

## Transfer of Chlorfenapyr Among Workers of *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) in the Laboratory

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**ABSTRACT** The potential for transfer of chlorfenapyr among subterranean termites was investigated using a donor-recipient (5:95 ratio) experiment. In one experiment, workers of *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae) were exposed to treated sand at 0, 50, 100, 250, and 500 ppm chlorfenapyr (wt [AI]/wt sand). Exposed workers were allowed to interact with untreated nestmates for 14 d, after which mortality was assessed. The three colonies responded differently to the treatments in this experiment. For two colonies, donor exposure rates of 500 ppm (as well as 250 ppm for colony B) chlorfenapyr resulted in significantly greater recipient mortality than controls. For colony C, donor chlorfenapyr exposure did not significantly influence recipient mortality. In a second experiment examining donor mortality over time, donor termites exposed to all test concentrations of chlorfenapyr (except for 0 ppm) suffered 100% mortality within 5 d. Analysis of donor termite body washes using gas chromatography indicated a linear uptake of chlorfenapyr by termites over the concentration range studied. Thus, for this concentration range, no upper limit (saturation plateau) of termite uptake for chlorfenapyr was reached.

**KEY WORDS** *Reticulitermes flavipes*, nonrepellent termiticides, toxicant transfer, chlorfenapyr, pyrroles

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The past decade has seen a change in the type of chemicals that are used for controlling or preventing termite damage to structures in the United States. Soil termiticides have been widely used for the prevention of structural infestation by termites (Su and Scheffrahn 1990; Grace et al. 1993; Gahlhoff and Koehler 2001), and this control method has remained unchanged for several decades (Su and Scheffrahn 1998). Although the applications have remained the same, the materials involved have changed. In keeping with the demand for chemicals that have less impact on human health and the environment, newer chemistries have come to the marketplace. Most traditional soil termiticides were applied expressly for the formation of a barrier beneath and around structures, and any complete penetration of the barrier resulting in wood damage by termites was considered a failure of the application (United States Environmental Protection Agency 1998). The newer chemistries include compounds that are applied at rates that are nonrepellent to termites and result in delayed termite mortality. Rather than making an impenetrable barrier to termites, such compounds ideally allow termites to move into treated areas, pick up lethal doses of the termiticide, and ultimately die away from the site of the application.

For the purposes of this study, the moving of exposed termites to other places was of greatest interest. As might be expected, exposed termites moving back

into the foraging tunnels will encounter unexposed (or untreated) individuals with whom social behaviors may take place. During these encounters, the exposed termite may pass on (or transfer) the termiticide to the untreated termite depending on the dose involved, and this may lead to secondary kill of the untreated individual.

The possibility for movement of termiticides from treated to untreated individuals is supported for a few compounds investigated (Ferster et al. 2001, Thorne and Breisch 2001, Ibrahim et al. 2003, Shelton and Grace 2003, Hu et al. 2005). In these investigations, neonicotinoid (imidacloprid), fiprole (fipronil), oxadiazine (indoxacarb), and spinosyn (spinosad) compounds as well as calcium arsenate dust (Ferster et al. 2001) have resulted in significant mortality of untreated subterranean or drywood termites in the laboratory. Repellent termiticides, such as permethrin, do not exhibit this trait (Shelton et al. 2005). Although this is a good start, there are many more delayed action nonrepellent compounds that also may be transferred from exposed to untreated termites. Previous studies have used a simple donor-recipient model, modifying methods originally designed for examining food passage among termites (e.g., Suárez and Thorne 2000). One interesting observation in an article working with transfer among *Coptotermes formosanus* Shiraki workers (Shelton and Grace 2003) was that not all colonies responded in a similar manner to identical treatments

