treatment effect ($F_{2,6} = 5.33$, $p = 0.047$; $R^2 = 0.64$), indicating that 64 % of the variance in captures was explained by treatment. Mean catches were 0.33 ± 0.58 moths (CI [–0.48 – 1.15]) for both hexane control and pheromone-baited traps, and 1.67 ± 0.58 moths (CI [0.85 – 2.48]) for virgin-female–baited traps. Tukey's HSD placed all treatments in a single homogeneous subset (group a), with overlapping CIs confirming no significant pairwise differences. The pooled $SD = 0.577$ and low predictive R^2 (19 %) highlight limited model generalizability. Week 2: Treatment effects were highly significant ($F_{2,6} = 36.33$, $p < 0.001$; $R^2 = 0.924$). Virgin-female–baited traps caught the most moths (4.33 ± 0.58; CI [3.52 – 5.15], group a), followed by pheromone-baited traps (2.67 ± 0.58; CI [1.85 – 3.48], group b) and hexane controls (0.33 ± 0.58; CI [–0.48 – 1.15], group c). Distinct Tukey groupings confirm significant pairwise differences and pooled $SD = 0.577$ indicates low within-treatment variability. Week 3: ANOVA remained significant ($F_{2,6} = 10.33$, $p = 0.011$; $R^2 = 0.775$, pred. $R^2 = 0.494$). Mean captures were 0.67 ± 0.58 (CI [–0.15 – 1.48]) for control, 2.33 ± 0.58 (CI [1.52 – 3.15]) for pheromone traps, and 2.67 ± 0.58 (CI [1.85 – 3.48]) for virgin-female traps. Tukey's HSD placed pheromone and virgin-female treatments together (group a), both significantly outperforming control (group b). The pooled $SD = 0.577$ supports these distinctions, showing the synthetic blend matches natural females by week 3. Week 4: A significant effect persisted ($F_{2,6} = 7.00$, $p = 0.027$; $R^2 = 0.700$, pred. $R^2 = 0.325$). Mean captures were 0.00 ± 0.33 (CI [–0.47 – 0.47]) for control, 0.67 ± 0.33 (CI [0.20 – 1.14]) for the optimized quaternary blend, and 1.00 ± 0.33 (CI [0.53 – 1.47]) for virgin-female traps. Tukey's grouping showed both active lures (groups a,b for blend; group a for females) significantly outperformed

control (group b) but did not differ from each other. The pooled $SD = 0.333$ indicates sustained efficacy of the synthetic mix into week 4.

Discussion

To enhance monitoring and management of H. cunea in Iran, we used GC–MS to analyze the pheromone profiles of northern populations. Four compounds were tentatively identified as preliminary candidates: (Z,Z,Z)-9,12,15-octadecatrienoic acid, (Z,Z)-9,12-octadecadienoic acid methyl ester, (Z)-9-octadecenoic acid methyl ester, and octadecanoic acid methyl ester. These compounds emerged as potential key volatile components in the pheromone profile of *H. cunea*. The structures of our four candidate pheromone compounds—fatty acids and their methyl esters—strongly suggest a role for insect fatty acid metabolism in their biosynthesis. In the pheromone gland, de novo fatty acid synthesis is followed by chain elongation and Δ-desaturation steps catalyzed by gland-specific desaturases, generating polyunsaturated precursors. These precursors are then oxidized by fatty-acyl reductases and methylated by pheromone gland methyltransferases to produce the final alcohol, aldehyde, and ester pheromones. (Jurenka, 2004; Moto *et al*., 2004).

