SURVIVAL ANALYSIS for the DETECTION of LOW-LEVEL INSECTICIDE RESISTANCE

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Abstract This study compares statistical methodologies for the detection of low levels of insecticide resistance in the German cockroach. Results show that logistic regression and resistance ratios are relatively ineffective in distinguishing modest differences between strains, whether dose-mortality or time-mortality data are obtained. An improved method involves proportional hazards modeling, known as Cox regression. This method distinguished small differences in fipronil tolerance between a field collected and an insecticide-susceptible strain.

Key Words *Blattella germanica* fipronil logistic regression resistance ratios Cox regression hazard ratios

INTRODUCTION

Over the last fifty years, the German cockroach, *Blattella germanica* (L.), has developed resistance to almost all insecticides that have been used against it (Cochran, 1995). As early as 1952, infestations were found resistant to chlordane (Heal et al., 1953), even though this insecticide had been available for only a few years. Following the failure of chlordane, a variety of other insecticides, with differing modes of action, were used. Ultimately, many of these lost their efficacy owing to resistance. A consequence of perpetual transition from one insecticide to another has been that many cockroach populations exhibit resistance to multiple insecticides (Holbrook et al., 1999).

In recent years, interest in insecticide resistance in the German cockroach has decreased (Robinson, 1999). This may be due to the effectiveness of insecticides in bait formulations. Baits may slow the development of resistance by killing with high doses of insecticide. Nevertheless, concern about resistance remains justifiable, because some currently used insecticides have modes of action identical to those of compounds used in the past. And cross-resistance can result in early failure (Cochran, 1995). For example, fipronil has a mode of action similar to chlordane (Ghiasuddin and Matsumura, 1982; Hosie et al., 1995).

The history of insecticide resistance in cockroaches has suggested implementation of resistance management programs (Cochran, 1995). For this strategy to be successful early detection of resistance is necessary (Roush and Miller, 1986). But the statistical and modeling approaches used to measure resistance, such as logit and probit analysis, in conjunction with resistance ratios, may be inadequate (Roush and Miller, 1986). Our results suggest that survival analysis is more appropriate.

MATERIALS and METHODS

Cockroaches and Insecticide

All cockroaches, except where noted otherwise, were kept at 27 ± 1.0 °C, under a 12:12 (L:D) hour photoperiodic regime, and provided rat chow (Purina Mills, St. Louis, MO) and water. Insecticide-susceptible, lab strain cockroaches were acquired from American Cyanamid. Four

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field strains: Hi-Al, GC-On, BL-Ra, and Jo-Or, were collected in North Carolina in 1997 and 1998 (Table 1). Adult males were used in experiments; upon eclosing, they were collected from cages and transferred to small plastic boxes. They were reared under the same conditions as their source colonies and given rat chow, water, and a cardboard shelter. Adults of all strains used in experiments were at least 14 days old, but no more than 49 days old.

Fipronil [(±)-5-amino-1-(2,6-dichloro-a,a,a-trifluoro-r-tolyl)-4-trifluoromethylsul-phinylpyrazole-3-carbonitrile], at 94.9% purity, was obtained from Aventis Environmental Science (Montvale, N.J.). All solutions containing the insecticide were made with pesticide grade acetone (VWR, South Plainfield, N.J.).

Dose-Mortality and Time-Mortality Assays

Dose-mortality curves were established for each of the five strains. Fipronil, from 1.5 to 12 ng, was topically administered in 1 µl acetone between the mesothoracic coxae. Cockroaches were anesthetized with carbon dioxide before applications of insecticide. Three sets of ten males from each strain were treated; all treated insects were maintained in groups of ten in 145 x 20 mm plastic Petri dishes, with rat chow and water. Mortality was recorded after 72 hours. Lab strain cockroaches were dosed with 10 separate amounts of fipronil; field strain males were treated with 7 different quantities. Thirty males from each strain were treated with acetone containing no insecticide.

Time-mortality data was obtained by topically dosing 4 sets of 12 males from all strains with 18.5 ng fipronil. Treated insects were placed in 145 x 20 mm plastic Petri dishes with food and water. Mortality was recorded hourly for the first 24 hours, then at longer intervals through 96 hours. Separate cohorts of 48 males from each strain served as controls and were treated with acetone.

Data Analysis

Logistic regression, or logit analysis, were used to characterize the relationship between mortality and fipronil dose, and mortality, and time. The dose and time, in both types of models, were transformed by taking their natural logarithm before parameters of the models were estimated with PROC LOGISTIC in SAS® 6.12 (Stokes et al., 1995). Pearson's c^2-statistic was used to assess how well logistic models fit data. Since acetone-treated insects did not die, correction for control mortality was not required in any of the analyses.

