Changes over Time in Activity Patterns of *Reticulitermes speratus* (Blattodea: Rhinotermitidae) Fed Fast- or Slow-acting Termiticides

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This study aimed to determine the insecticidal effectiveness of commercial fast- and slow-acting termiticides against *Reticulitermes speratus* by observing the changes in its activity patterns over time. Both the Petri dish and planar arena methods were used to evaluate colony mortality and activity changes over time associated with each termiticide, and the evaluation methods were compared. A colony elimination pattern was observed weekly in *R. speratus* colonies that ingested fast- or slow-acting termiticides using the planar arena method; however, the results showed that the Petri dish method was not suitable for evaluating slow-acting termiticides with an insecticidal effect that appeared after 3 weeks or more. In the colony-level evaluation, using the planar arena method, termites that ingested fipronil bait became sublethal within 1 week and died inside the 2 m plastic tube connected to the bait feeding site. The dead termites accumulated in the plastic tube and blocked access to the bait feeding site. In contrast, termites that ingested bait treated with bistrifluron or hexaflumuron during the first 3 weeks of the experiment spread throughout the colony. Decreased colony activity and immune mechanism collapse were observed in all screens and subnests, and more than 95% of termites in the colony had died after 10 weeks.

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INTRODUCTION

Subterranean termites build one or more habitats under or inside trees, and these chambers are inter-connected by a tunnel network. Therefore, the exploration and observation of community feeding patterns in subterranean termites are limited owing to the lack of visibility. *Reticulitermes* spp. is a pest that is widely distributed throughout the world, including Asia, North America, and Europe, and it causes serious damage to housing and wooden heritage properties (Rust and Su 2012). *Reticulitermes speratus* is mainly found in Far East Asian countries such as Korea and Japan. (Han *et al.* 1998; Nakayama *et al.* 2004; Im and Han 2021). Researchers in Korea and Japan have studied the insecticidal effects of both fast- and slow-acting termiticides (including pyrethroid, phenyl pyrazole, and benzophenylurea) in laboratory settings to find an effective method for eliminating *R. speratus* (Kubota *et al.* 2007; Kim and Chung 2017).

Laboratory studies investigating insecticidal effects have primarily used the Petri dish method because it is convenient for visually observing the insecticidal effects of the termiticides (Chouvenc *et al.* 2011), and this method has been selected for evaluating termiticides' ability to eliminate *R. speratus* regardless of whether the termiticide was fast- or slow-acting (Kim *et al.* 2010; Kim and Chung 2017; Im and Chung 2019; Yoon *et al.* 2021; Im *et al.* 2021). The JIS K 1571 Petri dish method (The subterranean termite standard test method of the Japan Standards Association, JSA) is mainly used to evaluate termiticides in Korea and Japan (Japanese industrial standard association, 2010). Although this method immediately confirms the insecticidal effect on termites directly exposed to termiticides, it does not confirm elimination at the colony level. For this reason, termite swarming is frequently observed from April to May every year even in wooden buildings treated with termiticides whose insecticidal effect has been confirmed by the Petri dish method (Im *et al.* 2021). Chouvenc *et al*. (2011) noted that termiticide contact patterns in a limited space, such as in a Petri dish, may be different from contact patterns in the field, and different patterns may emerge when eliminating colonies due to differences in termiticide transmission.

Fast-acting termiticides are needed to quickly control termites invading wooden buildings, and the Petri dish method is suitable to verify this. Although another appropriate method is needed to experimentally evaluate whether termiticides can effectively eliminate colonies, no studies have been conducted to confirm the colony removal effect of termiticides against *R. speratus*. To check whether *R. speratus* colonies are effectively removed by termiticides, it is necessary to introduce the planar arena method as applied to the study of *Coptotermes formosanus, C. gestroi* (Su 2005; Chouvenc 2018). The planar arena method is an experimental method that confirms the insecticidal effect of a termiticide at the colony level of subterranean termites in the laboratory stage.

Accordingly, the present study was conducted to determine the insecticidal effects of commercial fast- (Fipronil) and slow-acting (bistrifluron and hexaflumuron) termiticides on *R. speratus* colonies using both the Petri dish and planar arena methods. And the mortality and colony activity changes over time for each termiticide were evaluated by the planar arena method, which also allowed verifying the weekly activity status and colony elimination patterns of *R. speratus* fed fast- and slow-acting termiticides.

