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Research

Role of Cuticular Hydrocarbons in German Cockroach (Blattodea: Ectobiidae) Aggregation Behavior

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Abstract

Aggregation can be adaptive by providing protection from predators, facilitating thermoregulation, and expediting the location of food, shelter, and mates. German cockroaches *Blattella germanica* L. (Blattodea: Ectobiidae), are obligatory commensals in human-built structures, where they aggregate in crevices during the day. The source of the aggregation pheromone that drives this behavior and its chemical identity remain unclear. Cuticular hydrocarbons (CHCs) in feces have been proposed to serve as aggregation pheromone, but this function has not been investigated in relation to visual and tactile cues that mediate aggregation. Our objective was to delineate how CHCs in the feces and on the cockroach body operate in conditions that reflect the German cockroach's ecology—either applied to shelters, representing fecal deposition, or to previously extracted cockroaches, representing shelter co-habitation with other cockroaches. Cockroaches and feces-conditioned filter papers were extracted, CHCs were purified by flash chromatography, and two-choice behavior assays were performed with first instar nymphs. Our results confirmed that nymphs preferred to rest within feces-conditioned shelters. However, purified CHCs did not elicit more aggregation than solvent-treated control shelters. Nymphs significantly preferred to rest in shelters that contained a CHC-free dead female, but the addition of CHCs to the female did not enhance aggregation. Nymphs preferred to aggregate with the CHC-free female over CHC-treated shelters. Finally, a methanol extract of feces was highly effective at eliciting aggregation, contesting previous reports that fecal CHCs serve as aggregation pheromone. We assert that CHCs play a minor, if any, role in the aggregation behavior of German cockroaches.

Key words: hydrocarbons, aggregation, pheromone

Many species of insects form active aggregations, resting aggregations, or both. Active aggregations generally coincide with feeding periods and may serve to overcome the host, protect the aggregates from predators and parasitoids, or thermo- and hygro-regulate (Parrish et al. 1997). Aphids (*Aphis varians* Patch) (Hemiptera: Aphididae), for example, aggregate on substrates to decrease the risk of predation (Turchin and Kareiva 1989), bark beetles that attack healthy trees aggregate to overcome tree defenses (Gitau et al. 2013), and necrophilous maggots thermoregulate in mass aggregations to accelerate their development (Aubernon et al. 2016). Resting aggregations are usually formed during the inactive periods in places presenting environmental conditions that fulfill the ecological requirements of the species (Wertheim et al. 2005). For example, choice of rest sites can be guided by microclimatic factors such as temperature and humidity, and physical features such as size, color, and texture of the substrate. In both types of aggregations, environmental and social cues orient insects toward each other, focusing them to gather at a preferred site (Wertheim et al. 2005, Imen et al. 2015). In insects, group formation is often guided and sustained by aggregation pheromones, which may attract conspecifics to preferred sites and cause arrestment at the site (Wertheim et al. 2005, Imen et al. 2015).

Most cockroach species are nocturnal and aggregate in communal shelters during the day (Schal et al. 1984, Bell et al. 2007). Many factors affect aggregation behavior in the German cockroach, *Blattella germanica* L. (Blattodea: Ectobiidae), including preferences for certain sized shelters (Berthold and Wilson 1967), texture and vertical versus horizontal orientation (Bell et al. 1972), presence of conspecifics (Koehler et al. 1994), aggregation pheromones (Ishii and Kuwahara 1967), and other factors (e.g., Rust et al. 1995). A variety of chemicals have been proposed as aggregation pheromones of the German cockroach, including rectal pad secretions (Ishii and Kuwahara 1967), short- and medium-chain volatile fatty acids in feces (Ritter and Persoons 1974, McFarlane and Alli 1986, Wada-Katsumata et al. 2015), alkylamines and blattellastanoside A and B (Sakuma and Fukami 1990, 1993), and cuticular hydrocarbons (CHCs; Sreng et al. 1998).

Cuticular hydrocarbons comprise a thin apolar layer on the outer surface of insects. They have many functions, including as a protective barrier from microorganisms and water loss

(Howard and Blomquist 2005, Blomquist and Bagnères 2010). Hydrocarbons also play important roles as semiochemicals, including as nestmate and caste recognition pheromones in social insects (Lahav et al. 1999, van Zweden and d'Ettorre 2010, Funaro et al. 2018), as sex pheromones and pheromone precursors (Ferveur 2005, Jurenka et al. 2017), and as aggregation pheromones in some insects. For example, (*Z*)-10-heneicosene was found to be attractive to both male and female *Drosophila virilis* Sturtevant (Diptera: Drosophilidae) and considered to be part of their aggregation pheromone (Bartelt and Jackson 1984).

