INSECTICIDE RESISTANCE MECHANISMS IN THE

HONEY BEE, *APIS MELLIFERA* L.

by

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ABSTRACT

Pollinating insects would benefit considerably from some degree of resistance to chemicals used in crop protection. However, despite several literature reports of pesticide resistance in these insects, resistance mechanisms have not been studied in detail and remain poorly understood. Previous work had indicated considerable variation in resistance levels between colonies of the honey bee, *Apis mellifera* L.; I undertook studies to investigate the mechanisms behind this variation.

The acute toxicities of four insecticides were assayed in seven different colonies, and these results were related to colony levels of mixed-function oxidase and glutathione transferase enzymes. Linear regression equations were derived for the relationship between both enzyme systems and the acute toxicity of diazinon, propoxur, and aldrin. No significant relationship was found between either enzyme system and the acute toxicity of carbaryl.

Having established the dependence of resistance on enzyme activity, I wanted to assess factors that could affect enzyme levels. The schedule of temporal polyethism characteristic of honey bees causes dramatic shifts in behaviour and physiology as bees transfer from in-hive duties to field duties such as foraging. Field bees come into contact with environmental toxins more frequently than hive bees, and I predicted an increase in

detoxifying enzyme levels in older workers. This was supported by the finding that the specific activity of both enzyme systems increased in older worker bees. These changes in enzyme activity are not simply related to the process of aging, as shown in experiments using colonies initiated with only young workers. Increased specific activity was seen only in foragers, indicating a significant relationship between enzyme activity and behavioural status.

Effects of colony environment were also assessed using cross-fostering methodology. Cohorts of related workers introduced into foster colonies exhibited considerable variation in enzyme activity after 21 days; this was negatively correlated with colony population, but positively correlated with the ratio of brood/adults. The plasticity of response in cross-fostered cohorts suggests that detoxifying enzyme activity has a strong environmental component. These findings are discussed in the context of selection for insecticide resistance based on enzyme activity levels.

DEDICATION

For Mary-Anne, James, and Jeffrey, with very much love.

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

Pollinating insects are an indispensible part of modern agriculture. The list of insect-pollinated crops includes pomme fruits (apples and pears), stone fruits (peaches, cherries, apricots, and nectarines), citrus fruits (oranges, lemons, limes, and grapefruits), berries (strawberries, raspberries, blueberries, and cranberries), and numerous other crops. Much of modern agricultural production is completely dependent on adequate insect pollination.

The most important insect pollinator is the honey bee, *Apis mellifera* L., which pollinates in excess of $20 billion worth of crops annually in North America (Levin 1983; Winston and Scott 1984). It is especially important in areas of large monocultures where high pollinator densities are required, as honey bees can be provided in large numbers to meet pollination demands.

The requirements for adequate honey bee pollination are often at odds, however, with the practice of chemical pest control. The use of chemicals for the management of pest insects has often resulted in a drastic reduction in the number of all pollinators, not just honey bees. Numerous instances of bee poisoning are outlined in the National Research Council of Canada Report, "Pesticide-Pollinator Interactions" (Anonymous 1981). Other reviews outline the effects of chemical control agents on pollinator populations in the United States (Johansen 1977; Atkins et al. 1975) and Britain (Stevenson et al. 1978).

There are many reasons why honey bees are affected so severely by pesticide usage. Most obviously, bees are insects and as such are profoundly affected by any insecticide with broad spectrum activity. Bees are often simply "in the way" of control programs that kill a large percentage of the insect fauna in a given area.

An added problem is the sensitivity that honey bees exhibit towards insecticides. Bees are significantly more sensitive to many pest control chemicals than are the pest insects themselves. For example, carbamate insecticides are consistently more toxic to honey bees than to pest species (Vinopal and Johansen 1967). The reasons for this increased sensitivity are unclear, but may relate to the limited development of resistance mechanisms in these insects.

The development of insecticide resistance in target pest insects has been a major factor in the success or failure of insect control programs. By 1980, 428 species of insects and mites had developed significant resistance to insecticides (Georghiou and Mellon 1983). This has resulted in higher rates of application in order to achieve the required level of pest control, or switching to chemicals with efficacy as yet unaffected by resistance. However, resistance development to newly-introduced chemicals often occurs rapidly, sometimes within a single season. Interestingly, only three of the 428 resistant species (0.7%) are in the order Hymenoptera (Georghiou and Mellon 1983).