Documented mechanisms include monomorphic variation—shifts in the relative proportions of shared components, such as an increased level of trienoic acid, to fine-tune species-specific attraction—and polymorphic variation—appearance of novel functional groups like methyl esters to enhance signal specificity (El-Sayed *et al*., 2003). Geographic divergence in sex pheromone composition—observed in *Helicoverpa armigera* (Hübner) populations across Spain, China, and Australia (Gao *et al*., 2020)—directly impacts male trapping efficacy, mirroring broader patterns of spatial (geographic) and temporal (interannual) pheromone variability in related species like *Heliothis virescens* (Fabricius) and *H. subflexa* (Guenée) (Groot *et al*., 2009). Environmental factors, particularly nutrients derived from host plants, drive this plasticity through two distinct mechanisms. The first mechanism is direct modulation, in which the diet provides precursor molecules, such as fatty acids, that are essential for pheromone biosynthesis (Blaul *et al*., 2014). The second mechanism is indirect allocation, whereby nutritional intake influences resource allocation tradeoffs and favors pheromone production over other metabolic demands (Hock *et al*., 2014). Pheromone divergence linked to host plant variation has been empirically demonstrated in *Zeiraphera diniana* (Guenée). Populations feeding on larch (*Larix* spp.) utilize (E)-11-tetradecenyl acetate as a sex pheromone, while those associated with cembrian pine (*Pinus cembra*) produce (E)-9-dodecenyl acetate to attract males (Emelianov *et al*., 2001). Similar patterns are hypothesized

in other species, such as the maize and rice strains of *Spodoptera frugiperda* (J. E. Smith) (Groot et al., 2008) and *Cydia splendana* (Hübner) (Bengtsson et al., 2014). Such divergence may represent an incipient stage of reproductive isolation, driven by ecological specialization on distinct host plants. Geographic variation in host plant suitability may induce larval physiological stress, potentially altering adult pheromone biosynthesis or composition. For instance, although *H. armigera* exhibits broad host plant plasticity, distinct regional preferences are evident. Australian populations prefer tobacco and maize, Spanish populations primarily favor tomato, and Chinese populations avoid tomato, likely due to allelochemical incompatibility (Liu *et al*., 2004; Barthel *et al*., 2015).

Environmental factors such as temperature, humidity (Raina, 2003), photoperiod (Gemeno and Haynes, 2001), host plant chemistry (Reddy and Guerrero, 2004), and interspecific olfactory cues (Groot *et al*., 2010) may also influence the composition of pheromone released by females. Duménil *et al*. (2014) found intraspecific diversity in the sex pheromone of female *Cydia pomonella* L. both between females from the same population and between different populations, while genetic divergence among Iranian *Ectomyelois ceratoniae* (Zeller) populations correlates with morphometric changes linked to pheromonal traits (Mozaffarian *et al*., 2007). Together, these cases illustrate how environmental pressures and natural selection foster both phenotypic plasticity and adaptive evolution in moth pheromone biosynthesis. Despite extensive research, the underlying drivers of pheromone diversity remain only partially understood, though migration patterns and sympatric overlap with species sharing similar pheromone blends are hypothesized to influence the emergence and maintenance of interpopulation variation (Gemeno *et al*., 2000). However, Johansson and Jones (2007) investigated the role of sex pheromones in mate selection and demonstrated significant variability in these signals among populations. Pheromone plasticity occurs in two main forms. Quantitative variation involves differences in the ratios of pheromone components within a population (Balmer *et al*., 2018), whereas qualitative variation refers to the emergence of structurally novel compounds (Groot *et al*., 2010).

This is the first report on fall webworm pheromone components in Iran, however, experiments conducted on populations in the United States, Europe, New Zealand, and China demonstrated that this species included various local pheromones (Yarmand *et al*., 2009). Different compositions detected in the Iranian population of this species may indicate geographically specific sex pheromone compositions originating from the reproductive isolation of *H. cunea*, which is also true for other Lepidoptera, when geographic isolation led

to variances in sex pheromone compositions (Groot *et al*., 2009; Blassioli-Moraes *et al*., 2017). Su *et al*. (2008) evaluated pheromone gland extracts and concluded that *H. cunea* populations in China, Hungary, and New Zealand might have originated from different strains. Yang *et al*. (2017) investigated genetic associations between invasive populations (Iran, China, Japan, and Korea) and native populations (USA), revealing that the U.S. haplotypes MY1 and OH1 were absent in Asian populations. In contrast, populations from Guilan (Iran) and Jilin (China) exhibited nine novel haplotypes not found in the United States, suggesting that colonization in these regions involved multiple introduction events.

