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MEASURING THE SUSCEPTIBILITY OF CANEGRUBS TO CHLORPYRIFOS INSECTICIDE

by

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SUMMARY

This project sought to develop an efficient bioassay and biochemical tests to measure the susceptibility of canegrub populations to the insecticide chlorpyrifos. Both are required to detect, to monitor, and to manage resistance to chlorpyrifos.

Bioassays are essential at present and biochemical assays will be needed in the event of resistance. Both tests underpin efficient use of insecticide to manage canegrubs infesting sugarcane.

In this project, Mr Chandler developed a bioassay system more efficient than any used previously, and used it to measure the susceptibility of 10 canegrub species to chlorpyrifos. The data were reviewed for evidence of resistance. The effects of grub size and soil type on insecticide activity were assessed, in order to relate laboratory measurements to in-field product performance.

In a parallel program, Dr Rose developed tissue preparation methods and a biochemical assay for a key enzyme involved in resistance to chlorpyrifos, and measured enzymes from canegrubs with a range of tolerance to chlorpyrifos.

In this report, Mr Chandler proposes a basis for Insecticide Resistance Management (IRM) as a part of Integrated Pest Management (IPM) for efficient canegrub control by cultural, biological and chemical means for the Australian sugar industry.

1.0 INTRODUCTION

Canegrubs cause annual sugarcane crop losses of (approximately) $4M in Australia; additionally, at least $6M is spent on insecticide to protect crops from damage, and sugarcane processors are denied potential revenue. The soil-inhabiting larvae prune roots from sugarcane plants, starving them for moisture and nutrients and decreasing yield or destroying the crop. Unsupported plants fall over, pulling stems from the soil and leaving insufficient material to provide healthy ratoon crops.

A row-band application of a granular, controlled-release (CR) insecticide formulation (suSCon®Blue, Incitec Ltd now Crop Care Australasia, Brisbane; chlorpyrifos AI 140 g/kg) applied during furrow cultivation is currently the only registered product and treatment capable of controlling canegrubs on more than 80% of farms. The granules protect the crop for 2 - 3 yr, after which it is unprotected, and mostly untreatable. Under this scenario, chlorpyrifos-resistant canegrub populations could cause massive crop losses.

Tests are needed to determine if insecticide control failures are attributable to 'resistant' canegrubs. Insecticide bioassays are needed to confirm the onset of resistance, and quantify its effect on control. Once resistance is confirmed, biochemical assays can be used to monitor effectiveness of resistance management programs and decide future strategies. These biochemical assays are more rapid and efficient for those purposes than insecticide bioassays.

2.0 BACKGROUND

Resistance develops if survival of the least susceptible portion of a population treated with insecticide over several generations results in tolerant genotypes selectively becoming a significantly greater proportion of the genome than when exposure commenced (Roush and Daly, 1990). Practically, resistance occurs once the non-susceptible proportion of the population becomes sufficiently large that effective control with insecticide is no longer possible (Ball, 1981; Roush and Miller, 1986). Natural tolerance of an insecticide, whilst having the same effect, must not be confused with acquired resistance. The most common cause of resistance is due to increased activity of enzymes that negate the mode of action of the insecticide (Scott, 1990).

Bioassays can be used to measure the susceptibility of populations and species to insecticide. A bioassay system that mimics field exposure of an insect to an insecticide is usually a reliable guide to field performance of a product (Roush and Miller, 1986). Such a bioassay is versatile, the same information being used to diagnose control failures and to monitor for and manage insecticide resistance.

Biochemical assays for activity of the enzymes which disrupt insecticide function are efficient resistance management tools (Brown and Brogden, 1987). Biochemical assays have practical advantages over insecticide bioassays for detecting and monitoring resistant populations; they are more precise, more rapid, and require far fewer test subjects than bioassays with living insects (ffrench-Constant and Roush, 1990).

However, both insecticide bioassays and enzyme assays must be developed to take advantage of efficiencies in the biochemical assay system. Conventional bioassays are needed to first establish baseline susceptibility to the pesticide and to confirm if resistance develops. Enzymes responsible can be identified and quantified once resistance occurs. Rapid biochemical tests for enzyme activity can then be developed to measure the proportion of resistant individuals in populations, as a guide to the genetics of resistance inheritance, likely management programs, and their effect. Bioassays are needed to validate biochemical assays in terms of insecticide performance and field-effectiveness.

3.0 OBJECTIVES

This project sought to develop systems to monitor or detect resistance to the insecticide chlorpyrifos in whitegrub infestations in sugarcane. A secondary role was to prepare a basic insecticide resistance management (IRM) philosophy for the canegrub control program.

Objectives given in the (1992-amended) Research Plan were:

(1) Develop analyses for three enzyme systems associated with the effectiveness of the insecticide chlorpyrifos in canegrubs; these were mono oxygenases, glutathione-S-transferases and esterases.
(2) Quantify the activity of these enzymes for canegrub species with a range of responses to chlorpyrifos.
(3) Identify and quantify processes by which canegrubs are poisoned with chlorpyrifos from suSCon® Blue granules.
(4) Further develop bioassays relevant to the mode of action of suSCon® Blue insecticide, and use the most efficient system to measure the susceptibility of a range of canegrub species.
(5) Produce a draft insecticide resistance management strategy for canegrub control with chlorpyrifos.

Dr Rose was unable to complete objective #2 for two of the three enzymes, because of equipment failure leading to loss of the prepared samples and the non-availability of further specimens from the same populations when this occurred. All other objectives were completed.

4.0 DIFFICULTIES ENCOUNTERED

Previous BSES experience demonstrated the immense cost and impracticality of culturing large numbers of normal, healthy canegrubs in controlled environments. Thus our testing was restricted to times when canegrubs were available in fields.

Field collection was usually difficult and labour-intensive, and so we were unable to collect sufficient insects to compare all bioassay methods using single species or populations or growth stages. Thus, we had to compare a single method with each of the other methods in paired tests that required fewer insects, prolonging the test method selection process.

We did not detect populations which had developed resistance. Thus we were unable to identify enzymes involved in resistance, or develop specific insecticide resistance management programs based on the dynamics of resistance inheritance.

Freezer failure and loss of liquid nitrogen resulted in loss of the tissue preparations for both esterase and glutathione-S-transferase determination, after monooxygenase activity had been measured.

5.0 RESEARCH METHODOLOGY

Bioassays and other studies on field-collected grubs were conducted in the laboratory and constant-temperature facility at Meringa Experiment Station. Recommendations for bioassay methods and laboratory practice set down by Busvine (1971) and Robertson and Preisler (1992) were followed when ever possible. Full bioassay details will be reported separately; outlines are given here.

Living grubs from some of the same populations were sent to Sydney University, where tissue preparation methods and biochemical enzyme assays were modified to suit canegrub gut samples.

5.1 Chlorpyrifos acquisition

Full details of experiments on chlorpyrifos movement from suSCon® Blue granules and acquisition by canegrubs are given in Chandler(in prep. a).

Chlorpyrifos moving from granules was analysed in soil-sections 0.1-2.0, 2.0 - 4.0, 4.0 - 8.0, and 8.0 - 14.0

mm above, below, and beside horizontal or vertical single-layer bands of granules.

The zone of insecticidal effect in soil above granules was estimated by restricting canegrubs' access to within 0.5, 2.5, 4.5, 8.5, or 14.0 mm of three suSCon® Blue granules. Grubs were excluded with metal mesh (1.5 mm aperture, 0.4 mm thick) set above the granules.

The extent of contact with granules to kill *Lepidiota frenchi* grubs was simulated by allowing large third instars to burrow past single suSCon® Blue granules placed centrally every 10 cm along 40 cm long X 2.5 cm diameter (split) PVC cylinders packed with soil. After 14 d, each chamber was opened along the midline and the condition, position and prior movements of each grub observed. In fields, the proximity of dead, poisoned or paralysed canegrubs to suSCon® Blue granules was observed.

The possibility of grubs biting and ingesting fragments of granules was tested by microscopically examining gut contents of poisoned canegrubs from field trials and laboratory bioassays. Bodies were simmered for 12 - 48 h in 5% potassium hydroxide solution; the gut content was collected by washing the remaining tissue through a 100 mm aperture sieve. Granules collected next to dead or poisoned grubs in the field and from bioassay tubes were microscopically examined for bite-marks.

5.2 Bioassay dosing methods

Exposure. Grubs were exposed to chlorpyrifos by six methods, simulating key aspects of chlorpyrifos acquisition by canegrubs in field soil.

(1) burrowing in a range of chlorpyrifos concentrations in sand,
  (a) for the duration of the bioassay (≥30 d), or
  (b) for a range of periods (0.3 h - 5 d), or
  (c) for 3 d;
(2) burrowing in soil with controlled-release insecticide granules of different sizes and ages for 3 d;
(3) topical application of chlorpyrifos in 1 mL acetone solvent directly to the integument of each canegrub; and
(4) burrowing in sand injected with a 5 mL droplet of chlorpyrifos at a range of concentrations for 3 d.

Formulations. Dosages for all except the granule-applied treatment were prepared by dilution from a 1% stock concentrate of Lorsban® EC (chlorpyrifos 500 g/L). The concentrate was diluted in water for treatments applied to sand or soil. For the topically-applied treatments, the concentrate was dissolved in analytical-grade acetone. For the granule-applied treatments, commercial suSCon® Blue granules with technical chlorpyrifos in an ethlene-vinyl-acetate (EVA)-based matrix were used;.

Application. Sand-incorporated insecticide dosages were diluted in sufficient water to bring the medium to 10% moisture. The solution was sprayed onto the sand in a rotating mixer.