There have been several reports of insecticide resistance in honey bees. DDT resistance was reported in California in 1960 (Atkins and Anderson 1962). However, subsequent experiments with bees from these "resistant" strains demonstrated no further resistance development after two generations of selection, and the level of DDT resistance was no different from that in "susceptible" Louisiana strains (Graves and Mackensen 1965). Resistance to carbaryl was also reported after eleven generations of selection (Tucker 1980), but no further studies were conducted on these bees to evaluate other desirable traits. In each case, no attempt was made to determine resistance mechanisms.

Clearly, resistance development has been limited in honey bees when compared with many other insects. This is likely attributable to several factors. Firstly, as social insects with a physical caste structure consisting of queens, workers, and drones, only the worker bees are directly exposed to pesticide pressure under most circumstances. Thus, selection pressure on the queen is indirect. Secondly, honey bee colonies reproduce by swarming, resulting in a population growth rate many times slower than that of pest insect species. Rates of resistance development are directly related to the number of reproductive individuals in the population (Georghiou 1983), so the mode of honey bee reproduction does not favour rapid resistance development.

Thirdly, polyandry and subsequent sperm mixing in the spermatheca of the queen gives rise to multiple patrilines in each colony (Laidlaw and Page 1984; Page and Metcalf 1982). Thus, resistance genes present in any single drone are diluted up to 17-fold (Winston 1987) in the overall colony population. Even if the queen is homozygous for resistance alleles, only a small proportion of her female offspring would end up in the homozygous condition, and corresponding high resistance levels would not arise.

A final factor that mitigates against the development of insecticide resistance in honey bees is a result of common beekeeping practices. In many areas, beekeepers start their colonies anew each season from packages. Colonies exhibiting any level of resistance are destroyed in the fall and the resistance genes are lost. In addition, the requeening of colonies every 1-2 years with non-resistant strains, while making sense in beekeeping terms, virtually ensures that resistant lines will be discontinued. Both of these factors, in conjuction with the premium beekeepers put on preventing colony reproduction by swarming, make resistance development in honey bees unlikely and, combined with the biological factors mentioned earlier, explain why bees remain so sensitive to insecticide poisoning.

Resistance to insecticides is based on a number of factors, including behaviour (avoidance and repellency), physiology (elevated levels of excretion), morphology (thickened cuticle providing a penetration barrier), and biochemistry (altered

target enzymes and increased levels of detoxication enzyme systems). Resistance due to enhanced detoxication capacity is probably the major type of resistance detected in field populations. This increased metabolism is due to elevated levels of a number of different enzymes. Examples of detoxifying enzymes include cytochrome P_{450}-linked microsomal oxidases (also known as mixed-function oxidases or polysubstrate monooxygenases), glutathione transferases, carboxylesterases, and epoxide hydrolases (Dauterman and Hodgson 1978). These enzymes often work in conjunction with each other to greatly increase levels of xenobiotic detoxication.

Biochemical resistance mechanisms appear to be more common in herbivorous pests than in non-target insects. This may be due to pre-adaptation of herbivores for metabolizing toxins because they encounter toxins regularly in their diet in the form of plant allelochemicals. Krieger et al. (1971) showed that mixed-function oxidase levels in the guts of caterpillars were significantly correlated with the feeding habits of the species; polyphagous species had higher enzyme levels than did oligophagous species, which in turn had higher levels than monophages. The authors speculated that these enzyme levels had been adjusted through natural selection to detoxify a wide range of allelochemicals likely to be encountered by the polyphagous species. In other words, polyphagous herbivores are pre-adapted by their feeding patterns to be more tolerant of environmental contaminants such as pesticides.

The primary importance of biochemical detoxication reactions in determining insecticide resistance levels, and the limited occurrence of resistance in honey bees, suggests a possible connection between the two. Even though honey bees have active detoxication systems (Gilbert and Wilkinson 1974; Yu et al. 1984), no attempt has been made to relate enzyme activity to resistance levels on a colony-by-colony basis. The determination of quantitative relationships between detoxication and resistance would enable the prediction of colony resistance to several insecticides on the basis of enzyme activity, and would further our understanding of resistance development in honey bees and other insects as well.

The overall objective of my thesis work was to investigate the mechanisms underlying variation in resistance levels in honey bee colonies. Specifically, I wanted to establish relationships between resistance levels and colony detoxication activity, and was interested in identifying factors that would influence the activity of detoxication enzymes, such as worker age, behavioural status, and environmental surroundings.

