

The Role of Cuticular Hydrocarbons in the Intraspecific Sexual Recognition of *Cyclocephala Barrerae* (Coleoptera: Melolonthidae)

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Abstract

A mixture of hydrocarbons of different molecular weights covers the Coleoptera cuticle, protecting the insects from desiccation and, in some cases, providing information about their physiological state. Courtship and mating in *Cyclocephala barrerai* Martínez start upon the male antennae touching the thorax and elytra of the female. The male antennae of *C. barrerai* have sensilla that detect hydrocarbons; however, the role of these compounds in the mating behavior of this beetle species remains unknown. Here, we analyzed the behavioral responses of both sexes of *C. barrerai* to conspecific cuticular hydrocarbons (CHCs) and identified them to determine how their composition varies according to sex and age. CHCs were extracted from laboratory-reared *C. barrerai* individuals by immersion in hexane. We measured the percentage of adults who responded to CHCs extracts applied to a minimal visual component, paper filter, an inert decoy, and to the carcass of a conspecific stripped of CHCs. We identified and quantified the CHCs using gas chromatography coupled with mass spectrometry. Males exhibited arrest, courtship, and copulation behaviors to CHCs extracted from females. The extracts contain nine compounds, six of which were identified and quantified. Insects emerged from the pupa with a similar amount of CHCs, and they differentiate as the gonads mature. Our results suggest that CHCs from *C. barrerai* females function as a contact sex pheromone.

INTRODUCTION

Cuticular hydrocarbons (CHCs) are high molecular weight organic compounds located mainly in the outer cuticle of insects (Drijfhout et al. 2009). The primary function of CHCs is to protect insects from desiccation (Gibbs and Pomonis 1995). Furthermore, the diversity and abundance of CHCs may be related to the physiological state of the organism (e.g., gonad maturation stage, age, sex, mating status, health, etc.), thus acting as a chemical information channel and actively participating in intraspecific recognition (Howard and Blomquist 2005; Gibbs 2015).

The cuticular hydrocarbons that mediate the sexual behavior of insects are classified as contact sex pheromones. So, the insect antennae must contact the conspecifics to detect the pheromone (Wang 1998). In the Cerambycidae family, for example, the male beetle is arrested on a dummy that has been impregnated with CHCs extracted from conspecific females; the male then attempts to mount the dummy, grasping it with his legs, and exposing the aedeagus (Fukaya et al. 2000; Zhang et al. 2003; Spikes et al. 2010). In this family, the CHCs profile exhibits sexual dimorphism (Zhang et al. 2003; Barbour et al. 2007; Mutis et al. 2009; Spikes et al. 2010).

The study of CHCs of species in the Melolonthidae family (as classified by Cherman and Morón 2014) has received limited attention. In five species of the subfamily Melolonthinae, several allene-type CHCs have been recorded; however, it is currently unknown whether they have an intraspecific recognition function (McGrath et al. 2003). Within the subfamily Dynastinae, evidence indicates that CHCs may play a role in intraspecific sex recognition among species of the genus *Cyclocephala*. For instance, before copulation, *Cyclocephala putrida* Burmeister males touch the females' abdomen or pygidium with their

antennae (Saldanha et al. 2020). *Cyclocephala distincta* Burmeister males discriminate between females and males by touching them with their antennae, recognizing females and initiating copulation (Nóbrega et al. 2022). The cuticle of *Cyclocephala cearae* Höhne, *Cyclocephala celata* Dechambre, *C. distincta*, *Cyclocephala fosteri* Endrödi, *Cyclocephala ohausiana* Höhne, and *Cyclocephala paraguayensis* Arrow is covered with hydrocarbons, predominantly linear alkanes and unsaturated hydrocarbons. Among these six *Cyclocephala* species, each CHCs profile is unique, and only the CHCs profile in *C. ohausiana* exhibited sexual dimorphism (Santos et al. 2024). Nevertheless, the role of CHCs in mating has not been empirically demonstrated in any *Cyclocephala* species.

Cyclocephala barrerai Martínez is distributed in central Mexico (Aragón et al. 2001; Morón 2001) and spends most of its life cycle as larvae, buried in the soil and feeding on roots and organic matter (Morón and Deloya 1991; Morón 2010; Morón et al. 2014). A laboratory-rearing program for *C. barrerai* has been established (Sanchez-Cruz et al. 2025a), ensuring a continuous supply of insects of known characteristics for experimentation. Both sexes of *C. barrerai* are sexually mature at 18 d (Sanchez-Cruz et al. 2022), while sexual activity is observed at 25 d (Sanchez-Cruz et al. 2025b). During mating, males touch the cuticle of the female's thorax or abdomen with their antennae before initiating courtship and subsequent copulation (Sanchez-Cruz et al. 2025b). The male antennae of *C. barrerai* are dominated by placodea IV sensilla, which detect straight-chain hydrocarbons (Sanchez-Cruz et al. 2024). This information suggests that the CHCs of *C. barrerai* are involved in intraspecific sexual chemical communication.

In this study, we demonstrate that only female CHCs elicit sexual behavior in male *C. barrerai*. Furthermore, the CHCs profiles of adult *C. barrerai* are sexually dimorphic, and sexually mature beetles have a specific CHCs profile.