Cuticular hydrocarbons are naturally found on the cuticle and in the feces of *B. germanica* which contaminate their shelters, and they have been implicated as an aggregation pheromone (Sreng et al. 1998, Lihoreau et al. 2012). Recent studies of cockroach aggregation pheromones have confounded substrate-deposited chemicals (e.g., feces-produced) that do not require the presence of a cockroach, with cues that stimulate joining a cockroach or a group. In this study, we sought to determine the function of CHCs on the shelter (contaminated with compounds from feces and the body) and on the cockroach body, and investigate the relationship between CHCs and other aggregation cues. We focused on filter paper shelters because in a previous report CHCs on shelters elicited aggregation of nymphs (Sreng et al. 1998). Because CHC signals can also emanate from the body surface of conspecifics, we also assessed the effectiveness of CHCs when applied to pre-extracted insects. Therefore, we separated our queries about the roles of CHCs in aggregation behavior into two ecological contexts: a single nymph given a choice of two variously treated shelters, or a nymph given a choice to shelter with a treated female within a treated shelter. In our experiments, other features of the shelters (e.g., darkness, texture) were held constant. We sought to re-assess the behavioral activity of CHCs as an aggregation pheromone in the German cockroach by testing CHCs and other chemicals as aggregation agents. Based on recent findings (Wada-Katsumata et al. 2015), we hypothesized that polar lipids might play more prominent roles in guiding aggregation behavior than CHCs. Behavioral experiments confirmed that German cockroach nymphs preferred to shelter under feces-contaminated shelters, but CHCs did not elicit aggregation behavior. Conversely, more polar lipids from conditioned filter papers were significantly more effective at stimulating aggregation than CHCs. Understanding aggregation behavior in cockroaches can inform innovative pest management strategies for mitigating their adverse health effects in the indoor environment.

Materials and Methods

Cockroaches

The insects used in these experiments were from a laboratory strain of *B. germanica* (Orlando Normal, collected in a Florida apartment over 70 yr ago). They were reared on food pellets (Purina No. 5001 Rodent Diet, PMI Nutrition International, St. Louis, MO) and water in cotton-stoppered vials at 27 ± 1°C, 40–70% RH, and 12:12 (L:D) h photoperiod. Adult females were virgin of known ages, as indicated below, and first instars used in behavioral assays were ~1 d after hatching.

Female-Conditioned Filter Papers

Conditioned filter papers were prepared by allowing four groups of 20 adult females (0–1 d old) to condition (touch and defecate upon) filter papers for 7 d, representing the vitellogenic period when food consumption is high (Schal et al. 1997). Each Petri dish (14 cm diameter) contained 20 females, rodent chow, water in a cotton-stoppered glass vial, and an accordion-folded filter paper (Whatman #1, 24 cm, Pittsburgh, PA).

Extraction and Fractionation of Female-Conditioned Filter Papers

After conditioning, the filter papers were immersed in 10 ml of chloroform in 20 ml glass vials, vortexed intermittently for 5 min, and the chloroform was transferred to a clean vial. This process was repeated two more times and the chloroform extracts were combined. The extracts were reduced to approximately 0.1 ml using a gentle stream of high purity nitrogen. The filter paper extract was then separated into two fractions: CHCs and polar lipids. The extract was loaded onto a dry silica gel (70–230 mesh, EM Science, Gibbstown, NJ) column (Pasteur pipette loaded with 0.5 g silica gel, washed with dichloromethane, dried with nitrogen, activated 60 min at 110°C) and dried under a stream of nitrogen. The walls of the vial were washed twice with 0.5 ml of chloroform, reduced to approximately 0.1 ml, and loaded onto the column. The silica gel was then dried with a stream of nitrogen. The column was first eluted with 5 ml hexane into a glass vial to obtain CHCs (hexane fraction). Then the column was eluted with 5 ml ethyl acetate and 5 ml methanol which were combined in a glass vial to obtain more polar lipids. A total of four columns were used during the experiment (one for each conditioned filter paper), yielding four vials for each fraction. Each fraction was reduced to about 0.2 ml and all four hexane fractions transferred to the same 1.5 ml vial and all four polar fractions were transferred to another 1.5 ml vial. The walls of the vials were washed twice with clean hexane (CHC fractions) or chloroform (polar fractions) and the washes were combined in the respective 1.5 ml vial. The solvent was reduced to near dryness and 1 ml of hexane or chloroform was added so each vial contained 80 females-7-d-equivalents (FEs) in 1 ml of solvent, or 12.5 µl per FE.

Additionally, four new conditioned filter papers were prepared using 80 adult females (0–1 d old, 20 females per filter paper), as described above. These filter papers were extracted sequentially using three different solvents: chloroform, methanol, and water. Each filter paper was first immersed in 10 ml of chloroform in a 20 ml glass vial, then vortexed twice for 2 min each. The filter paper was removed and allowed to dry under the hood overnight. The chloroform was removed and transferred to a new glass vial. The walls of the vials were washed twice and added to the 20 ml vial. The four extracts were blown down to ~2 ml and combined into one vial. The combined extract was then blown down with nitrogen to 1 ml. This extraction process was repeated using methanol, and the filter paper was again allowed to dry overnight. The conditioned filter papers were finally extracted with water and the extract was reduced using a vacuum concentrator (Jouan RCT 60, Thermo Scientific, Waltham, MA). All the extracts were kept in a −30°C freezer for behavior assays.

