**(<3500 words)**

**The strength of sexual signals predicts same-sex paring in termites**

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**Abstract**

Same-sex sexual behavior (SSB) is an enigma in behavioral ecology as it does not result in reproduction, contrasting with normal heterosexual behavior. Proximately, the loss of sexual signals is thought to be critical in the evolution of SSB as smaller sex differences may lead to indiscriminate mating. However, if animals engage in SSB even after recognizing the partner as the same-sex, sexual signal can enhance SSB as in heterosexual pairing. Here we show that the strength of sex pheromone is associated with the frequency of same-sex pairing in two *Coptotermes* termites. In termites, mating pairs engage in tandem runs, where a male follows a female that produces sex pheromones. We found that the female-female tandem was more common in *C. formosanus* whose females produce more pheromones. On the other hand, male-male tandem was more common in *C. gestroi*, whose males usually follow females with less pheromone. Furthermore, female-female tandem was more common than male-male tandem in *C. formosanus*, while female-female and male-male tandem were equally observed in *C. gestroi*. These results suggest that the strength of sexual signals predicts the same-sex pairing in a sex-specific manner. The proximate mechanism of SSB can be diverse, reflecting their heterosexual context.

**Keywords**: homosexual behavior, movement coordination, pheromone, same-sex sexual behavior, social insects

**Introduction**

Same-sex sexual behavior (SSB) is widespread among diverse animals with considerable variations across taxa [1–3]. In most species, the SSB is considered the result of mistaken identity [3,4], while in some cases, SSB provides adaptive value by making the best of a bad job with the shortage of heterosexual partner [5,6]. In either case, the occurrence of SSB is dependent on the mode of mating strategy in regular heterosexual contexts and is strongly affected by the accuracy and carefulness of sex identification of the mating partner [7]. Sexual communication, which is mediated via sex-specific attracting signals (e.g., sex pheromones), underlies such sexual identification of the other sex. Therefore, even if the SSB does not have adaptive value, the evolutionary patterns of sexual signals could shape the diversity of SSB across species, as a by-product. However, the role of sex-specific signals in SSB has remained unexplored.

As signal senders and receivers play different roles in mate pairing (refs), the effect of sexual signals on SSB should differ between sender-sender pairs and receiver-receiver pairs. For example, strength of sexual signals has the opposite effect on the sender-sender SSB and receiver-receiver SSB. In the species with weak sex-specific signals, more frequent SSB between receivers is expected, compared with the species with strong signals [8]. With small sexual differences, receivers can locate other receivers as well as senders, which may also provoke mistaken identity of the sex of partner. On the other hand, more SSB between signal senders could be possible in the species with strong signals. It is rarer to observe SSB between senders as they are usually passive sex during mating [9]. But with strong signals, it is easier for even senders to find other senders than other receivers. Thus, the strength of sexual signals is expected to modify the relative occurrence of receiver-receiver and sender-sender SSB across species.

Mate pairing in neoisopetran termites (termites here after) provides an ideal model system to study the evolution of SSB. Termites form life-long monogamous pairs to establish colonies [10]. During a brief period, alates (winged adults) disperse from their nests. Both females and males land on the ground, shed their wings, and run to search for a mating partner. Upon joining, a pair performs a tandem run. The male follows the female, maintaining contact in a highly coordinated manner while seeking a suitable site for colony foundation. Although tandem running often involves communication via sex pheromones, same-sex tandem run can be observed in either sex [11,12]. Sex pheromone should play different roles between female-female pairs and male-male pairs. In male-male pairs, same-sex tandem can happen once one male started to follow another male. Thus, SSB can happen because of mistaken identity. This implies that species with weak sex pheromones provoke male-male tandem pairing. On the other hand, female-female tandem cannot happen because of mistaken identity. As the sex role is fixed (females do not follow males) [12,13], female-female tandem runs initiate after one female changed sex role in advance. In this situation, sex pheromones of females rather facilitate same-sex tandems because females can easily follow another female.

In this study, we compared the same-sex tandem running behavior in two *Coptotermes* termites: *Coptotermes formosanus* Shiraki and *Coptotermes gestroi* Wasmann. These two species share the same chemical for sex pairing pheromones [14] and can form heterosexual tandem runs [15,16]. However, the quantity of pheromones is different between these two species, where *C. formosanus* has 10 times more pheromones than *C. gestroi* [14]. Therefore, we predict that male-male tandem is more frequent in *C. gestroi* than *C. formosanus*, while female-female tandem is more frequent in *C. formosanus* than *C. gestroi*.