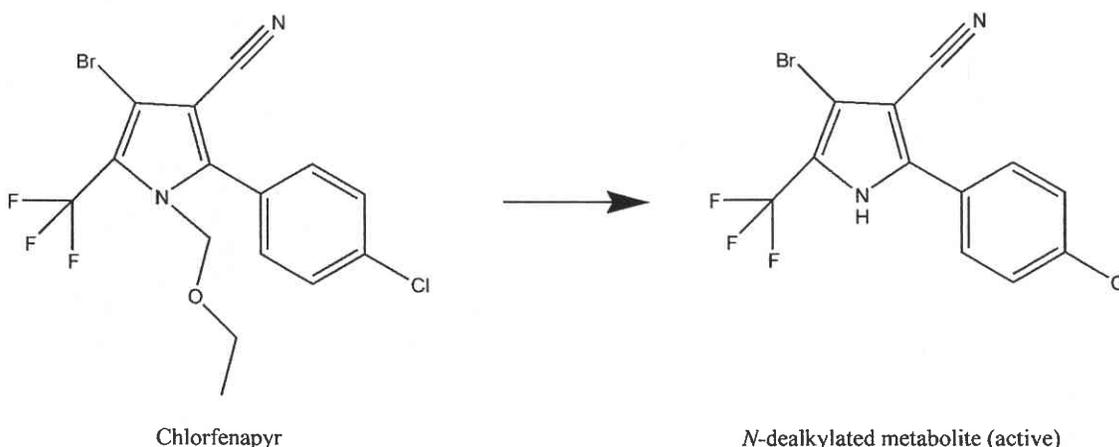


Fig. 1. Chlorfenapyr. (A) proinsecticidal parent compound and (B) active metabolite after monooxygenase activation (adapted from Black et al. 1994).

(same concentration series of the same pesticides). The influence of colony also may be an important consideration for transfer among other rhinotermitid individuals as well.

Chlorfenapyr, 4-bromo-2-(4-chlorophenyl)-1-(ethoxymethyl)-5-(trifluoromethyl)-1*H*-pyrrole-3-carbonitrile, is a newly registered pyrrole soil termiticide produced by BASF Corporation. It is sold as a soluble concentrate formulation under the name Phantom. Chlorfenapyr is a derivative of the natural product dioxapyrrolomycin obtained from an actinomycete *Streptomyces* spp. (Black et al. 1994). Chlorfenapyr must be activated by monooxygenase removal of the *N*-ethoxymethyl group into an active metabolite (Fig. 1; Black et al. 1994). This metabolite affects the mitochondria, inhibiting ATP production by disrupting the proton ( $H^+$ ) gradient in oxidative phosphorylation (Black et al. 1994). It is marketed as a delayed action, nonrepellent compound, making it a candidate for transfer among termites. We hypothesized that chlorfenapyr would have the same capacity for transfer as other delayed action nonrepellent termiticides.

This article describes three experiments investigating the transfer of chlorfenapyr. The model insect for this study was the Eastern subterranean termite, *Reticulitermes flavipes* (Kollar), an economically important pest of North American structures (Su and Scheffrahn 1990). The first experiment examines the possibility of (and possible influence of colony on) transfer of chlorfenapyr. The second experiment examines the length of time exposed termites survive and are able to serve as "donors" in simple laboratory bioassays. The third experiment quantifies the amount of chlorfenapyr adhering to exposed individuals and therefore the amount available for passage to untreated individuals after a given exposure time at various concentrations.

#### Materials and Methods

**Termites.** Groups of *Reticulitermes flavipes* (Kollar) were collected from colonies in fallen pine logs on

both the Mississippi State University John W. Starr Forest and the Noxubee National Wildlife Refuge within 10 miles of Starkville, MS. The logs were sectioned into 0.3–0.6-m lengths and stored in metal trash cans ( $\approx 113$  liters) for up to 3 mo in the laboratory. Trash cans containing termites remained at ambient laboratory temperatures ( $\approx 22$ – $24^\circ C$ ) until extraction. Termites were identified using the keys of Scheffrahn and Su (1994) and Hostettler et al. (1995).

**Insecticides.** All treatments were performed by allowing termites to walk on treated sand in a plastic disposable petri dish (7 cm in diameter, Fisherbrand, Fisher, Pittsburgh, PA). All calculations used a standardized amount of sand placed in the petri dishes (25 g), and a standard amount of solution (6 ml; 24% moisture) to fully saturate the sand. Calculations were made for mixing a solution such that 6 ml of the solution delivered 500 ppm (wt [AI]/wt sand) chlorfenapyr in 25 g of sand. The remaining treatment solutions (see below) were obtained through serial dilution from the 500 ppm stock. All three experiments used 0, 50, 100, 250, and 500 ppm (wt [AI]/wt sand) concentrations of chlorfenapyr. For comparison, Phantom is applied at a rate of 115 ppm chlorfenapyr in trench applications applied using a 0.25% solution (highest label rate).