EXPERIMENTAL

Termite Collection

Logs inhabited by termites were collected from the Chungbuk National University campus forest and suburban experimental forest and stored in the laboratory. Following the method of Im and Han (2021), termites in the log were transferred to a wet cardboard scroll without dismantling the log and used in the experiment. The termites used in this study were identified as *R. speratus* via molecular biology analysis.

Petri Dish Method Based on JIS K 1571 (2010)

The Petri dish method was conducted according to the JIS K 1571 method (2010) in an acrylic box (Fig. 1A). To maintain relative humidity above 90%, a thick paper towel wetted with distilled water was placed on the bottom of the box (Fig. 1B), and a 3 mm diameter hole was drilled into the lid of the box for ventilation. The bioassay arena was prepared by diluting a dental plaster (Yoshino Co., HI-Stone, JPN) on a transparent acrylic

column (diameter, 80 mm, height, 60 mm) according to the manufacturer's recommended ratio (Fig. 1C). A sample holder was placed at the bottom of the acrylic column to prevent the insecticide from leaching into the bottom surface of the wooden sample (Fig. 1D).

Pine specimens (10 mm × 10 mm × 20 mm) were extracted by soaking in distilled water for 8 h, and then dried at 60 °C. for 16 h total of 10 times. Three termiticides were used in this experiment: fipronil (10 ppm), bistrifluron (5,000 ppm), and hexaflumuron (5,000 ppm). The three insecticides used in the experiment are termiticides that have been used in colony elimination to remove *R. speratus* colonies in Korea, and the concentration used is the manufacturer's recommended concentration. After the diluent was applied to the pine specimen for which the weathering operation was completed, it was volatilized for 8 h and placed in a sample holder inside the acrylic column (Fig. 1E).

A total of 165 units (150 units containing up to three instar workers + 15 units containing one soldier) were placed inside the acrylic column. The experiment was conducted for three weeks at a temperature of 23 ± 2 °C to suit the habitat environment of *R. speratus*. Dead termites were immediately removed to prevent mass extinction due to methane and mold growth. The experiment was repeated five times for each type of termiticide and mortality was measured at intervals of 2 to 3 days.

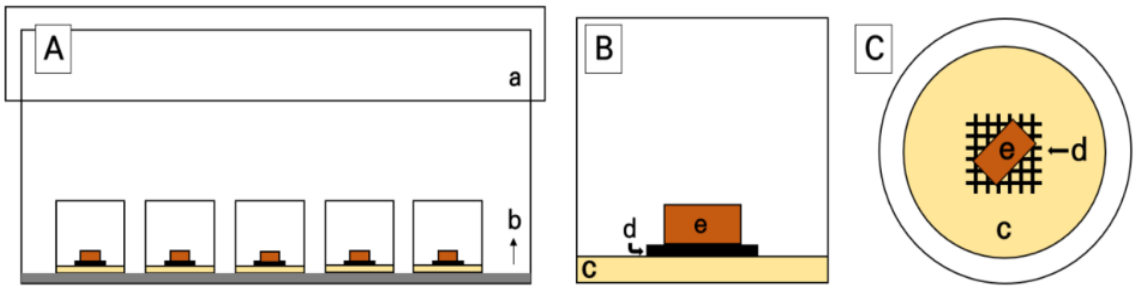

Fig. 1. Petri dish method (JIS K 1571) design used to evaluate the insecticidal effect of termiticides A: interior layout of the experimental space for maintaining humidity (dimensions: 220 × 400 × 150), B, C: side and top views, respectively, of the acrylic column. a: plastic box for experiment, b: paper towel + water, c: dental plaster, d: sample holder, e: wood sample + termiticide

Colony Establishment and Termiticide Treatment in Extended Planar Arena Method

The planar arena method was designed to fit the characteristics of *R. speratus* by modifying the methods of Su (2005) and Chouvenc (2018). Unlike the previous study that studied *C. formosanus*, a shelter (plastic jar) of a planar arena was attached to create a sub-nest inside, creating an environment more similar to a natural colony of *R. speratus*. Each screen was composed of a 1 mm transparent acrylic plate overlapping two transparent acrylic plates (150 × 150 mm, thickness of 2 mm) so that termite activity could be visually observed and photographed. The active ingredient (AI) jar containing the insecticide-treated bait was placed between the 3rd and 4th screens and connected to them by flexible plastic tubes (Fig. 2).

