Even- and odd-numbered fatty acids used as worker arrestant aggregation pheromone components in the Formosan subterranean termite *Coptotermes formosanus*

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Abstract

Pheromones play a pivotal role in coordinating collective activities. Recent research has demonstrated that workers of the Japanese subterranean termite, *Reticulitermes speratus*, secrete an aggregation pheromone that induces quick assemble and prolonged stay at the pheromone source for nestmate workers, facilitating efficient dead-wood digestion and colony expansion. The question remains: do other termite species also utilize aggregation pheromones? In this study, we report that workers of the Formosan subterranean termite, *Coptotermes formosanus*, use a mixture of fatty acids as part of the pheromone components to maintain worker aggregation for extended periods of time. Through bioassays and gas-chromatography mass-spectrometry (GC-MS) analysis, we determined that a mixture of four fatty acids including palmitoleic acid, palmitic acid, (*Z*)-10-heptadecenoic acid, and stearic acid possesses arrestant activity but little or no attractant activity. Also, the inclusion of cholesterol and cuticular hydrocarbons reinforces only its arrestant activity. Further, GC-MS analysis of extracts of wood and soil, which serve as termite food, indicated that the pheromone components other than palmitic acid are not derived from their food, and GC analysis of extracts from different parts of worker's body revealed that these components were most likely produced in the midgut and excreted to the outside of the body via the hindgut. These findings suggest that the aggregation pheromone components and their functions in *C. formosanus* are largely different from those in *R. speratus*. This research provides insights into the differences among termite species in the development of chemical signals triggering aggregation behavior.

Introduction

Aggregation behavior is evolutionarily advantageous, providing benefits such as protection, mate choice, and resource exploitation (Parrish and Edelstein-Keshet, 1999). Numerous studies have shown that a variety of gregarious insects, including cockroaches, stink bugs, bed bugs, locusts, fruit flies, bark beetles, and longhorn beetles, utilize aggregation pheromones for mate selection, group foraging, and collective gathering (Wertheim et al., 2005). In social insects (e.g., ants, bees, wasps, and termites), aggregation is observed in diverse scenarios such as foraging, swarming, queen cluster formation, colony defense, nest building, and thermoregulation. Their advanced self-assemblages enable more intricate collective behaviors than observed in non-social insects (Anderson et al., 2002).

Termites live mainly in dead wood, soil, or mounds that they built themselves (Bucek et al., 2019). They create colonies with vast numbers of siblings and exhibit a notable caste-based division of labor. For instance, the worker caste displays coordinated behaviors such as migration, nest building, recruitment, and foraging (Eggleton, 2011). When foraging workers discover a new food source, they recruit their nestmates and then congregate for extended periods to exploit and colonize this source. While past research has hinted at chemical signals that temporarily attract workers, such as phagostimulant agents (Reinhard and Kaib, 2001), corpse recognition cues (Reinhard et al., 2003; Sun et al., 2017), and trail pheromones (Bordereau and Pasteels, 2011), compounds causing prolonged aggregation remained unidentified. However, a recent study identified such an aggregation pheromone in the Japanese

subterranean termite *Reticulitermes speratus* (Mitaka et al., 2020). This pheromone consists of a mixture of an aromatic compounds, cuticular hydrocarbons (CHCs), fatty acids, and cholesterol. Although aggregation pheromone consists of two types of components: attractant components, which attract distant individuals, and arrestant components, which keep individuals at the odor source (Kennedy, 1978), the mixture synergistically functions both as an attractant and as an arrestant. It is postulated to facilitate swift aggregation, leading to effective dead-wood digestion and colony expansion (Mitaka et al., 2020). As termites such as *R. speratus* and others need to assemble when colonizing new wood to prevent pathogen infections and efficiently share nutrients and symbionts (Eggleton, 2011; Rosengaus et al., 2011), it is conceivable that other species use aggregation pheromones. Nevertheless, this hypothesis remains to be validated.

The Formosan subterranean termite, *Coptotermes formosanus*, stands out as a significant wood pest due to its vast distribution (Rust and Su, 2012; Bradshaw et al., 2016). Hence, prior research has delved into the mechanisms of lignocellulose digestion (Ke et al., 2011; Li et al., 2012; Tarmadi et al., 2018), feeding preferences (Chen and Henderson, 1996; Cornelius et al., 2002; Hapukotuwa and Grace, 2011), and foraging (Cornelius and Osbrink, 2011; Hapukotuwa and Grace, 2012) in this species. Worker-attracting elements have been of particular interest, with assumptions that a pheromone secreted by a worker could attract and hold other workers in place (Raina et al., 2005). Given the close relation between *C. formosanus* and *R. speratus* (Bucek et al., 2019), and the observed collective feeding behavior of *C. formosanus* workers against new wood (Raina et al., 2005), it is plausible that *C. formosanus* workers might also use an aggregation pheromone for grouping. Yet, such a pheromone remains unidentified.

In this study, we compared the aggregation activity of worker hexane extracts and its fractions from *C. formosanus*, narrowed down the candidates for the pheromone components, and assessed the dose-dependent response and duration of the pheromone mixture. In addition, we investigated whether termites obtain these pheromone components from their diet and which parts of the worker body secrete the pheromone components.

Materials and Methods

Termites

Incipient colonies of *C. formosanus*, established from alates of a singular matured colony, were used. The mature colony, colleced in May 2013 from a secondary forest in Yoshihara, Mihama-cho, Hidaka-gun, Wakayama Prefecture, Japan, was kept in a plastic bathtub at 28°C in the laboratory. For their sustenance, we provided commercially procured, dried square timbers of Japanese red pinewood (*Pinus densiflora*). Between 2013 and 2016, incipient colonies were annually established by pairing a male and a female dealate, both of which emerged from the primary mature colony. These colonies were maintained for approximately a year in 90 mm plastic dishes with BPC medium (Mitaka et al., 2023) and topped with pinewood chips (50 × 50 × 5 mm). Upon a notable increase in worker numbers, each colony in the plastic dish, with its lid opened, was transferred to a larger plastic container (321 × 232 × 223 mm) containing

moistened soil (Tsuchitaro, Sumitomo Forestry Landscaping Co., Ltd., Tokyo, Japan) and *P. densiflora* timbers. Ventilation holes, covered with wire gauze to deter escapes, were situated at the upper part of a side surface of the container. These containers, sealed with glass plates, were consistently kept at 28°C. For this study, from April 2019 to March 2022, we utilized seven such established colonies (colony codes: KU130604–5, KU130604–11, KU141013–5, KU141013–6, KU141013–40, KU141013-X, and KU160602–2). Given that worker numbers in these colonies ranged widely (from around 1500 to more than 4000 workers), worker crude extracts, as described later, were primarily taken from colonies with higher worker counts.