The adaptive features of *H. cunea* in its invaded range may have enhanced its colonization success. The species' broad host-plant range, encompassing over 600 plant species, combined with its climatic adaptability—evidenced by regional variations in voltinism and larval instar development (4–6)—probably drives divergent evolution in pheromone composition. This divergence may arise from differences in host-derived chemical precursors and local environmental conditions (Rezaei *et al*., 2006; Gomi *et al*., 2007; Yarmand *et al*., 2009).

Our identification of a regionally optimized 4:2:2:3 pheromone blend for Iranian *H. cunea* offers a potent, environmentally benign tool for integrated pest management. When deployed in mass-trapping or mating-disruption programs, these highly specific lures can improve monitoring accuracy, reduce reliance on broad-spectrum insecticides—thereby lowering growers' production costs—and preserve beneficial arthropods while mitigating chemical contamination of soil, water, and air. Critically, this approach also minimizes pesticide residues in food crops and surrounding ecosystems, safeguarding human health and ecosystem services—an outcome of particular importance in mulberry-sericulture systems, where conventional insecticide use threatens silk production (Edosa *et al*., 2019). The superior efficacy of our locally tailored blend over non-local formulations underscores the necessity of region-specific pheromone strategies. Given the ecological significance of these findings, further research should investigate the underlying physiological and metabolic pathways driving the production, release, and reception of these pheromone compounds in *H. cunea*, ensuring a comprehensive understanding for more precise pest control applications.

Conclusions

This study provides the first characterization of a region-specific female sex pheromone blend in northern Iranian populations of *H. cunea*, revealing marked divergence from previously reported pheromone blends in other regions. Such differentiation likely reflects adaptive responses to geographic isolation, local host-plant phytochemistry (and associated

volatile profiles), and microclimatic constraints—all critical drivers of pheromone biosynthesis and perception. Critically, such blend variation can significantly undermine the effectiveness of pheromone-based control strategies (e.g., trap-and-release, mating disruption), demonstrating that compositional shifts transcend theoretical interest and directly impair control efficacy. Consequently, elucidating the ecological and molecular foundations of these divergences is essential. Future research should prioritize electrophysiological validation of key pheromone components through EAG/GC-EAD analyses, comparative transcriptomics of pheromone-biosynthetic enzymes across clinal populations, and mechanistic studies exploring host-plant phytochemistry's role in modulating pheromone biosynthesis. Field validation must integrate these efforts, combining microclimate-specific dose–response assays with longitudinal monitoring to establish quantitative links between lure efficacy, spatiotemporal population dynamics, and host-plant distribution gradients. Ultimately, a deeper understanding of the geographic and ecological drivers underlying pheromonal diversification in *H. cunea* will not only advance the theoretical framework of insect chemical ecology but also support the development of sustainable and effective IPM strategies.

