



ECOSYSTEMS

Behavioral traits and sexual recognition: multiple signaling in the reproductive behavior of *Cyclocephala distincta* (Melolonthidae, Cyclocephalini)

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Abstract: Night-foraging cyclocephaline scarab beetles rely on floral structures of specific plant hosts for food and shelter, as well as mating sites. Although the role of floral fragrances as long-range attractants in these interactions has been elucidated, the mechanisms that mediate close-range mate discrimination in aggregations are still unclear. We recorded the mating-oriented behavior of male *Cyclocephala distincta*, focusing on the influence of contact signaling and movement over mate selection in a series of controlled bioassays. Roughly half of the males chose a conspecific female over another male, readily engaging in copulation upon initial contact. The remainder males required more experience, acquired through successive mounts on both females and males. Eventually, all focal males invested in copulation with females. When faced with the choice for a live or an inert conspecific female, male *C. distincta* preferred the former in 76% of cases, although we also recorded sexual investment on inert females (10% of cases). In paired experiments with an inert female or a male, nonetheless, focal males significantly opted for the opposite sex, and that included mating with the inert females. Innate characteristics of the females of *C. distincta* are evaluated by males synergistically, and not separately, in discriminating potential sexual partners.

Key words: behavior, *Cyclocephala*, interspecific, intraspecific, mating, reproduction.

INTRODUCTION

Cyclocephaline scarabs (Melolonthidae, Cyclocephalini) are among the most diverse and ubiquitous groups of anthophilous insects in the neotropics, with over 500 documented species (Moore et al. 2018). Nightly active, they are attracted to flowers by scent, in search of shelter, feeding resources and mating opportunities (Albuquerque et al. 2016, Hoe et al. 2016, Maia et al. 2018). Such is the chemically-mediated specificity of cyclocephaline scarabs to their preferred hosts that it has been hypothesized that some species might have

suppressed the use or even lost the ability to produce long-distance sexual and aggregation pheromones (Gibernau et al. 1999), which have nonetheless been described for several closely related scarabaeoid taxa (Haynes & Potter 1995, Koppenhofer et al. 2008). Aggregations of cyclocephaline scarabs in a single large inflorescence of an aroid (Araceae) or a palm (Arecaceae) can sum up to hundreds, even thousands of individuals (Henderson 1986, Gottsberger & Silberbauer-Gottsberger 1991).

The gathering of large numbers of individuals in a specific location functions as a pre-sexual selection filter (Thornhill & Alcock

1983), being commonly observed among many insect species (Schowalter 2016). Aggregations are often influenced by the emission of pheromones, but non-targeted species might also respond (Bernhardt 2000, Wyatt 2014). Herbivorous species in particular are also known to respond to allelochemicals released by their plant hosts (Turling & Erb 2018) and in a number of specialized pollination systems these semiochemicals are paramount for the reproductive success of flower-visiting insects, functioning as aggregation cues (Maia et al. 2013, Núñez et al. 2005).

Various studies have revealed that sexual recognition between partners may occur based even on discreet traits, that could be behavioral (Thornhill & Alcock 1983), morphological (e.g. size, color, Li et al. 2017) or chemical (e.g. sex pheromones; Quero et al. 2017, Darragh et al. 2017). However, sexual recognition is not error-proof (Bailey & Zuk 2009, Scharf & Martin 2013). Male-male mounting is common in the reproductive behavior of several species of Coleoptera, notably when sexual dimorphism is inconspicuous (Martin et al. 2015, Kepner et al. 2017, Rodriguez-Soana et al. 2018, Songvorawit et al. 2019). In male-male mountings, the rejection behavior of the mounted partner, as well as the lack of a fitting anatomical structure to accommodate the aedeagus, are obvious hindrances to effective copulation attempts (Bailey & Zuk 2009). Male-male mountings are most likely to occur when many conspecific individuals are found in a limited space, a situation in which mating recognition might not function adequately.

Despite recent advances in the characterization of chemical communication of cyclocephaline scarabs and their plant hosts (Dötterl et al. 2012, Maia et al. 2013, 2018, Pereira et al. 2014), knowledge about the sexual behavior of these insects is surprisingly limited. Moreover,

a still lacking thorough investigation of female-male interactions may provide new insights for the development of alternative methods for controlled management of pollinating and pest species. There are actually only three studies describing aspects of the mating behavior of *Cyclocephala* spp. (Potter 1980, Haynes & Potter 1995, Rodrigues et al. 2018) and none of them focused on the mechanisms of contact mate discrimination, an important step in avoiding unnecessary sexually oriented investment and minimizing energy expenditure (Thornhill & Alcock 1983).