**Methods**

*Termites and experimental arena*

We collected alates using a light-trapping system at dusk between 27-29 March for *C. gestroi* and 21-22 April, 1-2 May for *C. formosanus* in 2021 in Broward County (Florida, USA) during synchronized dispersal flights. All alates were collected at a single site. We brought the alates to the laboratory and maintained them on wet cardboard at 28°C. We used individuals who did not shed their wings by themselves to prevent any prior experience of tandem runs. After inducing shedding their wings, we observed their behaviors within 12 h. Each individual was used only once.

We performed all observations in an experimental arena made by filling a Petri dish (ø = 150 mm) with moistened plaster. The Petri dish had a clear lid during observations. A video camera (XXX) above the arena was adjusted so that the arena filled the camera frame. We introduced a pair of the termite into the arena. Each pair was recorded for 30 minutes in 30 frames per second (FPS). In total, we obtained 49, 61, and 44 videos for female-male, female-female, and male-male in *C. formosanus*; and 40, 40, and 45 videos for female-male, female-female, and male-male in *C. gestroi*. We extracted the coordinates of the centroids of termite movements from all obtained video, using the video-tracking system UMATracker [17]. We down sampled all coordinates to a rate of five FPS for subsequent analyses. We measured the diameter of dish and body length of two termites in pixel for each video, using a python program (link to github).

*Tandem analysis*

To compare the duration of tandem run between pair combinations and species, we automatically determined that pairs were in tandem when the distance between their centroids was within the sum of their body length. This simplest threshold is used in a previous study [18], and using other complex definitions (e.g., [16,19]) did not change the conclusion. We did not count very short interactions (< 5 seconds within two body length) as tandem runs and small separation (< 2 seconds) during tandem as interruptions.

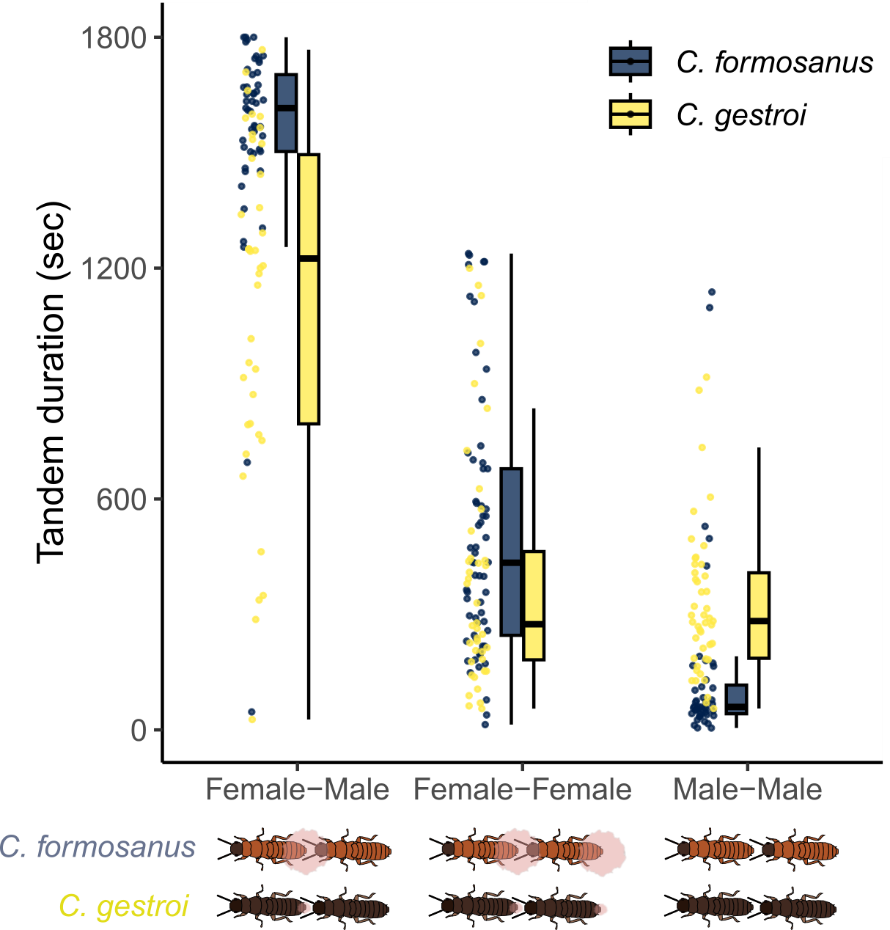
To compare the duration of tandem runs between species and combinations, we used mixed-effects Cox models [coxme() function in the coxme package in R [20]], with pair combination (or species) treated as a fixed effect and the ID of each video as a random effect. We compared the durations between species for each combination (heterosexual, female–female, or male–male) and among combinations for each species, separately. We also compared the proportion of the total time spent performing tandem runs across different pair combinations (or species), after transforming proportional data using logit-transformation after adding 0.01 to the observed proportions to avoid infinite values [21]. We used Welch t-test to compare them between species for each pair combination, while one-way anova with Tukey’s HSD to compare among combinations for each species.