Dry silica sand (40–100 mesh, Fisherbrand, Fisher) was measured into 100-g aliquots in a 946-ml recloseable plastic bag (Hefty One-Zip, Pactiv Corp., Lake Forest, IL). One bag was made and labeled for each concentration (treatment) in the study. To each bag, 24 ml of the appropriate solution was pipetted, and the sand was hand mixed for 1 min. The sand was then emptied into a disposable aluminum foil cake pan (20.0 by 20.0 by 4.5 cm, Durable, Inc., Schaumburg IL), labeled with the concentration, and placed into a darkened vacuum hood to dry for 4 d. At the end of 4 d, the sand was measured into disposable plastic petri dishes in 25-g aliquots, labeled according to colony and concentration (for experiment 1, each colony was treated separately; see below), and covered until test

initiation day. These dishes were used for exposing donor termites to the insecticide.

**Donor Treatment.** All experiments used similar methods in the treatment of exposed or donor termites. First, petri dishes containing the treated sand were uncovered, and 6 ml of deionized water was added. Dishes were set aside for 4 h to allow for evaporation. Stained termites were counted into groups of 30 individuals by colony and concentration, and each group added to the appropriate dish of treated sand for 1 h. Then, termites were removed and placed in disposable petri dishes each containing a clean, dry filter paper (Whatman no. 2, Whatman International, Ltd., Maidstone, United Kingdom), covered, and allowed to interact for 30 min. This was done to remove any grains of treated sand that might have adhered to the termites during treatment, preventing contamination of either the arena or the sample (experiment 3). Depending on the experiment, a number of donor termites were counted from the petri dishes and added directly to either the arenas, or to the sample vials (experiment 3).

**Potential for Transfer (Experiment 1).** Experiment 1 was a simple donor-recipient bioassay where treated termites (donors) were allowed to interact with untreated termites (recipients) in a plastic screw top jar arena. Evidence of transfer was inferred from recipient mortality at the end of the study (14 d). Because it was necessary to distinguish donors from recipients at the end of the study, donors were marked. Termites were marked using filter papers (grade #2, 9.0 cm in diameter, Whatman International, Ltd.) stained with 0.5% Sudan red 7B (Sigma, St. Louis MO; Su et al. 1991). One week before the start of the test, a small group of termites ( $\approx 200$ – $300$ , mixture of workers and soldiers) were extracted from each laboratory colony. These termites were placed in 9.0-cm-diameter glass petri dishes lined with two stained filter papers, each moistened with 1 ml of deionized water. Petri dishes were covered and placed in an unlit incubator at  $25 \pm 1^\circ\text{C}$  and  $\approx 75\%$  RH for 7 d.

Arenas for experiment 1 consisted of plastic screw top jars (eight by 10 cm) filled with 150 g of silica sand (Fisherbrand, Fisher) and moistened with 30 ml of deionized water (20% moisture). Because each jar represents an experimental unit, jars were labeled with the colony, concentration of donor exposure, and replicate number. To provide food during the experiment, a rectangle of aluminum foil (3.0 by 2.5 cm) with a wafer of *Pinus L. spp.* (southern yellow pine, 2.5 by 2.0 by 0.5 cm) was placed on top of the sand. Once complete, with the addition of water arenas were stored in an unlit incubator at  $25 \pm 1^\circ\text{C}$  and  $\approx 75\%$  RH for 24 h until tested.

On the day of the test, termites were extracted from each colony. A group of 95 workers was added to each arena (jar) according to labeled colony affiliation. These termites were the unexposed, or recipient, termites in the study. Five unstained workers from each colony were set aside for individual body mass determination. Stained termites (workers only) were treated as donors and then added to jars in groups of

five according to colony and concentration on the jar labels. Thus, the ratio of termites was five treated to 95 untreated in each jar. Jars were then returned to the incubator for 14 d, with shelf assignment determined using a random number table. After 14 d, the jars were disassembled, and surviving recipient and donor termites were counted and recorded.