CHAPTER II

INTERCOLONY VARIATION IN DETOXICATION ACTIVITY: RELATIONSHIP TO

DIAZINON TOXICITY AND SEASONAL FLUCTUATIONS

It has been known for many years that honey bee colonies vary widely in their ability to withstand insecticide exposure (Tahori et al. 1969). However, the mechanisms behind this variation have not been investigated. Honey bee colonies, with large numbers of sterile workers, provide a readily accessible supply of non-interbreeding populations that can be used to study relationships between colony characteristics and insecticide resistance.

The marked sensitivity of honey bees to insecticide poisoning may be due to a lack of detoxication enzymes, as indicated by low synergist ratios with carbaryl (Metcalf et al. 1966). However, subsequent studies have demonstrated active mixed-function oxidases in worker honey bees (Gilbert and Wilkinson 1974); other detoxifying enzyme systems such as glutathione transferases, esterases, epoxide hydrolases, and DDT-dehydrochlorinase are active as well (Yu et al. 1984).

Mixed-function oxidase enzymes, also referred to as polysubstrate monooxygenases or cytochrome P-450-linked monooxygenases, are the most important of these detoxification systems. These enzymes function by introducing one atom of molecular oxygen into a wide variety of lipophilic substrates, rendering these compounds more polar and water-soluble, and expediting their excretion from the body, often in the form of glutathione conjugates.

Despite the presence of these active metabolic detoxication systems, insecticide poisoning remains a serious problem to honey bees in the agricultural ecosystem. The selection of honey bee strains exhibiting some degree of insecticide resistance would therefore be of considerable benefit to North American agriculture.

The objective of this study was to examine possible relationships between colony mixed-function oxidase activity and intercolony variation in susceptibility to the organophosphate insecticide diazinon. I also investigated the possibility of constructing predictive models of insecticide resistance based on detoxifying enzyme activity, and examined seasonal fluctuations in these enzyme levels.

Materials and Methods

Chemicals

Sources of insecticides used in this study were: aldrin and dieldrin (analytical grade, >99%), Shell Chemical (New York, N.Y.); diazinon (technical, 98%), Later Chemicals (Richmond, B.C.). Other chemicals were of the highest quality available, and were purchased from commercial suppliers.

Adult worker honey bees were obtained from colonies maintained at one apiary site at Simon Fraser University, Burnaby, B.C. Bees were shaken off frames from the top super of each two-super colony, and kept in the laboratory for ca.12 h before being used for enzyme assays and acute toxicity determinations. All colonies had healthy, laying queens for the duration of the experiment.

Enzyme Assays

Mixed-function oxidase activity was assayed using aldrin as the substrate. Intact midguts were used as the enzyme source due to the presence of an inhibitor that is released when midgut tissues are homogenized (Gilbert and Wilkinson 1974, 1975). Midguts were dissected directly into cold 0.1 M potassium phosphate buffer, pH 7.4, and kept on ice until required for enzyme assays.

Midguts were incubated in a shaking water bath for 15 min at 40°C (optimum reaction temperature, Gilbert and Wilkinson 1974). The reaction mixture consisted of 20 midguts (including contents); 10 ml 0.1 M potassium phosphate buffer, pH 7.4; and 100 μg aldrin in 0.1 ml methyl cellosolve. Aldrin and dieldrin were extracted with 3 x 10 ml of acetone/hexane (1:1), and analyzed by electron-capture gas chromatography (Tracor 550 Gas Chromatograph). Amounts were quantified by comparing peak heights with injections of known quantities of analytical

standards.

Each of five colonies was assayed five times. On a given day, one sample was assayed from each colony; on the next day (2 or 3 days later), the procedure was repeated, with the sampling order being randomly assigned. Samples were always taken in late afternoon or early evening. This sampling procedure was designed to reduce diurnal variation in enzyme activity which could obscure colony differences. Colonies were sampled at three times of the year: fall, 1984 (October and November); spring, 1985 (February and March); and summer, 1985 (June - August).

This sampling protocol revealed no significant differences in enzyme levels within individual colonies over the 3 week course of a set of assays. The procedure was therefore modified slightly in Summer, 1985, with a single colony being assayed during each 3 week period. This enabled the assay of glutathione transferase enzymes at the same time, as well as the expansion of the study to include three additional insecticides. These experiments are described in detail in the next chapter.