MATERIAL AND METHODS

Insects

Cyclocephala barrerai adults of known age, genetics, sex, mating status, and gonadic maturation used in this work were obtained from a laboratory colony described elsewhere (Sanchez-Cruz et al. 2025a). Briefly, the organisms belonging to F2 were reared individually from egg to adult on a nutrient substrate containing soil and organic matter (leaves and dry wood), to which grass roots were added when they reached the L3 stage. The substrate was constantly moistened with bottled water. The adults remained individually in the substrate throughout gonadal maturation until CHCs extraction or bioassays.

Cuticular Hydrocarbons Extraction from Virgin *Cyclocephala barrerai* Adults at Different Ages

Cuticular hydrocarbons were extracted from virgin adults reared individually. According to Sanchez-Cruz et al. (2022), adults aged 25 d old were considered sexually mature (n = 19 per sex), and 0, 6, and 12 d (n

= 5 females and n = 3 males) were used as proxies for gonadal maturation stages.

The CHCs extracts of *C. barrerae* were obtained using the modified method of Peralta-Falcón et al. (2019). *C. barrerae* adults were individually housed in glass Petri dishes (90 mm diameter x 15 mm height) and subsequently placed in a freezer at -2°C for 2 h. The carcasses were removed from the Petri dishes and placed in a glass desiccator without a lid with silica gel at the bottom. The carcasses and the silica gel were separated by a glass grid. The desiccator containing the carcasses was placed in a drying oven at 45°C for 15 min. The carcass of a *C. barrerae* was subsequently placed in a 2 mL amber glass vial containing 1.5 mL of HPLC-grade hexane (Karal, Mexico). The vial was shaken for 5 min in a Vortex (Scientific Industries, USA). Finally, the liquid recovered from the vial was transferred to a clean one and concentrated to 200 µL using nitrogen.

Response of *Cyclocephala barrerae* Virgin Adults to Cuticular Hydrocarbons from Conspecifics

We used 25-27-d-old virgin adults during their sexual activity period (Sanchez-Cruz et al. 2025b). The bioassay room was at 24°C and 60% RH, illuminated with a red light at 0.02 lux (Her-410 digital lux meter, Steren, China). Bioassays were recorded with a webcam (pro C920, Logitech, USA) placed above the bioassay arena. The videos were processed using Blender software (version 2.9, Blender Foundation, Netherlands).

Glass Petri dishes (90 mm diameter, 15 mm height) were used as experimental arenas. The CHCs were placed on a solid surface (see below) on the periphery of the arena where *C. barrerae* would walk, find, and touch them. The experimental arenas were washed with glass soap (Hycel, HYCLIN-PLUS, Mexico), rinsed with tap and distilled water, and dried at 200°C for one hour before and after each bioassay.

The *C. barrerae* adult was introduced to the periphery of the arena on the opposite side of the stimulus and observed for a maximum of 15 min. Behaviors were classified according to Sanchez-Cruz et al. (2025b) as follows: Walking, the organism walks around the bioassay arena without any apparent direction; Quiet, the organism remains in the same position without locomotion, occasionally contracting and extending its antennae; Leave the bioassay arena, the organism leaves the Petri dish; Arrest, a behavioral response indicating overt interest in a solid surface with CHCs; Courtship behavior, characterized by antennal rubbing, mounting the substrate, and extension of the aedeagus; and Copulatory behavior, in which the male extends the aedeagus and inserts it into the female carcass.

A variable amount (see below) of the 200 µL concentrated extract was placed directly into the corresponding solid surface using a PCR micropipette. The vessel impregnated with the CHCs extract was left at 26°C for 3 min before being introduced into the arena to volatilize the solvent. A similar amount of hexane was used as a control.

We quantified the response of the insect toward the CHCs in three different bioassays:

1. **Cuticular hydrocarbons on a glass slide.** A 20 x 20 mm glass slide was treated with 5 μ L of CHCs extract (one female equivalent), which completely covered the slide. The response was considered positive when the insect was arrested on the plate. We observed the behavior of virgin males in response to the following stimuli: virgin female CHCs (n = 20), virgin male CHCs (n = 10), and hexane (control, n = 10), and the behavior of virgin females towards virgin male CHCs (n = 10) and the control (n = 10).
2. **Cuticular hydrocarbons on a glass dome.** A 13 mm diameter x 5 mm high glass dome was covered with 30 μ L of CHCs extract (one female equivalent). We recorded the behavior of 20 males towards CHCs of virgin females, the behavior of 10 males toward CHCs of virgin males, and the behavior of 10 females or males towards the glass dome without CHCs (control).
3. **Cuticular hydrocarbons on a female carcass.** The *C. barrerae* female was euthanized via freezing at -2°C for two hours. Soft tissue was removed by making a small incision in the abdomen with an entomological pin followed by the immersion of the female body in 70% ethanol for 12 months. The container holding the bodies was agitated gently every month, and the ethanol was replaced every four months. The female carcasses were retrieved from the ethanol solution and dried at room temperature. The female carcasses were rinsed individually in 1.5 mL of solvent and shaken in a vortex for five minutes. The body was rinsed three times in hexane and three times in methanol. The female carcass was entirely coated with 30 μ L (one female equivalent) of CHCs extracts. The objective of this bioassay was to ascertain whether any mechanical stimulus generated by the female's structures was involved in male courtship behavior, or whether it was the CHCs that evoked courtship behavior. Therefore, we observed the response of 20 virgin males toward the female carcass coated with virgin female CHCs and the response of 10 virgin males to a female carcass without female CHCs. If a male inserted the aedeagus into the female's body, the female was dissected searching for any transferred fluids.