Topically-applied dosages were delivered at the end of a blunt needle and applied to the cuticle between the third pair of legs of each grub in a 1 mL drop of acetone solution. Each drop was metered from a ground-glass syringe set in a micrometer measuring device.

For granule-applied treatments, granules of different sizes and ages were placed in soil in bioassay tubes, 2-24 mo before the biosassay; insecticide dosages in soil were estimated analytically from a sample of tubes for each treatment.

For microdroplet-injection treatments, 5 mL of water emulsion was injected into packed sand approx 10 mm above the base of each bioassay tube. A Hamilton CR-700-200 repeating dose syringe (Hamilton Co, Reno, Nevada) fitted with a blind-ended, side-ported needle was used to measure and deliver each dosage. The needle was wiped after each refill to remove adhering insecticide.

5.3 Selecting a bioassay method

Six bioassay systems with canegrubs exposed to chlorpyrifos were tested. Preliminary bioassays with all methods were run to gain experience and improve their efficiency. Two methods were relatively impractical and were rejected. The most consistent bioassay method in the preliminaries was selected as the putative standard for pairwise comparison with the other three methods in a series of tests on similar groups from the same populations, for at least three species of canegrubs. The less efficient method in each set of

comparisons was rejected and the more efficient method used for the next comparison.

Population responses with each bioassay were analysed using Probit analysis (Finney, 1978) with the computer-program POLO-PC (LeOra Software). Five statistics of the analyses (Robertson and Preisler, 1992) were used to assess the more efficient bioassay in each paired comparison.:-

1. The **slope** of the fitted response regression line. Bioassays with a higher slope to the response line are considered to be more sensitive (Tabashnik and Cushing (1987); Hinkle, Sheppard and Nolan (1985)).
2. The value of the statistic '**t**', a ratio of the slope of the response line to its standard error, indicating the strength of the response as a function of the treatment. **'t'** must be greater than 1.96 for the response to be significant (P=0.05). The response with the higher **'t'** is more likely to be a function of the treatment alone.
3. The "goodness of fit" **(χ^2)** predicted by the linear model; χ^2 values less than tabulated for the appropriate degrees of freedom are essential for use of the probit model.
4. The heterogeneity factor (**HF**) of the response (the ratio of the χ^2 of the residuals and the degrees of freedom) is a guide to the nature of the response. **HF** values less than 1 are desirable; higher values could indicate independent variables not accounted for in the model. It was reasoned that analyses for two or more test methods on the one population would probably all indicate heterogeneity if it were attributable to population genetics; alternatively, a higher **HF** with one test method than another may indicate greater variability inherent in using that method.
5. The index of significance of potency estimates (the statistic '**g**') defines the confidence intervals of the response-level dosages; '**g**' must have a value less than 0.5 at the required probability level for accurate calculation of the dosage limits. The bioassay with the smaller **'g'** was regarded as more precise.

A series of bioassays with the best method against comparable populations was analysed for consistency, was another feature of the POLO-PC program to test for similarity of slope and intercept between the populations (Chandler, in prep. b).

5.4 Bioassay procedures

Larvae: Third instars were collected from field infestations. Each was held with 10-20 g of its native soil in a 62 X 20 mm diameter phial, and fed small cubes of carrot or sugarcane stem. Each larva was weighed (±0.05 g). Larvae were separated around the median weight category into 10 equal-number weight-ranges. One randomly-selected larva from each weight-range was included in each test group. Responses of each larva were individually recorded.

Species tested. Populations of *Antitrogus rugulosus* (Blackburn), *A. consanguineus* (Blackburn*), A. parvulus* Britton*, Lepidiota frenchi* Blackburn, *L. negatoria* Blackburn, *L. crinita* Brenske, *L. consobrina* Girault, *L. picticollis* Lea, *L. noxia* Britton and *Dermolepida albohirtum* (Waterhouse) were used to compare bioassay methods and measure chlorpyrifos susceptibility.

Test medium*:* All bioassay method comparisons, except for the granule-applied treatment, were conducted in clean, washed, and sieved (<1 mm aperture) river sand at 10% (dry wt) moisture; sand is relatively inert and unlikely to influence the response. Granule treatments were applied in Mission series (Smith and Murtha, in prep.) clay-loam soil at 12.5% (dry wt) moisture. After exposure to the treated medium, each grub was moved for observation into a clean tube with 20 g of its native soil (untreated) at 12.5% moisture. Tests were conducted at 24°C.

Responses to microdroplet-applied chlorpyrifos were compared in sand and three far-northern soils (Smith and Murtha, in prep.) viz a Mission series red-brown clay-loam, a Pin Gin series red kraznozem, and a Thorpe series dark, sandy, light-clay loam.

Test arenas*:* Each bioassay tube (62 X 20 mm diameter) was packed with 20-25 g of firmly compressed sand or soil, leaving a chamber at the top to receive a grub. One grub was placed in each tube. All tubes were arranged on rectangular 30 X 24 cm trays. An inverted tray was set over each tray of tubes, and the packed tray of tubes stored in a clean plastic bag which was not sealed. Heavy weights were placed on the trays to stop grubs from pushing sand or soil from the tubes or from escaping.

Dosages: A preliminary bioassay with ≈50 insects was used to estimate the effective dosage range for each

population before the main bioassay. At least six treatments, most between Lethal Concentrations for 50% (LC_{50}) and 95% (LC_{95}) of the population, and one causing complete mortality were used in each bioassay.

***Numbers of test subjects**:* If possible, there were at least 120 treated larvae (20 per treatment), plus 10-20 untreated larvae in each bioassay.

***Observations**:* Each grub was inspected on (or near to) days 3, 7, 10, 14, 21, and 28 after treatment. The onset of incapacitation (inability to burrow), paralysis (inability to move in response to a gentle touch, or locked mandibles), or death were recorded for each grub. Observations were terminated if control mortality exceeded 10%.

***Untreated controls**:* Untreated control insects were subjected to the same handling and medium as the treated insects. Additional control insects were treated with emulsifiers and/or solvents at the maximum rate used to confirm that these did not kill or disable grubs.

***Data analysis**:* Population responses were analysed using the computer software POLO-PC (LeOra Software). The program was used to provide performance statistics about each bioassay, to estimate response-level dosages (with confidence intervals) for each population, to compare responses of different populations, to compare responses of different weight-range larvae for three species, and to compare population responses in sand and soil.

5.5 Measurements

***Bioassay methods**:* The five bioassay statistics, **slope, 't'**, **χ^2**, **HF**, and '**g**' were estimated for two or more bioassay methods on the same population of canegrubs. Statistics from the microdroplet-injected treatment were compared with values from the granule-applied treatment, topically-applied treatment, and sand-incorporated treatment, using the criteria outlined above.

***Consistency between estimates**:* The responses of eight populations of *D. albohirtum* which had been exposed to chlorpyrifos treatment, and three populations each of *A. consanguineus* and *A. parvulus*, none of which had been exposed to chlorpyrifos, were compared for consistency within species.

***Relative susceptibility of canegrub species**:* The susceptibility to chlorpyrifos of 23 populations representing 10 canegrub species was measured using the microdroplet-injected bioassay system.

***Effects of body-weight on susceptibility**:* Responses of individuals from three or four body-weight ranges were combined and analysed for three populations of *A.consanguineus*, three of *A. parvulus,* and six of *D. albohirtum*. Prior to this, combined analyses of the data for the individual populations within each species showed that the slopes of the responses to chlorpyrifos were not dissimilar.

***Susceptibility in sand and soil**:* The susceptibility to chlorpyrifos of two populations of *D. albohirtum* and one each of *A. parvulus* and *A. consanguineus* was measured in sand and one or more soils.

***Susceptibility changes with exposure**:* Susceptibility of three populations of *L.crinita*, one from a site not previously exposed to chlorpyrifos and two collected 4 years apart from an area previously treated with chlorpyrifos, was compared.

The susceptibility of six populations of *D. albohirtum* collected from sites treated previously with suSCon® Blue, but where granules were detoxified completely due to their age, was compared with the susceptibility of 'survivors' from treated first-ratoon cane where the insecticide was poisoning at least a portion of the grub population.

5.6 Enzymes associated with resistance

All grubs sent for enzyme bioassay were freshly collected from fields where, suSCon® Blue insecticide granules were no longer releasing chlorpyrifos due to their age, or where no insecticide had been used. "Survivors" from the bioassays with chlorpyrifos were not tested in this project.

***Enzyme preparation*:** Two *Antitrogus parvulus* and *A. consanguineus* grubs (which are relatively small) were combined for each tissue sample preparation. With all the other species tested, a single grub was used in each preparation. Canegrubs were weighed, then decapitated and fluid allowed to drain away. Using a pair of scissors, the grubs were cut ventrally from anterior to posterior and the alimentary canal gently teased out. The fore and mid gut was cut off and dissected longtitudinally. The contents of these were discarded along with the entire hindgut. All remaining tissue was placed in 0.1 M phosphate buffer (hereafter referred to as buffer), pH 7.5, containing 1 mM EDTA and 10 mM GSH. Tissue was placed in a crucible with 10 mL buffer and 20% PVPP, and cut into small pieces with scissors, then ground with a mortar. The homogenate was strained through MIRA cloth and centrifuged at 38,000g for 5 sec. The supernatant was collected and made up to 27 mL; 1 mL of this was taken for esterase determination and stored in liquid nitrogen. The remaining 26 mL was made up to 27 mL and centrifuged at 210,000g for 20 min. From the supernatant, 1 mL was taken for GSH-transferase determination and stored in liquid nitrogen. The pellet was resuspended in 5 mL buffer containing 1 mM EDTA and 20% glycerol after washing with the same mixture. This suspension (usually 1 grub/ mL) was used for determination of cytochrome P_{450} and of aldrin epoxidation.