Chemicals

n-Hexane, silica gel (C-200), diethyl ether (hereafter, DEE), 5% HCl methanol solution, *n*-tetradecanoic acid (myristic acid), *n*-hexadecanoic acid (palmitic acid), (*Z*)-9-octadecenoic acid (oleic acid), *n*-octadecanoic acid (stearic acid), and cholesterol were purchased from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan. (*Z*)-9-hexadecenoic acid (palmitoleic acid), and (*Z*)- and (*E*)-10-heptadecenoic acid were purchased from Funakoshi Co., Ltd., Tokyo, Japan. *n*-Pentadecanoic acid, *n*-heptadecanoic acid (margaric acid), and (5*Z*,8*Z*,11*Z*,14*Z*)-icosa-5,8,11,14-tetraenoic acid (arachidonic acid) were purchased from Tokyo Chemical Industry Co., Ltd.

Extraction and isolation of active compounds from workers and their food

To extract chemicals from worker termites, 1200 individuals from each colony were extracted in a 13 mL glass vial with 9 mL hexane for 24 h. Crude extracts were derived from four colonies: KU130604–5, KU130604–11, KU141013–5, and KU160602–2. These extracts, with an equivalent concentration of about 133 workers/mL, were transferred to fresh glass vials and stored at –30°C until needed. A 1 mL aliquot of the crude extract was subjected to chromatography on a silica gel column (6 mm inner diameter) containing 0.5 g silica gel. Elution of the column was sequential, starting with hexane (3 mL), followed by three DEE concentrations in hexane (3 mL each of 10% DEE, 30% DEE, and 50% DEE), and concluded with pure DEE (3 mL). Aliquots of 90 µL from each fraction were evaporated to 10 µL (equating to 0.4 workers/µL) with a gentle nitrogen stream. Subsequent chemical identification was performed through gas chromatography-mass spectrometry (GC-MS) analysis. The leftover portion from each fraction was stored at –30°C.

In addition, to ascertain if aggregation pheromone components are derived from food or autonomously produced by the workers, termite food sources (soil and *P. densiflora* timber sawdust) underwent hexane extraction for 24 h. Specifically, 1 g dried soil (Tsuchitaro) was extracted in a 10 mL glass vial containing 5 mL hexane, while 1 g dry *P. densiflora* sawdust was extracted in a 10 mL glass vial containing 10 mL hexane. Each resultant crude extract was concentrated into a 1 mL hexane solution and transferred to a

new vial. From each of these extracts, 500 µL was chromatographed similarly to the worker samples, using the silica gel column. Fractions were concentrated to 50 µL aliquots (equivalent to 10 g/µL) with a rotary evaporator, followed by GC-MS analysis. Residual fractions were preserved at –30°C.

GC-MS analysis

GC-MS analyses were executed on a JMS-Q1500GC (JEOL Ltd., Tokyo, Japan) in conjunction with an Agilent Technologies 7890B GC system (Agilent Technologies, Santa Clara, CA, USA), equipped with a DB-1MS column (30 m × 0.25 mm × 0.25 µm, Agilent Technologies). The column temperature was held at 50°C for 5 min, increased to 300°C at 20°C/min, and held at 300°C for 5 min. One portion (1 µL) of the solution was injected per sample. The injector was operated in splitless mode, utilizing helium as the carrier gas at 1 mL/min, and the injection port was consistently held at 250°C. MS data acquisition conditions were as follows: 50 µA ionization current, 70 eV ionization energy, 2 kV accelerating voltage, and a scan range from 40–500 *m/z*. The GC-MS system was maneuvered by the msPrimo System Controller ver. 1.06 (JEOL Ltd.). Data interpretation was facilitated by the Escrime ver. 2.04 software (JEOL Ltd., https://www.jeol.co.jp/en/products/detail/JMS-Q1500GC.html). Candidate compounds were predicted from the Mass Spectral Library (NIST11). Pheromone components were identified according to retention time and mass spectra, in comparison with commercial standards.

Methyl esterification

To discern the geometrical isomer of 10-heptadecenoic acid from worker extracts, fatty acid methyl esters was prepared prior to GC analysis. The 100 µL 30% DEE fraction obtained from KU141013–5, alongside 0.1 mg of both *cis*- and *trans*-10-heptadecenoic acid standards, were individually dissolved in 1 mL 5% HCl methanol solution within a 10 mL glass centrifuge tube. These tubes were subsequently heated at 80°C for 30 min. Upon cooling, the product within each tube was extracted using hexane in three 500 µL portions. Then the combined hexane fractions (1.5 mL) were concentrated via a rotary evaporator (30% DEE fraction to 11 µL, and both (Z)- and (E)-10-heptadecenoic acid to 100 µL) before undergoing GC analysis.

GC analysis was conducted on a GC-2014 (Shimadzu Corporation, Kyoto, Japan) paired with a flame ionization detector set to 220°C. One portion (1 µL) of the hexane fraction was injected per sample. The injector was operated in splitless mode, with the injection port temperature maintained at 210°C. An HP-88 column (30 m × 250 µm × 0.25 µm, Agilent Technologies) was used. Helium served as the carrier gas, flowing at 1 mL/min. The column temperature was held at 50°C for 5 min, increased to 210°C at 10°C/min, and held at 210°C for 1 min. The geometrical isomer of methyl 10-heptadecenoate in the 30% DEE fraction was identified by comparing its retention time with that of the methyl-esterified commercial standards.

Bioassay

Two-choice bioassays (Fig. 1A) were conducted as described in a previous study (Mitaka et al., 2020). Prior to the bioassays, around 2,000 termites were housed in a 140 mm plastic dish with a moistened filter paper (90 mm in diameter) under dark conditions at 25°C for 2 days. After this acclimation period, groups of 30 workers were introduced into 90 mm plastic dishes containing two filter paper sections (30 mm in diameter) positioned on opposing sides of the dishes, which had sanded surfaces. The 'sample' paper was treated with an extract (or fraction or authentic standard solution), while the 'solvent' paper was treated with only a solvent. Both papers were air-dried for roughly 5 min to evaporate solvents, before closing the lid of the dish. In the positive control setup, 7.5 µL crude extract (1 worker equivalent) sourced from KU141013–5 was added to the sample papers. In the negative control, 10 µL hexane was added to the sample paper. For other treatments (including the negative control), unless otherwise mentioned, 10 µL solutions (1 worker equivalent) were applied to the sample papers. The solvent papers in each treatment received an equal volume of solvent. Every bioassay was replicated 10 times per colony for every treatment, and workers from two different colonies were utilized for each treatment (totaling 20 replications per treatment). Vertical photographs of each dish were taken using a digital camera (EOS Kiss Digital N + EFS 60 mm f/2.8 Macro USM, Canon, Tokyo, Japan) to document the count of termites on both the sample and solvent papers. Worker counts on each paper were noted after a 5 minute in all behavioral tests, except when indicated differently.