Analysis and Identification of the Cuticular Hydrocarbons of *Cyclocephala barrerae* Adults

The analysis and identification of the CHCs extracts were performed according to the methodology of Peralta-Falc3n et al. (2020). A 2 μ L aliquot of the CHCs extract was injected into a 7890A gas chromatograph (GC) (Agilent, United States of America). The column was an HP-5MS 19091S-433 (Agilent, USA) of 30 m, 0.25 μ m inner diameter, 0.25 μ m thick. The injection port was operated in a 2:1 split mode at a temperature of 250°C. Hydrogen at 2 mL/min was the gas carrier. The oven started at 150°C for 2 min and increased 5°C/min to 308°C.

The mass spectrometer (MS) 5975C (Agilent, USA) operated with electron ionization (70 EV) in SCAN mode and 40 to 400 atomic mass range. Compounds were identified by retention time and comparison of their mass spectra with the NIST/EPA/NIH spectral library version 2.0 (NIST, USA). Compound identities were confirmed using pure chemical standards obtained diluted in HPLC-grade hexane: heneicosane (99% pure), Z-(9)-tricosane (99% pure), tricosane (99% pure), heptacosane (99% pure),

nonacosane (99% pure) were purchased from Sigma Aldrich (St. Louis, MO, USA) and pentacosane (97% pure) from AK Scientific (Union City, CA, USA).

Chemical standards were used to calculate the amount of CHCs using a calibration curve. Four known concentrations of each standard were injected. The areas under the curve were plotted and the equation of the line was calculated according to equation A (Harris, 2010):

Equation A

$$C = \frac{A - b}{m}$$

C is the compound concentration in the CHCs extract, A is the area under the curve obtained from the compound in the CHCs extract, b is the ordinate to the origin and m is the slope. These results indicate the amount of CHCs injected into the GC, 2 μ L. To know the individual amount of a particular compound for each organism, dissolved in 200 μ L, the amount obtained from equation A is multiplied by 100.

Statistical Analysis

A comparison of the quantity of CHCs in extracts from individuals of the same sex and across different ages was performed using Analysis of Variance (ANOVA) followed by a Tukey's multiple comparison test. Comparison of the quantity of each compound between females and males was performed with a Student's t-test. Preliminary analyses indicated that some datasets did not meet the assumption of normality. Therefore, different transformations were evaluated to meet a normal distribution, including the natural logarithm, base-10 logarithm, square root transformation, and the Box–Cox transformation (λ) (Box and Cox, 1964). All data met the assumption of homogeneity of variance. All data presented are original data. All statistical analyses were performed in Sigma plot 14.0., Systat Software, Inc., USA) at $\alpha = 0.05$. All presented data are mean $\pm$ standard error of the mean (SEM).

RESULTS

Response of Cyclocephala barrerae Adults to Cuticular Hydrocarbons from Conspecifics

1. **Cuticular hydrocarbons on a glass slide.** On glass slides coated with the CHCs of conspecific females, 85% of the males exhibited arrest behavior (supplementary video 1). Among these males, 11.6% also exhibited walking behavior, 11.6% remained quiet, and 23.5% left the bioassay arena during the bioassay. In contrast, 10% of the males remained quiet throughout the bioassay, and 5% left the arena without exhibiting any other behavior. On glass slides coated with the CHCs of conspecific males (supplementary video 2), 80% of the males exhibited walking behavior. Among these males, 37.5% also left the bioassay arena, 25% remained quiet, and 25% exhibited arrest behavior. Additionally, 20% of the males remained quiet. One hundred percent of the females exhibited walking behavior, and 60% of these females also exhibited quiet behavior. In the control treatment, 100% of the males exhibited walking behavior. Among these males, 20% also exhibited

quiet behavior, and 20% left the bioassay arena during the bioassay (supplementary video 3). On glass slides coated with the CHCs of conspecific males, one hundred percent of the females exhibited walking behavior, and 60% of these females also exhibited quiet behavior (supplementary video 4). Among females in the control treatment (supplementary video 5), 80% exhibited walking behavior, and 20% exhibited quiet behavior.

2. **Cuticular hydrocarbons on a glass dome.** Ninety-five percent of male *C. barrerai* exhibited courtship behavior when exposed to glass domes coated with female CHC extracts (supplementary video 6). Among the males that displayed courtship behavior, 42% alternated courtship with walking around the bioassay arena and repeatedly returned to the dome containing female CHCs to resume courtship, whereas 5.2% left the bioassay arena after attempting to court beneath the glass dome. Only one male (5%) exposed to female CHCs did not exhibit courtship behavior; instead, it walked around the bioassay arena and then left. In the control treatment, 100% of the males exhibited walking behavior (supplementary video 7). Of these, 50% continued walking until the end of the bioassay, 40% left the bioassay arena, and 10% exhibited quiet behavior until the end of the observation period. Among males exposed to glass domes coated with male CHC extracts, 90% exhibited walking behavior within the bioassay arena, and 50% exhibited arrest behavior at the glass dome. Of these males, seven remained quiet near the glass dome (supplementary video 8), whereas the remaining two interacted with the dome, moved their antennae, remained quiet beside the dome, and then moved away (supplementary video 8). 75% males exhibited quiet behavior, followed by either walking or arrest behavior.
3. **Cuticular hydrocarbons on a female carcass.** Ninety-five percent of *C. barrerai* males exhibited sexual behavior when exposed to carcasses containing cuticular hydrocarbons from conspecific females. Of these males, 85% exhibited the behavioral sequence of courtship followed by copulation (supplementary video 9), 5% exhibited courtship behavior only, and 5% copulated without prior courtship. The remaining 5% (one male) walked around the bioassay arena without exhibiting any additional behavior. No males exhibited courtship or copulatory behavior in the control treatment. Nevertheless, they approached the female carcass, contacted it with their antennae, and continued walking throughout the arena (supplementary video 10). 90% of the males exhibited walking behavior, of which 40% also exhibited quiet behavior, and 20% left the bioassay arena during the assay. The remaining 10% exhibited quiet behavior until the end of the bioassay.