**Duration of Donor Survival (Experiment 2).** A second experiment was designed to answer the question of the length of time donors survive after treatments. This experiment estimates donor survival after treatment by limited exposure to treated soil and being returned to naïve nestmates. Because of these methodological differences, data may not be comparable with standard termiticide mortality assays using constant exposure or direct application. Because daily examinations of survival would be necessary, the previous plastic jar arena would not be useful. Instead, this study used petri dishes lined with two filter papers (Whatman no. 2, Whatman International, Ltd.), moistened with 2 ml of deionized water. To provide food during the study, a wafer of *Pinus spp.* wood (2.5 by 2.0 by 0.5 cm) was placed on top of the filter papers in the dish. A single *R. flavipes* colony was used for this study. Donor staining and treatment were performed as described for experiment 1; 10 donors and 20 recipients were added to each dish. Dishes were arranged randomly on a shelf in an unlit incubator at  $25 \pm 1^\circ\text{C}$  and  $\approx 75\%$  RH. Each dish was removed to count the surviving donors daily until no donors survived.

**Amount of Chlorfenapyr on Donors (Experiment 3).** For this study, termites were collected from a single *R. flavipes* colony obtained and maintained as described above. Termites (100 workers in each of three replicates) were placed on chlorfenapyr-treated sand. Termites were treated as donors (as described for experiment 1), after which they were placed in vials containing 2.0 ml of 1:1 hexane:acetone, and extracted for 24 h. The termites were vacuum filtered, and the solute was reduced to 0.5 ml under a stream of ultrahigh purity grade nitrogen in a Rapid Vap (Labconco Corp., Kansas City, MO) set at  $32^\circ\text{C}$ .

Residues of the parent compound were analyzed using an Agilent 6890 gas chromatograph equipped with an Electron Capture Detector (ECD) using Chemstation software (Agilent Technologies, Palo Alto, CA). The parameters of the residue analysis method were as follows: injection volume, 1  $\mu\text{l}$ ; carrier gas, helium; makeup gas, nitrogen; injector temperature,  $250^\circ\text{C}$ ; detector temperature,  $300^\circ\text{C}$ ; oven program,  $60^\circ\text{C}$  initial temperature with a  $20^\circ\text{C}/\text{min}$  increase to  $250^\circ\text{C}$ . An Agilent Ultra-1 methyl siloxane column (25 m by 0.32 mm by 0.52  $\mu\text{m}$ ) with a 1.0 ml/min flow of helium was used. Retention time of chlorfenapyr was 14.449 min.

**Statistical Analyses.** Experiment 1 used a randomized complete block design with colony and concentration as the variables of interest. There were three colonies  $\times$  five concentrations  $\times$  five replicates for 75 total experimental units in this assay. Recipient mortality data were arcsine square-root transformed and

Table 1. Mean  $\pm$  SEM recipient mortality of three *R. flavipes* colonies, 14 d after donor exposure to various concentrations of chlorfenapyr (experiment 1)

Concn (ppm)	Colony	% recipient mortality	% donor mortality <sup>a</sup>
0	A	18.11 $\pm$ 1.74a	100.0 $\pm$ 0.0
	B	16.63 $\pm$ 1.64ab	100.0 $\pm$ 0.0
	C	7.16 $\pm$ 2.32c	96.0 $\pm$ 4.0
50	A	22.95 $\pm$ 1.77a	96.0 $\pm$ 4.0
	B	20.84 $\pm$ 3.67acf	100.0 $\pm$ 0.0
	C	14.53 $\pm$ 1.92a	80.0 $\pm$ 11.0
100	A	22.32 $\pm$ 2.27a	96.0 $\pm$ 4.0
	B	22.74 $\pm$ 6.36af	100.0 $\pm$ 0.0
	C	12.00 $\pm$ 3.80ac	68.0 $\pm$ 8.0
250	A	26.74 $\pm$ 2.37ad	96.0 $\pm$ 4.0
	B	29.05 $\pm$ 3.53ef	100.0 $\pm$ 0.0
	C	9.89 $\pm$ 2.53bc	92.0 $\pm$ 4.9
500	A	38.32 $\pm$ 4.52ef	92.0 $\pm$ 4.9
	B	27.79 $\pm$ 2.32e	100.0 $\pm$ 0.0
	C	13.26 $\pm$ 3.59bcd	96.0 $\pm$ 4.0

Bars with the same letter are not significantly different from all others (Tukey's HSD).

