The Effect of Variables on Laboratory Termite Testing: Part I—Worker:Soldier Ratio

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Abstract

The objective of this study was to determine if the same soldier:worker ratio could be used in the eastern subterranean termite (*Reticulitermes flavipes*) and the Formosan subterranean termite (*Coptotermes formosanus*) in standard tests. Native (*R. flavipes*) and introduced (*C. formosanus*) subterranean termite species were tested in an American Wood Protection Association E1 standard laboratory test. Statistically equivalent weight losses were found as long as the ratio was within 10 percent of the rate required by the standard.

The development of biologically sound procedures for evaluating the efficacies of wood preservative formulations for protecting wood from subterranean termites is essential. Annually, damage to wood-frame structures in the United States by these organisms, together with wood and soil treatments to prevent and/or control them, amounts to over a billion dollars (Su 1994). There are several species of subterranean termites in the United States, but this study focused on an introduced species, the Formosan subterranean termite (FST), *Coptotermes formosanus* Shiraki, and *Reticulitermes* spp., a genus native to the United States with the most economically important species being the eastern subterranean termite (EST), *Reticulitermes flavipes* Kollar.

The aggressive behavior of *C. formosanus* and its night dispersal flights, attraction to lights, large colony size, ability to colonize wood without soil contact, and high expansion rate into new territory are a concern to those currently or potentially in the projected dispersal path of *C. formosanus*. The northern limit of migration is projected to be at 35°N latitude, which is roughly the state line between Mississippi and Tennessee (Amburgey 2008). Damage is rarely detected until severe loss has occurred, due largely to nighttime dispersal flights, attraction to lights, and the large size of *C. formosanus* colonies, often located within structures being attacked. *Coptotermes formosanus* have also been found to move at faster rates while using less energy than the native *Reticulitermes* spp. (Shelton and Appel 2001). Because of the threat of economic damage and biological aspects that differ from those of *Reticulitermes* spp., new strategies, products, and environmentally safe chemicals are being developed to help control the FST. New wood protection products are also being evaluated at a greater rate now, especially since the voluntary restriction of chromated copper arsenate in 2004 (Barnes 2008).

With the development of new products comes the need for accurate and up-to-date testing procedures to evaluate the efficacies of these products. Research has expanded the knowledge base concerning *C. formosanus* and has been instrumental in understanding its biology. Research on *C. formosanus* has revealed differences in the behavior of the FST compared with that of *Reticulitermes* spp., such as varying population numbers along with different soldier proportions and other behavioral traits (Haverty 1979). With these variations, evaluation of testing procedures specified in American Wood Protection Association (AWPA) Standard E1 (AWPA 1997), when using FST as the test species, is needed to evaluate accurately whether a product qualifies for use as a control method. The test methods were studied

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extensively in this research, and changes made in parameters of Standard E1 are suggested to better suit the behavior of the FST as well as species of *Reticulitermes*.

Species of *Reticulitermes*, especially *R. flavipes,* are also economically important insects. *Reticulitermes flavipes* is ubiquitous, readily found throughout the state of Mississippi as well as in many other areas throughout the United States. The biology of *C. formosanus* differs in many ways from that of *R. flavipes*. As mentioned earlier, differences include colony size, and alate size and color, as well as other behavioral traits such as night dispersal flights. In the United States, tests to determine the efficacies of new biocides and/or products usually are conducted with *Reticulitermes* species, but a few companies choose to test only against the FST since it is typically the more aggressive of the two species. Additionally, it is assumed that if a product controls *C. formosanus*, it will control *R. flavipes*.

Tests in this study were conducted to determine if the methodology specified in the standard was manageable and easily verified. AWPA Standard E1 is a 28-day laboratory test that is used primarily to determine the efficacy of wood preservatives or products against subterranean termites. This test is very important in initial screening to determine if preservatives and products are viable and warrant further testing (i.e., field testing). Testing is accomplished by placing either one or two preservative-treated test blocks or products into a test jar with an appropriate amount of sand, water, and termites.