The termiticide types and concentrations used were the same as those used in the Petri dish method. Termiticides were dissolved in acetone, inoculated onto cardboard (20 mm × 20 mm, 200 μL each), and dried for 24 h. In accordance with Im and Han (2021), 200 μL of a 0.25% Nile Blue A solution was also inoculated onto the dried cardboard and

injected into the AI jar as bait. The control was inoculated with only acetone and 0.25% Nile Blue A, volatilized, and added to the AI jar.

The planar arena was 20 m in length and the flexible plastic tube connecting the planar arena was 4 m in length (Fig. 2B). *R. speratus* termites (12,000) were placed in 11 different spots (main nest + 10 subnests) seven times for one week. This procedure was repeated thrice for each termiticide.

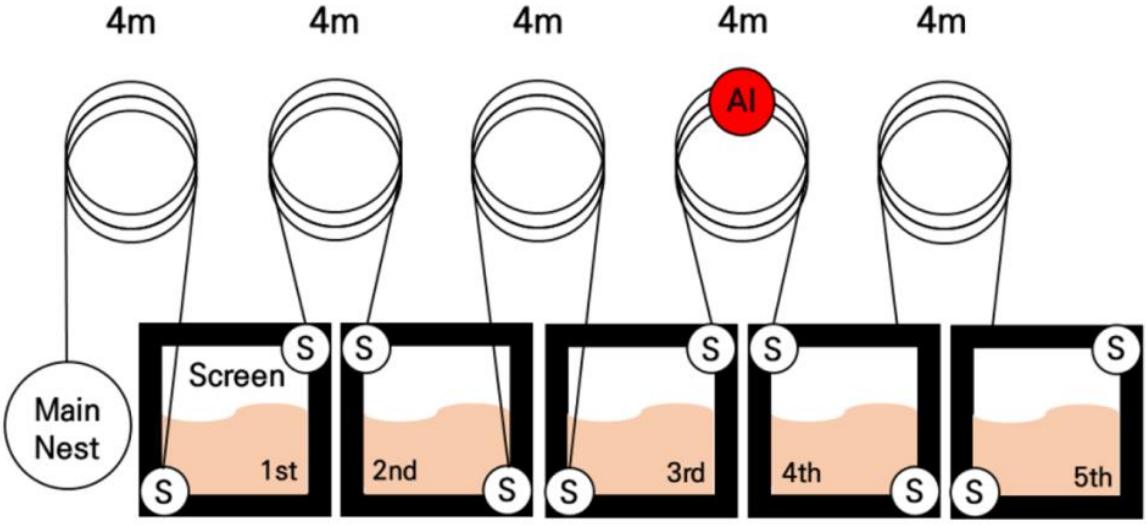

Fig. 2. Design of the planar arena for observing termite activity and elimination patterns. Main nest: a central *R. speratus* colony nest (≈12,000 termites) was reared in an 11.5 L container connected by clear plastic tubing to the first planar arena. Subnest (S): Supplementary habitat representing a characteristics subnest of *R. speratus*. Screen: a planar arena comprising silica sand and located 4 m from the central nest. AI: space where termites fed on termiticide-treated bait for the experiment. Each plastic tube had an inner diameter of 4 mm and a length of 4 m, and the total length of the planar arena was 20 m.

Calculation of Mortality

In the experiments using the Petri dish method (JIS K 1571), worker mortality was determined at 2- to 3-day intervals for three weeks. In the planar arena experiments, the number of remaining workers and soldiers were counted after 10 weeks, when the device was dismantled. Differences in the total number of colonies and ratio of soldiers according to termiticide type and concentration were investigated. Comparisons of all variables were performed through analysis of variance (ANOVA) using SPSS 26(IBM, 2021), and Tukey post hoc comparisons were employed where applicable. The results are presented as mean ± standard deviation (SD).

Relative Termite Activity in Planar Arenas

The method described by Chouvenc (2018) was used to measure the activity of *R. speratus* colonies over time. To verify the effect of termiticides on colony activity over time, the screens of all planar colonies were photographed weekly from week 1 to week 10. The weekly photographs were then used to count the number of termites in PhotoScape 3.7, after calibration. The number of termite populations in the same planar arena in each week was determined using the formula log (X+1) to calculate activity because the subterranean termites partially and temporarily gather in large numbers and then disperse. Termite numbers in all planar arenas were determined by taking photographs each week,

counting the termites, and then substituting the X in log (X+1) with these numbers to graph the log values for each count.