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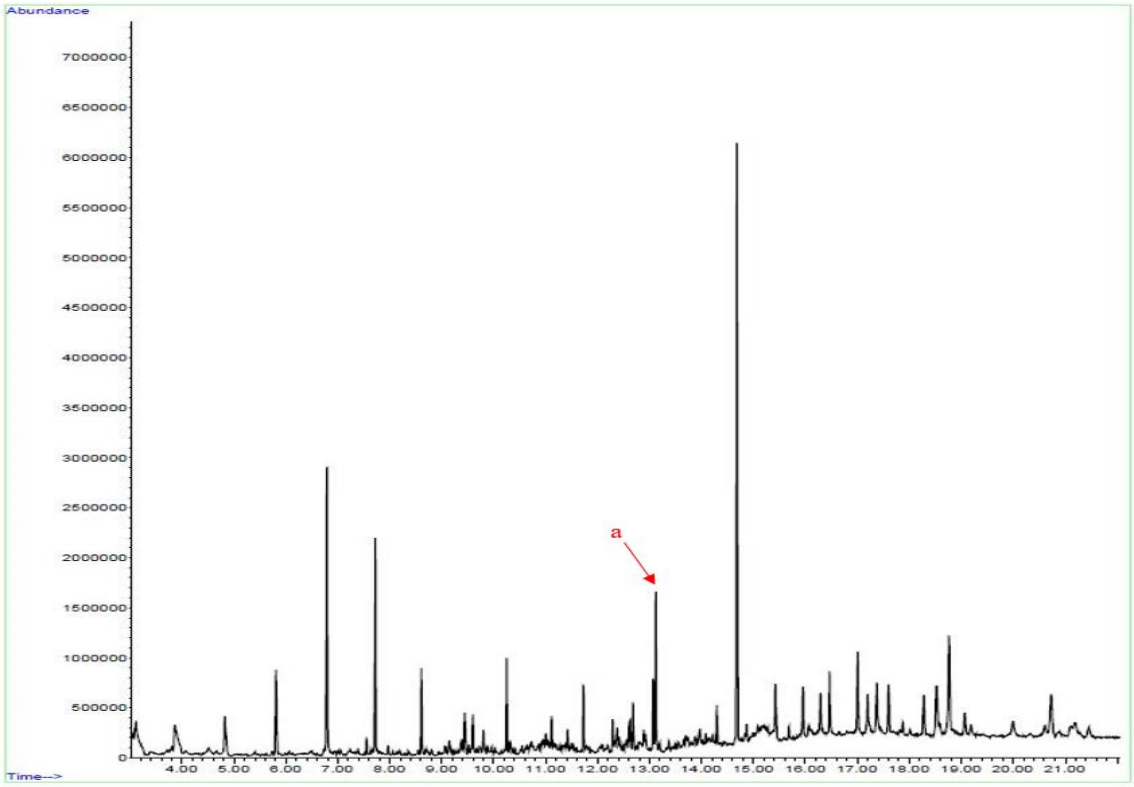


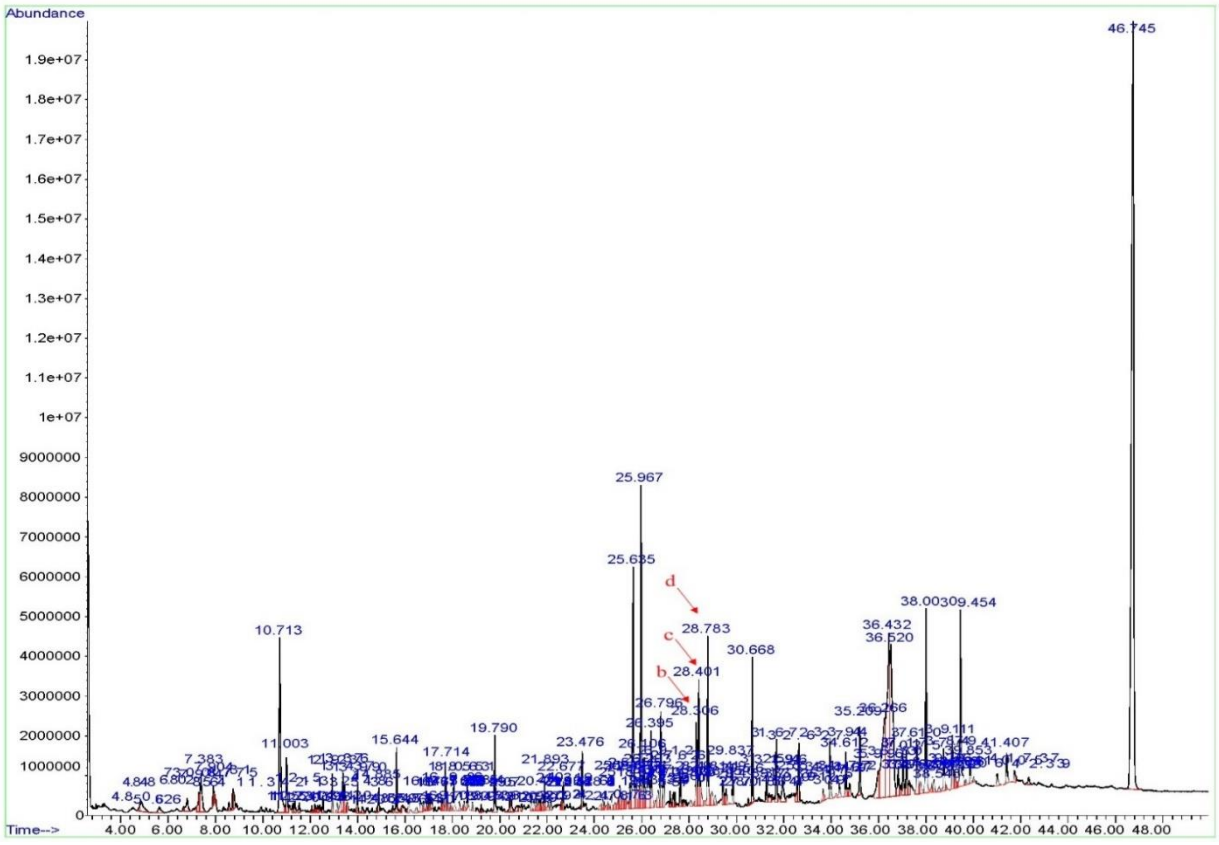
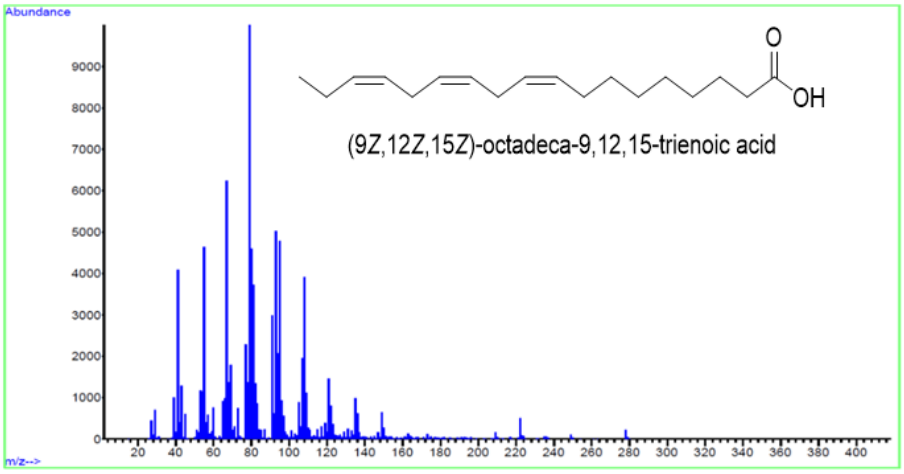