Here, we address these knowledge gaps through behavioral observations of *Cyclocephala distincta* Burmeister, 1847 in controlled bioassays within an experimental arena. We expect that (1) errors in sexual recognition will be observed in the sexual behavior of *C. distincta*; (2) mounted males will exhibit rejection behavior during male-male mountings; and (3) the movement of female *C. distincta* will exert positive influence on males' mating decisions. In this scenario, *C. distincta* seems to be an adequate model to test the role of selected traits in sexual recognition. Female and male *C. distincta* form dense aggregations in inflorescences of their plant hosts (Voeks 2002). As in the case of other *Cyclocephala*, sexual dimorphism is mostly restricted to the dilated foretarsi of males (Endrödi 1985). Large swarms of *C. distincta* are attracted to 2-alkyl-3-methoxypyrazines that dominate the floral scent compositions of different species of Neotropical palms, including *Attalea* spp. *Acrocomia* spp. *Aphandra natalia* (Balslev & A.J. Hend.) Barfod and *Bactris gasipaes* Kunth (Maia et al. 2018).

MATERIALS AND METHODS

Field collections

Female and male *C. distincta* were collected in the wild during the months of December 2016 and March-April 2017 at a private Atlantic Forest reserve, property of Usina São José S/A Sugarcane Company, in the municipality of Igarassu, northeastern coast of Brazil (7°45'10" S, 34°58'43" W; 110 m.a.s.l.). Beetles were attracted to flight-interception traps baited with a 1:1 mixture of 2-isopropyl-3-methoxypyrazine and 2-sec-butyl-3-methoxypyrazine (250 uL each in 250 mL of 1:50 solution of deionized water and ethanol) (refer to Maia et al. 2018 for details). The insects were collected manually and placed in plastic containers (250 cm³) containing pot soil and kept individualized until the observations were made at the Laboratory of Applied Entomology of the Federal University of Pernambuco under controlled environmental conditions (25 ± 2°C; 12h photophase). All observations took place between 18:00h and 23:00h, the period of activity of *C. distincta* (Albuquerque et al. 2016).

Behavioral assays

Behavioral assays were conducted in an experimental arena that consisted of capped clear glass petri dishes (60 x 15 mm), rinsed with ethanol. For each replicate, a new set of clean petri dishes was used. Observations occurred in a dark environment, using a red-light flashlight imperceptible to the insects.

From initial assessment of the sexual behavior of captivity reared *C. distincta*, based on the observation of 25 male-female pairings, several steps were observed, from approaching until effective copulation (see Figure 1). However, only the following steps were considered, for practical purposes: **1)** mounting - male climbs over the other individual, firmly holding on to the partner's elytra with the foretarsal claws

for a period longer than 10 s; **2)** eversion - male aedeagus is everted; and **3)** copulation - male introduces aedeagus in the orifice at the terminal portion of the abdomen of the paired individual (either the vagina on females, or the anus on males), and repeatedly moves its head dorsoventrally, while simultaneously contracting the abdomen. For each replicate, we followed the focal animal method (Altmann 1974) for a period of 20 min, or until copulation took place. Gathered data took into consideration the number of mountings performed by males in each replicate and the number of eversions,