Cuticular Hydrocarbons Profile of Cyclocephala barrerai

The CHCs of *C. barrerai* identified and quantified by chemical standards that appeared in all replicates of 25-d-old sexually mature adults are listed in Table 1. The CHCs profiles of females and males of *C. barrerai* consist of six higher alkanes, four compounds are straight chain, and (Z)-9-tricosene is the only isomer.

TABLE 1. CUTICULAR HYDROCARBONS IDENTIFIED IN 25-D-OLD *Cyclocephala barrerai* ADULTS

RT	Compound	CAS	Chemical formula	MW g/mol
12.61	Heneicosane	629-94-7	C ₂₁ H ₄₄	296.57
15.52	(Z)-9-tricosene	27519-02-4	C ₂₃ H ₄₆	322.61
15.97	Tricosane	638-67-5	C ₂₃ H ₄₈	324.63
19.15	Pentacosane	629-99-2	C ₂₅ H ₅₂	352.69
22.1	Heptacosane	593-49-7	C ₂₇ H ₅₆	380.74
24.9	Nonacosane	630-03-5	C ₂₉ H ₆₀	408.80

RT = retention time, CAS = chemical abstracts service, MW = molecular weight, g/mol = grams per mole.

The female's CHCs profile shows a significant difference in the amount of six compounds ($F = 69.85$; $df = 5,108$; $p < 0.001$). Tricosane is the hydrocarbon with the highest amount (Fig. 1). (Z)-9-Tricosene and heptacosane are the second hydrocarbon with the highest amount (Fig. 1). Heneicosane and heptacosane have similar amounts and are different from the rest of the hydrocarbons (Fig. 1). Finally, pentacosane is hydrocarbon with the lowest amount (Fig 1).

The male's CHCs profile shows a significant difference in the amount of the six compounds ($F = 118.51$; $df = 5,108$; $p < 0.001$). Tricosane and (Z)-9-tricosene are the hydrocarbons with the highest amount (Fig. 1). Heneicosane and heptacosane have similar amounts and are different from the rest of the hydrocarbons (Fig. 1). Nonacosane is different amount to the rest of the CHCs (Fig. 1). Pentacosane was the hydrocarbon with the lowest abundance (Fig. 1).

The amounts of heneicosane ($t = 8$; $df = 36$; $p < 0.01$), (Z)-9-tricosene ($t = 7.7$; $df = 36$; $p < 0.01$), and tricosane ($t = 5$; $df = 36$; $p < 0.001$) were significantly higher in males than in females (Fig. 1). The amounts of pentacosane ($t = 1.5$; $df = 36$; $p = 0.13$), heptacosane ($t = 1.1$; $df = 36$; $p = 0.24$), and nonacosane ($t = 1.2$; $df = 36$; $p = 0.2$) did not differ between sexes (Fig. 1).

Fig. 1 Mean amount ($\pm$ SEM) of cuticular hydrocarbons present in 25-day-old females and males of *Cyclocephala barrerai*. Gray bars represent females, and white bars represent males. Capital letters compare the results within females, and lowercase letters within males. Bars of the same color followed by the same letter are not statistically different (*Tukey*, $p > 0.05$). Bars within the same compound marked with an asterisk are statistically different (*Student's t-test*, $p < 0.05$). The bars are the mean, and the error bars are the standard error of the mean

Three additional hydrocarbons were preliminarily identified in CHCs extracts from adult *C. barrerai* (Table 2); the 2-methyltricosane was present in 85% of female and male extracts, but its identity could not be confirmed against chemical standards. The presence of hexacosane was verified using chemical standards; its amount is less than 0.002 $\mu\text{g}/\text{individual}$ and was found in 50% of female extracts and 10%

of male extracts. The 17-pentatriacontane appeared in 70% of female extracts and in all male extracts, but its identity could not be confirmed using chemical standards.

TABLE 2. PROPOSED IDENTITY OF COMPOUNDS NOT IDENTIFIED BY CHEMICAL STANDARD OR DUE TO LOW FREQUENCY OF OCCURRENCE IN THE CUTICULAR HYDROCARBON PROFILES OF FEMALES AND MALES OF *Cyclocephala barrerai*

RT	Compound	CAS	Chemical formula	MW g/mol
17.0	2-Methyltricosane	1928-30-9	C ₂₄ H ₅₀	338.6
20.6	Hexacosane	630-01-3	C ₂₆ H ₅₄	366.7
31.7	17-Pentatriacontene	6971-40-0	C ₃₅ H ₇₀	490.9

RT = retention time, CAS = chemical abstracts service, MW = molecular weight, g/mol = grams per mole.