Extraction of Cockroaches

We extracted females because they contain more CHCs than any other life stage (Jurenka et al. 1989), and females spend more time in association with first instar nymphs (Lihoreau et al. 2012). Fourteen groups of 20 adult females each (5–6 d old) were placed in 20 ml glass vials and frozen at −30°C. Each vial was then allowed to thaw to room temperature. The insects were covered in hexane (~10 ml), gently swirled for 2 min, and the hexane was transferred to a clean glass vial. This process was repeated twice, and the hexane extracts were combined. The hexane extract and extracted females were kept in a −30°C freezer for behavior assays. To purify CHCs, the extract was applied to a silica gel column, as described above, CHCs were eluted with 5 ml hexane, the solvent reduced to near dryness and

2.8 ml of hexane was added to the vial, yielding 280 FEs of CHCs in 2.8 ml, or 1 FE per 10 µl.

Quantification of CHCs

Aliquots of extracted filter papers, extracted females, and their respective CHC fractions were analyzed by gas chromatography. An internal standard (*n*-hexacosane = *n*-C26, 10 µg) was added to each vial, the solvent was evaporated to dryness and the residue taken up in 100 µl hexane in a glass insert within a 1.5 ml GC autoinjection vial. One of 100 µl was injected in pulsed splitless mode using a 7683B Agilent autosampler into a DB-5 column (20 m × 0.18 mm internal diameter × 0.18 µm film thickness, J&W Scientific, Folsom, CA) in an Agilent 7890 series GC (Agilent Technologies, Santa Clara, CA) connected to a flame ionization detector (FID) with ultra-high purity hydrogen as carrier gas (0.75 ml/min constant flow rate). The inlet was held at 300°C, FID at 320°C, and the column was held at 50°C for 1 min, increased to 320°C at 10°C/min, and held at 320°C for 10 min. Total peak area was used for the calculation of total CHC amount.

Behavior Tests

Binary-choice bioassays were conducted in disposable 60 × 15 mm plastic Petri dishes (Falcon-Corning, Corning, NY) using individual first instar nymphs to avoid signaling among nymphs. A single nymph was placed between two folded tent-shaped filter paper shelters, each 2 × 2 cm (4 cm^2) (Fig. 1A and B). Unless otherwise indicated, one shelter was either a conditioned filter paper (positive control) or treated with an extract, and the other shelter was treated with the respective solvent only. The assays were conducted at 27°C and under the same 12:12 (L:D) h cycle as rearing conditions. Fluorescent lights were on during the photophase, when cockroaches tend to rest within shelters, and a red headlight was used to observe the assays during the scotophase. The assays were set up late in the photophase, and the position of each nymph within the Petri dish was recorded 24 h later (in the photophase), allowing insects 12 h of nighttime activity before settling to shelter for the day. Control assays were completed to evaluate any directional bias in the assay conditions by giving individual nymphs a choice between two hexane-treated shelters.

In other assays, solvent-extracted females were placed inside a shelter to determine if they could guide nymphs to choose specific treatments. Females that had been extracted with hexane were extracted again in chloroform for 5 min, the chloroform extract was discarded, and the extracted females were allowed to dry overnight in a fume hood. This step was repeated in order to ensure there were no attractive substances on the extracted female body. In these aggregation assays, we tested various combinations of an extracted (CHC-free) female placed under a shelter, with either the female or the shelter treated with various extracts. The treatments were 1) CHC-free female in a hexane-treated shelter versus hexane-treated shelter; 2) CHC-free female in a hexane-treated shelter versus CHC-treated shelter; 3) CHC-free female in a hexane-treated shelter versus CHC-free female treated with CHCs in a hexane-treated shelter; and 4) CHC-free female in a CHC-treated shelter versus CHC-free female treated with CHCs in a hexane-treated shelter.

In assays of sequentially extracted conditioned filter papers, nymphs were given a choice between a shelter treated with each respective extract and another shelter treated with the corresponding