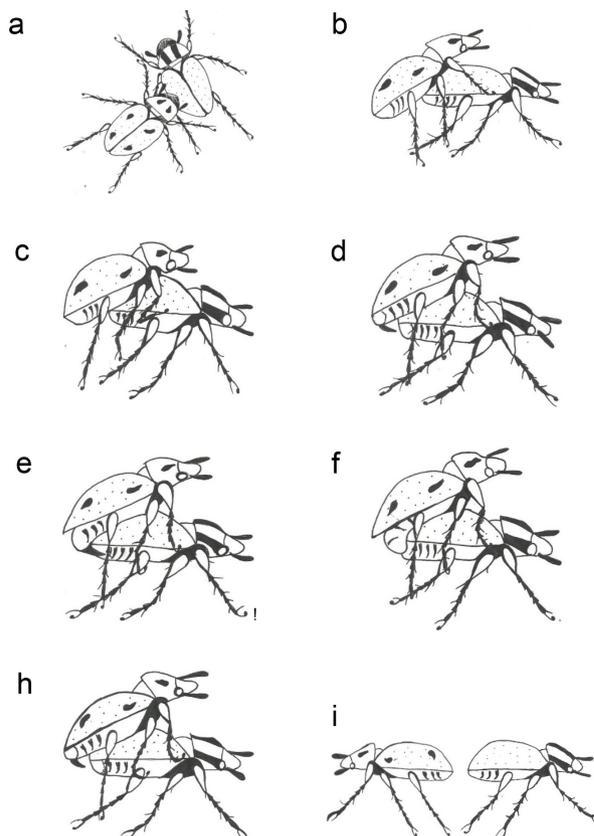


Figure 1. *Cyclocephala distincta* mating sequence (n = 25). **a** male approaches a female and rubs the antennas on her elytra; **b** male rises in the female; **c** male mounting the female; **d** male everts his aedeagus; **e** male inclines abdomen in relation to female pigidium; **f** copulation; **g** male withdraws and retracts the aedeagus; **h** disassembles and separation of individuals.

interpreted as a conclusive choice of a sexual partner in *C. distincta*, as it precedes copulation.

Sexual distinction

The first behavioral assay was conducted with the aim of understanding how male *C. distincta* behave when faced with more than one conspecific mating option. For this, the arena was set with two males and one female ($\text{♂}_{\text{focal}} + \text{♂}_{\text{option}}$ and ♀_{option} ; $n = 42$). A focal male individual was randomly chosen in each replicate and marked with watercolor ink (Acrilex®, São Bernardo do Campo, Brasil) at the base of the right elytrum. Preliminary observations with marked and unmarked females showed that the use of paint did not influence the sexual preference of males in relation to the number of mountings ($X^2 = 2.2857$, $df = 1$, $p\text{-value} = 0.1306$, $n = 25$).

Male behavior during male-male mountings

The second behavioral essay was designed to verify whether the movement of the females could influence males' mating decision. To this end, males were subjected to a two-choice test with one live female and one inert female. Inert insects were killed by freezing (removed from the cages and placed -24 °C refrigeration). Refrigerated individuals were removed from the freezer 30 min before the beginning of the tests, so that extreme body temperature differences would not influence male behavior ($n = 50$).

From the results of the second bioassay, we questioned ourselves whether males of *C. distincta* would be able to correctly identify a conspecific inert female from an inert male.

Female movement influence on males' mating decisions

A third behavioral test was then designed in two distinct situations: **a)** male paired with an inert female and; **b)** male paired with an inert male.

For each of the treatments, 50 repetitions were performed.

Data analysis

Data were first subjected to the Shapiro-Wilks normality test. Because they did not exhibit a normal distribution, we used X^2 -tests to test for differences in the frequencies of the behavior of males in each bioassay. All analyses were performed with R software v.2.1.4 (R Development Core Team 2019).

RESULTS

When approaching a conspecific individual, whether of the same or the opposite sex, male *C. distincta* characteristically used their antennae for body contact, seeking the terminal portion of the elytra of the potential partner and performing the same recognition steps: approaching, touching the elytra of the other individual with the antennae, walking over the other individual and mounting the other individual. Events often followed this specific order, but in ca. 10% of the cases mountings were observed immediately after a male bumped into another individual. Mountings could also take place following initial antennal contact with another individual. These behaviors are summarized in Figure 1, Table I.

Sexual distinction

For the $\text{♂}_{\text{focal}} + \text{♂}_{\text{option}}$ and ♀_{option} treatment, in 52% of the replicates focal males were able to find and copulate with a female upon initial contact, following a single mount. However, in the other 48% of the cases males required a variable number of attempts to engage in copulation, as they would either be unsuccessful upon mounting the female (23.8%) or attempt to mount another male (21.4%).

Table I. Simplified ethogram of the copulation repertoire of *Cyclocephala distincta* Burmeister 1847, adapted from original descriptions by Souza (2013) following experimental observations made in the laboratory.

BEHAVIORAL ACT	ACT DESCRIPTION
TOUCH*	Male touches the head and / or margins of the elytra with the antennae
CLIMB*	Male climbs over another individual.
MOUNT	Male holds on to the other individual's elytra with the foretarsal claws.
DRAG*	Individual moves around while mounted by another male
EVERSION	Male displays aedeagus.
GLANCE*	With aedeagus everted, male tilts the abdomen towards another individual's pygidium.
COPULATION**	Male inserts aedeagus into another individual's pygidial opening, moving the abdomen repeatedly.
REMOVE*	Male removes the aedeagus from the pygidial opening of the partner.
RETRACT*	Male aedeagus is retracted to the abdominal cavity.
Guard	Male remains mounted over another individual for a few minutes following aedeagus retraction.
LEAVE	Male dismounts from the other individual and moves away.