Change in Cuticular Hydrocarbon Profiles Through Gonadal Maturation in Adults of Cyclocephala barrerai

The amount of heneicosane ($F = 7.17$; $df = 3, 30$; $p > 0.001$), tricosane ($F = 6.4$; $df = 3, 30$; $p < 0.05$), pentacosane ($F = 14.9$; $df = 3, 30$; $p < 0.001$), heptacosane ($F = 13.9$; $df = 3, 30$; $p > 0.001$) and nonacosane ($F = 17.9$; $df = 3, 30$; $p > 0.001$) changed significantly during the gonadal maturation of *C. barrerai* female (Fig. 2). Heneicosane decreases on day 6 and remains low until day 25 (Fig. 2A). Tricosane peaks on day 0, decreases on day 6 and increases slightly in the following days (Fig. 2C). The amount of pentacosane increases slightly on day 6, reaching its highest peak on day 12 (Fig. 2D); subsequently, the amount decreases on day 25 to a similar level to that on day 0. Heptacosane shows a gradual increase throughout the gonadal maturation process (Fig. 2E). The amount of nonacosane increases gradually from day 0 to day 25 (Fig. 2F). Although the amount of (Z)-9-tricosene in females did not demonstrate significant differences ($F = 2$; $df = 3, 30$; $p = 0.127$), it is observed that the amount decreases on day 6 and remains at this level until day 25 (Fig. 2B).

The amount of heneicosane ($F = 3.4$; $df = 3, 24$; $p > 0.05$), (Z)-9-tricosane ($F = 4.73$; $df = 3, 24$; $p > 0.05$), tricosane ($F = 11.6$; $df = 3, 24$; $p > 0.001$) and heptacosane ($F = 25.3$; $df = 3, 24$; $p > 0.001$) in *C. barrerai* males changed significantly during gonadal maturation; in tricosane and heptacosane cases, the amount increased gradually during gonadal maturation (Fig. 2 C, E). In the case of heneicosane and (Z)-9-tricosane the concentration remains stable for a period of zero to 12 days and increases when they reach sexual maturity, although significant differences were reported in the ANOVA analysis, the Tukey test did not show significant differences (Fig. 2AB), The amount of pentacosane ($F = 0.2$; $df = 3, 24$; $p = 0.89$), and nonacosane ($F = 2$; $df = 3, 24$; $p = 0.12$) did not change significantly during gonadal maturation. In the case of (Z)-9-tricosane (Fig. 2B) and pentacosane (Fig. 2D), the amount of the compounds remained similar during days 0, 6, and 12 and increased when the organisms reached sexual maturity. In the case of nonacosane, amount increased gradually throughout the maturation period (Fig. 2F).

No significant differences were found between females and males on day 0 in the amount of heneicosane ($t = 0.06$; $df = 6$; $p = 0.95$), (Z)-9-tricosane ($t = 0.09$; $df = 6$; $p = 0.92$), tricosane ($t = 1.4$; $df = 6$; $p = 0.18$), pentacosane ($t = 0.8$; $df = 6$; $p = 0.45$), heptacosane ($t = 0.5$; $df = 6$; $p = 0.58$), and nonacosane ($t = 1.3$; $df = 6$; $p = 0.2$) (Fig. 2A-F). The amount of heneicosane ($t = 8.91$; $df = 6$; $p < 0.05$), (Z)-9-tricosane ($t = 8.3$; $df = 6$; $p < 0.001$), heptacosane ($t = 4.5$; $df = 6$; $p < 0.05$), and nonacosane ($t = 2.9$; $df = 6$; $p < 0.05$) day 6 were significantly higher in males than in females (Fig. 2A, B, E, F).

On day 12, the amount of heneicosane was significantly higher in males than in females ($t = 2.78$; $df = 6$; $p < 0.05$) (Fig. 2A). We found no significant differences in female and male amount on day 12 of (Z)-9-tricosane ($t = 1.4$; $df = 6$; $p = 0.18$) (Fig. 2B), tricosane ($t = 1.2$; $df = 6$; $p = 0.24$) (Fig. 2C), pentacosane ($t = 2.1$; $df = 6$; $p = 0.07$) (Fig. 2D), heptacosane ($t = 0.26$; $df = 6$; $p = 0.8$) (Fig. 2E), and nonacosane ($t = 0.1$; $df = 6$; $p = 0.85$) (Fig. 2F). The results of the sexually mature *C. barrerai* presented previously (Fig. 1) show the comparison of the compounds on day 25.

Fig. 2 Mean ($\pm$ ESM) amount of cuticular hydrocarbons in males and females of *Cyclocephala barrerai* at different ages. The gray bars represent females, and the white bars represent males. Capital letters compare the results for females, and lowercase letters compare the results for males. Bars of the same color followed by the same letter are not statistically different (*Tukey*, $p > 0.05$). The bars represent the mean, and the error bars represent the standard error of the mean

DISCUSSION

The results of this study are the first to demonstrate that CHCs play a role in the mating behavior of any species of *Cyclocephala*. Specifically, we found that CHCs are crucial for intraspecific sexual recognition of *C. barrerai*, with female CHCs eliciting courtship and copulation behaviors in conspecific males. The bioassay results are consistent with the findings of a previous study, which demonstrated that the antennal lamellae of *C. barrerai* possess olfactory neurons in the placodea IV sensilla that exhibit a positive physiological response when exposed to hydrocarbons identified in the cuticle (Sanchez-Cruz et al. 2024).