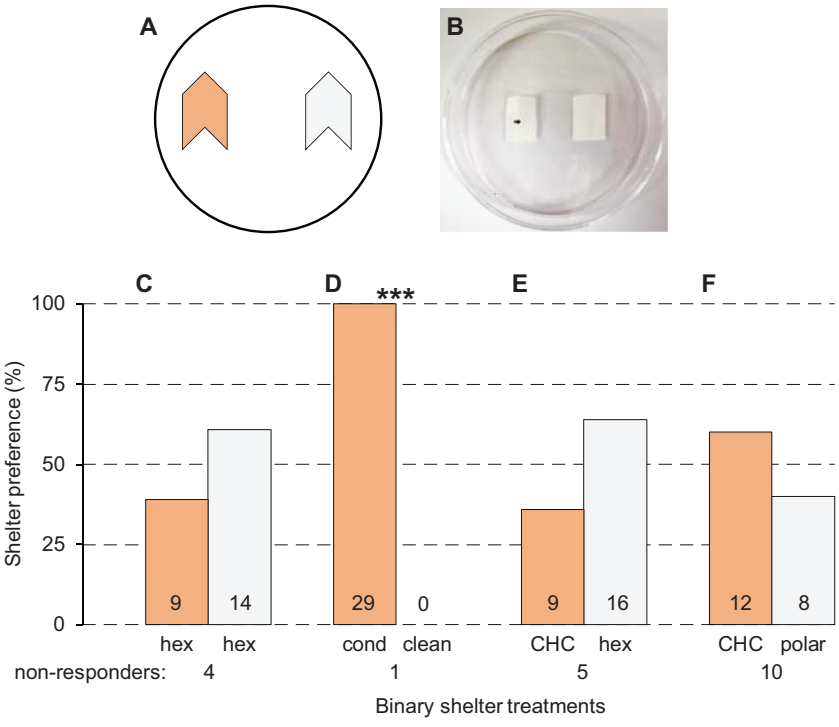

Fig. 1. Binary behavioral assay design and aggregation preferences of first instar nymphs under filter paper shelters in two-choice assays. Individual first instar nymphs were tested in Petri dish assays (A, B) with two filter paper shelters treated with the specified materials. Treatments included hexane versus hexane (C), female-conditioned shelters versus clean shelters (D), CHCs obtained from conditioned shelters versus hexane (E), and CHCs obtained from conditioned shelters versus polar lipids obtained from conditioned shelters (F). Shelter choices (%) are shown, along with number of assays with individual nymphs resulting in preference for each shelter, and number of assays resulting in nymphs resting outside either shelter. Assays with *** indicate $P < 0.001$; in other assays $P > 0.05$.

solvent. The three extracts were then recombined, and behavior assays were conducted between a filter paper shelter treated with the combined extracts and a filter paper shelter treated with the three solvents (chloroform, methanol, and water).

Statistics

Differences in aggregation between the two filter paper shelters were tested using a chi-square test of independence ($\alpha = 0.05$) performed in Microsoft Excel.

Results

CHCs on Females and Female-Conditioned Filter Papers

We recovered 159.8 ± 8.57 µg total CHCs per female cockroach ($n = 6$). Therefore, 1 FE of female extract or CHC fraction applied to a filter paper shelter represented 40 µg/cm^2. We recovered less CHCs from filter papers conditioned for 7 d by 20 adult females (3.24 ± 7.38 µg per female), so 1 FE of conditioned filter paper extract applied to a shelter represented 0.81 µg/cm^2.

Aggregation in Female-Conditioned Shelters

First instar nymphs were used in behavior assays (Fig. 1A and B) because they are highly motivated to aggregate during the photophase (Ishii and Kuwahara 1967). Moreover, we used a single nymph in each assay to preclude interactions among nymphs that may result in some nymphs following early responders. First instar nymphs sheltered equally under each of two hexane-treated filter paper shelters (chi-square test, $\chi^2 = 1.0869$, $P = 0.2971$, $n = 26$) (Fig. 1C), indicating that our environmental assay conditions did not provide nymphs any inadvertent directional cues. In four replicate assays, the nymph remained outside both shelters. Nymphs significantly preferred to rest under filter paper shelters conditioned (defecated upon) by adult cockroaches ($\chi^2 = 29$, $P < 0.0001$, $n = 29$) (Fig. 1D), indicating that feces and other secretions from adult females contained a highly effective aggregation pheromone.

Aggregation in Shelters Treated With Extracts and Fractions

Cockroach CHCs can guide conspecific aggregation behavior under two distinct ecological contexts—as components of feces and as components of the cuticular lipids of conspecifics. In two-choice assays, first instar nymphs sheltered slightly more under hexane-treated shelters than under shelters treated with 1 FE, or 159.8 µg, of CHCs (40 µg/cm^2) purified from the female cockroach body, although there was no significant difference between these two choices ($\chi^2 = 1.96$, $P = 0.1615$, $n = 25$) (Fig. 1E). This suggested that CHCs, which are found at a lower concentration in the feces, would fail to elicit aggregation alone.

We then tested whether more polar lipids extracted from conditioned filter papers would provide better stimuli for aggregation behavior than CHCs. We first examined fractions of chloroform-extracted conditioned filter papers. First instar nymphs did not show a significant preference for shelters treated with either 1 FE of the CHC fraction or of the more polar fraction ($\chi^2 = 0.8$, $P = 0.3711$, $n = 20$), with the nymph in 10 of 30 replicate assays not resting in either shelter (Fig. 1F). Finally, we sequentially extracted the conditioned filter papers in three solvents of increasing polarity: first with chloroform, then methanol, and lastly with water. First instar nymphs significantly preferred to rest within shelters treated with 1 FE of the methanol extract over shelters treated with methanol only ($\chi^2 = 21.16$, $P < 0.0001$, $n = 25$) (Fig. 2B). They showed no significant preferences between shelters treated with either 1 FE of the chloroform or water extracts and shelters treated with the respective solvent controls, chloroform ($\chi^2 = 0.3333$, $P = 0.5637$, $n = 27$) (Fig. 2A) and water ($\chi^2 = 1.5$, $P = 0.2207$, $n = 25$) (Fig. 2C). First instar nymphs also significantly preferred 1 FE of the recombined extract of chloroform, methanol, and water over the combined chloroform, methanol, and water solvent-treated shelter ($\chi^2 = 13.37$, $P = 0.0003$, $n = 27$) (Fig. 2D).