Grayscale follows the mating stages: pre-copulation, copulation and post-copulation, respectively. * As originally proposed by Souza (2013). ** Changes in act description.

The number of focal males that were able to find a female and successfully engage in copulation at their first attempt was not significantly different from the number of those that failed ($X^2 = 0.952$, $gl = 1$, $p = 0.7576$) (Figure 2). Among positive cases of copulation, 9.5% required five mounting attempts to finally engage in a successful copulation (Figure 3). Eventually, focal males in all but one of the replicates copulated with a conspecific female. There was no record of same-sex copulation.

Male behavior during male-male mountings

When mounted by another same-sex individual, ca. 20% of the males in the ♂_{focal} + ♂_{option} and ♀_{option} treatment behaved aggressively and attempted to shake off the other male by kicking and flouncing incessantly until disengagement. Mounted females, on the other hand, showed apparent little to no resistance to the engaging male, and also moved slower and less intensely when compared to mounted males.

Female movement influence on males' mating decisions

Males faced with the choice of either a live or an inert female showed preference for the former in 76% of replicates ($X^2 = 17.894$, $gl = 1$, $p < 0.05$). Nonetheless, 10% of individuals engaged in copulations with inert females (Figure 4).

When paired only with an inert female, males in 50% of the replicates performed mountings. All mountings were followed by eversions, but in only four replicates the males engaged in copulations. Faced with an inert male, only 8% of the focal males performed mountings. Among inert males, three eversions were observed; none of them resulting in effective copulation. When the two treatments were compared, all sexually oriented behaviors were significantly more frequent in different-sex pairings (number of mountings – $X^2 = 15.207$, $gl = 1$, $p < 0.05$) (Figure 5).

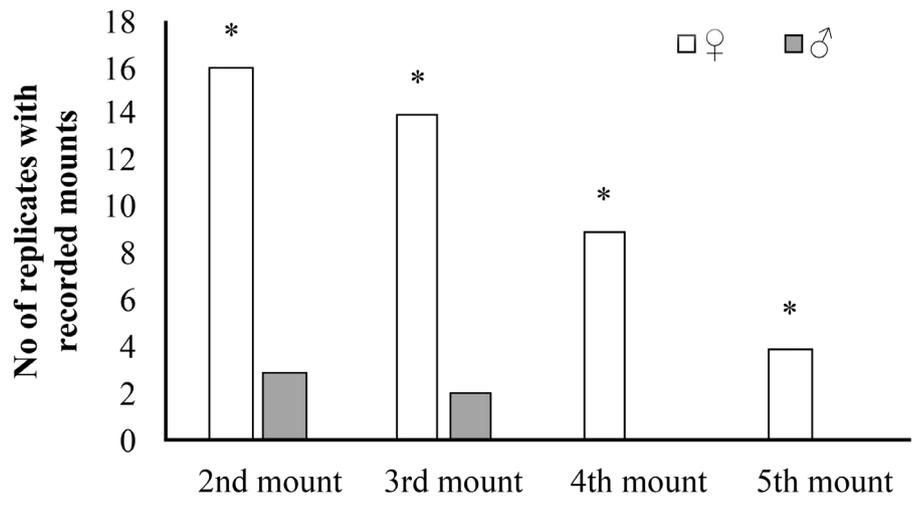


Figure 2. Mountings performed by males of *Cyclocephala distincta* in double-choice tests with conspecific males and females.