Maximum likelihood estimates (Allison, 1999) of model coefficients, together with their variances and covariances, were used to calculate LC_{50}s, LC_{95}s, LT_{50}s, and LT_{95}s, as well as 95% confidence intervals of these measures (Collett, 1991). Resistance ratios and their estimated fiducial limits were determined according to methods outlined by Robertson and Preisler (1992). All such ratios provided information comparing a single field strain to the insecticide-susceptible

Table 1. Strains of cockroaches, and where and when they were collected

Strain	Collection location	Date collected	Dwelling
Hi-Al	Mebane, Alamance Co.	1/29/98	H
GC-On	Jacksonville, Onslow Co.	2/18/98	M
BL-Ra	Asheboro, Randoph Co.	11/25/97	M
Jo-Or	Chapel Hill, Orange Co.	1/26/98	A

Cockroaches were collected in four counties of North Carolina from mobile homes (M), an apartment within a building consisting of four residential units (A), and a single-family dwelling (H).

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one. In the case of dose-mortality models, confidence intervals of resistance ratios that did not encompass the value one indicated that significantly more insecticide was needed to kill 50 or 95% of males from field-strains than males from the lab strain. The analogous interpretation for serial time-mortality models was that it took significantly longer for 50% or 95% of field-strain to die in comparison to lab-strain.

Time to mortality data from the five strains was used to construct a proportional hazards model (Cox regression model) containing four coefficients, one representing each of the field strains. Exponentiation of the coefficients yielded hazard ratios, which quantified the instantaneous probability of death of a field-strain male relative to that of a lab-strain one. A hazard ratio of less than one indicated that males of a field strain were dying more slowly than those of the lab strain. All model parameters were estimated with PROC PHREG in SAS (Allison, 1995). Since the exact time of an insect's death was unknown, but occurred within a certain interval of time, an exact likelihood method was used to estimate regression coefficients (Allison, 1995). The decision threshold for rejecting all null hypotheses was a = 0.05.

RESULTS

A common method measuring resistance in German cockroaches involves the construction of a model describing the relationship between the dose of an insecticide and mortality of treated insects after a prescribed period of time. Doses of fipronil that would kill greater than 0% but less than 100% of insects in all five strains were applied to adult males, and mortality was recorded in 72 hours. The data were used to construct logistic regression models (Table 2).

The lab strain had the least tolerance for fipronil, having the lowest LC_{50} and LC_{95} of all strains. Strain Hi-Al was slightly more tolerant of the insecticide, and the others showed even greater levels of resistance. Model fit was poor ($P < 0.05$) for three strains: Hi-Al, BL-Ra and Jo-Or, suggesting that a simple logistic equation was insufficient to describe the relationship between dose and mortality. Resistance ratios were determined using information from the models (Table 3). There was no discernable difference between any of the strains in the amount of fipronil predicted to kill 50% of insects. The highest resistance ratio at the LC_{50} was 1.99 for the Jo-Or strain, and its fiducial limits encompassed the value one. At the LC_{95} differences were found between two strains, BL-Ra and Jo-Or, and the lab strain.

Table 2. Results of logit analyses on the relationship between insecticide dose and insect mortality in 72 hours

		Model parameters[a]		Lethal concentrations[b]		Model fit		
Strain	n	intercept ± SE	slope ± SE	LC_{50} (95% CI)	LC_{95} (95% CI)	χ^2	df	*P*
Lab	300	-7.07 ± 0.84	8.91 ± 0.98	2.21 (2.13–2.30)	3.08 (2.86–3.31)	8.81	8	0.36
Hi-Al	210	-5.71 ± 0.86	5.18 ± 0.74	3.01 (2.81–3.23)	5.32 (4.51–6.27)	25.77	5	0.0001
GC-On	210	-6.10 ± 0.85	5.17 ± 0.69	3.26 (3.04–3.49)	5.76 (4.91–6.76)	1.11	5	0.95
BL-Ra	210	-4.17 ± 0.59	3.36 ± 0.44	3.46 (3.11–3.86)	8.33 (6.49–10.69)	12.30	5	0.031
Jo-Or	210	-3.46 ± 0.56	2.33 ± 0.34	4.41 (3.83–5.07)	15.6 (10.7–22.6)	13.86	5	0.017

Fipronil, in varying amounts, was topically administered to adult males of the five strains, and all insects were inspected for mortality 72 h later. Insects of the lab strain were exposed to one of ten doses of fipronil, 30 insects per dose, whereas those of the other strains were treated with one of seven doses, still at 30 insects per dose.