The blocks can either be tested with a ''no-choice'' or ''choice'' method. The ''no-choice'' method (one single test block) is used primarily to determine the toxic threshold of chemicals or products to termites and to determine the repellency if no other choice is available. The ''choice'' method contains two test blocks, one test sample and one untreated control. It is used primarily to determine if termites are repelled from the test product if a favorable choice is also available. This standard was first promulgated as a standard in 1972, as Standard M12-72 (AWPA 1972), and was first changed to E1-72 in the 1991 AWPA Book of Standards (AWPA 1991). This standard was first revised in the 1997 Book of Standards (AWPA 1997), which is the version used for this research. Subsequent revisions occurred in 2009, 2013, 2015, 2016, and 2017 (AWPA 2017).

For new products to be accepted into the marketplace, each product must pass multiple tests, including the AWPA E1 test, or sometimes its counterpart, American Society for Testing and Materials (ASTM) Standard D3345 (ASTM International 2004). ASTM standard D3345 parameters are very similar to those of AWPA E1. The most relevant variation is the location of the test block within the testing jar. In the ASTM standard, the test block is placed at the bottom of the jar and covered with moist sand, while in the AWPA method the block is place on top of the sand.

Many variables can affect the outcome of tests when dealing with biological factors. In the AWPA E1 test method, none is more important than the termites themselves. There are multiple opportunities to alter the outcome of test results by not being consistent throughout the testing procedure. This paper, the first in a series, will evaluate the role that soldier:worker ratio plays in the outcome of test results.

Tests (Haverty 1976) indicated that *C. formosanus* has a specific physiological mechanism for regulating soldier proportions. This study indicated that *C. formosanus* has been known to maintain a very high proportion of soldiers to workers, as high as 38 percent in some cases. While excess soldiers were likely eliminated by cannibalism in some colonies used for lab experiments, excess soldiers often succumb from microbial contamination because of starvation. It is believed that workers only feed sufficient soldiers to defend the colony. *Coptotermes formosaus* maintains numerically steady proportions of soldiers to workers within expansive limits (Haverty 1976). Many authors have suggested that there are behavioral and physiological mechanisms for maintaining caste ratios under the control of ''ectohormones,'' later identified as juvenile hormones (Haverty 1976, Mao et al. 2005). These pheromones are distributed through trophallaxis, which is the transfer of food or other substances among colony members. The authors stated that if the pheromone level is too high, soldiers are cannibalized; if it is too low, soldiers can be produced. Juvenile hormones are considered the most valuable regulator of the caste structure in termite colonies (Mao et al. 2005). This study showed evidence that soldier caste proportions help regulate caste differentiation in workers by regulating juvenile hormone levels.

A 10 percent soldier population for laboratory test groups of *C. formosanus* has been found (Smythe and Mauldin 1972). Some authors (King and Spink 1974) report around an 11 percent field soldier population for *C. formosanus* during the first two and a half years of colony establishment. Others found that foraging termites from two FST colonies had soldier percentages of 5.0 and 4.7, respectively (Haverty 1976). These studies were likely some of the data used for determining the current ratio of worker to soldiers used in the AWPA E1-97 standard. Time of collection also has effects on the soldier:worker ratios collected. One study (Howard and Haverty 1981) found that soldiers were most numerous immediately prior to alate flights and the least abundant during the winter season. This was also seen during termite collection for this study. Due to the differences in ratios found above, it was important that tests be conducted to verify the ratio range required for species of *Reticulitermes* and *C. formosanus*.

The AWPA standard was changed in 1997 (AWPA 1997) to 5 percent soldiers for *R. flavipes* and kept at 10 percent soldiers for *C. formosanus*. Section 9.2 of AWPA E1-97, the standard on which this research was based, stated that requires soldier:worker ratios of 360:40 for *Coptotermes* and 380:20 for *Reticulitermes*. The current tests were conducted according to AWPA standard E1-97 (AWPA 1997) except for the soldier:worker ratio.