Finally, for each separation event, we obtained the movement speed of both termites at after 5 second of separation and calculated the absolute difference of movement speed. We compared the difference of movement speed using linear mixed-effects models [lmer() function in the lme4 package in R (ref)], as the same approach with mixed-effect Cox model as above.

All data analyses were performed using R v4.3.0 [22], and source codes are available at github().

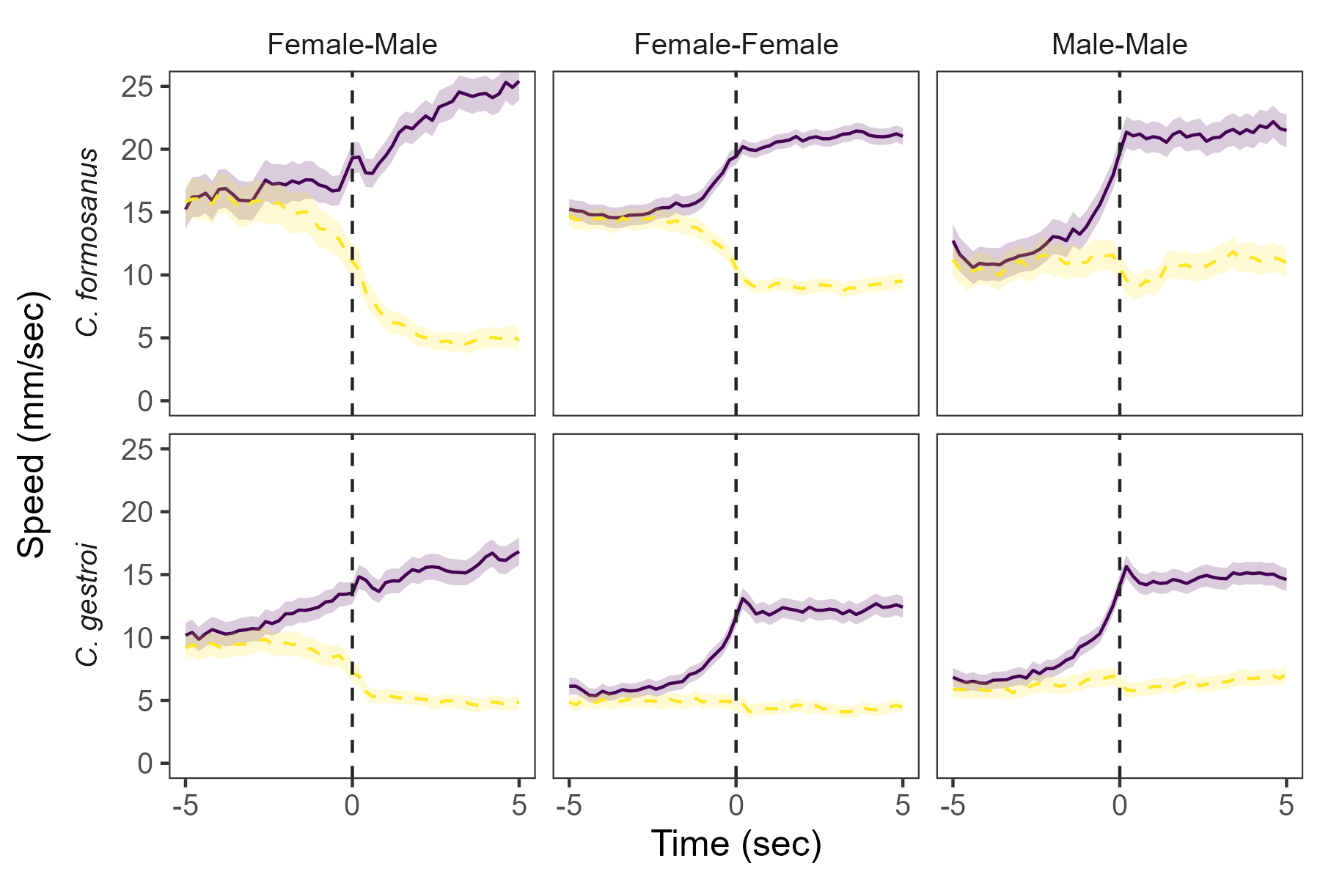
**Results**

The species difference of tandem running behavior depended on the pairing combinations. In heterosexual pairing, *C. formosanus* showed more stable tandems (coxed-model, Figure S1) and spent longer time for tandem runs than *C. gestroi* (t-test, Figure 1). Somewhat similar pattern was observed in female-female pairing, where *C. formosanus* showed more stable tandems than *C. gestroi* (coxed-model, Figure S1), without difference in the time spent in tandem runs (t-test, Figure 1). On the other hand, male-male pairing showed the opposite pattern, where *C. gestroi* showed more stable tandems (coxed-model, Figure S1) and spent longer time for tandem runs than *C. formosanus* (t-test, Figure 1). In either species, same-sex pairing was much more unstable than heterosexual pairing, with no difference between female-female pairs and male-male pairs in *C. gestroi*, while larger difference between female-female pairs and male-male pairs in *C. formosanus*.



**Figure 1.** Comparison of the total time spent in tandem among pairing combinations and species. The sex attracting pheromone is visualized below, with female leader releasing it and *C. formosanus* having larger quantity than *C. gestroi*.

After separations, all combinations showed speed differences between partners. As shown in previous studies, male followers sped up while female leaders slowed down after separation to enhance the probability of reunions. Although, similar movement differences were observed in same-sex pairings, the levels of difference between partners were smaller ().



**Figure 2.** Movement speed of termites before and after separation. Separation timing was determined when the distances between partners were larger than the sum of their body length. Solid lines indicate a partner moved faster, while dashed lines slower. Lines and shaded regions indicate mean ± 95CI.