Figure 1. The results of GC-MS sets of crude pheromone gland extracts from a female *H. cunea*. Identification of peaks: a: (Z, Z, Z)-9, 12, 15-Octadecatrienoic acid, b: (Z, Z)-9, 12-Octadecadienoic acid methyl ester, c: (Z)-9-Octadecadienoic acid methyl ester, d: Octadecanoic acid methyl ester.



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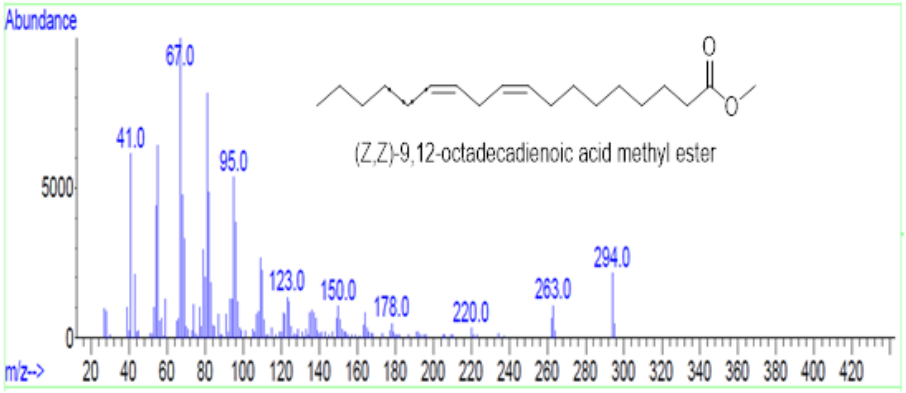