Aggregation in Shelters Occupied by Conspecific Females

We tested whether aggregation responses could be influenced by CHCs on the surface of a cockroach within the shelter. The presence

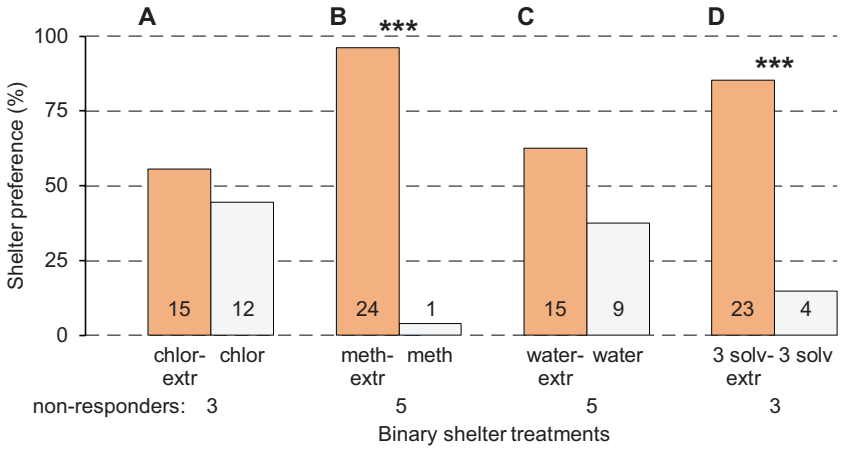

Fig. 2. Recovery of aggregation preference with solvent extraction of adult-conditioned paper. Individual first instar nymphs were tested in two-choice assays with filter paper shelters treated with various solvent extractions of female-conditioned filter papers. Conditioned papers were extracted sequentially with chloroform (chlor-ext) (A), methanol (meth-extr) (B), and water (water-extr) (C); the extracts were applied to filter paper shelters, and paired with shelters treated with the respective solvent as control. In (D), the three extracts were recombined (3 solv-extr) and assayed against the combined solvents (3 solv). Shelter choices (%) are shown, along with number of assays with individual nymphs resulting in preference for each shelter, and number of assays resulting in nymphs resting outside either shelter. Assays with *** indicate $P < 0.001$; in other assays $P > 0.05$.

of a dead female within the shelter guided nymphs to that shelter. First instar nymphs sheltered significantly more in a shelter containing a thoroughly solvent-extracted CHC-free female cockroach than under either a hexane-treated shelter with no cockroach ($\chi^2 = 12.45$, $P < 0.0001$, $n = 29$) (Fig. 3A) or a shelter treated with 1 FE of CHCs (159.8 µg, or 40 µg/cm^2) with no cockroach ($\chi^2 = 4.4815$, $P = 0.0343$, $n = 28$) (Fig. 3B). These results showed that the presence of a CHC-free cockroach alone could be a stronger aggregation stimulus than CHCs applied to the shelter.

To further investigate the role of female-associated CHCs, a solvent-extracted female was treated with 1 FE of CHCs and placed under a hexane-treated shelter and compared to an extracted (CHC-free) female treated with hexane only under a hexane-treated shelter. There was no significant difference in the aggregation preference of the first instar nymphs ($\chi^2 = 0.1429$, $P = 0.7055$, $n = 29$) (Fig. 4A). Moreover, direct comparisons of the two ecological contexts in which CHCs could operate revealed that first instar nymphs slightly, but not significantly, preferred to shelter with the CHC-treated female within a hexane-treated shelter over a CHC-free female within a CHC-treated shelter ($\chi^2 = 2.2857$, $P = 0.1306$, $n = 28$) (Fig. 4B). Thus, CHCs associated with both conspecifics and with the shelter were ineffective at guiding aggregation of individual cockroaches.

Discussion

A variety of cues and signals can guide the decision of cockroaches to seek shelter, where to shelter and with whom to aggregate. The aggregation of cockroaches in shelters is considered a self-organizing process (Deneubourg et al. 2002), where for an individual cockroach, the larger the number of sheltering neighbors, the more likely the individual is to stop and stay beside them (Garnier et al. 2009). Therefore, an individual cockroach is more likely to choose to aggregate under a shelter where other cockroaches are already present, and the decision to join a group has a density-dependent element. This tendency for collective and density-based decision-making can challenge studies of the proximate mechanisms that underlie aggregation behavior because of the inherent difficulties of separating cues that guide individual decisions from those that guide the group. Although individual nymphs and groups of nymphs may exhibit similar preferences in simple aggregation assays with conditioned shelters (e.g., Rivault and Cloarec 1998), the group may compromise our ability to assess the relative contributions of various cues and signals to this preference. Therefore, in this study we used individual nymphs, rather than groups of nymphs, to eliminate three related limitations of group assays: 1) collective decisions by a group may violate assumptions of independence of individual behavioral choices and statistical tests; 2) the presence of a group can alter the characteristics of the shelter, especially when shelter features are suboptimal (e.g., too light, too large); and 3) groups of nymphs are much more likely to defecate and thus alter the quality of the shelter during long-duration assays (e.g., 24 h), which are common in such studies. The tendency of cockroaches to aggregate together as a group is clearly documented in studies—all members of a group tend to shelter together on one of two equally suitable shelters (e.g., Ame et al. 2004, Saïd et al. 2005), making it impossible to disentangle individual responses to shelter-associated cues (e.g., fecal pheromone) from group-associated cues. This is particularly evident when shelter characteristics are inadequate for cockroach aggregation. Individual cockroaches may ignore poor quality shelters, but they accept the same shelter as a group (e.g., Sempo et al. 2009), likely because the group itself altered the shelter characteristics (e.g., darker, more shadows, more crevices).