[a]The intercept and slope parameters are for logistic models in which the independent variable is natural logarithm of dose. Shown with all values are their standard errors (SE).

[b]Lethal concentrations are expressed in nanograms per insect. Confidence intervals (CI) for these values are shown in parentheses.

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Table 3. Resistance ratios obtained from dose-mortality and time-mortality logistic models

	Lethal concentration[a]		Lethal time[b]	
Strain	RR_{50} (95% CI)	RR_{95} (95% CI)	RR_{50} (95% CI)	RR_{95} (95% CI)
Hi-Al	1.36 (0.28–2.45)	1.73 (0.53–2.93)	1.26 (0.21–2.30)	1.35 (0.25–2.45)
GC-On	1.47 (0.39–2.56)	1.87 (0.68–3.07)	1.29 (0.24–2.34)	1.58 (0.47–2.69)
BL-Ra	1.57 (0.44–2.69)	2.71 (1.41–4.01)	1.55 (0.51–2.59)	1.73 (0.63–2.83)
Jo-Or	1.99 (0.83–3.15)	5.06 (3.59–6.53)	1.52 (0.46–2.57)	2.22 (1.06–3.38)

[a]Resistance ratios (RR) for lethal concentration were essentially the LC_{50}s and LC_{95}s of the four field strains, divided by the LC_{50} and LC_{95} of the lab strain; all these estimates can be found in Table 2. Shown in parentheses are confidence intervals (CI) for the ratios.

[b]Resistance ratios for lethal time are determined from data in Table 4 and were calculated in a similar manner as RRs for lethal concentration.

Another method of characterizing resistance is dosing insects with a predetermined amount of insecticide and recording mortality. Time-mortality data of this kind was gathered from insects of all strains after they had been dosed with 18.5 ng fipronil, an amount approximately 5-fold the LC_{99} of the lab strain (Table 4). The lab strain exhibited the greatest susceptibility to the treatment; 50% of these insects were predicted to die within 11.3 hours, and 95% within 16.3 hours. Model fit was generally better than for dose-mortality data (Table 2), but the logistic curve described poorly the relationship between time and mortality in the susceptible strain.

Resistance ratios were determined at both the LT_{50} and LT_{95}, and none of these exceeded 2.22 (Table 3). The only strain showing evidence of greater time to death than the lab strain was Jo-Or. In all others, the fiducial limits of resistance ratios included the value one.

Proportional hazards modeling, or Cox regression, was applied to the same time-mortality data that had been analyzed in constructing the logistic models in Table 4. A likelihood ratio test showed that regression coefficients representing the four field strains significantly improved the fit of the model (χ^2=72.173, 4 df, $P < 0.0001$), and each coefficient was, in itself, significant (Wald statistic in Table 5). These results justified the inclusion of all four coefficients in the final model.

Hazard ratios are generally considered the most readily interpretable statistics that can be garnered from proportional hazards models. The hazard ratios associated with all of the strains evaluated here were smaller than one in magnitude (Table 5), and none of their 95% confidence intervals encompassed this value. This result provided strong evidence that all field strains were dying at a significantly slower rate than the lab strain. The instantaneous probability of death of an

Table 4. Results of logit analyses on mortality of fipronil-treated insects over time

	Model parameters[a]		Lethal times[b]		Model fit		
Strain	intercept ± SE	slope ± SE	LT_{50} (95% CI)	LT_{95} (95% CI)	χ^2	df	P
Lab	-19.44 ± 1.68	8.02 ± 0.69	11.3 (10.9–11.6)	16.3 (15.2–17.4)	17.85	8	0.022
Hi-Al	-17.62 ± 1.31	6.65 ± 0.49	14.2 (13.7–14.6)	22.1 (20.7–23.5)	9.46	12	0.66
GC-On	-13.72 ± 0.98	5.13 ± 0.36	14.5 (14.0–15.0)	25.8 (23.9–27.8)	7.34	15	0.95
BL-Ra	-17.66 ± 1.26	6.17 ± 0.44	17.5 (17.0–18.0)	28.2 (26.2–30.3)	15.64	14	0.34
Jo-Or	-11.15 ± 0.84	3.93 ± 0.30	17.1 (16.4–17.9)	36.2 (31.8–41.3)	8.59	16	0.93

A set amount of fipronil, 18.5 ng, was topically administered to adult males of the five strains, and insects were subsequently inspected for mortality at frequent intervals through 96 hours.