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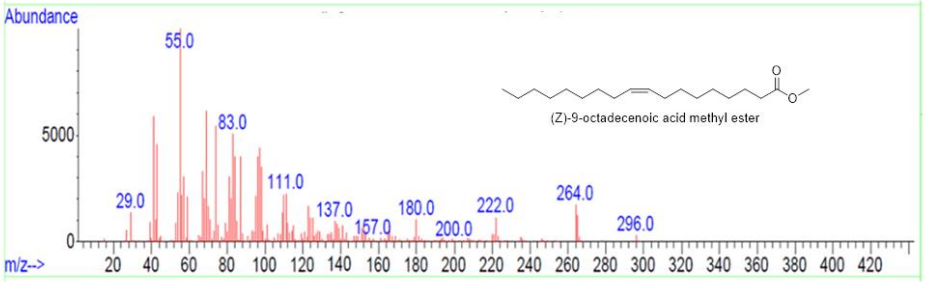

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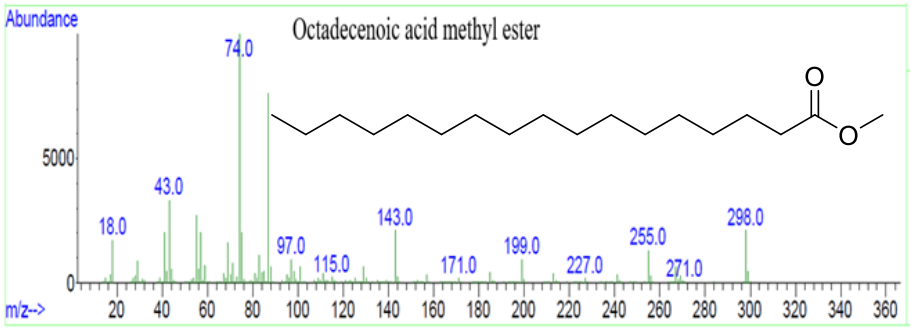

d

Figure 2. MS spectrum of the compounds: (a) (Z,Z,Z)-9,12,15-Octadecatrienoic acid; (b) (Z,Z)-9,12-Octadecadienoic acid methyl ester; (c) (Z)-9-Octadecadienoic acid methyl ester, and (d) Octadecenoic acid methyl ester.

