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## Scientific Note

# Do cuticular hydrocarbons influence mate recognition in the palm florivore *Cyclocephala distincta* Burmeister, 1847 (Melolonthidae: Cyclocephalini)?

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**Abstract.** Cyclocephaline beetles often congregate in vast numbers on the flowers and inflorescences of their host plants, where precise sexual partner identification is essential for reproductive success. Recognizing that lipophilic compounds on the external cuticle function as contact pheromones in many beetle taxa, we investigated the role of cuticular hydrocarbons (CHCs) in sexual recognition in *Cyclocephala distincta* Burmeister, 1847 (Melolonthidae: Cyclocephalini) - a pollen-feeding species associated with Neotropical palms. Although the CHC profile of *C. distincta* is highly species-specific when compared to congeners, it lacks sex-specificity. To explore the potential role of CHCs in mediating sexual behavior, we conducted bioassays using hexane extracts from both male and female specimens, which included reintroducing CHCs to solvent-washed individuals, exchanging extracts between sexes, and applying cuticular extracts from female *C. distincta* to female *Cyclocephala paraguayensis* Arrow, 1904 (Melolonthidae: Cyclocephalini), a sympatric congener. Removal of CHCs sharply reduced copulatory attempts by focal males towards both conspecific and heterospecific females, whereas reapplication of *C. distincta* extracts only partially restored them, and did so irrespective of the sex or the species of the donor. Rather than encoding species or sex identity, CHCs appear to act as permissive cues that license the initiation of mating attempts, with accurate mate identification depending on additional, presumably mechanotactile, signals.

**Keywords**: semiochemicals, Coleoptera, sexual recognition, mating behavior.

Anthophilous beetles use floral scents as allelochemical cues for aggregation and mating (Maia et al. 2018). In species relying on volatile organic compounds (VOCs), confined aggregations necessitate short-range sexual recognition mechanisms (Pureswaran & Poland 2009), often mediated by contact sex pheromones, notably cuticular hydrocarbons (CHCs) (Chung & Carroll 2015). CHCs are long-chain hydrophobic molecules (C21-C50) synthesized in abdominal oenocytes, providing protection and facilitating close-range recognition (Blomquist et al. 2018). Their specificity influences mate recognition and reproductive isolation (Chung & Carroll 2015).

Cyclocephaline beetles (Melolonthidae, Cyclocephalini) are specialized pollinators and florivores attracted to host plant floral VOCs (Maia et al. 2013). *Cyclocephala distincta* Burmeister, 1847, a Neotropical species, aggregates on palms (*Acrocomia*, *Attalea*) in Atlantic Forest remnants (Scariot et al. 1991; Maia et al. 2018). While aspects of its biology are studied (Nóbrega et al. 2022), its reproductive behavior remains poorly understood. Laboratory pair-choice experiments show male *C. distincta* touch potential mates before mounting, achieving ~50% accuracy in initial conspecific female recognition (Nóbrega et al. 2022), suggesting contact cues mediate recognition. Nonetheless, Santos et al. (2024) found no significant sex differences in the cuticular profiles of *C. distincta*, which are dominated (~97% of the total composition) by C21-C31 linear alkanes and (*Z*)-alkenes, with esters such as ethyl oleate present only in trace amounts (<1%).

We tested whether CHCs function as contact pheromones in *C. distincta*. Specifically, we posed the following questions: (1) Does CHC removal reduce male copulatory behavior, and is it restored upon reapplication? (2) Does applying *C. distincta* CHCs to the sympatric *Cyclocephala paraguayensis* Arrow, 1904 alter male responses? We predicted that CHC removal would reduce male copulatory attempts and that reapplication of conspecific extracts would restore them. If CHCs additionally encode species identity, extracts applied to a heterospecific female should not elicit conspecific-level responses; if instead they act as a general permissive cue for close-range mating attempts, the sex and the species of the donor should be immaterial.

Adult *C. distincta* were collected (December 2016; March-April 2017) in Igarassu, Brazil (7°45'10" S, 34°58'43" W) using semiochemical-baited flight-interception traps (see Maia et al. 2018 for details). Beetles were housed in soil-filled containers at 27 °C ± 2 °C (12:12 photophase; Nóbrega et al. 2022).