Assays: Cytochrome P_{450} The amount in the microsomal suspension was measured by the metyhod of Omura and Sato (1964). After bubbling CO through through the suspension in the sample cuvette, sodium dithionite was added to the reference and sample cuvettes. The spectrum from 400 - 510 nm was recorded. The extinction co-efficient used for cytochrome P_{450} was 91 nM^{-1} cm^{-1}.

Aldrin epoxidase: The procedure used was similar to that of Rose and Wallbank (1986). The 2.5 mL incubation mixture contained 1.7 mL buffer, 0.25 mL of a mixture of 1.1 µmol NADP, 12.1 µmol glucose-6-phosphate and 2 units of glucose-6-phosphate dehydrogenase in buffer, 0.5 nL of microsomal suspension and 274 µmol aldrin in 0.05 mL methyl cellosolve. Incubations were carried out with shaking at 30°C for 10 min. The reaction was stopped by the addition of with 2 mL of 3 M HCl, and 10 mL of hexane. After further shaking and centrifuging, the amount of dieldrin in the hexane layer was analysed by electron capture gas chromatography using a Varian model 3700 equipped with a Ni63 electron capture detector. The glass column, 2.0 m long x 2.0 mm inside diameter, was packed with 5.0% OV-210 on chromosorb WHP 80-100 mesh. Conditions employed were that the injector temperature was 181°C, the detector temperature was 230°C and the N_2 flow rate 50 mL/min. The peak height of a standard solution of dieldrin injected into the gas chromatograph after every fifth sample was used to quantify the results. Results were calculated as picomoles dieldrin produced per minute on a per individual basis, or per g tissue basis.

General esterase: Activity was measured with the substrate para-nitrophenyl acetate (PNPA) using a modification of the method of Mackness, Walker, Rowlands and Price (1983). The 3 mL incubation mixture contained 2.9 mL of buffer, 0.05 mL of substrate (0.12 M PNPA in ethanol) and 0.05 mL enzyme. The reaction was initiated by addition of substrate to the mixture at 30°C. The hydrolysis of PNPA shows the yellow colour of paranitrophenol generated, and was detected by the change in absorbance at 400 nm. The absorbance change was converted to nmole paranitrophenol produced, using the extinction coefficient of 17000 M^{-1} cm^{-1} for paranitrophenol.

Glutathione S-transferase: Activity was assayed by the method described by Rose and Wallbank (1986). The substrate was 1,2-dichloro-4-nitrobenzene (DCNB). The reaction mixture was prepared as follows: 0.1 mL of the supernatant (sample) or 0.1 mL of the homogenising buffer (reference) was added to 1.0 mL buffer (20 m glutathione in buffer) and the mixture gently shaken in quartz cuvettes. Mixtures were allowed to equilibrate at 35°C in a recording spectrophotometer for 4 min. Then, 0.01 mL of 150 m*M* of DCNB was added to both cuvettes, mixed, and replaced in the spectrophotometer. Activity was measured as the rate of change in absorbance over ca. 4 min at 344 nm and converted to nanomoles per minute per milligram of tissue, using an extinction co-efficient of 10 nM^{-1} cm^{-1} (Yu, 1982).

5.7 Insecticide resistance management strategy

Mr Chandler discussed recent developments for monitoring, measuring, and managing insecticide resistance (Croft (1990); Roush and Miller (1986)) in the context of the canegrub situation with several Australian experts in this field including: Dr Pat Collins, Queensland DPI, Brisbane; Dr Robyn Gunning NSW Agriculture, Tamworth; Dr Joanne Daly, CSIRO, Canberra; Dr H A Rose, University of Sydney; Dr Gary Fitt, CSIRO, Narrabri, NSW; as well as the entomology group at BSES. Plans for monitoring canegrub infestations to detect signs of developing resistance, and for managing canegrub control programs to minimise the opportunity for resistance to develop are suggested.

6.0 RESULTS

6.1 Processes of chlorpyrifos acquisition

Almost 85% of the chlorpyrifos in soil was within 2 mm (vertically) of horizontal bands of granules, and about 70% of chlorpyrifos was within 4 mm (horizontally) of vertical bands of granules (Table 1).

All *L. negatoria* canegrubs approaching 2.5 mm or less from three suSCon® Blue granules died or were paralysed; 38% (corrected for control mortality) of grubs excluded by 4.5 mm died; only 11-17% of grubs excluded by 8.5 mm or more died (Table 2). An attempt to repeat the excluder experiment with greyback grub (a more tolerant species) failed; the grubs destroyed the mesh excluders used in this bioassay. Nevertheless, <50% of greyback grubs with unrestricted access to granules were killed.

In the 40 cm long cylindrical tubes, the *L. frenchi* grubs' path was evident from disturbed soil in its wake. Five of the eight grubs died or were completely paralysed at the first granule. Two died shortly after passing the first granule but before reaching the second granule. One did not contact the first granule, but died in contact with the second granule. In several cases, the granules remained ***in situ***. Each grub in the four tubes without granules remained healthy.

Gut contents of only 1 of 25 poisoned and paralysed greyback grubs collected from fields contained a fragment of suSCon® Blue granule. In contrast, 65 corpses out of 120 recovered from bioassay tubes where grubs had access to granules for 7 d contained fragments of plastic. No field-collected granules from near poisoned grubs showed bite-marks, whereas many granules recovered from bioassays had been chewed.

6.2 Comparisons of Bioassay methods

For efficiency: Dosing methods using variable exposure times or continuous exposure for the duration of the bioassay were rejected from further testing after the preliminary bioassays. Timed-exposure dosages were impractical to administer, requiring multiple start and inspection times. Also, slow-to-burrow grubs caused uneven exposure within treatments, particularly with short treatment times; this was very obvious with one population of *L. crinita*. Continuous-exposure dosing was also impractical. There was poor discrimination between concentrations. Responses to less than 2 mg AI/kg took up to 35 d to have full effect, by which time mortality of untreated groups was often excessive (>20%). Also, long response time precluded preliminary tests to establish the effective concentration range.

Responses to insecticide doses from 3-days of exposure to treated sand, from granules in soil, and from topical application to the insect cuticle were statistically reliable in preliminary bioassays; techniques for each were reasonably practical, and were for 5 canegrub species improved after the preliminary bioassays had been conducted. All preliminary bioassays for 5 canegrub species with the microdroplet-injection dosing were statistically reliable (Table 3), so this method tentatively became the standard with which to compare each of the other three dosing methods in a series of paired bioassays.

Responses to three of four bioassays with granule-applied dosing were heterogeneous, and two also had non-significant slopes to the response lines (Table 4). In contrast, comparable bioassays with microdroplet-injected dosing were statistically reliable.

All three bioassays with topically-applied chlorpyrifos were less efficient by most criteria than comparable, successful bioassays with microdroplet-injection dosing (Table 5).

Three of five bioassays with exposure to sand-incorporated chlorpyrifos for 3 d had heterogeneous data, and all five had unacceptable **'g'** values (>0.5) (Table 6). In contrast, all five comparable bioassays with microdroplet-injected dosing were statistically reliable.

Thus, bioassays with the microdroplet-injected dosing system were more efficient than with the five other dosing systems.

For consistency: Responses to microdroplet-injected chlorpyrifos from six populations of *D. albohirtum* (Table 7) were consistent; slopes of the six regression lines did not differ significantly (χ^2= 5.04, df=5, P=0.41) and the fiducial limits for the calculated LC_{50} and LC_{95} dosages overlap. Response-slopes for three populations (Table 8) of *A. consanguineus* were similar (χ^2=1.82, df=2, P=0.40), as were the slopes for three populations of *A. parvulus* (χ^2=4.02, df=3, P=0.26).

6.3 Relative susceptibility of 10 species

Bioassay statistics and calculated LC_{50} and LC_{95} values for all populations of 10 species tested by the microdroplet-injection bioassay are detailed in Table 8. All tests were statistically reliable. Susceptibility of the ten species varied widely.

6.4 Effect of body-weight on susceptibility of three species

Three populations of *A. consanguineus* had similar (χ^2=1.82, df=2, P=0.4) slopes to their response regressions. The responses of all individuals to chlorpyrifos in three weight-ranges (0.9-1.6, 1.7-2.2, and 2.3-3.7 g) were similar. Slopes and intercepts of response-lines for each (Table 9) were similar (χ^2=2.89, df=4, P=0.57).

Three populations of *A. parvulus* had similar (χ^2=0.33, df=2, P=0.85) slopes to their response regressions. The responses to chlorpyrifos of all individuals in four weight-ranges (0.8-1.2, 1.3-1.5, 1.6-1.9, and 1.9-2.5 g) were similar. Intercepts of response-lines for each differed slightly (χ^2=10.99, df=6, P=0.088); the slopes of the lines (Table 9) were similar (P=0.25).

Five populations of *D. albohirtum* had similar slopes and intercepts (χ^2=9.28, df=8, P=0.319) to their response regressions. The combined responses to chlorpyrifos of individuals in four weight-ranges (2.6-3.9, 4.0-4.4, 4.5-4.9, and 5.0-5.7 g) were different. Slopes of the response-lines (Table 10) were significantly (χ^2=21.3, df=3, P=0.000) steeper for the largest larvae. LC_{50} dosages for all weight-ranges were similar, but LC_{95} dosages appear to decrease as larval weight increases.