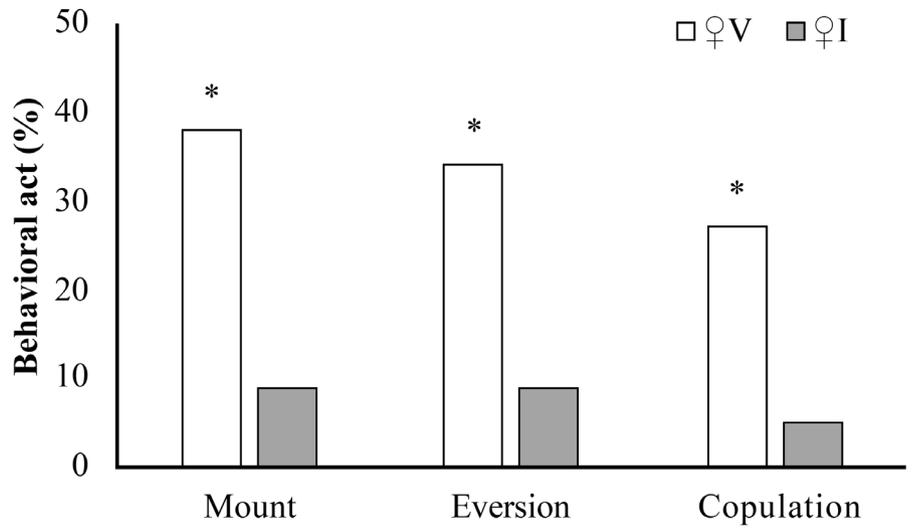


Figure 3. Mount, eversion and copulation performed by *Cyclocephala distincta* in double-choice experiments with conspecific live and inert conspecific females. V - live; I - inert. * refers to treatments without significant difference.

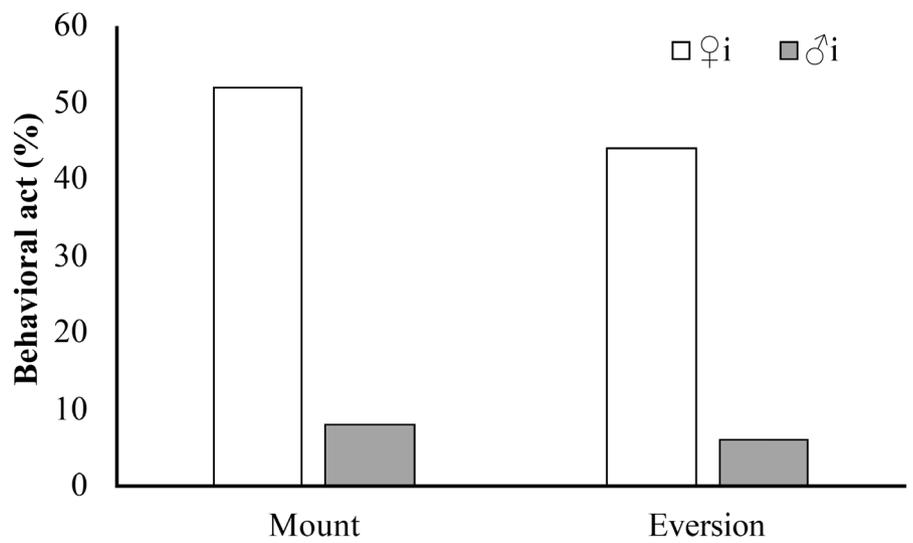


Figure 4. Group choices of *Cyclocephala distincta* males that performed more than one mount during observations.

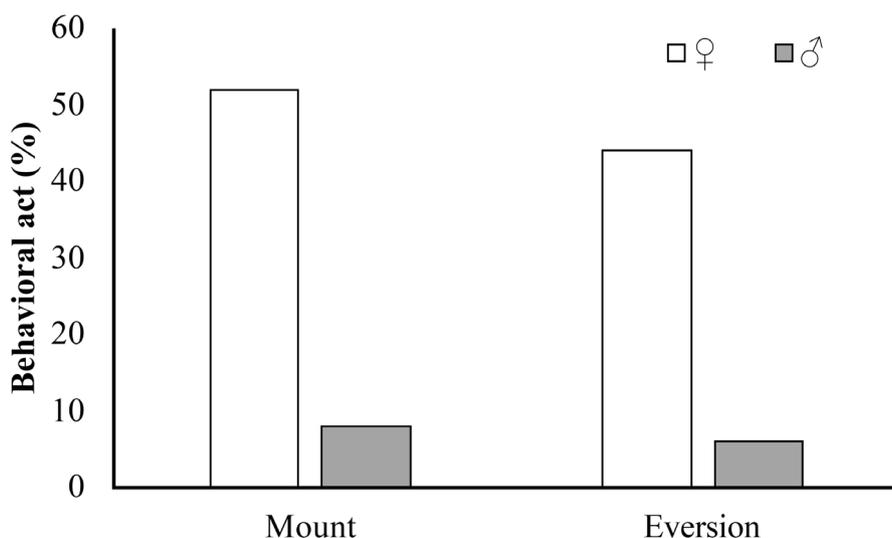


Figure 5. Quantification of selected sex oriented acts (mount and eversion) performed by focal males of *Cyclocephala distincta* paired with inert conspecific individuals.