[a]The intercept and slope parameters are for logistic models in which the independent variable is natural logarithm of time. Shown with all values are their standard errors (SE).

[b]Lethal times are expressed in hours, and 95% confidence intervals (CI) for these values are shown in parentheses.

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Table 5. Proportional hazards model on time-mortality data

Strain	Regression coefficient, b	SE of b	Wald statistic $\chi^2 = [b/\mathrm{SE}]^2$	P-value	$\mathrm{HR} = e^b$	95% CI of HR
Hi-Al	- 1.120	0.221	25.73	0.0001	0.326	(0.212–0.503)
GC-On	- 1.245	0.224	30.85	0.0001	0.288	(0.185–0.447)
BL-Ra	- 1.762	0.234	56.69	0.0001	0.172	(0.109–0.272)
Jo-Or	- 1.945	0.247	61.74	0.0001	0.143	(0.088–0.232)

The data used to construct the model were the same as those used in generating the logistic models in Table 4. Shown alongside each model coefficient is its standard error (SE); also displayed are hazard ratios (HR) and their 95% confidence intervals (CI).

insect from the Jo-Or strain was approximately one-seventh (hazard ratio: 0.143) that of an insect from the lab strain. Males of the most insecticide intolerant strain, Hi-Al, were found to be about one-third (hazard ratio: 0.326) as likely to die at any point in time as males from the lab strain.

DISCUSSION

Researchers in other disciplines are using survival analysis, especially proportional hazards (Cox) modeling, to analyze time-to-event data (Singer and Willett, 1991). Cox regression has been little used in insecticide resistance. The statistical power of the procedure allows for the detection of small differences in the rates of death of individuals. Cox regression has the benefit of yielding highly intuitive statistics, namely hazard ratios (Parmar and Machin, 1995). These can be used to compare directly the death rate of one group to that of a control. Hazard ratios are somewhat analogous to resistance ratios and can be readily used in their place for quantifying the magnitude of insecticide resistance. Hazard ratios, unlike resistance ratios, impart information on rates of mortality.

Traditional approaches to detecting resistance usually call for dosing insects with an insecticide and subsequently recording when they die, or alternately, identifying the percentage of insects that have died after a predetermined period of time. Once all data have been collected, probit or logit analyses are then used to assess whether insects, suspected to be resistant, are actually more tolerant of the insecticide than a known susceptible strain. The results obtained with probit and logit analysis are nearly identical (Allison, 1999). We no longer use probits but instead logistic regression because predictor variables can be more easily incorporated into logit models (Neter et al., 1996).

The ways in which probit and logit models are currently being used to analyze dose-mortality data are considered acceptable by most statisticians. Typically, different sets of insects are treated with varying amounts of insecticide, and the insects are inspected for mortality at a single point in time. In this design, death of an individual is measured once, and all observations on mortality are independent, an important assumption that must be met for probit or logit modeling (Robertson and Preisler, 1992). The independence assumption is frequently violated in investigations on insecticide resistance in German cockroaches, and other insects (Robertson and Preisler, 1992). In many studies, investigators record at several points in time the percentage of initial insects that have died. These data serve as the response variable in logit or probit analysis. This tactic is inappropriate, since the percentage of insects dead at any time is dependent on the percentage that was dead before that time. The data are correlated in such analyses because insects are being counted as dead more than once, a clear violation of the independence assumption (Robertson and Preisler, 1992).

Alternatives do exist to logit and probit analysis, ones that directly address the problem of correlation in serial time-mortality assays (Robertson and Preisler, 1992; Throne et al., 1995). One

approach is to use the complementary log-log model (Preisler and Robertson, 1989; Nowierski et al., 1996), which has not been used by researchers. The coefficients of a complementary log-log model are, for most time-mortality data collected in resistance studies, probably identical to those of a proportional hazards model (Allison, 1999). The discussion here could have been addressed from the perspective of the complementary log-log model.

Our results question the usefulness of logistic regression, and of probit analysis for the detection and quantification of resistance using time-mortality data. Although logit models tended to fit these kinds of data (Table 4), very little range was evident in LT_{50} and LT_{95} values across the tested strains. Resistance ratios did not show significant differences in the predicted time to 50% mortality between any of the four field strains and the lab strain. These results, coupled with those from the proportional hazards model showing all field-strain males dying at a much slower rate than lab-strain ones, lead us to conclude that logit and probit analysis should be replaced with Cox modeling in the analysis of time-to-mortality data. We used the same data to generate both logistic and Cox models, and obtained different results.