6.5 Influence of soil-type on susceptibility

Slopes and intercepts of response lines for *D. albohirtum* treated with chlorpyrifos in sand and three soil-types differ (χ^2 =94.5, df=6, P=0.000). LC_{50} to chlorpyrifos in Mission series clay-loam soil was three to four times the LC_{50} in sand for *D.albohirtum* and *A. consanguineus* (Table 11), and 12 times larger for *A. parvulus*. Chlorpyrifos was equally potent (Robertson and Preisler, 1990) against *D. albohirtum* in the Mission clay-loam and in the Pin Gin kraznozem, and only 0.5 as effective in the Thorpe series dark sandy-loam as in the other two soils.

6.6 Influence of prior exposure on susceptibility

Chlorpyrifos was 4.7 times (3.5-6.1) as potent for a previously unexposed population of *L. crinita* (Table 8) as for two previously exposed populations; the slopes and intercepts of the response-lines were significantly (χ^2=8.4, df=2, P=0.015) different.

Dermolepida albohirtum 'survivors' from treated fields where suSCon® Blue granules were releasing chlorpyrifos and effectively reducing grub numbers and damage, were as susceptible to chlorpyrifos as larvae from seven populations collected from fields where suSCon® Blue granules had ceased releasing chlorpyrifos, due to their age; slopes of the responses by all eight populations were similar (χ^2 =9.7 df=7

P=0.21).

6.7 Enzyme activity

Monooxygenase. Table 12 shows the amounts of cytochrome P450 and activity of aldrin epoxidase on a per gram individual basis.

Transferases and esterases. The methods described for measuring these enzymes were effective in preliminary tests. As stated previously, tissue preparations for these populations were lost due to freezer failure and loss of liquid nitrogen from the storage container, so measurements from the relevant populations were not possible.

7.0 DISCUSSION

Acquisition: In a simulation, highly susceptible *L. frenchi* canegrubs died at or close to a single granule. In a controlled bioassay, all *L. negatoria* canegrubs died after approaching to within 2.5 mm of three granules, but only 39% (corrected mortality) died if excluded to 4.5 mm or more from three suSCon® granules (Table 2). This suggests the 'grub-toxic' zone around suSCon® Blue granules is 2.5 - 4.5 mm for susceptible species such as *L. negatoria, and* probably less than 2 mm for chlorpyrifos-tolerant grubs such as *D. albohirtum* (Table 8), which (presumably) acquire insecticide from very close to one or a number of granules before there is a lethal effect.

Chlorpyrifos released from suSCon® Blue concentrates very close to the granule (Table 1). For standard-size granules (120/g), 10-60 mg chlorpyrifos normally surrounds each granule (Chandler, unpublished BSES data), depending on age. This represents concentrations of 280-1680 mg/kg if confined to within 1 mm of a 2 mm diameter sphere.

Grubs rarely ingest portions of granules in the field. However, they readily chew granules when confined in a bioassay tube without food. This suggests that in the field, grubs may manipulate and explore granules with their mouthparts but reject them without chewing if alternative food is available. **This raises the possibility that a 'palatable'or 'attractive' matrix which holds insecticide tightly (not releasing into the soil) could be a commercial prospect as a poisoned bait for long-term canegrub control.**

Bioassay method: Data suggest grubs die from transient contact with high chlorpyrifos concentration in soil within 1-2 mm of one or more suSCon® Blue granules, or from direct contact and manipulation of granule(s), but not from ingestion of granules. Therefore, the microdroplet bioassay was modelled on exposure to 5 mL (a sphere ≈ 2.0 mm diameter) of high concentration (50-10,000+ mg/kg) insecticide for 3 d.

The injected-microdroplet bioassay method was the most efficient for measuring susceptibility to chlorpyrifos. Responses to chlorpyrifos administered by this method were statistically reliable in 22 consecutive bioassays including 10 species (Table 8). There were several unreliable results with all of the other systems tested, including topical application of insecticide to the integument which has long been a standard method for testing insects (Busvine, 1971). Continuous exposure to relatively low concentrations in soil, a method commonly used for tests with white grubs (Lawrence, Ladd and Kwolek,1977; Villani Wright and Baker, 1988) and used in previous BSES bioassays, was also insensitive and impractical. Responses to the microdroplet bioassay from six populations of *D. albohirtum* (Table 7) and three each of *A. consanguineus* and *A. parvulus* (Table 8) were consistent within species.

The microdroplet-injection bioassay system is efficient, consistent within and across species, and repeatable. It is also rapid and simple, and relatively safe to administer. Equipment needed is cheap and easy to obtain. For a 150-insect bioassay, mixing insecticide and injecting sand-filled tubes takes (approx.) 2.5 h. Evaluation required three inspections, at days 4, 10-14, and 21-28, totalling approximately 6 h. Time to collect and sort larvae is extra, but remains the same regardless of what bioassay method is established. Responses on the fourth day were highly indicative; by the fourteenth day the response was almost complete.

Susceptibility and grub body-weight: Consistent response between large and small body-sizes of *Antitrogus* spp. grubs (Table 9), contrasts with the variable response between small and large *D. albohirtum* grubs (Table 10). Thus weight groups for susceptibility monitoring for greyback grub should be selected carefully. Individuals should be weighed before testing, and all test groups should be of similar weight

composition. Fewer precautions with *Antitrogus* spp. may be justified.

Where possible, mature larvae (those showing dark-cream colour from body fat, often quiescent and almost pre-pupal, and usually the largest weight-range in the population) should not be used for susceptibility baseline data or resistance detection programs, particularly if critical dose testing (Roush and Miller, 1986)is adopted. Mature larvae may behave differently (eg the weak burrowing response mentioned above for *L. crinita*) from actively growing larvae, and/or may also differ physiologically from growing larvae. Less insecticide is required to kill mature *Helicoverpa* larvae than younger, actively growing larvae, perhaps because body enzymes become less active in mature caterpillars (R. Gunning, NSW Dep Agriculture, Tamworth). Similar differences may occur with *Dermolepida*; however, the fact that the LC_{50} values remain similar for all weight-ranges (Table 10) is not explained by an age-related change in large larvae.

Susceptibility and the test substrate: Variation of response with soil type (Table 11) justifies the rationale of adopting sand as a standard testing medium for consistency. Comparison of responses in sand and soil helps to relate Lethal Concentrations in sand to minimum insecticide-release capabilities for suSCon® Blue granules to be effective in soil. Harris (1972) and Tashiro and Kuhr (1978) described effects of soil factors including organic carbon, sand:clay ratio, soil moisture and clay type on responses to insecticides, which were generally less effective in clay soils than in sandy soils. In our bioassays, LC_{50} values in sand were also lower than in soil (Table 11).

Species susceptibility relative to field treatment efficacy: The relative ranking of 10 species for susceptibility (Table 8) also reflects the commercial effectiveness of suSCon® Blue in their control. The most susceptible (*A. consanguineus*, *A. rugulosus*, *L. negatoria and L. frenchi*) are also those for which there are few complaints about poor control with chlorpyrifos granules. The three least susceptible species (*D. albohirtum*, *L. picticollis* and *L. noxia)* are the ones about which there are numerous examples of inefficient control that cannot be attributed to other factors such as defective application. There have been complaints about poor control with *A. parvulus* (an intermediate-susceptibile species), but observations and field-trials (Allsopp, McGill and Stringer, 1996) suggest control is efficient and effective if suSCon® Blue is applied correctly.

The similar ranking of these bioassays and commercial responses to suSCon® Blue treatment suggests that the microdroplet bioassay system is relevant for susceptibility monitoring, and for researching new insecticides, and perhaps for diagnosing chemical factors associated with poor control.

The calculated LC_{50} and LC_{95} dosages in sand are only a guide to the amount of chlorpyrifos necessary for suSCon® Blue granules to control each species of canegrub; dosages of chlorpyrifos in soil are 3-12 times the dose to elicit similar response-levels (Table 11) in sand.

LC_{50} and LC_{95} dosages varied 40-50-fold between the most susceptible (*A. consanguineus*) and the least susceptible (*L. noxia*) species. LC_{50} (soil) dosages for susceptible and intermediate species such as *A. consanguineus* (10 µg) and *A. parvulus* (43 µg) are within the production capacity of a single suSCon® Blue granule. LC_{50}(soil) dosages for *D. albohirtum* (range 51-107 mg chlorpyrifos, Table 11) are greater than the 10-60 µg which one suSCon® Blue granule produces for much of its effective life (KJ Chandler, unpublished field-survey report, 1996). Yet, 21 kg of granules/ha kills 80-90% of *D. albohirtum* grubs (Hitchcock, Chandler and Stickley, 1984; Chandler, McGuire, McMahon and Schultz, 1993; KJ Chandler, unpublished data) infesting a sugarcane crop. Two factors may explain the apparent disparity between the relatively low dosages available around granules and the relatively high field mortality of *D. albohirtum* grubs. First, grubs may have to contact more than one granule before poisoning occurs. Second, the majority of grubs may not be killed by suSCon® Blue until they become well-grown and more susceptible than in the early third instar. The necessity for multiple contacts before poisoning occurs will, by the laws of probability, prolong the period for which each grub is able to remain actively feeding. Similarly, well-developed grubs will have already caused considerable damage. Both factors will result in inefficient control (ie slow to kill, with substantial root damage) of *D. albohirtum* compared with control of the more susceptible species.

This situation suggests a fundamental weakness in attempting to control such a wide range of species with one product and a single application rate. Larger granules that sustain more insecticide in the soil, may improve control of tolerant species such as *D. albohirtum*. The reverse may also be true; smaller granules applied at lower rates (less product per ha) may provide adequate control of the highly susceptible species at lesser cost.