DISCUSSION

Roughly half of the tested male *Cyclocephala distincta* were successful in rapidly identifying then engaging in copulation with a conspecific female, whereas the other half needed to perform several mounting attempts before correctly identifying a mating partner. Indecisive or tentative mounts were short-lasting and followed by sequential mounts, and the males appeared to succeed upon prolonged contact with potential partners. Such behavior may be indispensable for some males to acquire sexual maturation and subsequent success in future mating attempts, significantly reducing (or eliminating) misidentification issues (Favila 1998). According to some studies, the individual’s age is an important factor in its sexual maturation and partner receptivity, indicating both experience and fitness (Thornhill & Alcock 1983). The individuals we used in our experiments originated from an heterogenous sampling, and we cannot precise their age or life histories. Those important factors influencing mate choice should be taken into consideration in future research through the application of rearing/breeding protocols (Souza et al. 2014).

Male-male mating attempts were observed in *C. distincta*, although no successful copulations were recorded. Incorrect sex-oriented choices made at the first attempt are common among different insect taxa (Serrano et al. 1991, Scharf & Martin 2013, Switzer et al. 2014). Such errors are often based on inaccurate evaluation of the partner’s physical traits (e.g. size; Solensky 2004). Among cyclocephaline beetles, such misidentifications could at least in part be attributed to inconspicuous sexual dimorphism (see review by Scharf & Martin 2013), as well as to the fact that the aggregation sites these insects use are often crowded and saturated with floral scent volatile kairomones emitted by their host plants (Maia et al. 2013, 2018).

Movement of the mounted conspecific individual proved to be important, although not absolutely necessary, for a male of *C. distincta* to properly identify it as an adequate sexual partner and invest in copulation. Females are not always receptive to the male (Arnqvist 2014). However, we believe that behavior does not appear to be crucial or unique to correct sexual recognition, as sexual advances, as already presented in Ginzel’s review (2010).

Our results suggest that male *C. distincta* distinguish conspecific females, even if they might require several attempts (or mountings) to ultimately engage in copulation. Although the sexual recognition system in *C. distincta* is not fully directional, as in some insect groups in which short-distance pheromones are indispensable (Steiger et al. 2007, Chung & Carroll 2015, Billeter & Levine 2015, Lane et al. 2015, 2016, Xue et al. 2016), innate traits or characteristics of female *C. distincta* appear to drive the sexual behavior of the male, as evidenced by the proportion of replicates in which focal males copulated with a female following the first mounting attempt.

There seems to be some level of hierarchy in the steps required for sexual recognition in *C. distincta*, as a number of traits and factors play major or minor roles in male mating choice. The traits that lead to correct mate identification have been addressed, in other studies, to identify the most important appealing characters for mating choice (Jang & Greenfield 1998, Wagner 1998). Several traits have been shown to influence mating decision, following a specific gradient of importance concerning a hierarchical step confidence, from the minor to the major reliability of the trait. For example, in *Drosophila melanogaster*, both chemical and acoustic cues are important for stimulating sexual receptivity; however, song is the crucial factor affecting mating decision (Dickson 2008). In the *Anoplophora malasiaca* (Thompson) beetle, males are able to recognize conspecific females in the host plant through proximal visual and olfactory cues, as well as by the influence of a sexual pheromone (Yasui 2009).

As observed among other Scarabaeoideae (Facundo et al. 1999, Arakaki et al. 2004, Ferreira et al. 2016, 2018, Rodrigues et al. 2016, 2017, 2018), male *C. distincta* exhibit the behavior of rubbing their antennae on another individual's elytra

while approaching it, an indication that there is contact recognition regardless of the absence of hard evidence of pheromones among the Cyclocephalini. Because it is a contact signal, possible candidates are cuticle hydrocarbons, known to be function as short-distance sex pheromones in many species of insects (Hunt & Sakaluk 2014, Chung & Carroll 2015, Kepner et al. 2017).

We have compiled convincing evidence that sexual discrimination and male mating choice in *C. distincta* depends on the behavior of the potential partner (movement and sexual receptivity), as well as on contact assessment mediated by cuticular pheromones. Further investigation should include the identification of cuticular hydrocarbons of *C. distincta* and their actual role in male sexual-oriented behavior.

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