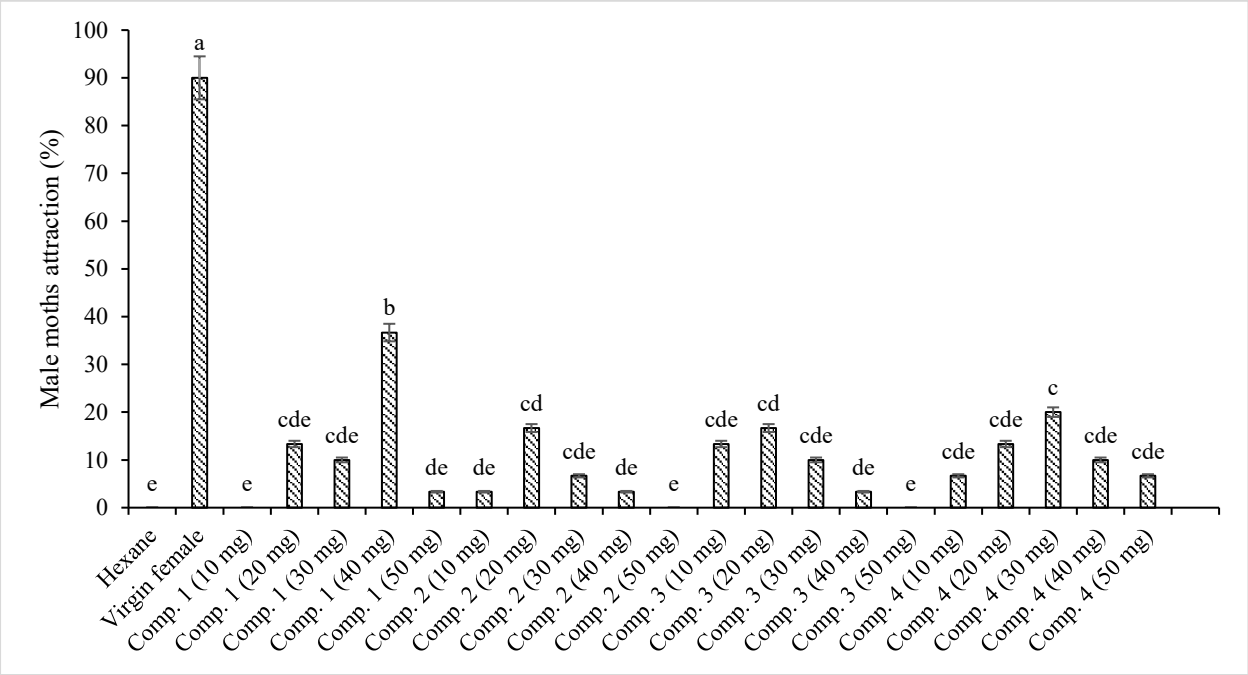

Figure 3. Attraction of *H. cunea* male to Hexane, virgin females and four individually tested compounds identified from the pheromone gland extract of virgin female, in wind tunnel bioassays. Different letters above the bars indicate a significant difference between treatments (one-way ANOVA followed by Tukey's test, $p < 0.05$). Comp.1: (Z, Z, Z)-9, 12, 15-Octadecatrienoic acid, Comp.2: (Z, Z)-9, 12-Octadecadienoic acid methyl ester, Comp.3: (Z)-9-Octadecadienoic acid methyl ester, Comp.4: Octadecanoic acid methyl ester.