Methods and Materials

Termite collection

Termites were collected from bucket traps located near active colonies at the McNeill Formosan Termite Research Facility, Pearl River County, Mississippi. The buckets were collected from multiple colonies after termites had thoroughly infested the untreated material. Once the buckets were collected, bucket material was stored in lidded garbage cans before being separated from the wood for testing. Termites were separated from the infested wooden material by delicately shaking the termites through a screen onto a clean tray for further collection. Once in the clean plastic trays, the termites and fine debris were further separated by laying damp paper towels over the termites and debris to remove the termites from any further debris. The termites were then counted using a vacuum aspirator that pulled the termites into a jar to be later transferred into the test containers. This sequence is shown in Figure 1.

Experimental groups of 400 termites (*R. flavipes* and *C. formosanus*) were established at various worker:soldier ratios to determine a favorable ratio for use with the AWPA Standard E1 test method and to determine the effect of soldier:worker ratios on overall wood consumption. Section 6.2.2 of AWPA Standard E1-97 (AWPA 1997) specifies that 5 percent soldiers for *R. flavipes* and 10 percent soldiers for *C. formosanus* be used per test unit. The only way to maintain this type of accuracy would be to count individual containers to verify this ratio. Time constraint is a disadvantage to counting all termites in individual containers, especially if large numbers of variables are tested.

Assembly of test units

In this experiment, three different soldier:worker ratio groups (Table 1) were counted and used to determine their effect on wood consumption. Tests on both *R. flavipes* and *C. formosanus* were conducted using replicates of 10. All test blocks were 100 percent untreated southern pine (*Pinus* spp., SYP) sapwood selected according to the E1 standard. The test blocks were first conditioned at 105°C for 2 days to reach an ovendry weight. The samples were allowed to equilibrate to room conditions for a minimum of 2 days prior to initiation of testing. Test bottle lids were loosely fitted to allow proper air exchange and were stored in a climate-controlled laboratory for the duration of testing.