Signals and Cues Associated With Shelters

In two-choice behavior assays with individual first instar nymphs, the nymphs significantly preferred shelters conditioned by adult females over unconditioned shelters, indicating that fecal, oral, and/or body secretions on the conditioned shelters, possibly including CHCs,

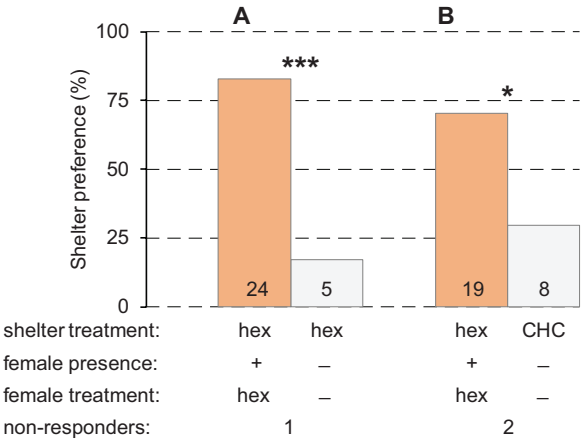

Fig. 3. Effect of presence of an extracted female within a shelter on aggregation preference of first instar nymphs in two-choice assays. Individual first instar nymphs were tested in assays with filter papers treated with female CHCs or hexane (hex) only. In (A), both shelters were treated with hexane, and a thoroughly extracted dead female was treated with hexane and placed in one of the shelters. In (B), one of the shelters was treated with female CHCs and the other shelter received an extracted female. Shelter choices (%) are shown, along with number of assays with individual nymphs resulting in preference for each shelter, and number of assays resulting in nymphs resting outside either shelter. Assays with *** indicate $P < 0.001$, * indicates $P < 0.05$.

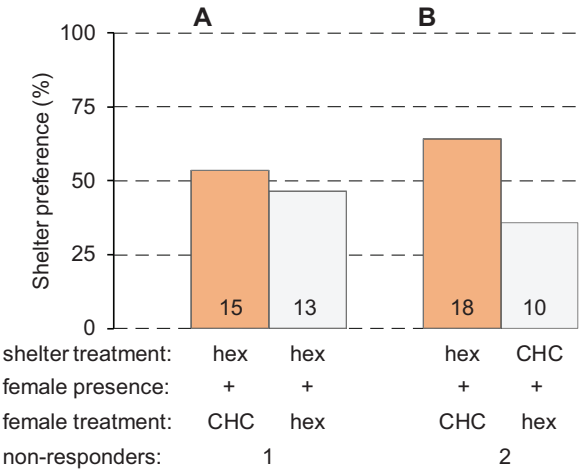

Fig. 4. Effect of presence of a CHC-treated female within a shelter on aggregation preference of first instar nymphs in two-choice assays. Individual first instar nymphs were tested in assays with filter papers treated with female CHCs or hexane (hex) only. In (A), both shelters were treated with hexane, and a thoroughly extracted dead female was placed in each shelter. One female was treated with hexane and the other with female CHCs. In (B), one of the shelters was treated with female CHCs and the other with hexane. Both shelters received an extracted female, but the female in the hexane-treated shelter was also treated with female CHCs. Shelter choices (%) are shown, along with number of assays of individual nymphs resulting in preference for each shelter, and number of assays resulting in nymphs resting outside either shelter. In both sets of assays $P > 0.05$.

serve as aggregation pheromone. Surprisingly however, nymphs rested equally in hexane-treated shelters as in shelters treated with chromatographically purified female CHCs. To compare the CHC fraction to more polar lipids, we extracted conditioned filter papers in chloroform and assayed nymphs with the CHC fraction and a polar fraction obtained from flash chromatography. Nymphs again rested equally in both shelters, and ~33% of first instar nymphs remained outside both shelters. These findings indicated that 1) CHCs were ineffective as an aggregation pheromone, and 2) chloroform failed to extract behaviorally active chemicals from conditioned filter papers.

