**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 (Bagemihl, 1999; Bailey and Zuk, 2009; Scharf and Martin, 2013). In most species, the SSB is considered as the result of mistaken identity (Monk et al., 2019; Scharf and Martin, 2013), while in some cases, SSB provides adaptive value as making the best of a bad job with the shortage of heterosexual partner (Mizumoto et al., 2016; Young and VanderWerf, 2013). 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 (Lerch and Servedio, 2021). 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 signal receivers play different roles in mate pairing, 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 (Pfau et al., 2021). 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 (Burgevin et al., 2013). 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 (Nutting, 1969, p. 19). 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 (Matsuura et al., 2002; Mizumoto et al., 2022). 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 as a result of mistaken identity. As the sex role is fixed (females do not follow males) (Mizumoto et al., 2022; Park et al., 2004), 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* and *Coptotermes gestroi*. These two species share the same chemical for sex pairing pheromones (Chouvenc et al., 2020) and can form heterosexual tandem runs (Chouvenc et al., 2015; Mizumoto et al., 2021). However, the quantity of pheromones is different between these two species, where *C. formosanus* has 10 times more pheromones than *C. gestroi* (Chouvenc et al., 2020). 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 of *C. formosanus* and *C. gestroi* using a light-trapping system at dusk between X and Y April 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 shed their wings by themselves and observed their behaviour within 12 h after the flight. 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 XX, YY, and ZZ videos for female-male, female-female, and male-male in *C. formosanus*; and XX, YY, and ZZ videos for female-male, female-female, and male-male in *C. gestroi*. We extracted the coordinates of termite movements from all obtained video, using the video-tracking system UMATracker (Yamanaka and Takeuchi, 2018). We down-sampled all videos to a rate of five FPS for subsequent analyses. All data analyses were performed using R v4.3.0 (R Core Team, 2023).

*Tandem analysis*

To compare the duration of tandem run between pair combinations and species, we automatically identified whether a pair was performing tandem run, following methods described in previous studies (Mizumoto et al., 2021; Mizumoto and Dobata, 2019). During observations, pairs were determined to be in one of three states: 1) tandem running, 2) interacting but not tandem running, and 3) searching (individuals in the pair are physically separated). We defined individuals in the pair as interacting (or tandem running) when the distance between their centroids was less than 7 mm (18). This distance slightly exceeds the average body length because termites in a tandem run are nearly in physical contact (18). An interacting pair was considered to be performing a tandem run only if they met the following criteria (56). First, the interaction needed to last for more than 2 s; a very short separation (<2 s) was not regarded as a separation event. Second, both termites needed to move more than 30 mm while interacting. After separation, we considered that individuals were engaging in separation search until they interacted again for more than 1 s. These thresholds (7 mm, 2 s, 30 mm) were determined based on previous studies (18, 56, 57). To assess the sensitivity of our analyses, we tested how modification of these thresholds affected the results (range: 6.5 to 7.5 mm, 1 to 3 s, 20 to 40 mm, respectively). These modifications slightly changed the proportion of the time spent in tandem runs, but the qualitative conclusion that same-sex tandems were not different from heterosexual tandems was consistent (SI Appendix, Fig. S2).

We compared the proportion of time spent performing tandem runs across different pair combinations using an LMM, with pair combination (heterosexual, female–female, or male–male) treated as a fixed effect and the ID of the original colony as a random effect. We transformed proportional data using logittransformation after adding 0.01 to the observed proportions to avoid infinite values (59). We also used a mixed-effects Cox model [coxme() function in the coxme package in R (60)], with pair combination treated as a fixed effect and the ID of the original colony as a random effect.

*Movement Analysis upon Separation*

When termites in a pair are accidentally separated, females pause while males move to enhance the chances to reunite [19]. As moving speed is related to reunion efficiency [26], we measured the change in movement speed, focusing on a time window around separation events. We compared movement speed between the last 2 s before separation and the first 2 s after separation. For each separation event, we measured the mean movement speed for both time windows. Then, we used linear mixed models (LMM), with the time window treated as a fixed effect and video ID included as a random effect. The model was fit for each combination of pairs. The likelihood ratio test was used to determine the statistical significance of each explanatory variable (type II test). Finally, we examined if re-encounter after separation resulted in a tandem run or not.

**Results**

**Discussion**

All of the previous studies on same-sex tandem runs in termites have focused on *Reticulitermes* termites (Li et al., 2013; Matsuura et al., 2002; Mizumoto et al., 2022). In *Reticulitermes* termites, same-sex pairing functions by providing survival benefits (Matsuura et al., 2004, 2002; Mizumoto et al., 2016) and is not result of mistaken identity (Mizumoto et al., 2022). 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 (Mizumoto et al., 2022). 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. Nevertheless, by clarifying the interspecific variation of non-adaptive tandem in Coptotermes, our study 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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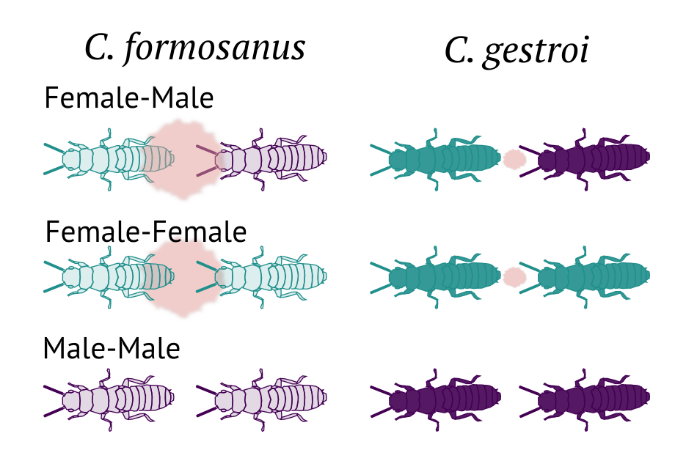
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**Figure 1.** Experimental scheme.

A picture containing chart

Description automatically generated

**Figure 2.** Comparison of the tandem duration among pair combinations and species.