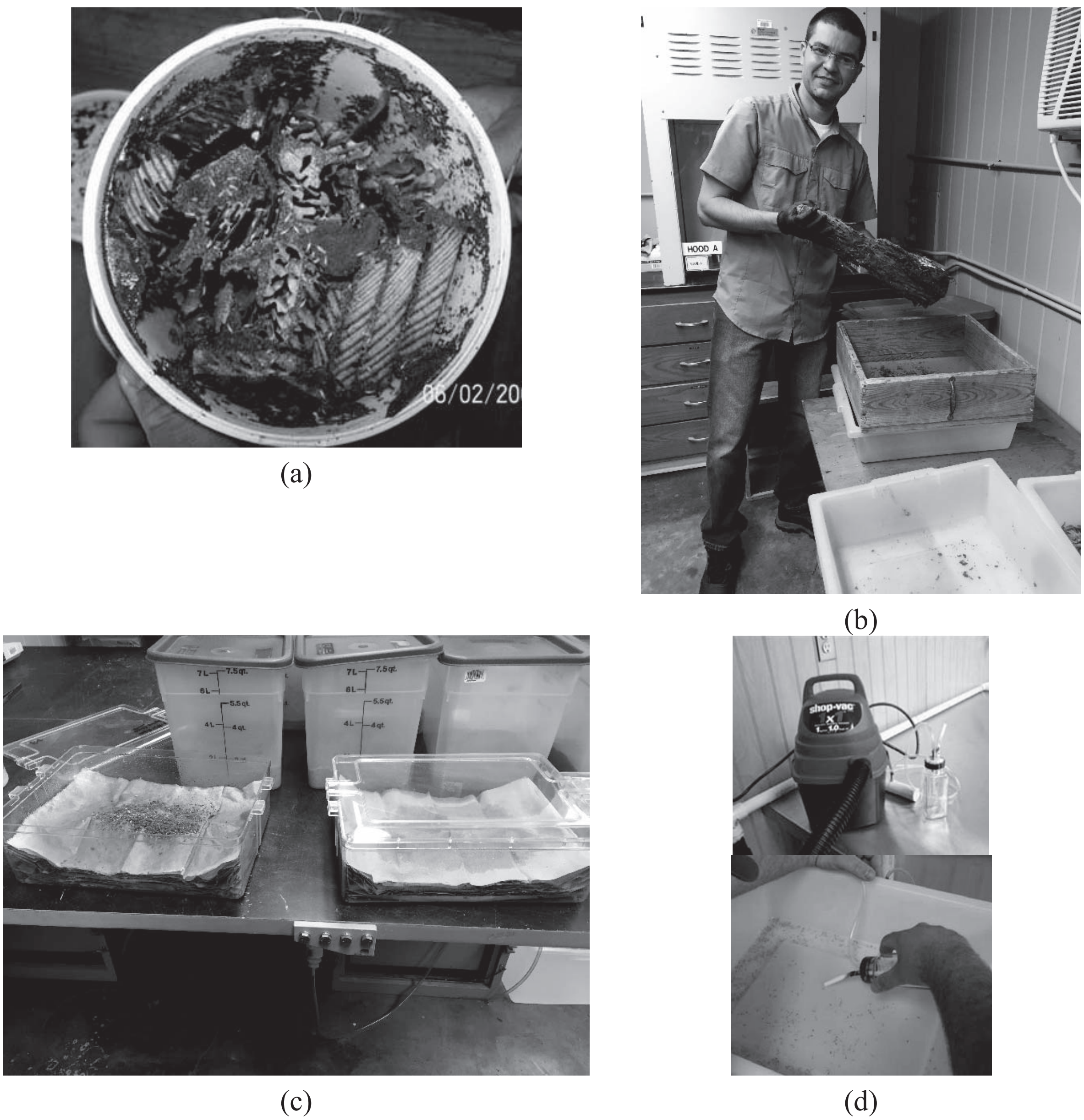

Figure 1.—Termites were collected from infested wood that was placed in (a) bucket traps located near active colonies and (b) gently separated from infested wood, (c) removed from debris with moist paper towels, and (d) collected and counted with a vacuum aspirator.

Table 1.—Test parameters to determine the effects of differing soldier: worker ratios of Reticulitermes flavipes *(EST) and* Coptotermes formosanus *(FST) on wood consumption in laboratory tests.*

Soldier:worker ratios (% workers)	Species	Replicates	Total bottles
0:400 (100)	EST, FST	10	20
20:380 (95)[a]	EST	10	10
40:360 (90)[b]	EST, FST	10	20
80:320 (80)	FST	10	10
0:0 (0)	No termites	20	20
Total			80

[a] Soldier:worker ratio of *R. flavipes* required by standard.
[b] Soldier:worker ratio of *C. formosanus* required by standard.

Tests with both species were conducted at the Mississippi State University Formosan Termite Research Facility located at McNeill, Mississippi.

SYP wafers (25 by 25 mm in cross-section by 6 mm in the longitudinal direction) were selected according to the standard. They were placed in 80 by 100-mm French square jars on top of the sand substrate. The jars were filled with 120 mL (approximately 150 g) of screened and washed silica sand and 30 mL of distilled water 2 hours prior to test blocks being introduced. The SYP wafers were introduced into the jars using a pair of tweezers with care to place each block touching two sides of the jar. Once the blocks were in place, 400 termites were added to each jar according to the ratios found in Table 1. Figure 2 is an example of jars after introduction of termites. After the 28-day exposure period, samples were removed from the bottles, cleaned to remove any residual sand or termite feces, oven-dried, and weighed; weight losses were then determined. Data were analyzed using analysis of variance and means compared using Tukey's test at $P = 0.05$.

Results and Discussion

Extensive damage was observed on all wood test blocks at the conclusion of testing (Fig. 3). Tukey's test showed no significant difference in weight loss of untreated SYP sapwood test blocks after 4 weeks of exposure when comparing 100 percent with 95 percent *R. flavipes* workers and no significant difference when comparing 95 percent with 90 percent *R. flavipes* workers. There was a significant difference, however, when comparing 100 percent with 90 percent *R. flavipes* workers (Fig. 4). Only eight jars were tested in the 90 percent *R. flavipes* worker test group and no jars in the 80 percent group due to difficultly in locating sufficient soldiers.