RESULTS AND DISCUSSION

Effects of Termiticides on *R. speratus* According to the Petri Dish Method

Table 1 shows the effects of fipronil, bistrifluron, and hexaflumuron on the mortality of *R. speratus* according to the Petri dish method. *R. speratus* workers exposed to fipronil had a 100% mortality rate after 5 days. On the other hand, *R. speratus* workers exposed to bistrifluron or hexaflumuron showed a mortality similar to that of the control group throughout the exposure period, reaching approximately 25% after the 3-week experimental period.

Fipronil is a phenylpyrazole-based chemical that is known to disturb the central nervous system of insects (Moffat 1993). Previous studies have confirmed that *R. speratus* exhibits a mortality rate of over 90% within 7 days after fipronil exposure (Kim and Chung 2017). Bistrifluron and hexaflumuron are known to prevent exoskeleton formation in insects by acting as chitin synthesis inhibitors (CSIs) (Xing *et al.*, 2014). When *R. speratus* workers were exposed to bistrifluron or hexaflumuron using the Petri dish method, approximately 3/4 of the workers survived for 3 weeks, which might be related to the molting cycle of *R. speratus* workers.

The mortality rate of the control group exceeded 30% within 3 weeks in the present study and it was also high in previous studies (Kim *et al.* 2014, 2020). This could be because the growth of microorganisms that may infect and kill termites is unavoidable on the long-term under a high-humidity environment. Indeed, Chouvenc *et al.* (2011) stated that the Petri dish method did not prevent the growth of microorganisms on the dead termites, as there was no substrate to bury the dead termites. In addition, Hughes *et al.* (2008) reported that termite mortality in the environmental conditions of Petri dishes was several times higher than that in termites' native habitat conditions.

Table 1. Mortality over Time in Each Termiticide-treated Group Using the Petri Dish Method

Days	Mortality (%)*			
	Control	Fipronil (0.001%)	Bistrifluron (0.5%)	Hexaflumuron (0.5%)
2	0.8±0.5^{a}	1.6±0.3^{a}	3.3±1.9^{a}	5.6±2.6^{a}
5	2.3±1.0^{a}	99.3±0.9^{b}	4.7±1.6^{a}	6.9±2.9^{a}
7	3.8±1.6^{a}	100.0^{b}	5.8±2.2^{a}	7.1±2.7^{a}
9	6.5±2.4^{a}	100.0^{b}	6.7±1.9^{a}	7.8±3.2^{a}
12	9.0±4.1^{a}	100.0^{b}	9.6±1.9^{a}	8.7±2.7^{a}
14	13.2±3.4^{a}	100.0^{b}	11.3±2.4^{a}	9.3±2.1^{a}
16	16.8±5.7^{a}	100.0^{b}	14.9±1.9^{a}	13.8±1.1^{a}
19	26.5±5.2^{a}	100.0^{b}	23.1±4.0^{a}	21.8±0.6^{a}
21	30.7±3.8^{a}	100.0^{b}	25.3±4.2^{a}	25.1±2.9^{a}

* The comparison in Table 1 was performed in the row direction according to each day.

Planar Arena Method for Colony Establishment and Treatment in Extended Arenas

Colony status at 10 weeks after termiticide treatment

Figure 3A shows the mortality of termites in the colony at 10 weeks after treatment with termiticides, and Fig. 3B shows the soldier ratio among the termites surviving in the colony at the same time. At 10 weeks after fipronil treatment, only ~20% of termites in the colony died, and the soldier ratio in the remaining termite colony was not significantly different from that of the control group. The soldier ratio observed in the control group is consistent with the results obtained by Matsuura (2002) (3.59 ± 2.50%) for 108 *R. speratus* colonies. Due to the acute toxicity of fipronil, the workers who ingested the fipronil bait died very quickly, and fipronil could not be transmitted to other termites in the colony.

On the other hand, in the colonies treated with bistrifluron or hexaflumuron, the termite mortality was greater than 95%. This is consistent with the results of previous studies (Tsunoda *et al.* 1998; Kubota 2011) in which bistrifluron and hexaflumuron were effective in eliminating *R. speratus* colonies. The soldier ratio in the surviving termite colonies was above 40% in both treatments, which was significantly different from that in the control group. Soldiers who did not molt were not affected by bistrifluron or hexaflumuron, and therefore survived relatively longer than workers. Accordingly, a high soldier ratio was obtained.