<sup>a</sup> Donor mortality was not subjected to statistical analysis.

subjected to the MIXED procedure in SAS (SAS Institute 1985) examining colony, concentration, and the colony  $\times$  concentration interaction as fixed effects. Means were separated using the differences of least squares means with Tukey's honestly significant difference (HSD) adjustment. Experiment 2 donor-mortality data curves were fit to time (in days) by using linear and nonlinear regression (exponential rise to maximum model), reporting significant models having the least number of coefficients, with SigmaPlot (SPSS Inc. 1998). Chlorfenapyr recovery data (experiment 3) were subjected to linear regression on donor exposure concentration using MINITAB (Minitab, Inc. 2003).

## Results

**Experiment 1.** Mean  $\pm$  SEM individual body masses for the three *R. flavipes* colonies were 2.17  $\pm$  0.09 mg (colony A), 2.07  $\pm$  0.1 mg (colony B), and 2.23  $\pm$  0.07 mg (colony C). The MIXED procedure indicated that the three colonies responded differently to the treatments ( $df = 2, 60; F = 31.64; P < 0.0001$ ). Concentration of donor exposure also significantly influenced recipient mortality for two of the three colonies ( $df = 4, 60; F = 5.31; P < 0.001$ ). The interaction of colony and concentration did not significantly effect recipient mortality in experiment 1 ( $df = 8, 60; F = 1.14; P = 0.3476$ ). Mean  $\pm$  SEM donor and recipient mortalities for each colony in experiment 1 are presented in Table 1. Mean separation procedures indicated that for colony A only the 500 ppm, and for colony B the 250 and 500 ppm donor exposure to chlorfenapyr resulted in significantly greater recipient mortality than the controls (0 ppm chlorfenapyr donor exposure).

**Experiment 2.** Daily mean  $\pm$  SEM percentage donor mortality from experiment 2 is presented in Fig. 2. As illustrated, the mortality curves of the chlorfenapyr treatments all follow a similar exponential rise to maximum function. Details of the simplest model (significant models having the least number of coefficients) describing each treatment are presented in Table 2, along with the linear model describing the control data.

**Experiment 3.** Mean amounts  $\pm$  SEM recovered from donors exposed to varying concentrations of chlorfenapyr are presented in Fig. 3. Amounts ranged from 0.0  $\pm$  0.0 (for 0 ppm exposure) to 18.09  $\pm$  5.53 ng per termite (500 ppm exposure). Recovered amounts regressed linearly with concentration of donor exposure (Fig. 3;  $r^2 = 0.75; F = 38.48; df = 1, 4; P < 0.001$ ), following the model:

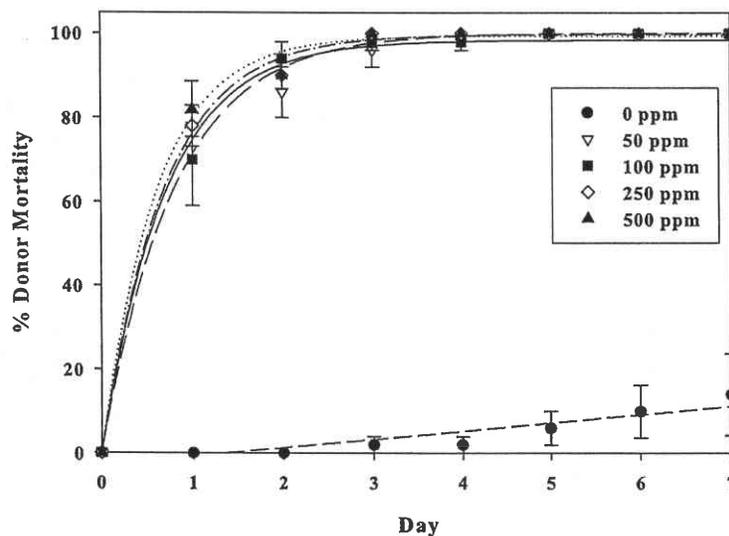


Fig. 2. Time-dependent mortality of donor termites treated with various concentrations of chlorfenapyr (experiment 2). Data are forced through 0 on day 0.