To ascertain aggregation pheromone components, we proceeded as follows:

(1) Attraction activity analysis among worker crude extract fractions

To evaluate the attractive activity across different fractions (hexane, 10% DEE, 30% DEE, 50% DEE, and DEE fractions), workers from two colonies (KU141013–5 and KU141013–6) were used. For each replicate, 22.5 µL fraction solution (1 worker equivalent) was applied to the sample paper. An equivalent volume of solvent was added to the solvent paper for each treatment.

(2) Subtraction test

To discern if all fatty acids identified via GC-MS analysis contribute to attraction, a subtractive approach was used. Attraction from a nine-fatty-acid mixture [myristic acid: *n*-pentadecanoic acid: palmitoleic acid: palmitic acid: margaric acid: (*Z*)-10-heptadecenoic acid: oleic acid: stearic acid: arachidonic acid = 150: 120: 260: 2740: 640: 1850: 26170: 1540: 100 (unit: ng per 10 µL hexane), equivalent to 1 worker] (Mix) was contrasted with six treatments each missing certain components: 1) A mixture excluding myristic acid, *n*-pentadecanoic acid, and arachidonic acid from the Mix, 2) A mixture excluding myristic acid, *n*-pentadecanoic acid, arachidonic acid, and oleic acid from the Mix, 3) A mixture of three saturated fatty acids [palmitic acid: margaric acid: stearic acid = 2740: 640: 1540 (unit: ng per 10 µL hexane)], 4) A

mixture of palmitic acid and margaric acid, 5) A mixture of margaric acid and stearic acid, and 6) A mixture of palmitic acid and stearic acid (detailed in Text S1 in electronic supplementary materials).

(3) Effects of unsaturated fatty acids

To establish the role of unsaturated fatty acids in pheromone activity, attraction from a four-fatty-acid mix [palmitoleic acid: palmitic acid: (*Z*)-10-heptadecenoic acid: stearic acid = 260: 2740: 1850: 1540 (unit: ng per 10 µL hexane)] (Mix A treatment) was compared to treatments missing one of the unsaturated fatty acids, specifically palmitoleic acid and (*Z*)-10-heptadecenoic acid. Each fatty acid combination represented an equivalent of 1 worker. We utilized workers from two colonies (KU141013–40 and KU141013-X) (additional details in Text S1 in electronic supplementary materials).

(4) Dose response of four fatty acids

To validate the attraction and arrestment effects of the previously mentioned four fatty acids, the efficacy of the Mix A solution was matched against a 10-fold concentrated version (Mix A × 10 treatment) and a 10-fold diluted version (Mix A × 0.1 treatment). Workers from the colonies KU141013–40 and KU141013-X participated in this test. During this experiment, worker counts on both sample and solvent papers were taken at intervals of 5, 60, 120, and 240 min.

(5) Incorporating fatty acids, CHCs, and cholesterol:

To assess if adding Mix A with compounds from the hexane and 50% DEE fractions could enhance its attractant and arrestant activities, we compared the activities of Mix A solution with that of mixtures of Mix A combined with CHCs and/or cholesterol. The Mix A with cholesterol solution (Mix A + Ch treatment) was composed of a 260: 2740: 1850: 1540: 40 (unit: ng per 10 µL hexane) blend of palmitoleic acid, palmitic acid, (*Z*)-10-heptadecenoic acid, stearic acid, and cholesterol. For the Mix A with CHCs solution (Mix A + CHCs treatment), a 750 µL hexane fraction from the worker crude extract (equivalent to 200 workers in 3 mL hexane) from colony KU141013–5 was placed into a 1.5 mL glass vial. Then this was evaporated under a gentle stream of pure nitrogen and redissolved in 500 µL the Mix A solution. Similarly, the Mix A combined with CHCs and cholesterol solution (Mix A + Ch + CHCs treatment) was derived from the evaporated hexane fraction redissolved in 500 µL Mix A + Ch solution. A 10 µL aliquot of each formulation corresponds to one worker's equivalent. Workers from colonies KU141013–40 and KU141013-X were engaged in this study. Counts of workers on the papers were recorded at 5, 60, 120, and 240 min intervals.

The degree of aggregation was gauged through an aggregation index (AI), defined as $(N_{Sample} - N_{Solvent})/N_{Total}$, where N_{Sample} is the worker count on the sample paper, $N_{Solvent}$ represents the count on the solvent papers, and N_{Total} is the total workers in a bioassay dish (i.e., 30 workers). The index spans from -1 to 1, with positive AI values indicates an aggregation response. For statistical analysis, we compared the proportion of the total number of workers on the sample papers to the total number of workers on both sample and solvent papers, using Fisher's exact tests. *P*-values were adjusted for

multiple testing based on the Bonferroni correction. Both statistical analysis and data visualization were executed in R software ver. 3.3.3 (R Core Team, 2016).

Detection of aggregation pheromone from each part of worker body

To estimate the site of secretion of the fatty acid components of the aggregation pheromone, we first dissected workers (from colony KU141013-6), which were fed with cellulose powder and soil for more than two months period, to separate them into head (including foregut), gut (midgut and hindgut), and the other residual parts (thorax and abdomen). Subsequently, to estimate which part of the gut contains particularly levels of the fatty acid components, we collected the guts of another workers (obtained from colony of the same origin as above) and separate them into midgut, paunch (the anterior part of hindgut), colon (the posterior part of hindgut), and rectum (the end part of hindgut). We pooled 20 individuals for each body part. Each body part was extracted in a 10-mL glass vial containing 1 mL of hexane for 24 h. Subsequently, the hexane crude extract (1 mL) was chromatographed on a silica gel column (6 mm inner diameter) containing 0.5 g of silica gel. The column was successively eluted with hexane (3 mL), and two concentrations of DEE in hexane (3 mL each of 10% DEE and 30%DEE). The 30%DEE fraction was concentrated to a 50-μL solution (equivalent to 0.4 workers/μL) using a rotary evaporator and then subjected to GC analysis for quantification. Fractionated solutions of each body part were measured five times, and the average of those measurements was taken as the content of each compound at that body part. GC analysis was performed on a GC-2014 (Shimadzu Corporation) with a flame ionisation detector (300°C). One portions (1 μL) of each sample were injected in splitless mode. The injection port temperature was 300°C. The column used was an DB-1HT (15 m × 0.25 mm × 0.25 μm, Agilent Technologies). The carrier gas was helium, with a flow rate of 1 mL/min. The column temperature was held at 50°C for 5 min, increased to 300°C at 20°C/min, and held at 300°C for 5 min. Compounds were identified according to the retention time compared to authentic standards. The total amounts of pheromone components in each body part extract were calculated by comparison with external standard curve (regression: adjusted $R^2 \geq 0.90$).