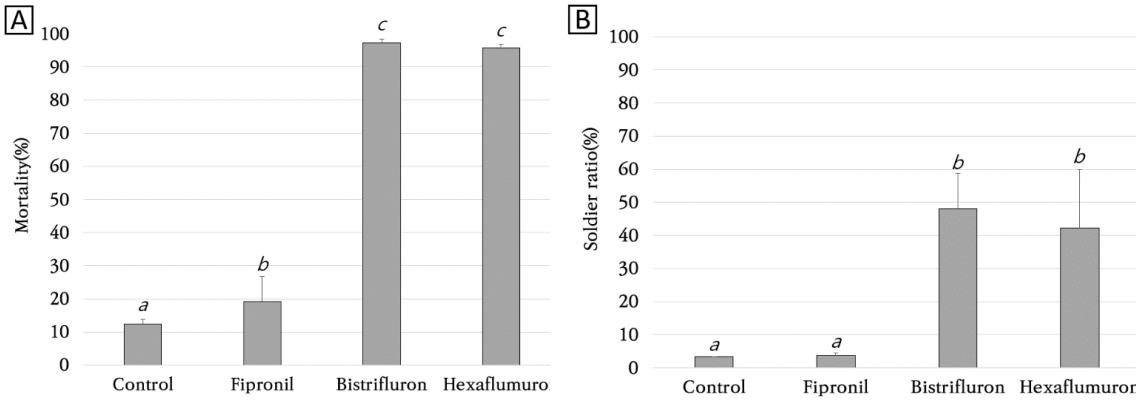

Fig. 3. Colony status at 10 weeks after termiticide treatment (mean ± SD). The main nest, subnest, and plastic tube were dismantled to determine the numbers of all termites. A: Mortality in *R. speratus* colony by treatment condition, B: Soldier ratio (%) in *R. speratus* colony by treatment condition (ANOVA, Tukey post hoc test, α = 0.05)

Colony activity of R. speratus over time in the planar arena

After 2 weeks of treatment with the termiticide fipronil, dead *R. speratus* were observed in screens C and D, 2 m away from the AI jar, and the activity of termites started to decrease. However, after 5 to 7 weeks, termites recolonized screens C and D and their activity began to recover (Figs. 4C, D). After 10 weeks, the relative termite activity in screen C, the closest to the AI jar, was 1.99 ± 0.19, indicating that termite activity was completely restored to its initial state (Fig. 4C). These results indicated that, in the early stage of exposure, termites died rapidly in the area close to where fipronil was placed, but over time fipronil exposure had little effect on the overall activity of *R. speratus* colonies. This suggests that the effective range of fipronil as an insecticide against *R. speratus* is not very wide, which is

consistent with the results of previous studies (Ripa *et al*. 2007; Saran and Rust 2007). It was demonstrated that the fipronil extermination effect had a range of up to 2 m against *R. flavipes* and *R. hesperus.*

In the case of *R. speratus* colonies treated with bistrifluron or hexaflumuron, their relative activities on screen C after 2 weeks were 2.33 ± 0.15 and 1.93 ± 0.26, respectively, showing no decrease in activity compared to the control group (Fig. 4C). However, after 3 weeks, the activity of the colonies began to decrease rapidly. At 6 weeks, the relative activities of the colonies treated with bistrifluron and hexaflumuron were 0.28 ± 0.40 and 0.32 ± 0.45, respectively, and after 8 weeks, the activity of all colonies converged to 0. This decrease in activity of *R. speratus* colonies was observed in all screens. These results indicated that termite workers who ingested bait containing CSI termiticides did not enter the sublethal state for at least 3 weeks. In addition, workers who ingested the termiticide during this period were able to freely move around the planar arena and spread the termiticide to all workers through trophallaxis and cannibalism.

In addition, bistrifluron was shown to decrease termite activity about 2 weeks earlier than hexaflumuron (Fig. 4). This means that even for CSI termiticides, the onset time of their efficacy differs depending on the insecticide type. This is consistent with the results of Kubota *et al.* (2006), who reported that the insecticidal effect of bistrifluron on *R. speratus* appeared 2 to 3 weeks earlier than that of hexaflumuron.