Dose-mortality assays, together with probit or logit analysis, are frequently used to detect resistance (Scharf et al., 1996; Valles, 1999), and our results would suggest these designs are preferable to serial time-mortality ones analyzed in a similar way. The LC_{50} and LC_{95} values, and their corresponding resistance ratios, spanned a wider range in dose-mortality assays. None of the RR_{50}s were significant and only the two highest RR_{95}s were. These results show the power and overall superiority of proportional hazards modeling. We conclude that researchers studying insecticide resistance would be well served in using experimental designs yielding data suitable for Cox regression.

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REFERENCES

Allison, P.D. 1995. Survival Analysis Using the SAS® System. Cary, N.C.: SAS Institute. 292 pp.

Allison, P.D. 1999. Logistic Regression using the SAS® System. Cary, N.C.: SAS Institute. 288 pp.

Collett, D. 1991. Modelling Binary Data. New York: Chapman & Hall. 369 pp.

Ghiasuddin, S.M., and Matsumura, F. 1982. Inhibition of gamma-aminobutyric acid (GABA)-induced chloride uptake by gamma-BHC and heptachlor epoxide. Comp. Biochem. Physiol. 73C: 141-144.

Heal, R.E., Nash, K.B., and Williams, M. 1953. An insecticide-resistant strain of the German cockroach from Corpus Christi, Texas. J. Econ. Entomol. 46: 385-386.

Holbrook, G.L., Roebuck, J., Moore, C.B., and Schal, C. 1999. Prevalence and magnitude of insecticide resistance in the German cockroach (Dictyoptera: Blattellidae). In: Robinson, W.H., Rettich, F., and Rambo, G.W., eds. Proceedings of the 3rd International Conference on Urban Pests. Czech Republic: Grafické závody Hronov.

Hosie, A.M., Baylis, H.A., Buckingham, S.D., and Sattelle, D.B. 1995. Actions of the insecticide fipronil, on dieldrin-sensitive and -resistant GABA receptors of *Drosophila melanogaster*. Brit. J. Pharmacol. 115: 909-912.

Neter, J., Kutner, M.H., Nachtsheim, C.J., and Wasserman, W. 1996. Applied Linear Statistical Models. 4th ed. Boston: Irwin Publishers. 1408 pp.

Nowierski, R.M., Zeng, Z., Jaronski, S., Delgado, F., and Swearingen, W. 1996. Analysis and modeling of time-dose-mortality of *Melaoplus sanguinipes*, *Locusta migratoria migratorioides*, and *Schistocerca gregaria* (Orthoptera: Acrididae) from Beauveria, Metarhizium, and *Paecilomyces* isolates from Madagascar. J. Invert. Pathol. 67: 236-252.

Parmar, M.K.B., and Machin, D. 1995. Survival Analysis: a Practical Approach. New York: John Wiley & Sons. 255 pp.

Preisler, H.K., and Robertson, J.L. 1989. Analysis of time-dose-mortality data. J. Econ. Entomol. 82: 1534-1542.

Robertson, J.L., and Preisler, J.L. 1992. Pesticide Bioassay with Arthropods. Boca Raton: CRC Press, 127 pp.

Robinson, W. 1999. Worries over German cockroach resistance fade. Pest Control Magazine 67(6): 40-42.

Roush, R.T., and Miller, G.L. 1986. Considerations for design of insecticide resistance monitoring programs. J. Econ. Entomol. 79: 293-298.

Scharf, M.E., Hemingway, J., Reid, B.L., Small, G.J., and Bennett, G.W. 1996. Toxicological and biochemical characterization of insecticide resistance in a field-collected strain of *Blattella germanica* (Dictyoptera: Blattellidae). J. Econ. Entomol. 89: 322-331.

Singer, J.D., and Willett, J.B. 1991. Modeling the days of our lives: using survival analysis when designing and analyzing longitudinal studies of duration and the timing of events. Psychol. Bull. 110: 268-290.

Stokes, M.E., Davis, C.S., and Koch, G.G. 1995. Categorical Data Analysis Using the SAS® System. Cary, N.C.: SAS Institute. 499 pp.

Throne, J.E., Weaver, D.K., Chew, V., and Baker, J.E. 1995. Probit analysis of correlated data: multiple observations over time at one concentration. J. Econ. Entomol. 88: 1510-1512.

Valles, S.M. 1999. l-cyhalothrin resistance detection in the German cockroach (Blattodea: Blattellidae). J. Econ. Entomol. 92: 293-297.

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