**Discussion**

We found an association between same-sex pairing behavior and sex pheromone quantity in two termite species with shared chemicals. First, female-female pairing was more stable in *C. formosanus*, the species with larger quantity of sex pheromones, where females could use sex pheromones to maintain stable movement coordination with the same-sex individuals. Because females usually are leaders in *Coptotermes* tandem, females need to play follower (male) roles before the initiation of same-sex pairing [12]. In this situation, female-female pairing is not a result of mistaken identity, rather could be adaptive response as observed in *Reticulitermes* termites [5,11] or non-adaptive consequence of genetic correlation (ref). On the other hand, male-male pairing was more prominent in *C. gestroi*, the species with smaller quantity of sex pheromones.

All of the previous studies on same-sex tandem runs in termites have focused on *Reticulitermes* termites [11,12,23]. In *Reticulitermes* termites, same-sex pairing functions by providing survival benefits [5,11,24] and is not result of mistaken identity [12]. However, our study illustrates the distinct pattern in *Coptotermes* termites, where same-sex tandem in *Coptotermes* termites is rather accidental. First, in *Reticulitermes* termites, same-sex tandem pairs were as stable as heterosexual tandem pairs and formed a long-term relationship [12]. In *Coptotermes* termites, on the other hand, even once they form a same-sex tandem pair, either female-female and male-male tandems were far less stable than heterosexual tandems. This suggests that *Coptotermes* termites do not adjust their movement patterns to maintain same-sex tandems. Such lower motivation can be seen in their movement patterns upon accidental separation. Therefore, we conclude that same-sex tandem in *Coptotermes* termites is less functional than *Reticulitermes* termites.

Even though the same-sex pairing of Coptotermes termites is not adaptive, our study on their interspecific variations shows the proximate explanation of the diversity in SSB.

**Data accessibility**

Data that support the findings of this study are available in XXX

**Authors’ contributions**

NM: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft

SBL: Methodology, Investigation, Data curation, Writing – review & editing

TC: Resources, Writing – review & editing

**Competing interests**

The authors declare no competing interest.

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Supplementary material for

**The strength of sexual signals predicts same-sex paring in termites**

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This file includes:

Figure S1

**A graph of two people

Description automatically generated**

**Figure S1.** Comparison of the duration of tandem running until separation across different pairing combinations and species. Kaplan–Meier survival curves were generated for each pairing combination. The marks for censored data are not shown. Shaded regions show 95% confidence intervals.