The male behavioral response toward the virgin female CHCs depends on the way they are presented. In Petri dishes, lacking visual or tactile stimuli, males were arrested on the slide, continuously searching for the female; however, no courtship behavior was exhibited. Wang (1998) suggests that CHCs traces on the grass or soil could act as a signal for *Anoplophora chinensis* (Forster) males to locate females. In contrast, when we used a glass dome impregnated with CHCs of *C. barrerai* females, mimicking the shape and scent of a female, males engaged in courtship and attempted to copulate with the glass dome. Males of *A. glabripennis* and *A. malasiaca* have been observed courting and attempting copulation with Eppendorf tubes and glass cylinders impregnated with female CHCs (Fukaya 2003; Zhang et al. 2003). CHCs function as contact pheromones, so there is likely a relationship between mechanoreception and the shape of the container holding the chemical stimuli.

Bioassays exposing CHCs on the female carcass confirmed that these compounds are responsible for eliciting courtship and copulatory behavior in *C. barrerae* males. Although the female body produces an apparent visual attraction, this is not sufficient to induce courtship or copulation. Similar observations have been recorded in various species within the Cerambycidae family, including *Neoclytus mucronatus mucronatus* F., *Megacyllene caryae* Gahan, *Megacyllene robiniae* Forster, *Plectrodera scalator* F. (Ginzel and Hanks 2003), *Prionus californicus* Motschulsky (Barbour et al. 2007), and *Mallodon dasystomus* Say (Spikes et al. 2010). Males of these species engage in copulation attempts with the inert bodies of females that have been impregnated with CHCs extracts. The deceased and solvent-washed female body of *C. barrerae*, treated with its CHCs extract, prompted the male to attempt copulation, including the insertion of the aedeagus through the female's pygidium. Nonetheless, transfer of a spermatophore or any other fluid did not occur due to the lack of soft tissue structures necessary to facilitate this process (Sanchez-Cruz et al. 2022).

The CHCs from *C. barrerae* males did not elicit any discernible behavioral response from other males. This finding contrasts with earlier observations of behavior in other beetle species, where male CHCs elicited conspecific fighting behavior (Scott et al. 2008; Ginzel 2010). The absence of aggressive behavior triggered by male CHCs can be attributed to the fact that *C. barrerae* males do not require a territory for mating. The primary objective of *C. barrerae* males is to locate females as quickly as possible, given the limited time frame of their mating period (Sanchez-Cruz et al. 2025b, c). Simultaneously exposing *C. barrerae* males to the CHCs of both females and males will enhance our understanding of the dynamics of male-female-male interactions during the mating process.

The CHCs profile of *C. barrerae* adults is composed of at least six identified compounds. The mixture of compounds that forms the CHCs profile of *C. barrerae* adults is specific compared to the reports of the other six *Cyclocephala* species studied. Still, individual compounds may be present or absent in the profiles of other species of this genus (Santos et al. 2024). For example, the CHCs profile of *C. cearae* lacks hexacosane, and the CHCs profile of *C. distincta* does not contain (Z)-9-tricosene (Santos et al. 2024). In Coleoptera, compound similarity is common, differing only in their ratios and amounts within the blend. Heptacosane, pentacosane, and (Z)-9-tricosene have also been reported in Cerambycidae species (Peschke and Metzler 1987; Fukaya et al. 2000; Zhang et al. 2003), although they are isomers at carbons 7 and 9 or have a methyl group.

(Z)-9-Tricosene is frequently reported as a constituent of an insect pheromone. For example, it is part of the sex pheromone of *Musca domestica* L. (Nelson et al. 1981; La-France et al. 1989; Carpita et al. 2012). In the ant *Solenopsis geminata* F., the amount of (Z)-9-tricosene varies between different castes (Hu et al. 2017). In beetles, this compound is part of the sex pheromone of the ground beetle *Anchomenus dorsalis* Pontoppidan (Bonacci et al. 2011), the staphylinid *Aleochara curtula* Goeze (Peschke and Metzler 1987), and the cerambycid *Anoplophora glabripennis* Motschulsky. In the latter species, this compound is present in higher proportions in females than in males (Zhang et al. 2003).

The chemical composition of CHCs in *C. barrerai* females and males is similar; however, the amounts of heneicosane, (Z)-9-tricosene, and tricosane are higher in males than in females, resulting in sexual dimorphism. In insects, CHCs profiles are often similar, but differences in the levels of specific compounds generating sexual dimorphism (e.g., Zhang et al. 2003; Hasan and Ansari 2016). The precise causes of sexual dimorphism in CHCs remain to be elucidated; however, it has been suggested that the synthesis of these compounds is associated with enzymatic and hormonal activity (Chung and Carroll 2015).

CHCs in males and females are present in low amounts when they emerge from the pupa, and their amount is similar between sexes, changing as the gonads mature. Only when adults reach sexual maturity, both sexes emerge from the soil looking for a mate (Sanchez-Cruz et al. 2022). The relationship between gonad maturation and CHCs profile is not fully understood. In females and males of the common housefly, *M. domestica*, both sexes emerge with the same CHCs amount, which changes as the gonads mature. Blomquist and Ginzl (2021) proposed that juvenile hormones regulate both processes. In the termite *Zootermopsis nevadensis* Hagen, the juvenile hormone triggers gonadal maturation activating other mechanisms that modify the CHCs profiles, especially when gametes are fully mature (Brent et al. 2016).