Results

Attractant efficacy of worker crude extract and fractions

We initiated our research by assessing the attractant efficacy of the worker crude extract and its fractions (hexane, 10% DEE, 30% DEE, 50% DEE, and DEE fractions) using a two-choice bioassay approach (Fig. 1A). When comparing AI scores across treatments, the 10% DEE and 30% DEE fractions exhibited higher attractant activities relative to other fractions (Fisher's exact test with Bonferroni correction, $P < 0.05$, Fig. 1B). We inferred that the primary active elements are contained within these two sections.

Fraction chemical composition

To identify potential pheromone components, every fraction from the four colonies underwent GC-MS analysis. Consequently, 18 compounds (compound **1–18**) were consistently observed across all fractions and colonies (Fig. 2 and Table 1). The hexane fraction contained CHC elements such as 3-methylpentacosane (**11**), *n*-hexacosane (**12**), *n*-heptacosane (**13**), 13-methylheptacosane (**14**), 2-methylheptacosane (**15**), 13-methyloctacosane (**16**), and 13-methylnonacosane (**17**). By contrast, the 10% DEE and 30% DEE fractions harbored both saturated and unsaturated fatty acids such as myristic acid (**2**), *n*-pentadecanoic acid (**3**), palmitoleic acid (**4**), palmitic acid (**5**), margaric acid (**6**), 10-heptadecenoic acid (**7**), oleic acid (**8**), stearic acid (**9**), and arachidonic acid (**10**). The geometric isomers of 10-heptadecenoic acid were also discerned, with the (*Z*)-isomer specifically identified via comparative GC analysis [retention time of 20.0 min for (*Z*)-isomer, 19.9 min for (*E*)-isomer, and 20.0 min for natural 10-heptadecenoic acid]. The 50% DEE fraction was distinctively characterized by cholesterol (**18**). Notably, the 10% DEE, 30% DEE, 50% DEE, and DEE fractions all contained butylated hydroxytoluene (BHT) (**1**), a stabilizer inherent in the DEE solvent. In summary, our chemical analyses suggest that any or all of the nine aforementioned fatty acids, cholesterol, and CHCs may constitute the aggregation pheromone component of *C. formosanus*.

Dose- and time-dependent activities of fatty acid components

To verify whether all nine fatty acids, including myristic acid (**2**), *n*-pentadecanoic acid (**3**), palmitoleic acid (**4**), palmitic acid (**5**), margaric acid (**6**), (*Z*)-10-heptadecenoic acid (**7**), oleic acid (**8**), stearic acid (**9**), and arachidonic acid (**10**), play a role in the pheromone activity, we used subtraction schemes. The outcome revealed that a specific mixture, termed Mix A, composed of palmitoleic acid (**4**), palmitic acid (**5**), (*Z*)-10-heptadecenoic acid (**7**), and stearic acid (**9**) (see Fig. S1 for mass spectra), is possibly the primary contributor to the aggregation activity seen in the fatty acid components of the aggregation pheromone (as elaborated in Text S1 in the electronic supplementary materials).

To test the dose- and time-dependent responses of Mix A, the Mix A solution, its 10-fold diluted solution, and its 10-fold concentrated solutions were presented to workers. Then we compared AI values among these doses at intervals of 5, 60, 120, and 240 min post-exposure. Interestingly, both Mix A and its 10-fold concentrated solution showed marginally elevated AI values compared to the 10-folded diluted Mix A and the negative control just 5 min post-exposure. Yet, as time elapsed, the AI values of Mix A and its 10-fold concentrated solution increased, nearly mirroring those of the crude extract (Fisher's exact test with Bonferroni correction, $P < 0.05$, Fig. 3). These results indicate that the Mix A has an arrestant activity rather than an attractant activity. In addition, during the experiment, workers on the filter paper simply stopped or walked and did not exhibit any special behavior such as feeding behavior.

Effects of compounds other than fatty acids on aggregation activity

To assess if compounds from the hexane and 50% DEE fractions, specifically CHCs (**11–17**) and cholesterol (**18**), could enhance the aggregation pheromone activity, we exposed workers to Mix A and to Mix A supplemented with CHCs (**11–17**) and/or cholesterol (**18**). Then the AI values were compared at intervals of 5, 60, 120, and 240 min. Initially, none of the mixtures displayed significant attractant activity at the 5 min mark. However, over time, the AI values for Mix A combined with CHCs (**11–17**) and/or cholesterol (**18**) increased and significantly surpassed those of Mix A alone (Fisher's exact test with Bonferroni correction, $P < 0.05$, Fig. 4). Notably, after 240 min, the Mix A + CHCs (**11–17**) + cholesterol (**18**) treatment exhibited the highest AI value (0.74) among all artificial pheromone blends, closely resembling that of the crude extract (0.84). This suggests that the inclusion of CHCs (**11–17**) and cholesterol (**18**) boosts the arrestant activity of Mix A, without necessarily improving its attractant activity.

Chemical analysis of termite foods

Our bioassays revealed that the aggregation pheromone of *C. formosanus* workers contains mainly four fatty acids (**4**, **5**, **7**, and **9**), with CHCs (**11–17**) and cholesterol (**18**) supplementally enhancing the arrestant effect. To verify whether these workers derive these compounds from their food sources (specifically pinewood and soil), we conducted a GC-MS analysis of all fractions from pinewood sawdust and soil extracts. The pinewood sawdust fractions contained a total of 37 compounds (compounds **1**, **5**, **8**, and **19–52**), which included terpenes, fatty acids, and resin acids (refer to Fig. S3 and Table S1 in the electronic supplementary materials). Among these compounds, palmitic acid (**5**) was also contained as one of the fatty acid components. This suggests that although it is not clear whether *C. formosanus* workers use wood-derived palmitic acid (**5**) as their aggregation pheromone components, biosynthesize palmitic acid (**5**) in their own bodies, or obtain palmitic acid (**5**) synthesized by gut symbionts, the other compounds such as palmitoleic acid (**4**), (*Z*)-10-heptadecenoic acid (**7**), stearic acid (**9**), CHCs (**11–17**), and cholesterol (**18**) are likely synthesized internally by the worker or through gut symbionts. Moreover, only BHT (**1**), a stabilizer present in the DEE solvent, was identified in all soil extract fractions (see Fig. S4 in the electronic supplementary materials).