For CHC extraction, beetles were placed in a container filled with fine-grain sand ($SiO_2$) for approximately 1 h to remove coarse surface impurities as they naturally burrowed. They were then euthanized by gradual freezing (-5 to -20 °C, 60 min) and immersed in *n*-hexane (2.5 mL), in batches of 10 same-sex individuals, for 2 min, as described in Santos et al. (2024). In total, 20 batches were extracted for *C. distincta* (10 female, 10 male) and 20 for *C. paraguayensis* females, each batch constituting an independent biological replicate; extractions were not additionally replicated within batches. A subset of these batches (five female and three male for *C. distincta*, two female for *C. paraguayensis*) was characterized by GC-MS and is reported in Santos et al. (2024); the remaining batches were prepared identically, from the same collections and season, but were not analyzed individually. Crude eluates were filtered through a borosilicate glass wool plug and concentrated under a laminar $N_2$ flow to 0.5 mL, from which aliquots were withdrawn for GC-MS characterization. The remaining eluate of each batch was further concentrated to a final volume of 25 µL and stored at -24 °C, yielding one working solution per batch. At this concentration 25 µL corresponded to the pooled cuticular load of 10 individuals, so that each 2.5 µL aliquot applied to a solvent-washed specimen delivered approximately one beetle-equivalent. Aliquots were applied with a microsyringe and the solvent was allowed to evaporate for 5 min before the specimen was offered to a focal male. A second 2-min hexane rinse

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ensured complete removal of lipophilic compounds from recipients.

Behavioral trials were conducted in Petri dishes (9 cm diameter) during peak beetle activity (18:00-23:00; Souza et al. 2015), pairing live focal *C. distincta* males with freeze-euthanized beetles under red-light illumination. Three assays (50 trials per treatment) were tested: (1) male response to CHC-removed conspecifics (female and male); (2) males paired with conspecifics having transposed CHCs between sexes; (3) male response to female *C. paraguayensis* with intact, removed, or replaced CHCs (from *C. distincta* females). Mounting and aedeagus eversion were recorded as copulatory attempts (Nóbrega et al. 2022). Each trial was scored as a binary outcome: aedeagus eversion by the focal male within the 20-minute observation window (1) or its absence (0). Treatment effects were analyzed with a generalized linear model (binomial family, logit link) with treatment as a single fixed factor and [female intact] as the reference level. Because each trial employed a different focal male, and extract batches were not tracked to individual trials, no grouping variable was available and no random effects were fitted. The overall effect of treatment was assessed by a likelihood-ratio test against the intercept-only model; individual treatments were compared with the control by Wald tests on model coefficients, with Holm correction across the eight comparisons. Five planned contrasts were tested as linear combinations of model coefficients and are reported uncorrected. Proportions are given with 95% Wilson intervals, and effects as odds ratios (*OR*) with 95% confidence intervals (*CI*). All statistical analyses were performed in R version 4.6.0 (R Core Team 2026).

Treatment strongly affected the likelihood of copulatory attempts (likelihood-ratio test: $\chi^2 = 29.4$, $df = 8$, $p < 0.001$). Focal males everted the aedeagus in 44% of trials with intact conspecific females (22/50; 95% *CI* 31-58%). Removal of CHCs reduced this response to 10% for conspecific females (5/50; *CI* 4-21%; *OR* = 0.14, *CI* 0.05-0.42, $p = 0.003$) and to 16% for conspecific males (8/50; *CI* 8–29%; *OR* = 0.24, *CI* 0.09–0.62, $p = 0.019$, relative to the control), confirming that lipophilic cuticular compounds are required for males to initiate mating attempts at close range (Fig. 1A).