The current understanding of the metabolic pathways involved in CHCs synthesis in insects suggests that the precursors of CHCs must be obtained through the diet and subsequently transformed into the final compounds (Chung and Carroll, 2015; Blomquist and Ginzl, 2021). The lack of adult feeding in *C. barrerai* (Sanchez-Cruz et al. 2022) suggests that CHCs precursors must be obtained during the larval stage.

In summary, traces of female CHCs prompt male mating behavior. These chemicals indicate the presence of a female, prompting courtship and copulation. The CHCs profile of adults of *C. barrerai* consists of at least seven compounds, with specific amounts for males and females. The CHCs amounts of adults emerging from pupae are similar but become more specialized upon sexual maturity.

Declarations

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Competing Interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Supplementary information

The following supporting information can be downloaded at <https://doi.org/10.17632/77wnrjbwvr.1>. Sanchez-Cruz, Abraham; Villa-Ayala, Patricia; Rojas, Julio C; García-Sosa, Paola R.; Robledo, Norma; Jiménez-Pérez, Alfredo (2026), "The role of cuticular hydrocarbons of *Cyclocephala barrerae* (Coleoptera: Melolonthidae) in intraspecific recognition ", Mendeley Data, V2.

Author Contribution

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Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information. The supplementary material includes the original videos documenting the behavioral bioassays, which can be accessed at Sanchez-Cruz, Abraham; Villa-Ayala, Patricia; Rojas, Julio C; García-Sosa, Paola R.; Robledo, Norma; Jiménez-Pérez, Alfredo (2026), "The role of cuticular hydrocarbons of *Cyclocephala barrerae* (Coleoptera: Melolonthidae) in intraspecific recognition", Mendeley Data, V2. DOI: <https://doi.org/10.17632/77wnrjbwvr.1>).