Secretion part of aggregation pheromone

The distribution of primary components of the aggregation pheromone (four fatty acids **4**, **5**, **7**, and **9**) was still unclear, although it is known that CHCs are distributed throughout insect body surface (Blomquist and Bagnères, 2010) and that cholesterol is contained within the bilayer of cell membranes throughout the body (Jing and Behmer, 2020). To estimate secretion part of these four fatty acids (**4**, **5**, **7**, and **9**), we dissected workers' bodies, extracted them by hexane, and quantified the amounts of the four

fatty acids in each body part. First, workers were dissected and analyzed in the head, thorax and abdomen, and gut, which revealed that the pheromone components were detected in all body parts but were most abundant in the gut (Fig. S5A and Dataset in the electronic supplementary materials). Subsequently, the gut was dissected and analyzed in the midgut, paunch, colon, and rectum, which revealed that all pheromone components were detected in the midgut, paunch, and colon, but were the most abundant in the midgut. In the rectum, all components except stearic acid were detected in trace amounts (Fig. S5B and Dataset in the electronic supplementary materials).

Discussion

Aggregation pheromones typically consist of an attractant and/or an arrestant component. The attractant attracts conspecific individuals from afar, while the arrestant induces a passing conspecific to stay near the pheromone source (Kennedy, 1978). Our study revealed that workers of *C. formosanus* utilize a blend of four fatty acids, namely, palmitoleic acid (**4**), palmitic acid (**5**), (*Z*)-10-heptadecenoic acid (**7**), and stearic acid (**9**), as at least part of the aggregation pheromone components, which primarily exhibits arrestant rather than attractant activities (Fig. 3). In the dose-response test for a mixture of four fatty acids (Mix A), the AI values of Mix A treatment and its 10-fold concentrated treatment are significantly higher than those of the negative control in the results after five minutes (Fig. 3), but they are not significantly different (these treatments have an AI value of about 0.05). However, after 60 minutes, the results show that all doses of Mix A have higher AI values than the negative control (negative control: AI = 0-0.1, Mix A treatments: 0.16 < AI < 0.64). Therefore, it can be said that the mixture of the four fatty acids has only an arrestant activity. In addition, when CHCs (**11–17**) and cholesterol (**18**) were added to Mix A, the AI values of Mix A with cholesterol and/or CHCs were slightly lower than that of Mix A alone at the 5-min result, whereas the AI values of Mix A with cholesterol and/or CHCs were significantly higher than Mix A alone after 60 min (Fig. 4), indicating that cholesterol and CHCs enhance only the arrestant activity of Mix A and have no significant effect on the attractant activity of it. Our results suggest that *C. formosanus* workers emit this fatty-acid-based pheromone to retain passing nestmate workers for prolonged durations, facilitating the long-lasting assembly of worker groups. This is consistent with previous research on *C. formosanus* that has reported that workers, upon entering a foraging zone, linger there longer than in non-foraging areas (Lee et al., 2021). The use of this aggregation pheromone, endowed with a pronounced arrestant activity, likely helps sustain the persistent collective actions observed in *C. formosanus*. In contrast to the aggregation pheromone of *R. speratus*, which has both attractant and arrestant activities and these activities were reproduced by an artificial pheromone blend (Mitaka et al., 2020), the mixture of fatty acids identified in this study has only an arrestant activity, and the mixture was not able to fully reproduce the activity of the worker crude extract even if cholesterol and CHCs are added to the mixture (Fig. 3 and 4). The lack of identical attraction and arrestant activity in the mixture identified in this study compared to natural worker extracts may be due to the lack of key compounds. It is possible that compounds undetectable or unidentified in our GC-MS analysis are also involved with the aggregation activities. Unraveling the identity of these compounds responsible for the attractant and arrestant functions will be an essential objective for subsequent studies.

C. formosanus workers use an odd-numbered fatty acid, (*Z*)-10-heptadecenoic acid (**7**), in addition to even-numbered fatty acids, including palmitoleic acid (**4**), palmitic acid (**5**), and stearic acid (**9**), for their aggregation pheromone (Fig. 3). By contrast, *R. speratus* workers solely utilize even-numbered fatty acids, specifically palmitic acid (**5**) and *trans*-vaccenic acid (Mitaka et al., 2020). While a previous study noted the presence of odd-numbered fatty acids in individuals of *C. formosanus* (Chen and Laine, 2016), their roles remained undefined until now. Typically, *de novo* fatty acid biosynthesis primarily yields palmitic acid (**5**), accompanied by traces of stearic acid (**9**). Fatty acids with >18 carbons originate from 2-carbon chain elongation in eukaryotes (Cook and McMaster, 2002). Hence, the biosynthesis of odd-numbered fatty acids presumably engages a unique pathway (Fromm and Hargrove, 2012). Also, palmitic acid is usually biosynthesized by extending the carbon chain of short-chain carboxylic acids (Ratledge, 2004; Rajasekharan and Nachiappan, 2010; Paiva et al., 2021), and termites obtain short-chain carboxylic acids by absorbing acetic acid produced by cellulose-metabolizing gut symbionts in paunch (Brune, 2014). Given that we found no trace of odd-numbered fatty acids in pinewood and soil extracts, both dietary staples for termites (Fig. S3 and S4 in the electronic supplementary materials) but found that fatty-acid pheromone components are detected primarily in the gut (especially midgut) and in small amounts throughout the body outside of the gut (Fig. S5 in the electronic supplementary materials), it is likely that *C. formosanus* workers may biosynthesize the fatty-acid pheromone components in their midgut glands or the inside of midgut from short-chain carboxylic acids, and then the workers excrete the fatty acids via the hindgut (consisted of paunch, colon, and rectum) to the outside of the body. Although only palmitic acid was found in the food pinewood, it may not have been biosynthesized by the workers themselves. The biosynthetic pathways of (*Z*)-10-heptadecenoic acid, palmitoleic acid, and stearic acid need to be investigated in detail in the future.