Changes in the appearance of termites exposed to termiticides

Colonies treated with fipronil started to show dead termites in the AI jar and the 2 m plastic tubes connected to both sides after 1 week. At 2 weeks of treatment, dead termites accumulated inside the connector, the passageway was blocked (Fig. 5A), and the colony was divided into two groups. This has also been observed in previous studies performed on the Formosan subterranean termite (Su *et al.* 1982; Fei and Henderson 2005; Woodrow *et al.* 2008; Wagner 2003). On the contrary, the colonies treated with bistrifluron or hexaflumuron continued to ingest the baits as shown in Fig. 5B. Termites stained with Nile Blue A were observed in all screens suggesting that CSI termiticides such as bistrifluron and hexaflumuron were widely spread throughout the colony by termites that ingested the bait from the AI jar.

After 6 weeks, egg piles and larvae were observed in the subnests and screens of the control and fipronil-treated colonies, as shown in Fig. 5C. This suggested that nymphs of fipronil-treated colonies could differentiate into secondary reproductives and lay eggs. In this regard, Matsuura *et al.* (2010) stated that several nymphs differentiate into such stages because volatile inhibitory pheromones that block differentiation are not secreted in the absence of primary queens or secondary reproductives in *R. speratus* colonies. However, even in colonies treated with bistrifluron or hexaflumuron, dead termites were observed after the 3rd week, and as time elapsed, termite carcasses were observed throughout the screens as shown in Fig. 5D.

In the main nest, workers died in the jackknife position while molting (Fig. 5E). This posture is observed when workers who ingest CSI termiticides die without coming out of the existing cuticle during the molting process (Kakkar and Su 2018). After 7 weeks, the activity of termites was significantly slowed down in all screens, and cannibalism was observed among workers in the subnest (cream color, Fig. 5F). Xing *et al.* (2014) reported that a large amount of uric acid accumulates due to CSI ingestion, explaining the cream-colored body of termites. Kubota (2011) noted that when bistrifluron bait ingestion slowed the activity of *R. speratus* workers, they were eliminated through cannibalism by fellow

workers. Therefore, the CSI component is believed to persist in the colony through trophallaxis and cannibalism between termite individuals.

When all nests and screens were dismantled after 10 weeks of experimentation, many secondary reproductives were observed in fipronil-treated colonies (Figure 5G). This indicated that the colony exposure to fipronil was blocked by the termites that died in the tunnel connecting the AI jar to the experimental set, and that the living termites continued to undergo class differentiation and colony development. This fact also explains why termite damage continues to occur in historical wooden buildings in Korea that are treated with fipronil bait devices. In contrast, parasitic insects such as mites were observed in colonies treated with CSIs as shown in Figure 5H. This means that colony elimination can be accelerated by parasites as well as insecticidal effects over time in CSI-treated colonies.