Table 2. Model details describing donor time-mortality curves from experiment 2

Donor exposure	<i>a</i>	<i>b</i>	Adjusted <i>r</i> <sup>2</sup>	<i>P</i>
0 ppm	1.98 ± 0.35	-2.67 ± 1.48	0.812	0.0014
50 ppm	98.43 ± 1.51	1.42 ± 0.14	0.991	<0.0001
100 ppm	100.11 ± 0.57	1.24 ± 0.04	0.999	<0.0001
250 ppm	99.80 ± 0.91	1.45 ± 0.09	0.997	<0.0001
500 ppm	99.43 ± 1.14	1.63 ± 0.14	0.995	<0.0001

All models except for the control (0 ppm) are exponential rise to maximum [donor mortality =  $a \times (1 - e^{-b \times \text{day}})$ ]. The control model is a simple linear model (donor mortality =  $a \times \text{day} + b$ ). *P*-values are reported for each whole model, not for individual estimates (*a* or *b*).

$$\begin{aligned} \text{Amount of chlorfenapyr} = \\ 0.037 (\pm 0.01) \times \text{donor exposure concentration} \\ + 0.09 (\pm 1.51). \end{aligned}$$

### Discussion

In the first experiment, laboratory bioassays using a simple donor-recipient model evaluated the possibility of transfer of chlorfenapyr among termites; chlorfenapyr is the only currently available pyrrole soil termiticide. The data indicate that donor exposure to chlorfenapyr significantly increased recipient mortality by the end of the 14-d study for two of the three colonies examined. These data support the hypothesis that chlorfenapyr is capable of being transferred from exposed termites to unexposed nestmates. However, aside from concentration of donor exposure, this response was clearly dependent on the colony involved. This is different from previous work (Shelton and Grace 2003) where differences among colonies were a matter of degree (i.e., all colonies were significantly effected by the treatments, but the colonies differed

significantly in level of response). Here, colony C recipient mortality did not significantly differ from controls for any level of donor exposure to chlorfenapyr. It should be noted that termites from all three colonies were of relatively similar physical size (as determined by fresh mass). Osbrink et al. (2001) noted differences among colonies of *R. virginicus* in termiticide susceptibility.

Comparisons to other studies are always problematic due to differences in methodology, but Shelton and Grace (2003) used very similar methods to those used here, although with a different termite (*C. formosanus*). Considering only the highest rate of chlorfenapyr donor exposure used in our first experiment (500 ppm) and only colonies A and B, the maximum recipient percentage of mortality was ≈11–20% (subtracting mean recipient control mortality from mean recipient mortality of the treatment). In their study, imidacloprid percentage recipient mortality ranged from ≈29 to 50% and from ≈21 to 45% for fipronil, both at 100 ppm donor exposure (using the same calculations, Table 1; Shelton and Grace 2003). There seems to be some overlap between fipronil and chlorfenapyr recipient mortality, except for the five-fold difference in concentrations used.

There have been no studies directly examining the effects of toxicant transfer (using soil-applied termiticides, not bait formulations) on termite populations in the field. In the laboratory, the concentrations necessary for chlorfenapyr transfer are above those that would result from by-the-label application (e.g., 115 ppm for perimeter treatments versus 250 or 500 ppm needed for transfer; Table 1), and not all termite colonies can be expected to exhibit the phenomenon, making the potential population effects via transfer of chlorfenapyr among *R. flavipes* a purely academic matter.