Cholesterol (**18**) enhances an arrestant activity of the aggregation pheromone of *C. formosanus* (Fig. 4). While steroids act as precursors for molting hormones in insects, the insects themselves typically cannot independently synthesize steroids. As a result, insects must source steroids either through their diet or symbiotic relationships (Clark and Bloch, 1959; Svoboda et al., 1978; Jing and Behmer, 2020). Our GC-MS analyses revealed that neither pinewood nor soil extracts, both fundamental foods for *C. formosanus*, contain steroids including cholesterol (**18**) (Figs. S3 and S4, and Table S1 in electronic supplementary materials). This suggests that *C. formosanus* workers likely obtain cholesterol (**18**) or its precursors from gut symbionts, allocating a portion of this cholesterol to the aggregation pheromone. It is considered that the gut symbionts might produce ample quantities of cholesterol (**18**) or its precursors and that surplus cholesterol (**18**) within the worker body is repurposed as a pheromone component.

The introduction of CHCs (**11–17**) to the fatty acid components amplified the arrestant activity of the aggregation pheromone (Fig. 4). This study utilized immature *C. formosanus* colonies, aged between 3 to 9 years post-laboratory establishment. Consequently, the worker count in these colonies ranged approximately from 1500 to 4000, significantly fewer than the million-plus workers observed in mature wild colonies (Su and Scheffrahn, 1988). Given this limitation in colony size, only a select few colonies could supply adequate workers for both extract preparation and assays. Hence, in the assay examining the effects of adding CHCs (**11–17**) to fatty acids (**4**, **5**, **7**, and **9**), the workers used for the assays were

different from the workers used to prepare crude extracts and hexane fractions. Yet, high arrestant activity was observed in response to both the crude extract and the mixture of fatty acids and CHCs (AI = 0.74–0.84, Fig. 4). This suggests that *C. formosanus* workers aggregate to the aggregation pheromone whether it contains nestmate CHCs or non-nestmate CHCs. While social insects are considered to exhibit distinct CHC composition ratios across colonies, aiding nestmate differentiation (Van Zweden and D'Ettorre, 2010), the precise role of CHCs in nestmate recognition remains debated. For *C. formosanus*, within the realm of aggregation behavior, workers might view CHC components as indicators of species-specific pheromone components rather than nestmate identifiers.

This research underscores that both *C. formosanus* and *R. speratus* produce aggregation pheromones. It is likely that such pheromones are prevalent at least within the rhinotermitid species range, with the pheromone components varying by species. Although *R. speratus* workers utilize CHCs, an aromatic compound, cholesterol (**18**), and even-chain fatty acids as their aggregation pheromone (Mitaka et al., 2020), *C. formosanus* workers utilize mainly the mixture of odd- and even-chain fatty acids (**4**, **5**, **7**, and **9**), with the addition of CHCs (**11–17**) and cholesterol (**18**) enhancing the arrestant activity (Fig. 3 and 4). These suggest that in termite workers, gut symbionts produce large amounts of short-chain carboxylic acid including acetic acid through cellulose metabolism (Brune, 2014) allowing workers to biosynthesize various types of long-chain fatty acids from the short-chain fatty acids and thus to divert the surplus long-chain fatty acids for aggregation pheromones. Considering that fatty acids are biosynthesized from cellulose metabolites, the surplus long-chain fatty acids excreted from their bodies may have initially functioned as a signal to nestmates of the presence of cellulose and eventually come to function as an aggregation pheromone to attract and arrest nestmates. Although it is now very likely that fatty acids are the basic component of aggregation pheromones, at least in rhinotermitid species, the structure of fatty acids used as pheromone components and the presence of non-fatty acid pheromone components (such as steroids, CHCs and other metabolites) vary among species probably because of differences in the chemical profile of foods and the metabolic pathways in each termite species. Differences in fatty acid composition and species-specific non-fatty acid components may play an important role in recognizing conspecific aggregation pheromone in termites. This research enriches our comprehension of the evolutionary trajectory of termite pheromone-driven aggregation behaviors.

Declarations

Supplementary information

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Author contributions

Y.M. designed experiments. K.M. collected termites. K.M. and Y.M. kept termites. Y.M. performed bioassays and statistical analyses and wrote the draft manuscript. Y.M. and T.A. performed chemical analyses. Y.M., K.M., and T.A. provided feedbacks and edits.

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Data availability

The data used in this papar are available in the supplementary information.

Competing interests

The authors declare no competing interests.

Ethic approval

Ethics approval was not required in this study.

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Tables

Table 1. Compounds in Fractions of the Whole-Body Worker Extract.

No	Kovats index	Compound name	Relative peak area (% Mean ± SEM)				
			Hexane frac.	10% DEE frac.	30% DEE frac.	50% DEE frac.	DEE frac.
1	1487	Butylated Hydroxytoluene	0.00 ± 0.00	28.08 ± 14.41†	0.22 ± 0.03	53.81 ± 10.95	84.52 ± 9.21
2	1788	Myristic acid*	0.00 ± 0.00	0.26 ± 0.26†	0.52 ± 0.08	0.00 ± 0.00	0.00 ± 0.00
3	1820	*n*-Pentadecanoic acid*	0.00 ± 0.00	9.73 ± 5.70†	1.77 ± 0.86	0.00 ± 0.00	0.00 ± 0.00
4	1900	Palmitoleic acid*	0.00 ± 0.00	8.64 ± 5.29†	0.49 ± 0.15	0.00 ± 0.00	0.00 ± 0.00
5	1924	Palmitic acid*	0.00 ± 0.00	8.64 ± 5.95†	5.52 ± 1.12	0.44 ± 0.44†	0.00 ± 0.00
6	1984	Margaric acid*	0.00 ± 0.00	2.08 ± 1.53†	5.89 ± 2.41	0.00 ± 0.00	0.00 ± 0.00
7	1997	(*Z*)-10-Heptadecenoic acid*	0.00 ± 0.00	2.48 ± 1.46†	4.13 ± 0.59	0.00 ± 0.00	0.00 ± 0.00
8	2110	Oleic Acid*	1.23 ± 0.96†	2.69 ± 1.92†	78.78 ± 1.12	7.18 ± 5.22†	12.96 ± 7.66†
9	2140	Stearic acid*	0.74 ± 0.74†	1.48 ± 0.92†	2.11 ± 1.07	0.00 ± 0.00	1.39 ± 0.81†
10	2285	Arachidonic acid*	0.00 ± 0.00	3.04 ± 2.32†	0.59 ± 0.18	0.00 ± 0.00	1.14 ± 0.78†
11	2577	3-Methylpentacosane	2.33 ± 0.33	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
12	2600	*n*-Hexacosane*	5.23 ± 3.24	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
13	2700	*n*-Heptacosane*	8.03 ± 2.20	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
14	2733	13-Methylheptacosane	41.73 ± 4.37	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
15	2762	2-Methylheptacosane	28.37 ± 2.28	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
16	2830	13-Methyloctacosane	2.92 ± 0.52	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
17	2930	13-Methylnonacosane	9.44 ± 1.60	32.89 ± 23.57†	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
18	3142	Cholesterol*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	38.58 ± 7.98	0.00 ± 0.00

*Identified compounds by comparing retention time and mass spectra with those of authentic standards. †The compound that was not common among all colonies in the fraction.