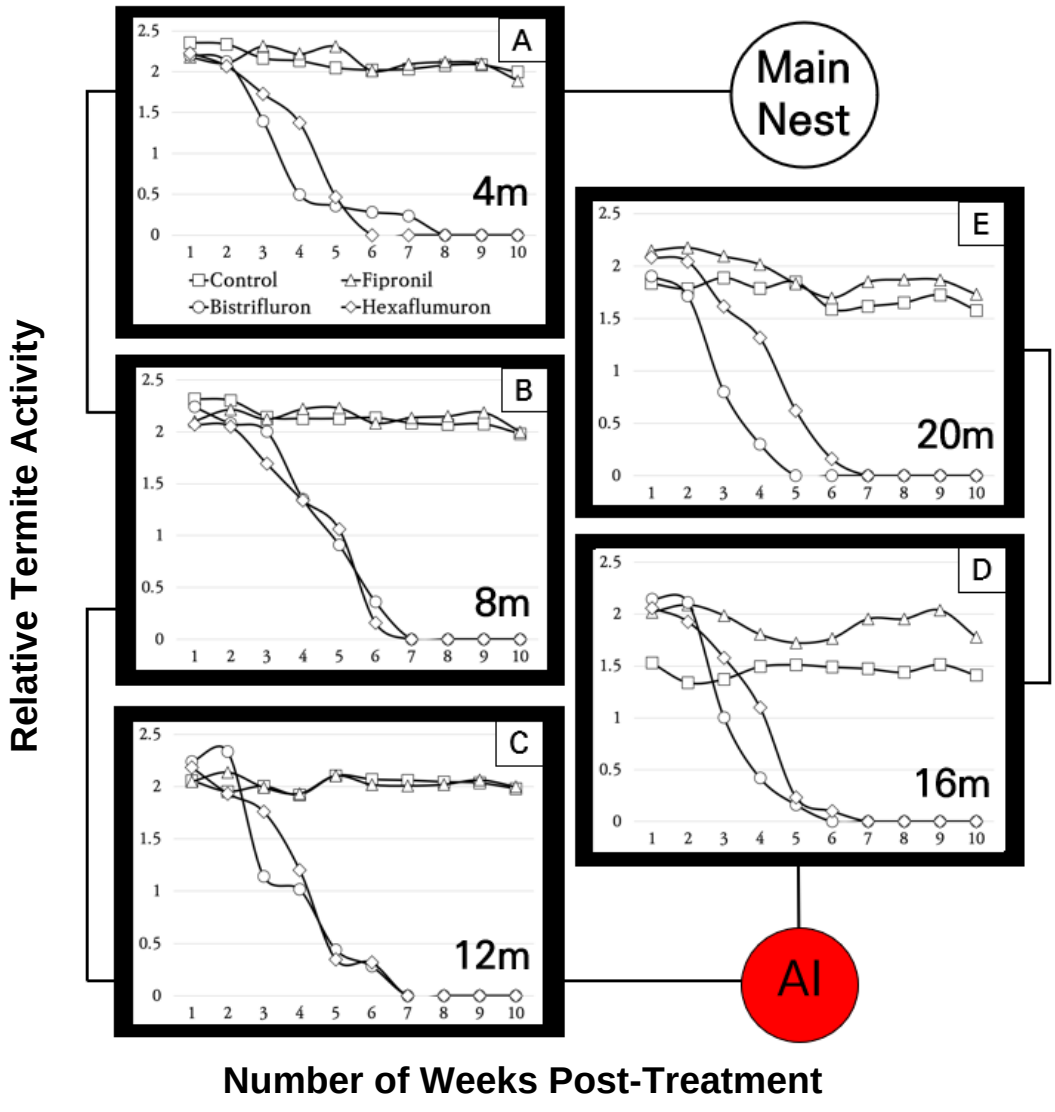

Fig. 4. Relative termite activity in planar arenas over the 10-week period after different termiticide treatments were applied. Activity under the control treatment is also shown. Considering the main nest as the starting point, each screen is then connected to the next by a 4 m plastic tube (black line), and 2 m plastic tubes connected screens C and D to the sides of the AI Jar. The line graph represents the relative termite activity (mean number of termites, data log (X + 1) transformed), n = 3 colonies (ANOVA, Tukey post hoc test, α = 0.05).