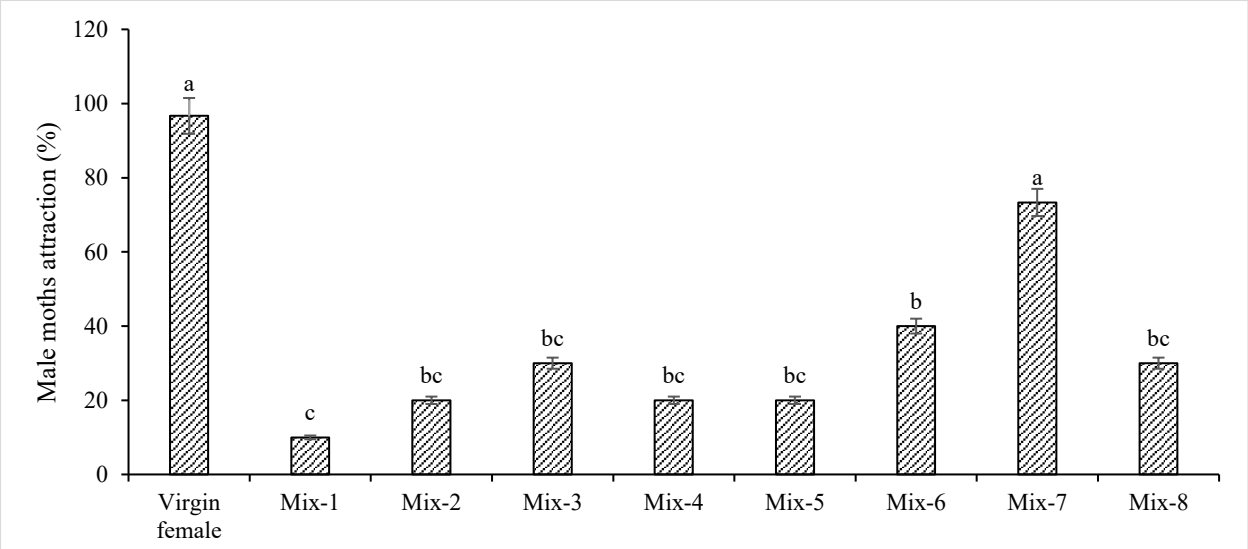

Figure 4. Attraction (% contact response) of *Hyphantria cunea* males to virgin females and various synthetic pheromone blends in wind tunnel assays. Attraction was measured as the proportion of males successfully reaching and contacting the pheromone source (100 cm upwind) within a 20-minute observation period. Binary blends: mix-1 (Comp. 1 + Comp. 2, 1:1); mix-2 (1:2). Ternary blends: mix-3 (Comp. 1–3, 1:1:1); mix-4 (2:1:1); mix-5 (Comp. 2–4, 1:1:1). Quaternary blends: mix-6 (Comp. 1–4, 1:1:1:1); mix-7 (Comp. 1–4, 4:2:2:3); mix-8 (Comp. 1–4, 2:2:2:2). Virgin females and a hexane control were included as positive and negative benchmarks, respectively. Different lowercase letters above bars indicate significant differences among treatments based on one-way ANOVA followed by Tukey's test ($P < 0.05$).

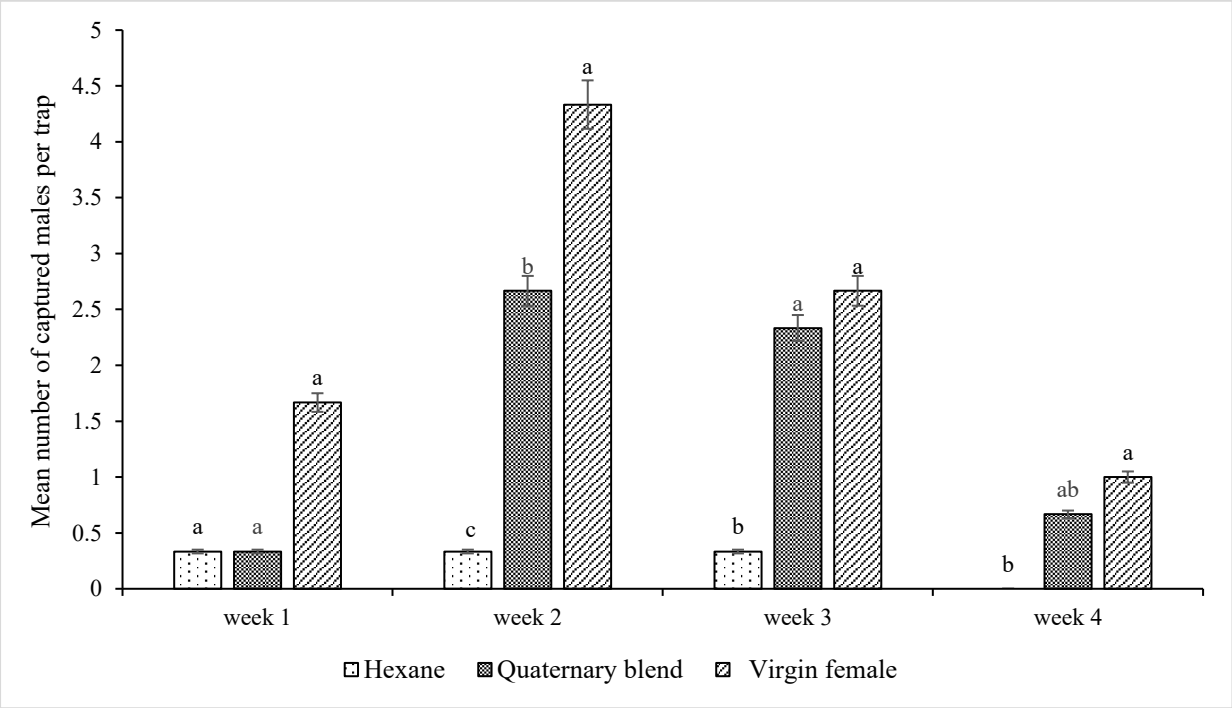

Figure 5. Mean number of *H. cunea* males captured in field experiments using three different treatments: blank, vial impregnated with hexane; twoday- old virgin females; quaternary blend at a ratio of 4:2:2:3, which contained the compounds (Z,Z,Z)-9,12,15-Octadecatrienoic acid, (Z,Z)-9,12-Octadecadienoic acid methyl ester, (Z)-9-Octadecenoic acid methyl ester and Octadecanoic acid methyl ester. Different lowercase letters above the bars indicate a significant difference between treatments (one-way ANOVA followed by Tukey's test, $p < 0.05$).