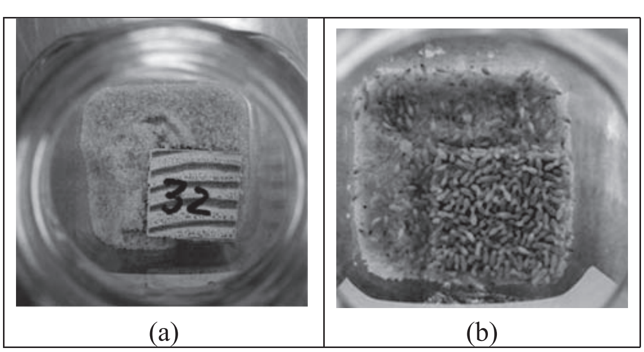

Figure 2.—Test block on sand (a) immediately after placement and (b) 30 minutes after placement.

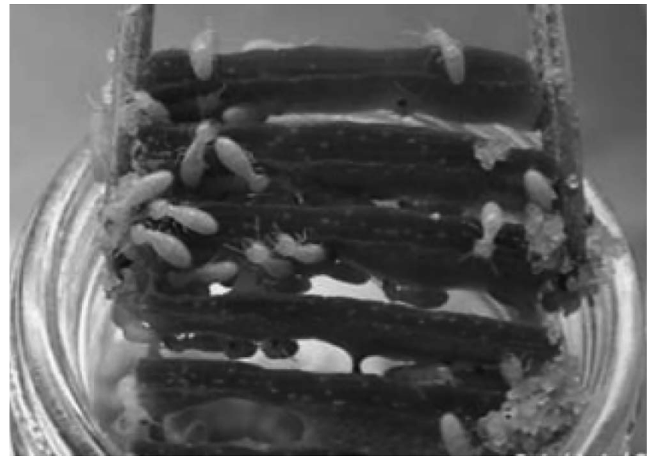

Figure 3.—Untreated southern pine sapwood block with extensive damage by Coptotermes formosanus *at the end of the testing period.*

With *C. formosanus*, there was a significant difference when comparing 100 percent workers with 90 percent and 80 percent workers. There was no significant difference, however, when comparing 90 percent with 80 percent *C. formosanus* workers (Fig. 5). Results obtained indicate that soldier:worker ratios within the limits tested did not have any adverse effect on outcome of test results.

Although Tukey's test indicated that there were significant differences within some of the ratio groups, the groups with significant differences are rarely used in testing. Ninety percent workers in the *R. flavipes* group were of significant difference when compared with 100 percent workers. That high percentage (10%) of native soldiers, however, is rarely found in field collections of termites and was difficult to find for use in this experiment. With *C. formosanus*, 100 percent workers yielded significantly higher weight loss, with no difference between 80 percent and 90 percent. As expected, increasing the proportion of termite workers progressively increased the consumption of test blocks. The results obtained from this

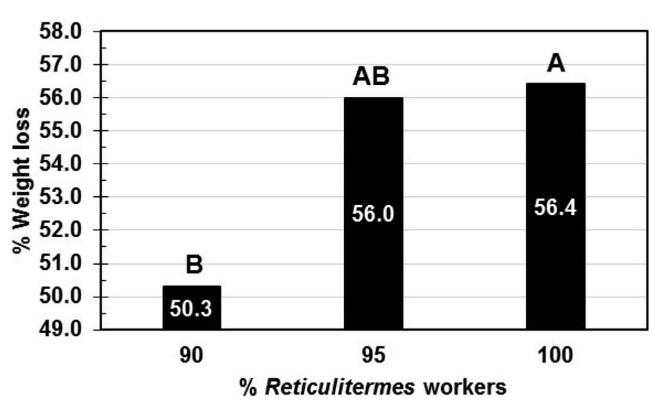

Figure 4.—Percentage of weight loss of southern pine sapwood blocks after 4 weeks exposure to varying Reticulitermes flavipes *soldier:worker ratios (means with the same letter are not significantly different at* $P = 0.05$*).*