Enzyme activity: ***Monooxygenase.*** In insects, the cytochrome P_{450} monooxygenases are the most influential enzymes involved in metabolism of chlorpyrifos. Assay conditions for canegrubs were optimised with respect to determination of cytochrome P_{450} and aldrin epoxidase in six canegrub species. In general, these measurements (Table 12) should correlate for each species, since the former provides an overall indication of the metabolising capacity of the system, whereas the latter measures the specific ability to oxidise a substrate, in this case aldrin to dieldrin. Other substrates could have been chosen, but aldrin has been widely used in numerous toxicological studies; the assay is extremely sensitive and therefore ideal where activity is low, as in canegrubs.

Generally, insect strains resistant to chlorpyrifos have much higher monooxygenase activity than do susceptible strains. There appears to be no direct correlation between monooxygenase activity (Table 12) and susceptibility of canegrubs to chlorpyrifos in sand (Table 8). Surprisingly, there appears to be an inverse relationship; the more chlorpyrifos-tolerant species have the lower monooxygenase levels, and the least tolerant species the higher enzyme levels. More specifically, *L. crinita* from the 91-22 collection, which had the second highest LD_{50} for chlorpyrifos of all species had the lowest amount of cytochrome P_{450} of those tested. However, the more critical test of comparing monooxygenase in susceptible and tolerant strains of one species were not performed in this project, as no contrasting strains of the one species were identified at the time.

Esterases and transferases. Unfortunately, no results were obtained with these enzymes due to equipment and storage failure, but the methods for enzyme preparation and assay procedures given here are suitable for determining the activities of these enzymes with canegrubs.

Evidence of resistance to chlorpyrifos: There is an indication of resistance amongst treated populations of *L. crinita*, the only species for which populations have been tested from areas previously treated and not treated with chlorpyrifos. The LC_{50} and LC_{95} doses for a non-exposed population bioassayed in 1992 are significantly ($P<0.05$) lower than for two previously-exposed populations measured in 1991 and 1995 (Table 8). The slope of the response-line for the 1991 population is significantly less ($P=0.015$) than for the 1995 population. Increasing LC_{50} and LC_{95} response-levels after initial selection and substantial steepening of response-lines after continued selection (Fig 1) are hallmarks of resistance (ffrench-Constant and Roush, 1990). The only data that do not conform to the expected pattern for "resistance" are the response-levels of the 1995 population; the LC_{50} dosage remained the same as in 1991 and the LC_{95} dosage decreased (Fig 1; Table 8). This is the reverse of what is expected if the proportion of the population with a resistance gene were increasing; there should be an increase in the LC_{50} dosage with no decrease in the LC_{95} as the response-line steepens.

No assumptions about resistance can be drawn for nine species until previously-exposed and unexposed populations (respectively) are bioassayed. All 'susceptibles' (*A. rugulosus, A. consanguineus, L. negatoria* and *L. frenchi),* an 'intermediate' species *(A. parvulus*), and the most 'tolerant' species (*L. noxia*) were from fields with no history of chlorpyrifos. All populations of *L. consobrina, L. picticollis*, and *D. albohirtum* were from previously-treated fields.

There was no evidence that *D. albohirtum* 'survivors' from two fields in which 1.5 yr-old granules were delivering chlorpyrifos but root damage was excessive were more tolerant than seven other populations from fields where granules were too old to contain any insecticide; all had a similar slopes to their response-lines (Table 8; $\chi^2=9.7$, df=7, $P=0.207$). The LC_{50} and LC_{95} response dosages for the 'survivors' were 14 and 114 mg, respectively, well within the range of other populations (Table 7). However, evidence of low chlorpyrifos concentration in the soil at these sites (Chandler and Erbacher, 1997) suggests poor control was most likely due to insecticide degradation and not to resistance.

7.1 Potential for resistance

It is possible that resistance to chlorpyrifos may never develop in various canegrub populations, even though some species are naturally tolerant or uncontrollable in practical terms.

Modern theories of insecticide resistance management (Roush and Tabashnik, 1990; Roush and Daly, 1990) maintain that resistance development is almost entirely dependent on the dynamics of the resistant genes within and between populations, and competitive "fitness" of individuals to pass on these genes, and

heritability of factors resulting in resistance. However, we know nothing about fitness and heritability until (if) resistance develops.

Earlier theories of whether or not resistance may occur used factors such as the slopes of response-lines, the dosages of insecticide applied, the frequency of insecticide application, the number of generations selected and the severity of selection. However, these are not reliable determinants. For example, slow generation turnover (eg univoltine or biennial life cycles of canegrubs) should result in very slow selection for resistance, and yet Roush and Daly (1990) quote species that develop resistance within only three or four generations. The factors listed above may determine the population dynamics of resistance, but not the probability of it occurring.

The initial frequency of resistance genes within populations is "of considerable fundamental interest" (Roush and Daly, 1990) in understanding the population genetics of resistance. The slopes of response-lines could indicate the proportion of tolerant or resistant genes in a population; shallower slopes indicating a higher proportion of 'relative tolerance' than steeper slopes. All canegrub species had relatively low slopes (Table 8) to their response-lines; however, low slopes could be an artifact of the bioassay, rather than an indicator of incipient resistance. Should we be more concerned about resistance in *A.parvulus* and *A. consanguineus,* with low slopes, than in other species?

It is not practical to specify a general increase in canegrub tolerance of chlorpyrifos that constitutes resistance. Even a minor increase in the LC_{50} for chlorpyrifos-tolerant species such as *D. albohirtum* and *A. parvulus* could result in populations becoming uncontrollable, or resistant in practical terms (Ball, 1981) to the dosages supplied by CR insecticide granules. Conversely, susceptible populations such as *A. consanguineus* would require much greater increases in LC values for populations to become uncontrollable.

The small amount of information about *D. albohirtum* 'survivors' from treated fields suggests they are not a tolerant portion of the population: their survival may be behaviourally-related or simply by chance, rather than a genetic expression of tolerance.

Application of slow-release insecticide has been classed a "high-risk" strategy for developing resistance (Day, 1983; Roush and Daly, 1990). However arguments to support this assertion, relative to the risk posed by other insecticide strategies against canegrubs, are ambivalent. Canegrub generations are annual or biennial. Thus, the frequency of selection for resistance with CR insecticides may not be any greater than if short-term insecticide formulations are applied for control whenever infestations develop. However, the frequency of selection with routine CR-insecticide treatment will be higher than if insecticide is curatively-applied only when grub numbers exceed an action threshold. Conversely, selection pressure in terms of the numbers of survivors from CR-insecticide treatment is relatively low: there is >85% mortality of greyback grubs from CR-insecticide (Chandler *et al*, 1993). Thus, whilst CR insecticide may invite resistance through frequent and severe selection pressure, it may also work against resistance by contributing relatively few 'survivors' to the gene-pool for resistance. The current alternative of control with 'knockdown' insecticide such as ethoprophos reduces grub numbers between 50 - 60% for some species such as *L. frenchi* (unpublished BSES data) and 78 - 86% for *A. parvulus* (Allsopp, McGill and Bull, 1992).

One aspect of the current CR insecticide system equates to a tactic (see below) to discourage resistance. Over 80% of greyback grub infestations in northern Queensland (unpublished CPPB/BSES survey data) develop in 'later' ratoons where aged (3-6 y) CR granules no longer exert selection pressure. Thus large numbers of non-selected individuals will dilute the influence of the relatively small numbers of survivors from 'earlier' crops where the CR-insecticide may be selecting tolerant individuals.

There are conflicting opinions about another aspect of CR insecticide release and its effect on resistance selection. Insecticide release declines and there is less control in the second and third years (Chandler, *et al*, 1993) as granules age. Traditionalists would suggest that lower insecticide concentration will subject large numbers of grubs to sub-lethal dosages which may favour selection for resistance. Modern tactics (see below) suggest the opposite: light selection allows some susceptibles to survive, diluting the potential for homozygous resistance.

7.2 IRM strategies and tactics for canegrub control

In the (extremely fortunate) absence of insecticide resistance *per se* amongst canegrubs, current IRM strategies must centre on manipulating selection pressure "so that increases in the frequencies of resistance

genes can be arrested or slowed" (Roush and Daly, 1990). These authors suggest two general strategies. The tactics for each may seem ambivalent; however "looking at these tactics strictly from the point of view of how the pesticide is used obscures their underlying genetic similarities" (Roush and Daly, 1990). Two strategies and the tactics are:

(A) "management by alternation, or multiple attack", which employs insecticide to reduce selection pressure and reduce the fitness of resistant individuals and survival of their genes in the population, and

(B) "management by moderation", which reduces the severity of selection with insecticide and allows for dilution of resistant genes by immigration of unselected individuals.

The tactics for each strategy are:

A. Reduce fitness of resistant insects when insecticide is applied.

1. Increase dose to kill heterozygotes or resistant homozygotes.
2. Use compounds that confer lower levels of resistance.
3. Treat the most vulnerable life-stage.
4. Use synergists to suppress detoxification mechanisms.
5. Mix pesticides of differing modes of action and metabolism.

B. Reduce the total selection pressure applied.

6. Lower insecticide concentration so some susceptibles survive.
7. Reduce the number of pesticide applications.
8. Use short-term chemicals and avoid slow-release formulations.
9. Do not treat all habitats of the pest; use spot-treatments.
10. Rotate pesticides so that not all generations are exposed to the same one, but avoid spatial mosaic treatments. (Presumably, mosaics of several pesticides?).

Roush and Daly's (1990) comments on the suitability of each tactic, plus comments on their relevance for canegrub management are discussed below.

Tactic #1 has environmental and financial constraints, but "may be useful in populations in which resistance is not yet a problem, where doses can be tightly controlled and uniform, and where resistance in heterozygotes (RS) is low". Roush and Daly (1990) suggest that increases in the frequency of resistant (RR) individuals may be very slow, if the concentration of insecticide kills heterozygotes (and resistance is recessive), if there are very few (RR) individuals, and if high numbers of susceptibles escape exposure and are able to mate with resistant genotypes to assure they will not produce RR offspring.