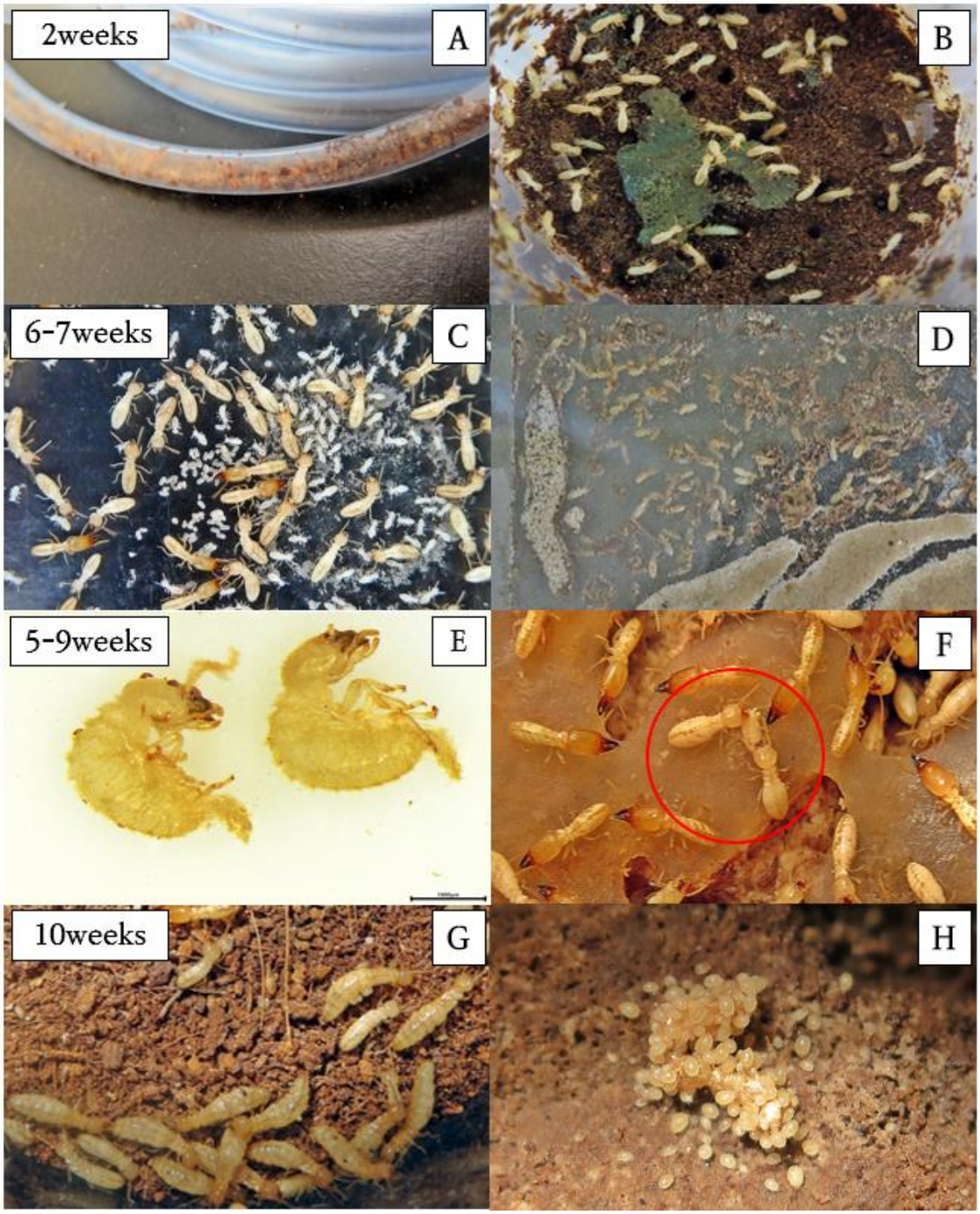

Fig. 5. Changes in *R. speratus* colonies over time during treatment with fast- and slow-acting termiticides. B: Termites feeding on a CSI bait with 0.25% Nile blue inside the AI jar. C: Egg piles, the larvae were seen on the screen after treating *R. speratus* with fipronil bait for 6 weeks. D: Dead termites on the screen after treating *R. speratus* with CSI bait for 3 weeks. E: The jackknife position of workers in CSI bait-treated *R. speratus*. F: Termiticide transfer by cannibalism in CSI bait-treated *R. speratus*. G: Secondary reproductives developed from nymphs in *R. speratus* colonies treated with fipronil for 10 weeks. H: Mites observed in CSI bait-treated *R. speratus* colonies with weakened immune mechanisms.

CONCLUSIONS

The insecticidal effects of fast- and slow-acting termiticides on *R. speratus* were evaluated in a laboratory environment using both the Petri dish and planar arena methods. The results revealed that the Petri dish method (JIS K 1571) was unsuitable for evaluating the effect of slow-acting termiticides, for which more than 3 weeks were required to confirm their insecticidal effects. Accordingly, the applicability of the evaluation methods of fast- and slow-acting termiticides was confirmed based on the insecticide effect time.

In the planar arena experiment, termites that consumed the fipronil bait were rendered sublethal within a week and died inside the 2 m plastic tubes connected to the bait feeding jar. Dead termites accumulated in the 2 m plastic tube and blocked continuous access to the bait space. These results suggest that even in field applications, re-invasion of bait feeding sites will be blocked because termites that die from fipronil exposure will accumulate in the tunnels of *R. speratus* nests.

In contrast, bistrifluron- or hexaflumuron-treated bait continued to be ingested by termites from the start of the experiment until week 3. Individuals were blue-stained after ingesting bait and were observed across all screens. After 7 weeks, there was an almost complete lack of termite activity on all screens due to the insecticide effect of CSIs, and a colony mortality of 95% was confirmed at the end of the 10-week experiment. These results suggest that the use of slow-acting termiticides such as bistrifluron or hexaflumuron can eliminate R. speratus colonies that damage wood within buildings and heritage properties.

In the present study, insecticide patterns of termiticides reported to have insecticidal effects on *R. speratus* were more specifically determined using mortality and activity measurements, as well as photography. The colony elimination pattern of each termiticide can be more accurately identified if the planar arena method used here is applied to the laboratory evaluation of termiticides administered to *R. speratus* in the future.

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