Figures

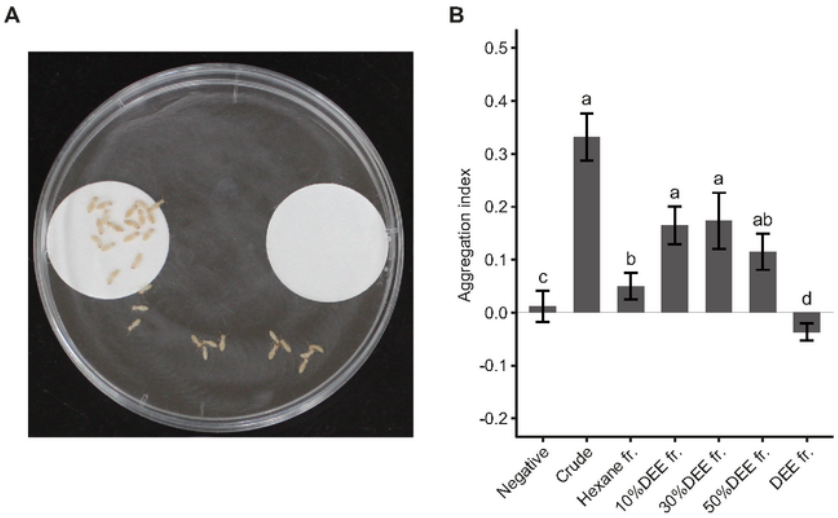

Figure 1

Two-choice bioassay for identifying active fractions. (A) A photograph of the two-choice assay when testing the attractant activity of the crude extract. More workers congregated on the filter paper where the crude extract was applied (left paper) compared to the filter paper treated with hexane (right paper). (B) Attractant activity comparison of the crude extract and its fractions after 5 min. The values denote the mean ± standard error of the mean (SEM) of 20 replicates (10 replicates × 2 colonies). Different letters

denote significant differences in the mean aggregation index among treatments (Fisher's exact test with Bonferroni correction, $P < 0.05$). The 10% and 30% DEE fractions exhibited higher attractant activity than other fractions and the negative controls.

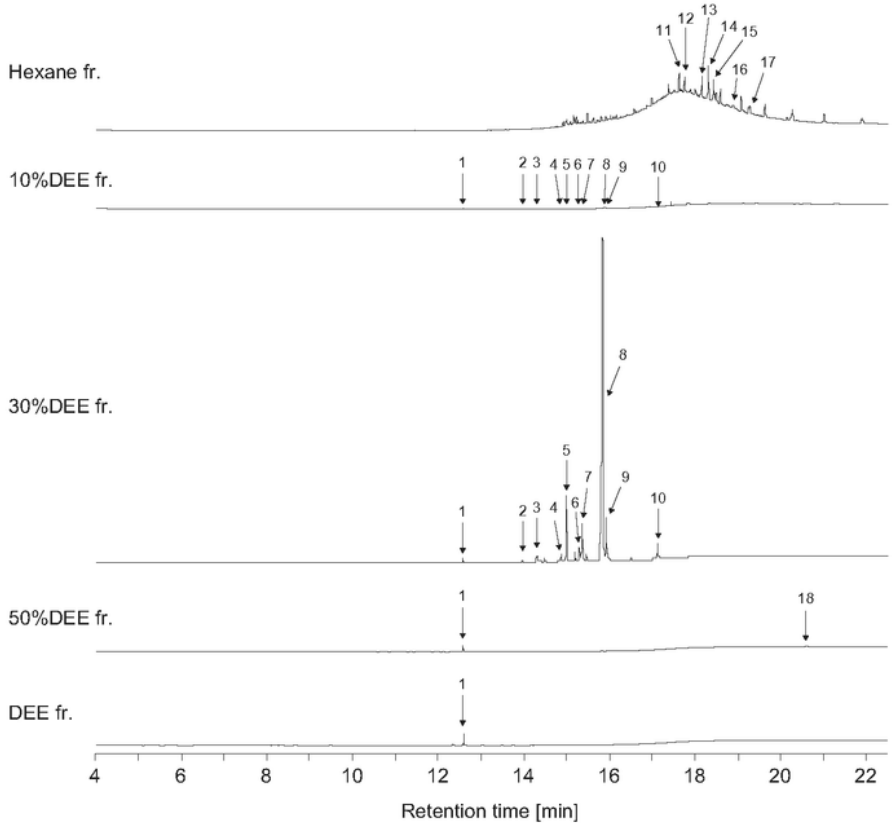

Figure 2

Representative gas chromatographs of the fractions. Chromatographs originated from colony KU141013–5. Numbers paired with arrows indicate distinct compounds. Peaks marked by arrows point to identified compounds detected in extracts from all colonies, whereas other peaks represent unidentified compounds, colony-specific compounds, or contaminants. Compound names are listed in Table 1.