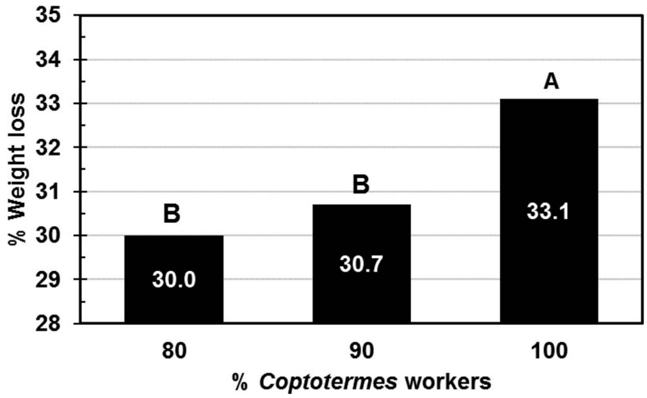

Figure 5.—Percentage of weight loss of southern pine sapwood blocks after 4 weeks exposure to varying Coptotermes formosanus *soldier:worker ratios (means with the same letter are not significantly different at* P = *0.05).*

test would indicate that the original soldier:worker ratios (not to exceed 10% soldiers) specified in the 1972 AWPA standard procedure would be an accurate, easy-to-maintain ratio for testing. Difficulty in maintaining a higher ratio is often due to the low availability of *R. flavipes* soldier termites and to the possibility of harming termites during counting of each individual test container prior to testing. Some termite tests should require counting out individual termites and maintaining exact ratios, especially when studying the biology of the termite species. When performing tests evaluating the efficacy of preservatives and products, such as AWPA Standard E1, which sometimes require upwards of 80,000 termites, a suitable method would be like that described in the ASTM Standard D3345 method (ASTM International 2004).

The ASTM Standard D3345 (ASTM International 2004) method is to use a known weight in each container and record the soldier:worker ratio (not to exceed 10% soldiers) and the number of termites per gram (e.g., on average, 1 g of *R. flavipes* equals approximately 400 termites). Alternatively, another method, like that described in AWPA E1, would be to count out 400 individual termites, record the soldier:worker ratio, then weigh and use that weight of termites in the test jars. Due to differences of ratios in colonies found in nature and in lab-maintained colonies as well as variation in collections for tests during various seasons of the year, this research has indicated that as long as soldier volumes do not exceed 10 percent per test unit for either species, test results should be within an acceptable range. Further studies should be done to determine mortality using both the counting and weighing method.

The test facility received tremendous damage and electric power losses from a hurricane during the conduct of this study. Temperature data indicated that during the *C. formosanus* testing, the mean temperature was 21.5°C, with a high temperature of 30.9°C and a low of 11.0°C. During *R. flavipes* testing, the mean temperature was 23.8°C, with a high temperature of 46.4°C and a low temperature of 16.8°C. The *R. flavipes* test was extended for 8 days past the E1-97 standard 28-day test period due to travel difficulties caused by the hurricane. This likely explains the greater feeding by *R. flavipes* compared with *C. formosanus* during this test. Power loss due to the storm was also responsible for the temperature fluctuations recorded during the 36-day period of testing for *R. flavipes*.

While the higher temperature, along with an increased testing period, likely caused increased consumption by EST, thus negating an accurate comparison between the two species of termites, ratio comparison within each species test group was still valid.

Summary and Conclusions

Not exceeding a 10 percent soldier proportion for both *R. flavipes* and *C. formosanus* did not adversely affect test results. Counting a representative sample of termites prior to testing should still be required to determine and record the ratios. Using 1 g of termites per container as specified in ASTM Standard D3345 method would limit any chance of a higher mortality in test bottles from the counting procedure and should not have any adverse results on the outcome of test results. Based on this research, the soldier:worker ratio has been changed in the current AWPA E1 standard (AWPA 2017) to 90 percent for both species.

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