Reapplication of conspecific extracts partially recovered the response, to 26% (13/50; *CI* 16-40%), which did not differ significantly from intact females (*OR* = 0.45, *CI* 0.19-1.04, $p = 0.061$) but also did not differ from CHC-free females once multiplicity was accounted for (*OR* = 3.16, *CI* 1.03-9.69, $p = 0.044$ uncorrected; Fig. 1B). Recovery was therefore incomplete, as would be expected if the hexane rinse also removes non-hydrocarbon cuticular lipids or alters cuticular texture, and if a fraction of the applied extract is lost or unevenly distributed. Critically, extracts from male and female donors were interchangeable: females bearing male-derived CHCs elicited the same response as females bearing female-derived CHCs (13/50 in both; *OR* = 1.00, *CI* 0.41-2.44, $p = 1.00$). Males bearing female-derived CHCs elicited attempts in 20% of trials (10/50; *CI* 11–33%; *OR* = 0.32, *CI* 0.13–0.77, $p = 0.047$). This is the behavioral counterpart of the compositional identity of male and female CHC profiles reported by Santos et al. (2024), and indicates that these compounds carry no sex-specific information.

The heterospecific trials point in the same direction. Intact *C. paraguayensis* females elicited attempts in 20% of trials (10/50; CI 11-33%), significantly fewer than intact conspecifics (OR = 0.32, CI 0.13-0.77, p = 0.047), so males are not indifferent to the identity of the female they encounter. Yet removing the CHCs of *C. paraguayensis* females lowered the response further, to 6% (3/50; *CI* 2-16%; *OR* = 0.08, *CI* 0.02-0.30, $p = 0.001$), and applying *C. distincta* female extracts to them raised it to 16% (8/50; *CI* 8-29%; *OR* = 0.24 relative to the control, $p = 0.019$) — a difference from CHC-free heterospecific females that our data do not resolve (*OR* = 2.98, *CI* 0.74-11.99, $p = 0.123$), and that in no case approached the conspecific control. Conspecific CHCs applied to a heterospecific body thus do not confer conspecific identity, although the lower dose per unit cuticular surface received by the larger *C. paraguayensis* females may have contributed to this outcome.

Taken together, these results are more consistent with CHCs acting as permissive cues than as species- or sex-recognition signals: their presence is a precondition for males to initiate mounting and eversion, but their specific composition — conspecific or heterospecific, male or female — does not determine whether an attempt occurs. Interspecific misrecognition arising from overlapping cuticular profiles has been reported in other insects (Bailey & French 2012; Shimomura et al. 2017). Discrimination must therefore rest on additional cues assessed during antennal contact and mounting (Leal 1998; Nóbrega et al. 2022), consistent with the ~50% initial accuracy of conspecific female recognition previously reported for this species (Nóbrega et al. 2022).

Our findings indicate that sexual recognition in *C. distincta* cannot be explained by cuticular hydrocarbons alone. CHCs are necessary for mating attempts to be initiated, but are neither sex- nor species-specific in their behavioral effect, and are best interpreted as permissive contact cues within an integrated, multimodal recognition system (Smadja & Butlin 2009; Xue et al. 2016). Three limitations qualify this