The sugarcane situation does not comply with all the constraints, particularly with the tolerant species, where aged granules are relatively ineffective and resistant heterozygotes will survive.

Tactic #2 recognises that use of compounds which do not select strongly for resistance or cross-resistance (resistance to one conferring resistance to another compound) should be preferred.

This tactic is largely academic for canegrub IRM at present, as resistance-prone compounds can not be identified until resistance develops, and elucidation of their heritability would be difficult and prolonged because of long canegrub life-cycles.

Tactic #3 attempts to weaken discrimination between genotypes, by not treating life-stages in which resistance mechanisms express strongly, or by treating RR genotypes only at a vulnerable stage.

The CR-insecticide system complies with one and violates the other of these recommendations, by remaining active all the time. We can not identify the most resistant or suceptible life-stages until resistant canegrub populations are confirmed. We have not been able to show any major difference in susceptibility between growth-stages of (III) instar grubs. Recent experiments (Allsopp, McGill and Stringer, 1996; Chandler and Erbacher, 1997, Chandler, *et al*., 1993, and unpublished data) suggest that grub control is actually less effective if granules are placed "shallow" (100 - 120 mm below the soil surface), in the zone preferred by the

young second or first instars (pers. communication, P.W. Walker, BSES, unpublished data) which are (theoretically) relatively susceptible, rather than at 150 - 200 mm where the (relatively tolerant) third instars move as they develop.

- **A susceptibility profile of earlier (eg second) instars for some species will help decide if control of difficult species can be enhanced by more determined efforts to target these stages, as well as helping in resistance management.**

Tactics #4 and #5, using synergists and mixtures to disrupt resistance mechanisms can only eventuate if resistance develops.

- **We know almost nothing of the effect of synergists or insecticide mixtures on canegrub mortality: this should be studied.**

Tactic #6 has the (genetic) weakness that a dose which some susceptibles survive will allow even greater survival of heterozygotes, unless resistance is completely recessive. Also, survival of a large proportion of susceptibles plus heterozygotes may incur economically unacceptable damage. Theoretical studies suggest this tactic is not viable on its own, but may enhance alternative controls, eg stabilising predator:prey relationships for biological control.

This tactic (unwittingly) already occurs when CR formulations lose potency as they age, and if some grubs do not contact the CR granules. Lower concentrations or dosages, perhaps with smaller CR granules, could be used deliberately in the hope of enhancing biological controls, especially where damage-potential is low. Perhaps greater emphasis on risk-assessment will allow certain regions where this tactic is practical to be identified.

- **The possibility of using sub-lethal poisoning to slow crop-damage, and yet render a generation of grubs susceptible to natural disease epidemics, and thereby increasing inoculum levels in the soil to infect future generations, should be investigated.**

Tactic #7 involves treating only the highest population density sites and accepting damage at lower density sites, so producing susceptibles that dilute resistance by interbreeding.

Areas with only moderate or low infestation potential, determined through monitoring, risk-assessment and cost-benefit analysis, would not be treated routinely with CR-insecticide in plant crops. Where and when heavy infestation becomes likely, CR-insecticide could be applied to new plant cane and in young ratoons. Alternatively, short-term insecticides or biological control agents could be applied, or a decision made to make no attempt at 'control' but to then apply cultural tactics which prevent or minimise further outbreaks.

- **Such tactics (along with #6) should be tried, especially in regions prone to the more 'tractable' species (eg. *L. frenchi* at Mackay).**

Tactic #8 attempts to kill only those individuals present in the target zone at the time of treatment with short-term actives, and to spare those from selection which are not in the critical target area or damaging the crop, and subsequent generations, and other life-stages. Persistent formulations may unnecessarily increase resistance selection by killing non-damaging insects, generations, and life-stages.

The logic behind this tactic is relevant to other crops and pests but may not fully apply to canegrubs, where there is only one generation per crop-cycle, there is no crop-stage where the grubs do not cause damage, and significant numbers of other life-stages such as adults are unlikely to be selected (poisoned) by treatments which control larvae.

Tactic #9 intends, by avoiding blanket treatment and not treating all habitats, to provide a pool of non-selected individuals to dilute heterozygotes and homozygotes in the population, and to destroy later life-stages (adults or their larvae) originating from survivors in treated areas by spot-treatment (eg in mating or feeding aggregations).

The controlled-release treatment strategy complies with this tactic in that non-selected canegrubs develop in older sugarcane fields where CR insecticide is no longer effective. Perhaps the tactic could be furthered by trap-crop strategies applied in areas that are mostly treated with insecticide. Beetles developing from survivors in treated fields would be aggregated on adult feeding plants (such as bananas) and subsequently poisoned, and/or their egglaying could be aggregated in highly attractive sacrificial trap-crops such as early-planted sugarcane or sorghum and the progeny then destroyed mechanically, without selection for resistance.

- **Spatial treatment mosaics (grid-pattern treatment) within each field, which may allow some unselected survivors (tactic #6) and encourage biological agents (eg diseases), plus economise on pesticide, should be tried.**
- **Grid-pattern insecticide treatment experiments were tried previously (KJ Chandler, unpublished), but infestations on which to measure the effect failed to develop, and the experiments should be repeated.**
- **Mosaics of (untreated) grub-tolerant cultivars interplanted with less tolerant cultivars protected with insecticide may comply with these objectives, and should be tried.**

Tactic #10 seeks to minimise selection for one resistance mechanism or gene, by alternating pesticides with different modes of action. Models suggest that spatial mosaics with different chemicals are not as effective as rotations over time.

This tactic is currently impractical because of the lack of effective alternatives, but is justification for current programs to develop a range of new canegrub treatment options and insecticide products.

7.3 Implications for insecticide resistance management (IRM)

Modern pest management principles treat IRM as a component of integrated pest management (IPM) (Roush and Daly, 1990; Croft, 1990). Because resistance is a dynamic and evolutionary consequence of selection by insecticide, it follows that processes which lessen the need for insecticide use minimise the rate at which resistant genotypes are selected and the proportion of resistant genotypes in the general population, and are the nub of IRM plans. Thus, objectives of an IRM for canegrub control are consistent with objectives in the industry's IPM plan to diversify and include cultural and biological strategies to minimise grub numbers.

However, strategic insecticide application is likely to remain essential for canegrub management. Thus, efficient insecticide technology, developing new or alternative insecticides and application practices, and maintaining efficiency of existing controls, are also essential components of IPM/IRM.

Maintaining an efficient IRM information base and warning capability for when/if resistance develops is also essential for efficient insecticide technology. This information already improves the efficiency of insecticidal controls.

Detailed resistance management plans for canegrubs are not possible at this stage, and may never be practical or even possible. The nature and heritability of resistance would be an essential pre-requisite for each species of grub for which a detailed IRM plan is required. These remain unknown until resistance is identified. Even if resistance were confirmed, it may not be possible to quantify heritability of resistance with classic studies; the long life-cycle of canegrubs and difficulty and cost of rearing larvae will ensure very slow progress.

8.0 Outcomes

- Developed tissue preparation and analytical methods to measure cytochrome P_{450} monooxygenases, esterases and glutathione S-transferases from canegrub bodies.
- Measured cytochrome P_{450} monooxygenase activity, which seems unrelated to the susceptibility of six species to chlorpyrifos.
- Showed a challenge with a 5 µL drop of chlorpyrifos mimics processes of canegrub control with suSCon® CR granules.
- Showed the microdroplet bioassay method was more efficient, practical, and reliable than five other bioassay methods.
- Measured susceptibility of 10 canegrub species to chlorpyrifos, to provide some baseline susceptibility data on which to judge future control problems and/or to measure change in susceptibility.
- Showed species varied widely in susceptibility, but estimates were consistent between similar populations.
- Showed the extent to which soil type and grub weight affect susceptibility to chlorpyrifos.
- Identified one canegrub species that may be developing resistance.
- Recommended directions for future research on this project.
- Established an insecticide resistance management (IRM) philosophy for canegrub control in the sugarcane industry.

9.0 Recommendations

Recommendations that follow on from the foundation provided with this research are listed, with priorities.

- **** Measure and compare susceptibility of previously treated or untreated populations to assess resistance potential of 10 canegrub species, especially the potentially troublesome *L. crinita.*
- **** Test populations for decreasing susceptibility where control problems are being experienced.
- **** Continue to develop grub controls with new chemicals and formulations, and alternative strategies: synergists and insecticide mixtures; non-releasing insecticide granules palatable to canegrubs; trap-crop and bait-crop tactics; combinations of products to alternate over time; new application methods (eg spatial mosaics, trickle tube).
- **** Continue to develop non-insecticidal controls for grubs: natural and introduced biological control agents; synergism of biological agents and insecticides; host-plant resistance options.
- *** Develop trap-crop and bait-crop tactics to manage cane-beetles (eg in the Burdekin) as an adjunct to control of larvae, and thus reduce numbers of individuals with selection for resistance.
- *** Develop integrated canegrub control tactics with minimal insecticide treatment for specific risk-assessment categories and areas (eg against *L. frenchi* at Mackay), to reduce the risk of selecting insecticide-resistant canegrubs. Develop cost-benefit analyses to evaluate potential for the new tactics.
- ** Watch developments in biochemical test methods for resistance enzymes, especially in the cotton industry.
- ** Measure susceptibility of in-field grub populations, as appropriate, also noting the local effectiveness of control.
- ** Measure susceptibility of second instars to chlorpyrifos, especially species difficult to control and those with two-year life-cycles; strategies that attempt to control the relatively non-damaging second-instars may improve insecticidal efficiency against some species and lessen the risk of resistance for all species.
- ** Develop and test discriminating doses for different species as sufficient susceptibility data become available.
- * Develop the microdroplet bioassay (in soil) as the standard method for screening new insecticides for use in CR formulations, and other purposes.