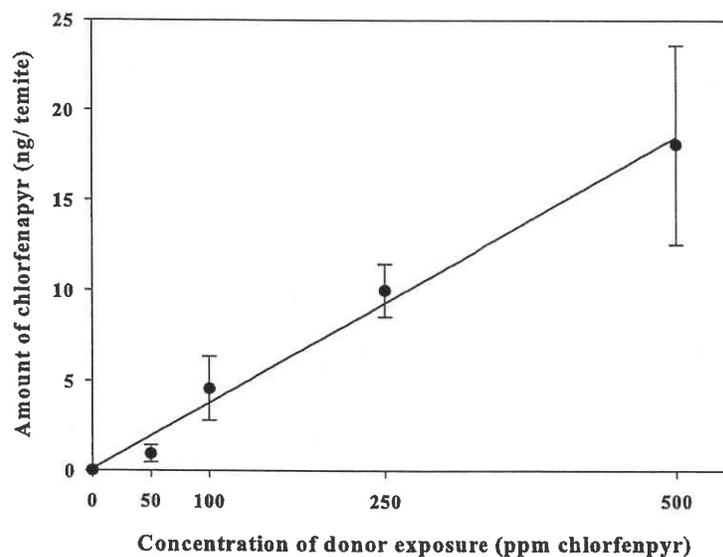


Fig. 3. Mean ± SEM amount of chlorfenapyr (in nanograms per termite) recovered from donor termites ( $n = 3$ ; 100 workers per replicate) treated with various concentrations of chlorfenapyr. Extractions were made using 1:1 hexane:acetone.

The second experiment was designed to estimate the survival time of donor termites, because most, if not all, donors were dead by the end of 14 d (Table 1). This is similar to data from other studies (Shelton and Grace 2003; 100 ppm donor mortality). Donor mortality over time models describing each concentration (except the controls) were very similar (Table 2; Fig. 2), with all estimates falling within 2 SEs of one another. From these data, it seems that most ( $\approx 80\%$ ; Fig. 2) donor termites exposed to as little as 50 ppm chlorfenapyr die within 24 h of exposure to the treated sand. Data from these experiments indicate that most donors survive for  $<2$  wk, depending on exposure concentration.

The donor mortality data from the second study provides some insight into the donor mortality observed in the first study. In the first study, donor mortality was very high (Table 1), which is not surprising because these termites are expected to die during the experiment. It is difficult to make observations from such small numbers of termites (only five donors per experimental unit); however, the donor control mortality is of some concern. When considered alone, it leads to the assumption that all donors died immediately as a result of handling or staining (not necessarily from chlorfenapyr) and were not available for long enough to provide toxicant transfer. However, by examining the data from the second study for the length of time that donors survived, we see that control termites indeed lasted much longer than those treated with chlorfenapyr. Donors treated with all concentrations of chlorfenapyr died within 5 d of study initiation, whereas the controls reached a mean mortality of  $14.0 \pm 9.8\%$  by 7 d. This indicates that even though there was an unfortunate level of control donor mortality in the first experiment, the treated donors were most likely already dead and no longer directly contributing to toxicant transfer before any handling or staining mortality associated with being a control donor.

In the final experiment, an assumption was made regarding the movement of termiticides from donors to recipients. It was assumed that termites pick up termiticides from the treated substrate (sand, in this study), and it is the termiticides adhering to the cuticle that are passed to recipients during grooming activities. As a result, the data provided represent only washes from the surfaces of the donor termites and not extractions of homogenized donors. Under the assumption above, and using information from experiment 1 (Table 1), it is apparent that very little chlorfenapyr is needed per donor ( $10.01 \pm 1.45$  ng per termite at 250 ppm; Fig. 3) for transfer to manifest as recipient mortality. Concentration-dependent recovery of materials is not infinitely linear—it is expected that eventually a plateau will be reached where increases in exposure concentration do not increase recovery of the material (saturation) because no more will fit on the surface area of the termite contacting the soil. Even with a 500 ppm upper end to the concentration bracket, no plateau was apparently reached for the amount of chlorfenapyr adhering to donor ter-

mites. However, because very few donors exposed to 500 ppm chlorfenapyr survived 14 d (Table 1), and recipient mortality at the higher doses increased, it is unlikely that any further increase in donor exposure would be biologically significant.

Results of these experiments indicate that chlorfenapyr is capable of being passed among nestmates of *R. flavipes* in the laboratory, but it is dependent on both concentration of donor exposure and colony origin of the termites. Termites exposed to sand treated with chlorfenapyr do not survive for long periods ( $\approx 80\%$  dead in 2 d); thus, the transfer effects must take place quickly to be of potential value for large-scale control measures. These data suggest that donors retain small amounts of chlorfenapyr to transfer lethal doses to nestmates. Other work remains; influences of altering donor: recipient ratios, minimum donor exposure concentrations necessary for transfer, amounts of parent and active metabolites of chlorfenapyr recovered from exterior (washes) versus interior (homogenate) extractions, behavioral means of transfer, and field-scale implications must still be addressed.

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