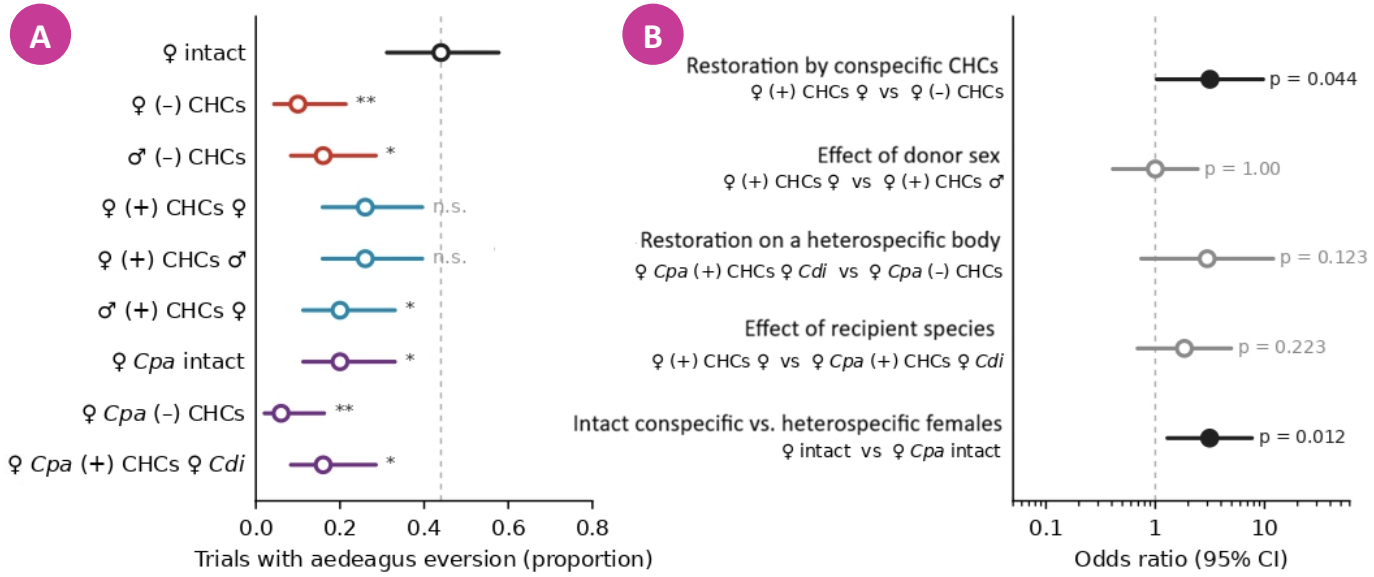



**Figure 1**. Copulatory attempts by male *Cyclocephala distincta* Burmeister, 1847 (Melolonthidae: Cyclocephalini) (aedeagus eversion within a 20-minute observation window; $n = 50$ trials per treatment). Treatments: intact freeze-euthanized conspecific females [female intact]; conspecific females [female (-) CHCs] or males [male (-) CHCs] devoid of cuticular hydrocarbons (CHCs); conspecific females or males with reintroduced CHCs from the same or the opposite sex [female (+) CHCs female; female (+) CHCs male; male (+) CHCs female]; intact freeze-euthanized *Cyclocephala paraguayensis* Arrow, 1904 (Melolonthidae: Cyclocephalini) females [female *Cpa* intact], the same devoid of CHCs [female *Cpa* (-) CHCs], or with reintroduced CHCs from *C. distincta* females [female *Cpa* (+) CHCs female *Cdi*]. a) Proportion of trials with aedeagus eversion (open circles) with 95% Wilson confidence intervals; the dashed line marks the [female intact] control, and asterisks denote Holm-adjusted differences from it (*$p < 0.05$; ** $p < 0.01$; n.s., not significant). b) Planned contrasts expressed as odds ratios with 95% confidence intervals on a logarithmic scale, with uncorrected p-values; the dashed line marks *OR* = 1; contrasts significant at $p < 0.05$ are shown in black.

interpretation. First, all trials used freeze-euthanized stimulus beetles, which cannot provide the dynamic cues — movement, stridulation, posture — that a live female would; our design therefore addresses only the static contact chemistry of recognition. Second, focal males were wild-caught, so their age, mating history and previous chemical experience were unknown, and extract batches were not tracked to individual trials, so among-batch variation could not be modeled; cuticular profiles of *C. distincta* nevertheless form a tight, well-supported cluster with no detectable sex differences (Santos et al. 2024), and this variation is expected to be minor relative to the experimental contrasts. Third, with 50 trials per treatment and response rates between 6% and 44%, only comparatively large differences are detectable, and the partial recoveries reported here should be treated as suggestive. Assays with live, CHC-manipulated females, and with fractionated or synthetic blends, will be needed to establish whether any component of the profile carries specific information.

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## Authors' Contributions

ACDM: Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Writing - review & editing, Supervision; WJTP: Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Writing - review & editing, Supervision; RLN: Investigation, Writing - original draft; AOSJ: Investigation, Data curation, Formal analysis, Visualization, Writing - original draft.

## Conflict of Interest Statement

No potential conflict of interest was reported by the authors.

## Ethical Approval

This study did not involve human participants or vertebrate animals and, therefore, did not require approval from an ethics committee under applicable institutional and national guidelines.

## Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## Generative AI Statement

Generative AI (ChatGPT) was used exclusively to assist with English language editing and grammar improvement. The AI tool did not contribute to the study design, data analysis, interpretation of results, or scientific conclusions. All content was reviewed and approved by the authors, who take full responsibility for the manuscript.

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