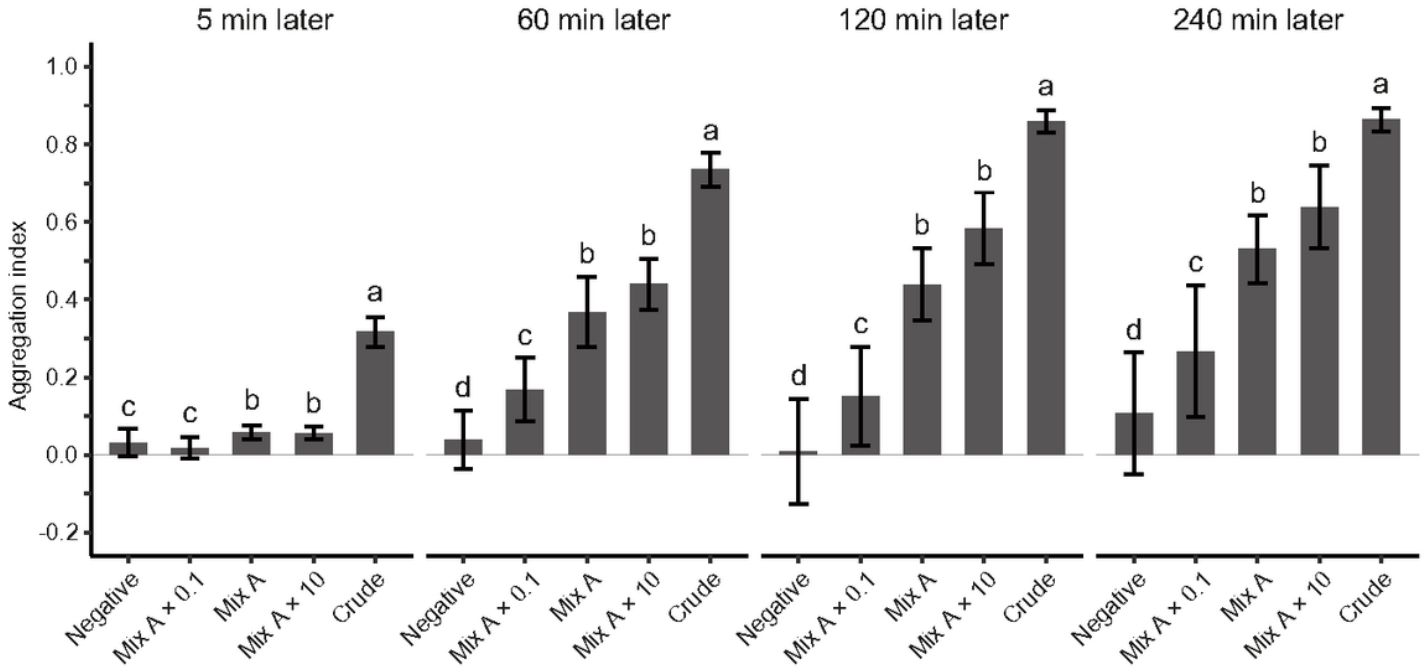

Figure 3

Dose-response of Mix A solution after intervals of 5, 60, 120, and 240 min. The values denote the mean ± standard error of the mean (SEM) of 20 replicates (10 replicates × 2 colonies). Different letters indicate significant differences in the mean aggregation index among treatments (Fisher's exact test with Bonferroni correction, $P < 0.05$). At 5 min, Mix A and its 10-fold concentrated solution exhibited marginally superior aggregation activity compared to the negative control. However, this level was notably lower than the crude extract. From 60 min onwards, AI values for Mix A and its 10-fold concentrated solution progressively rose, ultimately achieving approximately 63–73% of the crude extract's activity.

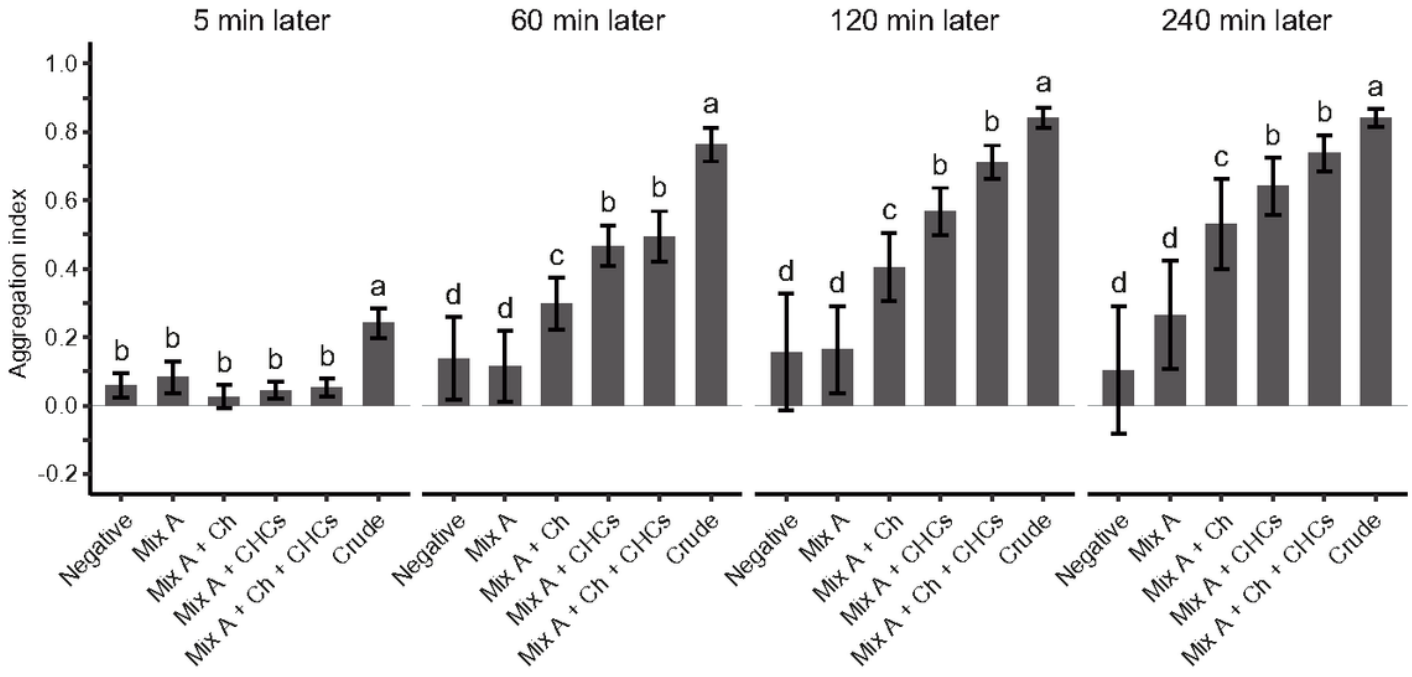

Figure 4

Attraction and arrestant activity comparison between Mix A and Mix A combined with cholesterol (Ch) and/or cuticular hydrocarbons (CHCs). The values denote the mean ± standard error of the mean (SEM) of 20 replicates (10 replicates × 2 colonies). Different letters indicate significant differences in the mean aggregation index among treatments (Fisher's exact test with Bonferroni correction, $P < 0.05$). At the 5 min mark, there was no significant difference in AI values among all treatments, with the exception of the crude extract. After 60, 120, and 240 min, AI values for Mix A combined with Ch and/or CHCs surpassed those of the lone Mix A treatment. Notably, the AI value for the Mix A combined with both Ch and CHCs approached about 87% of the crude extract's effectiveness.

Supplementary Files

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