The lack of behavioral activity in chloroform extracts of conditioned shelters suggested that more polar compounds should be considered. Therefore, we sequentially extracted female-conditioned filter papers with solvents of increasing polarity: chloroform, methanol, and water. Consistent with our previous assays, nymphs exhibited no preference for shelters treated with either the chloroform extract or the water extract versus shelters treated with the respective solvent. However, nymphs significantly preferred shelters treated with methanol extracts over shelters treated with methanol only, and shelters treated with the recombined three-solvent extracts also were significantly preferred over the solvent-treated shelters. These results suggested that polar lipids are more important than CHCs in inducing German cockroach aggregation, and these compounds were excluded from our hexane and chloroform extractions.

The literature on the role of CHCs in shelter choice by cockroaches is fragmentary and often contradictory. For example, American cockroach (*Periplaneta americana* L.) (Blattodea: Blattidae) nymphs were shown not to aggregate any more on cuticular dichloromethane extract-treated shelters than on solvent only (dichloromethane)-treated shelters (Saïd et al. 2005, Imen et al. 2015), but other dichloromethane extracts were used successfully to guide cockroaches to shelter with robots (Halloy et al. 2007). Several factors have contributed to these disparities. First and foremost, when insects are extracted, CHCs are often not separated from crude extracts of cuticular lipids, precluding a direct attribution of the behavior to CHCs because cuticular extracts may contain triglycerides, fatty acids, sterols, and other lipids, some of which effectively stimulate aggregation (McFarlane and Alli 1986, Scherkenbeck et al. 1999, Wada-Katsumata et al. 2015). Second, because compounds excreted in feces are often adsorbed to cuticular lipids (Ishii and Kuwahara 1967), behavioral responses to shelters treated with high doses of crude cuticular extracts (e.g., Imen et al. 2015) may be confounded by fecal compounds. Third, methodologies related to chemicals (e.g., extraction, fractionation, concentration) and assay procedures (e.g., individual vs group, photophase vs scotophase) vary considerably among studies. Because we aimed to investigate the role of nonvolatile CHCs, we allowed solvent extracts and fractions to evaporate to dryness, so it is possible that volatile compounds that may be important for aggregation were lost during these procedures. Notably, volatile carboxylic acids extracted from feces with methanol were highly effective aggregation cues in both olfactometer and sheltering assays (Wada-Katsumata et al. 2015). Finally, when groups of live insects are used in binary assays, it is impossible to separate the effects of CHCs and other semiochemicals from their feces that may differ because their gut microbiomes may differ (Wada-Katsumata et al. 2015, Kakumanu et al. 2018).

Signals and Cues Associated With Conspecifics

Our conclusion that CHCs from conditioned filter papers do not serve as an aggregation pheromone was confirmed in a second ecological context (CHCs on the cockroach body surface) using the CHCs of adult females placed either on shelters or on extracted CHC-free females. The premise of this assay paradigm was that CHCs on conspecifics within a shelter might bias the nymph's choice toward this aggregation site or the conspecific. In our assays, nymphs significantly preferred to rest under a shelter that contained a thoroughly extracted CHC-free female. They also preferred a CHC-free female over a female-free shelter treated with female CHCs. Furthermore, the addition of CHCs either to an extracted female within a shelter or to the other shelter containing a CHC-free female did not contribute to shelter preferences by nymphs. Overall, these results with females and their CHCs indicate that CHCs do not influence or bias the nymphs' preferences in two-choice assays.

Assay designs with a female in one shelter and not in the other shelter are inherently asymmetrical, and therefore difficult to interpret. Although it is possible that species-specific nonchemical signals from the solvent-extracted female guided the nymphs to shelter with her, we suspect that the most prominent cues that guided nymphs to the dead female were positive thigmotaxis and negative phototaxis. Cockroaches prefer to rest in dark, tight-fitting crevices that provide tactile stimuli to their ventral and dorsal surfaces (Berthold and Wilson 1967). The extracted female likely fragmented and reduced the open space within the shelter, darkened it, and provided the thigmotactic stimuli that nymphs seek. A similar confounding influence of thigmotaxis and phototaxis may be evident in aggregation tests using cockroach extract-treated robots. In two-choice aggregation assays in a large arena, *P. americana* preferred to assemble with extract-treated robots under a lighter, less attractive shelter than in a darker but empty shelter (Halloy et al. 2007). It is possible, however, that the robots simply improved the quality of the inadequate shelters in at least two ways unrelated to the extract or robot behavior. Moreover, because crude cuticular extracts were used to make robots more attractive, it is possible that non-CHC components of the extract played some role since cuticular extracts of *P. americana*, like other insects, contain some triglycerides, free fatty acids, sterols, and other lipids (Jackson 1972). These experiments, reported by Halloy et al. (2007), could not resolve the influence of multiple cues upon cockroach preferences. Our results with asymmetrical assays indicated that CHCs did not change the preference of *B. germanica* nymphs, suggesting that these compounds do not take precedence over other signals and cues in the cockroach's environment.