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Figures

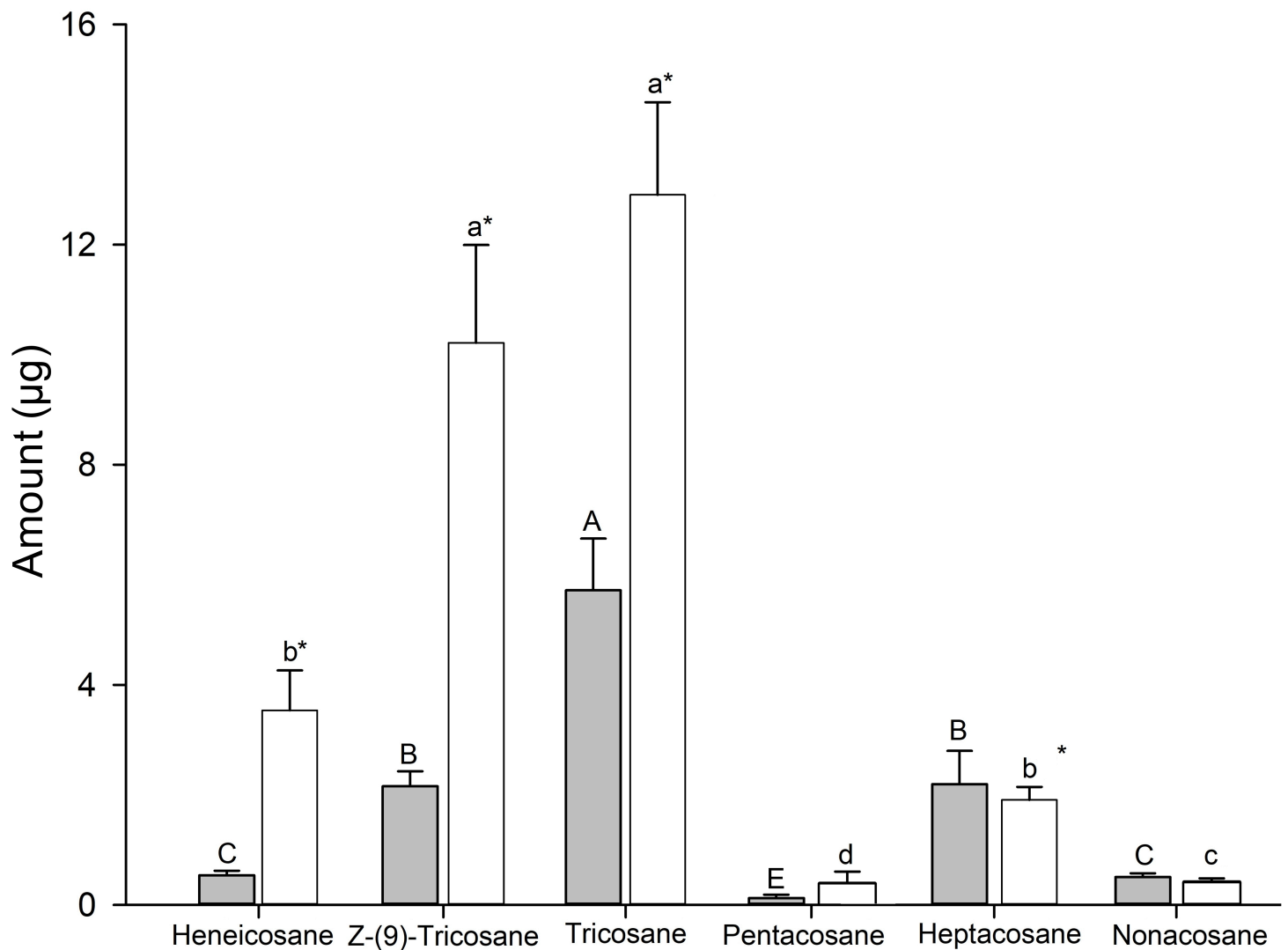


Figure 1

Mean amount ($\pm$ SEM) of cuticular hydrocarbons present in 25-day-old females and males of *Cyclocephala barrerai*. Gray bars represent females, and white bars represent males. Capital letters compare the results within females, and lowercase letters within males. Bars of the same color followed by the same letter are not statistically different (*Tukey*, $p > 0.05$). Bars within the same compound

marked with an asterisk are statistically different (*Student's t-test*, $p < 0.05$). The bars are the mean, and the error bars are the standard error of the mean

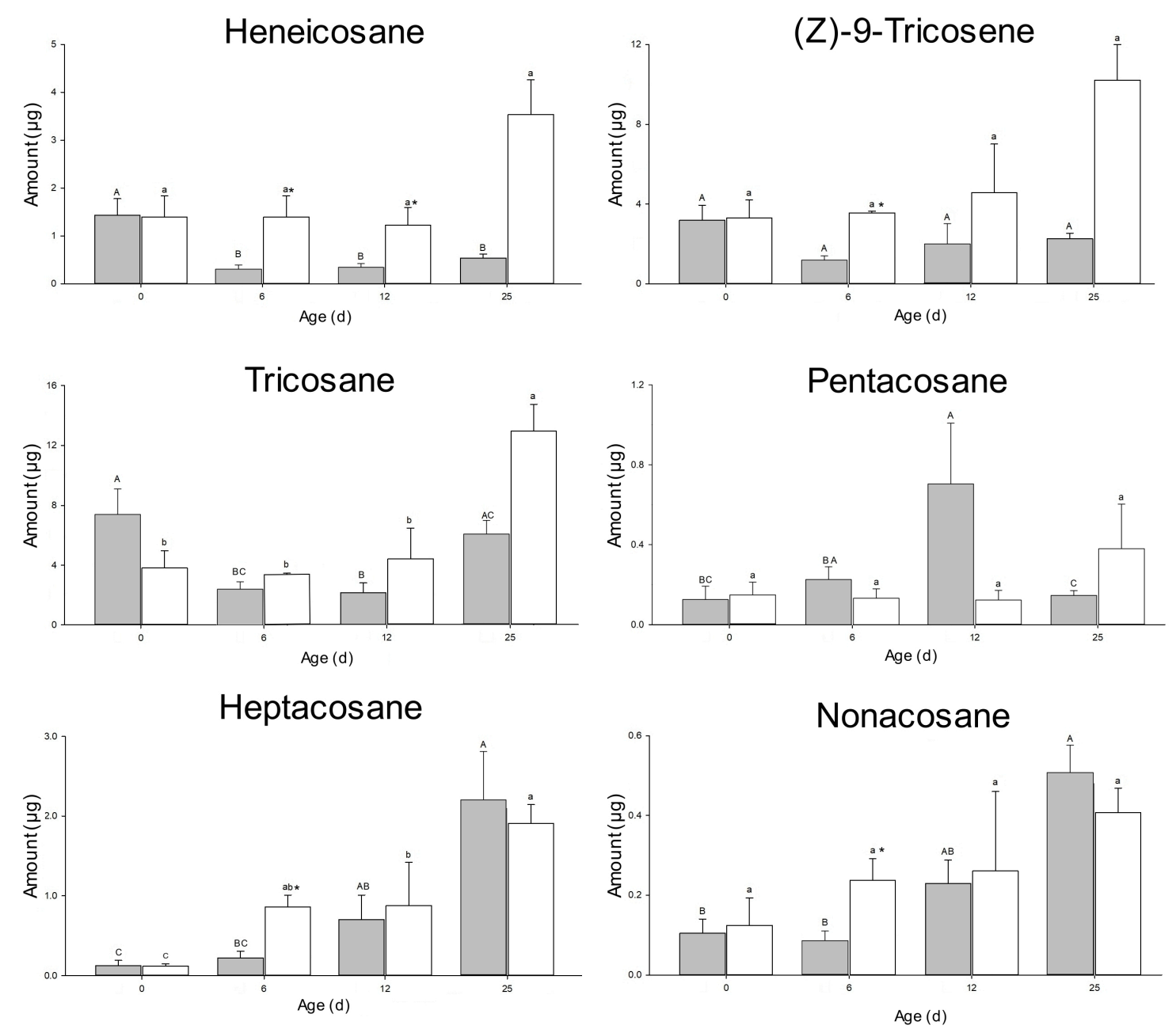


Figure 2

Mean (± ESM) amount of cuticular hydrocarbons in males and females of *Cyclocephala barrerai* at different ages. The gray bars represent females, and the white bars represent males. Capital letters compare the results for females, and lowercase letters compare the results for males. Bars of the same color followed by the same letter are not statistically different (*Tukey*, $p > 0.05$). The bars represent the mean, and the error bars represent the standard error of the mean