10.0 TECHNICAL SUMMARY

Soil analyses show that 10-60 µg chlorpyrifos is normally present about each suSCon® Blue controlled-release granule, of which about 75% is within 2 mm of the granules. Average concentrations are 280-1680 mg/kg soil; point concentrations are much higher. Bioassays and controlled experiments show that sugarcane whitegrubs (canegrubs) acquire poison by contact with and/or ingestion of toxic soil, or contact with granules, but not normally by ingesting fragments of granules.

A new bioassay was developed and proven more efficient than methods used previously, and standard methods used elsewhere. The new bioassay is relatively rapid, simple and safe to perform, and results are reproducible. The dosing system mimics field exposure of grubs, except that susceptibility is normally measured in sand, a standard medium, rather than in soil. Dosages that elicit similar mortality in cane-growing soils were 2-14 times higher than in sand.

Susceptibility measurements are consistent with field information on the relative ability of CR-chlorpyrifos to control the various canegrub species. Ten canegrub species differ widely in susceptibility to chlorpyrifos. *Antitrogus rugulosus, A. consanguineus, Lepidiota negatoria* and *L. frenchi* are the most susceptible. *Dermolepida albohirtum* (greyback grub) is one of the least susceptible. Chlorpyrifos dosages to kill greyback populations are 2-5 times as much as for the most susceptible group of species. A single CR-granule produces sufficient chlorpyrifos to kill ≈95% of the most susceptible canegrub species in soil, but only a third or a quarter of that needed for the least susceptible species.

There is disturbing evidence of *Lepidiota crinita*, a relatively susceptible species from Bundaberg, rapidly becoming highly tolerant or even resistant to control with suSCon® Blue. *L. crinita* is the only species for which both previously-exposed and non-exposed populations have been tested. Susceptibility data for nine other species are all from either non-exposed populations (six species, including the least susceptible), or from previously-exposed populations. Information from populations in the opposite category for each species is required to assess the likelihood of resistance to chlorpyrifos.

Resistance management for the future depends on developing new pest management systems for use in conjunction with the current system, and to avoid practices that encourage resistance to become entrenched in the population. Despite predictions about the widespread use of suSCon® Blue controlled-release insecticide leading to resistance, there are a number of features which operate against resistance becoming entrenched in sugarcane whitegrubs.

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12.0 REFERENCES AND PUBLICATIONS

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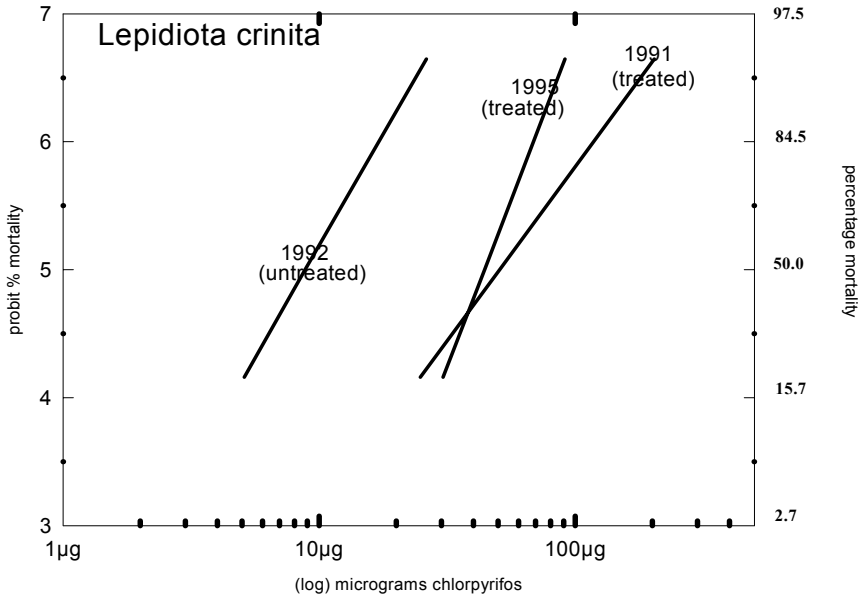
FIGURE

Figure 1: Responses of *Lepidiota crinita* to chlorpyrifos, applied by the microdroplet method; two populations were from nearby fields previously treated with suSCon® Blue, and one population from a distant, untreated field.



TABLES

Table 1: Proportion (%) of total chlorpyrifos in soil sections within, above, below and laterally from single-layer bands of suSCon® granules set horizontally or vertically.

Distance (mm) from granules	Horizontal layer			Vertical layer	
	above	below	within	laterally	within
0	-	-	75.2±5.1	-	19.3±1.8
0.1-2.0	4.9±0.8	4.8±1.1		29.1±3.9	
2.0-4.0	2.5±0.4	3.2±0.7		23.2±3.2	
4.0-8.0	2.8±0.8	2.3±0.5		24.6±1.7	
8.0-14.0	2.1±0.7	2.1±1.1		3.7±0.9	

± sample standard deviation (n=4)

Table 2: Progressive mortality of *Lepidiota negatoria* larvae after 3 d restricted access to three suSCon® Blue granules.

Treatment (Restriction)	Corrected mortality (%)(n=20)		
	Day 4	Day 13	Day 20
Direct access, no excluder in tube	10	50*	65*
Direct access, excluder 0.1 mm below granules	15	85*	85*
No direct access, excluded 0.5 mm above granules	15	85*	85*
No direct access, excluded 2.5 mm above granules	20	55*	65*
No direct access, excluded 4.5 mm above granules	0	30	45
No direct access, excluded 8.5 mm above granules	0	20	20
No direct access, excluded 14.0 mm above granules	0	5	25

∗ surviving larvae paralysed or immobilised.
Control mortality (%; n=20): Day 4=0%, Day 13=5%, Day 20=10%

Table 3 : **Statistics for responses to microdroplet-injected chlorpyrifos in sand for five canegrub species in six consecutive bioassays**.

Ref	Species	*n*	Slope (SE)	t	c^2	df	g (0.95)
91-5	*L. picticollis*	120	3.8 (0.6)	6.2	3.8	4	0.10
91-6	*A. consanguineus*	120	2.0 (0.5)	3.7	3.1	4	0.27
91-8	*A. rugulosus*	120	3.3 (0.5)	6.3	1.5	4	0.09
91-9	*D. albohirtum*	98	2.0 (0.4)	4.8	8.1	3	1.2
91-11	*D. albohirtum*	120	1.7 (0.4)	4.3	4.8	4	0.5
91-16	*A. parvulus*	60	1.5 (0.5)	3.0	3.1	4	0.4

Table 4: Statistics for responses to microdroplet-injected and granule-applied chlorpyrifos on canegrubs.

		MICRODROPLET-INJECTED IN SAND					GRANULE-APPLIED IN SOIL				
Ref	**Species**	***n***	**Slope (SE)**	**c^2**	**df**	**g (0.95)**	***n***	**Slope (SE)**	**c^2**	**df**	**g (0.95)**
89-1	*L. frenchi*	-					500	5.2 (0.7)	3.2	3	0.1
89-2	*L. negatoria*	-					540	$4.3\ (9.0)_{ns}$	342*	6	-
91-1	*L. consobrina*	100	3.2 (0.8)	0.7	3	0.2	480	$0.6\ (0.4)_{ns}$	126*	22	1.5
90-1	*D. albohirtum*	180	3.6 (0.8)	5.4	4	0.17	240	2.4 (0.5)	51*	19	0.2

* heterogeneous data: c^2 differs significantly (0.05) from the expected value.

$_{ns}$ slope not significant (**P** = 0.05)

Table 5 : Statistics for responses to microdroplet-injected and topically-applied chlorpyrifos on three canegrub species in paired bioassays.

		MICRODROPLET INJECTED IN SAND					DROPLET APPLIED TO GRUB INTEGUMENT				
Ref	**Species**	***n***	**Slope (SE)**	**c^2**	**df**	**g (0.95)**	***n***	**Slope (SE)**	**c^2**	**df**	**g (0.95)**
92-06	*L. noxia*	140	2.4 (0.4)	7.4	5	0.3	68	1.6 (0.5)	3.5	4	0.4
92-13	*L. crinita*	130	3.5 (0.6)	2.3	4	0.1	140	2.6 (0.7)	4.6	5	0.3
93-01	*D. albohirtum*	160	3.6 (0.7)	8.9	6	0.36	140	1.0 (0.25)	25.9*	5	2.3

(*) heterogeneous data: χ^2 differs significantly (0.05) from expected value.

Table 6: Statistics for responses to microdroplet-injected and sand-incorporated chlorpyrifos on five canegrub species in paired bioassays.

		MICRODROPLET INJECTED IN SAND					SAND-INCORPORATED				
Ref	**Species**	***n***	**Slope (SE)**	**c^2**	**df**	**g (0.95)**	***n***	**Slope (SE)**	**c^2**	**df**	**g (0.95)**
91-16	*A. parvulus*	65	1.8 (0.6)	3.5	5	0.43	75	3.5 (0.9)	6.0	6	1.0
91-22	*L. crinita*	159	2.7 (0.4)	3.3	6	0.09	120	1.8 (0.4)	9.9	4	0.9
92-01	*A. consanguineus*	160	1.4 (0.3)	6.0	6	0.24	140	1.7 (0.2)	18.8*	5	0.5
92-02	*D. albohirtum*	119	3.2 (0.9)	2.0	4	0.29	120	4.0 (0.6)	11.1*	4	0.6
92-06	*L. noxia*	140	2.4 (0.4)	7.4	5	0.32	139	1.5 (0.3)	22.4*	5	1.1

(*) heterogeneous data: c^2 differs significantly (0.05) from the expected value.