In symmetrical aggregation assays with *P. americana*, adults preferred to assemble with a cuticular lipid extract-treated robot under a shelter rather than in shelters containing a solvent-treated robot (Halloy et al. 2007). We performed a similar assay with *B. germanica* in this study, but with a solvent-extracted dead female rather than a robot. A nymph was given a choice to shelter with an extracted CHC-free female treated with CHCs under a hexane-treated shelter versus an extracted female treated with solvent only under a hexane-treated shelter. Nymphs exhibited no significant aggregation preference for either CHC-treated or CHC-free females. It is possible that this apparent discrepancy is related to species-specific differences (*P. americana* vs *B. germanica*), or to methodological differences such as the purity of CHCs, amount of CHCs used (both studies treated the substrate the same concentration per cm^2 as found on the respective cockroach cuticle), and the substrate on which CHCs were placed. Future investigations should conduct comparative studies using a standard methodology.

Aggregation Pheromone of *B. germanica*

The chemical identification of aggregation pheromone in *B. germanica*, and the role of CHCs as aggregation pheromone, has been

contentious since the early documentation of aggregation in this species. Ishii and Kuwahara (1967) observed that ether and methanol extracts of cockroaches and cockroach-conditioned papers elicited aggregation, but they concluded that the aggregation pheromone was associated with feces that contaminated the cuticular lipids of cockroaches. A series of investigations by multiple labs followed, most showing that the active compounds were fatty acids associated with feces (Ishii and Kuwahara 1967, Ritter and Persoons 1974, Scherkenbeck et al. 1999, Wada-Katsumata et al. 2015). Other compounds were also identified as attractants (volatile alkylamines) and arrestants (glycosylated steroids: blattellastanoside A and B) (Sakuma and Fukami 1990, 1991), but the behavioral activity of the latter has been contested (Scherkenbeck et al. 1999).

A series of reports, starting with Sreng et al. (1998), challenged the notion that polar lipids are involved, and instead concluded that CHCs comprise the aggregation pheromone of *B. germanica*. Unlike subsequent investigations by this team, however, Sreng et al. (1998) was the only study that separated CHCs from other cuticular lipids; therefore, this report deserves particular scrutiny. Using two-choice assays with groups of 20 first instar nymphs and filter paper substrates treated with various extracts, Sreng et al. (1998) found that dichloromethane and pentane extractions of cockroaches were most effective at inducing aggregation, while methanol extracts failed to do so. However, CHCs purified by flash chromatography from dichloromethane extracts failed to elicit aggregation. Sreng et al. (1998) nevertheless speculated that some CHCs were lost during the fractionation procedure, which eliminated their effectiveness. But several observations suggest otherwise. First, because *B. germanica* CHCs range from C27 (MW 379) to C32 alkanes and mono- and dimethyl alkanes (MW 463), they are essentially nonvolatile and easily recovered almost quantitatively from flash chromatography columns. Second, although methanol extraction is not an efficient method for recovering CHCs, the chromatograms in Sreng et al. (1998) show that the methanol extracts contained similar amounts of CHCs (with several linear and near terminally branched alkanes less represented), yet this fraction was inactive. Third, each filter paper substrate was treated with the extract of 15 sixth instar nymphs, and this amount far exceeds the amount of CHCs found in feces. Fourth, careful bioassay-guided fractionations have not found behavioral activity in the CHC fraction (Scherkenbeck et al. 1999). Fifth, there is ample evidence from other species of cockroaches (Jackson 1970, Tartivita and Jackson 1970, Jackson 1972) and from *B. germanica* (C.S., personal observations) that their cuticular surface contains fatty acids and other polar lipids, in addition to CHCs. Sixth, methanol extracts have proven effective in various olfactometer assays (Sakuma and Fukami 1985, Wendler and Vlatten 1993, Sakuma et al. 1997, Wada-Katsumata et al. 2015). Lastly, our results showed that CHCs, purified from either feces-contaminated filter papers or adult females, were ineffective at eliciting aggregation in *B. germanica*. We confirmed that CHCs were not effective at eliciting aggregation behavior by showing that purified CHCs were ineffective, CHCs applied to extracted CHC-free females did not increase her attractiveness to nymphs, and chloroform extracts that contain CHCs were ineffective.

In our assays, methanol extractions of feces-contaminated papers recovered a highly effective aggregation pheromone, as in previous studies (Kitamura et al. 1974, Wendler and Vlatten 1993, Miller et al. 1997, Scherkenbeck et al. 1999, Wada-Katsumata et al. 2015). We conclude that CHCs play a minor, if any, role in the aggregation behavior of the German cockroach. It is likely that the aggregation pheromone consists of attractants that guide the early stages of the aggregation process and arrestants that act through contact (Mori and Fukamatsu 1993, Sakuma and Fukami 1993, Rivault and Cloarec 1998). Feces-associated attractants, mainly carboxylic acids and amines, some of which may be microbial products, clearly play prominent roles as aggregation pheromone components. However, their importance relative to other chemicals, as well as to tactile (Lihoreau and Rivault 2008, Uzsák et al. 2014) and acoustic signals (Mistal et al. 2000), remains unknown. Nevertheless, in this study we showed that CHCs associated with either conspecifics or with the shelter were ineffective alone at guiding aggregation.

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