Table 7: Response statistics and susceptibility for six *D. albohirtum* populations to chlorpyrifos injected in sand.

Ref	n	Slope (SE)	c^2	df	LC_{50}(95% CL) (mg AI)	LC95(95% CL) (mg AI)	g (0.95)
91-09	98	2.0 (0.4)	8.2	3	37.6 (∞)	242(∞)	1.2
91-11	120	1.7 (0.4)	4.8	4	17.1 (4.8-28.4)	149 (82-813)	0.5
92-02	119	3.2 (0.9)	1.9	4	32.5 (16.7-45.2)	107 72-295)	0.3
93-01	160	3.6 (0.7)	8.9	6	33.6 (19.1-48.0)	97 63-397)	0.4
94-3a	140	2.8 (0.6)	3.8	5	23.9 (12.9-31.7)	92 69-173)	0.2
94-3b	120	2.4 (0.8)	3.6	4	23.6 (4.2-36.3)	111 (80-356)	0.4

Table 8 : Susceptibility of sugarcane white grubs to a 5 µL droplet of chlorpyrifos in packed sand

Ref	Species	Wt (range) (g.)	*n*	Slope (SE)	LC_{50}(95% CL) (µg AI)	LC_{95}(95% CL) (µg AI)	c^2	df
91-08	*A. rugulosus*	2.4 (1.3-3.4)	120	3.3 (0.5)	2.0 (1.5-2.5)	6.4 (4.6-11)	1.5	4
91-06	*A. consanguineus*	2.7 (2.0-3.7)	120	2.0 (0.5)	0.5 (0.2-0.8)	3.5 (2.1-12)	3.0	4
92-01	*A. consanguineus*	2.0 (1.5-2.6)	160	1.4 (0.3)	2.3 (0.7-4.1)	35 (14-311)	6.0	6
94-01	*A. consanguineus*	1.4 (0.9-2.0)	220	1.9 (0.2)	1.2 (0.6-2.0)	9.1 (4.9-28)	13.9	9
93-04	*L. negatoria*	2.8 (1.4-4.0)	248	2.5 (0.3)	6.2 (4.6-8.0)	29 (22-43)	4.5	9
96-03	*L. frenchi*	2.7 (1.4-3.6)	240	3.3 (0.4)	8.8 (7.2-10.4)	28 (21-44)	11.0	9
91-16	*A. parvulus*	1.7 (1.3-2.5)	65	1.5 (0.5)	3.4 (0.9-6.1)	40 (16-2205)	3.1	4
91-21	*A. parvulus*	1.6 (0.8-2.6)	100	1.7 (0.3)	12.4 (2.8-28.2)	117 (45-3879)	14.8	8
93-05	*A. parvulus*	1.2 (0.5-2.1)	280	1.5 (0.2)	14.3 (8.6-21.4)	171 (87-644)	16.6	12
91-22	*L. crinita 1991* *	2.1 (1.0-3.3)	159	2.7 (0.4)	50.6 (38.4-67.1)	204 (135-418)	3.3	6
92-12	*L. crinita 1992*	1.9 (1.1-3.3)	140	2.6 (0.7)	6.9 (3.0-9.4)	30 (20-89)	4.6	5
95-07	*L. crinita 1995* *	2.2 (1.1-3.3)	150	5.2 (0.8)	44.2 (38.3-49.7)	91 (77-121)	6.4	9
91-01	*L. consobrina* *	2.9 (2.0-4.0)	100	3.2 (0.8)	20.7 (13.0-27.5)	66 (46-157)	0.7	3
91-05	*L. picticollis* *	2.7 (1.9-3.8)	120	3.8 (0.6)	26.2 (20.2-31.4)	70 (56-103)	3.8	4
8 pops.	*D. albohirtum* *♦	4.2 (1.3-6.5)	1007	2.4 (0.2)	39.0 (26.0-58.0)	187 (122-313)	73.1	46
92-06	*L. noxia*	2.3 (1.5-3.3)	140	2.4 (0.4)	66.9 (30.4-109)	327 (178-1935)	11.11	6

♦ Eight populations, including six in Table 7, combined; Slopes similar (χ^2=9.6, df=7, P=0.2)

(*) Populations exposed to chlorpyrifos (suSCon® Blue).

Table 9 : The effect of grub body weight on susceptibility of *A. consanguineus and A. parvulus* to chlorpyrifos.

A. consanguineus	**grub weight-groups(g)**		
	0.9-1.6	**1.7-2.2**	**2.3-3.7**
n	126	132	116
Slope (SE)	1.54 (0.42)	2.0 (0.3)	1.5 (0.3)
LC_{50}(95% CL), mg [AI]	1.1 (0.2-2.2)	1.4 (0.9-2.0)	1.0 (0.5-1.5)
LC_{95}(95% CL), mg [AI]	13.0 (6.3-81.6)	8.9 (5.4-22.5)	11.72 (5.5-77.4)
c^2	6.73	5.90	6.75
df	7	8	5
Relative tolerance	1	1.1 (2.1-0.6)	0.9 (1.6-0.5)

A. parvulus	**grub weight-groups (g)**			
	0.8 - 1.2	**1.3 - 1.5**	**1.6 - 1.9**	**1.9 - 2.5**
n	109	134	95	94
Slope (SE)	1.1 (0.28)	1.3 (0.32)	1.2 (0.28)	2.0 (0.44)
LC_{50}(95% CL), mg [AI]	7.0 (2.3-12.8)	10.5 (1.3-26.8)	14.6 (5.7-45.1)	17.5 (7.6-34.4)
LC_{95}(95% CL), mg [AI]	221.6 (77.7-4088)	211.1 (62.6-62034)	298.9 (74.3-193882)	119.3 (52.9-1541)
c^2	8.7	27.2	31.4	23.3
df	11	16	16	16
Relative tolerance	1	1.4 (3.3-0.6)	1.8 (4.7-0.8)	2.4 (6.8-1.0)

Table 10 The effect of grub body weight on susceptibility of *D. albohirtum* to chlorpyrifos.

	grub weight-groups (g)			
D. albohirtum	**2.6 - 3.9**	**4.0 - 4.4**	**4.5 - 4.9**	**5.0 - 5.7**
n	148	146	186	144
Slope (SE)	1.57 (0.37)	2.42 (0.38)	3.00 (0.5)	5.27 (0.9)
LC_{50}(95% CL) (mg AI)	12.9 (4.2-21.9)	29.9 (21.9-37.7)	27.5 (19.5-34.3)	31.4 (22.3-39.0)
LC_{95}(95% CL) (mg AI)	144.5 (80.9-543.0)	142.2 (99.8-263.9)	97.2 (75.3-149.7)	64.3 (49.6-118.9)
c^2	4.37	7.05	4.3	12.2
df	8	8	8	8
Relative tolerance	1	1.6 (2.5-1.0)	1.3 (2.1-0.8)	1.3 (2.1-0.8)

Table 11 : Susceptibility of *Dermolepida albohirtum, Antitrogus parvulus and A. consanguineus* to chlorpyrifos in sand and three soil-types.

	D. albohirtum (91-11)				**D. albohirtum (93-01)**	
	Sand	**Soil #1**	**Soil #2**	**Soil #3**	**Sand**	**Soil #1**
n	120	120	120	140	160	160
slope (SE)	1.8 (0.4)	5.1 (0.9)	3.0 (0.5)	2.2 (0.4)	3.6 (0.7)	2.7 (0.5)
LC50(95% CL) (mg AI)	13.8 (1.9-24.8)	51.5 (42-62)	51 (18-87)	85 (54-149)	34 (19.1-48)	107 (57-167)
LC95(95% CL) (mg AI)	114.2 (61-1185)	108 (86-162)	177 (99.9-1934)	466 (244-4626)	97 (62.7-397)	433 (252-1885)
c^2	4.3	0.5	10.1	7.9	8.9	8.4
df	4	4	4	5	6	6

	A. parvulus (91-16)		**A. consanguineus (92-01)**	
	Sand	Soil #1	Sand	Soil #1
n	6o	70	160	100
slope (SE)	1.5 (0.5)	1.5 (0.4)	1.5 (0.3)	1.3 (0.3)
LC50(95% CL) (mg AI)	3.4 (0.9-6.1)	43.8 (18.9-71)	2.6 (0.8-5.3)	10.3 (4.5-20.6)
LC95(95% CL) (mg AI)	40.0 (16.0-2205)	511.6 (228-5696)	33.6 (13.9-276)	198 (74-1837)
c^2	3.1	1.6	6.0	2.1
df	4	5	6	3

#1 Mission series, reddish-brown clay-loam
#2 Pin Gin series series, kraznozem
#3 Thorpe series, dark sandy light-textured clay-loam.
Soils classification after Smith & Murtha (in prep.).

Table 12. Monooxygenase activity in six species of sugarcane white grubs

Species	Av grub wt (g)	n [a]	Cytochrome P_{450} [b] (/g body wt)	Aldrin epoxidase [c] (/g body wt)
A. consanguineus	1.86	4	115 ± 25	92 ± 18
A. parvulus	1.57	6	96 ± 38	58 ± 45
L. negatoria	2.94	4	139 ± 34	nd
L. crinita	2.40	4	65 ± 15	nd
L. frenchi	2.83	3	206 ± 55	65 ± 15
D. albohirtum	3.95	6	85 ± 20	nd

[a] number of enzyme preparations
[b] picomoles
[c] picomoles per minute
nd not determined