Enzyme activity data were analyzed using the analysis of variance, Student Newman-Keuls test, and linear regression of LD_{50} on aldrin epoxidase activity.

Toxicity Tests

Worker bees were treated on the dorsal surface of the thorax with 1 ul of technical diazinon diluted in acetone. Insects were lightly anaesthetized with CO_2 to facilitate handling. Fifty insects were treated at each of 5 doses, plus an acetone-treated control, and all tests were replicated at least twice. Data, corrected for control mortality (Abbott 1925), were analyzed using probit analysis (Finney 1971). The criterion of nonoverlapping 95% confidence limits was used to determine significant differences between LD_{50}s.

Results

Enzyme Assays

Significant differences in aldrin epoxidase activity were found between colonies at all three times of the year ($P < 0.05$; Table 1). Activity levels were comparable to those found by Gilbert and Wilkinson (1974) and Yu et al. (1984). Enzyme activity within a colony also changed significantly at different times of the year in four out of five cases ($P < 0.05$). These four colonies had lower enzyme levels in the spring of the year, and three colonies reached their highest levels in the summer. Colony IV showed no significant differences between seasons.

Table 1. Aldrin epoxidase activity in five colonies at three times of the year.

Colony	Summer	Fall	Spring
I	44.92 ± 2.25a	41.43 ± 3.19ac	37.52 ± 2.25a
II	35.46 ± 1.62bc	33.28 ± 3.07bc	24.89 ± 1.43b
III	38.02 ± 1.57bc	48.25 ± 2.10a	28.95 ± 2.35bc
IV	33.81 ± 1.27b	31.32 ± 2.87b	29.98 ± 2.48bc
V	40.67 ± 0.97ac	43.34 ± 1.95a	34.80 ± 2.22ac

Means within columns followed by the same letter are not significantly different (P = 0.05; Newman-Keuls test [Zar 1984]). Means within rows underlined by a common line are not significantly different (P = 0.05; Newman-Keuls test [Zar 1984]). pmoles of dieldrin per minute per midgut; $\bar{x}$ ± SEM of five replicates.

Table 2. Acute toxicity of diazinon to five colonies at three times of the year.

Hive	Season	n	LD_{50}[a]	95% CL[a]	Slope ± SEM
I	Summer	500	0.154	0.146-0.162	8.13 ± 0.25
	Fall	500	0.144	0.138-0.150	12.13 ± 1.51
	Spring	500	0.127	0.121-0.135	6.15 ± 0.71
II	Summer	500	0.140	0.134-0.146	10.20 ± 1.16
	Fall	500	0.131	0.125-0.137	11.15 ± 0.18
	Spring	750	0.107	0.103-0.111	10.41 ± 0.41
III	Summer	500	0.137	0.131-0.144	10.30 ± 0.53
	Fall	500	0.170	0.138-0.209	1.78 ± 0.16
	Spring	750	0.103	0.099-0.107	8.16 ± 0.31
IV	Summer	500	0.128	0.122-0.134	12.41 ± 0.45
	Fall	500	0.121	0.113-0.129	10.66 ± 1.00
	Spring	750	0.101	0.098-0.105	8.90 ± 0.38
V	Summer	500	0.155	0.148-0.162	13.60 ± 0.25
	Fall	500	0.135	0.131-0.139	13.35 ± 0.72
	Spring	750	0.127	0.123-0.131	8.86 ± 0.36

[a]LD_{50} and CL expressed as micrograms per bee.

Significant differences in diazinon toxicity were found between colonies at all three times of the year (Table 2). Colonies I and V, which had higher levels of resistance in the spring, also had higher resistance in the summer than colonies II, III, and IV. Colony III showed a high level of resistance to diazinon in the fall of 1984, but this was lost by the following spring. All five colonies were the most susceptible to diazinon in the spring after overwintering.

Relationship Between Enzyme Activity and Acute Toxicity

There was a significant linear relationship between the acute toxicity of diazinon and colony mixed-function oxidase activity (Fig. 1). The r^2 value, indicating 84% explained variation, suggests that colony oxidase activity could be used as a good predictor of resistance to diazinon.

Discussion

My results indicate that variation in mixed-function oxidase activity in honey bee colonies is a significant factor in determining resistance to diazinon. Significant variation exists in both enzyme activity and diazinon toxicity. Mean aldrin epoxidase activity ranged from 24.89 pmoles dieldrin/ min per midgut to 48.25 pmoles/ min per midgut, a difference of 94%. LD_{50} values for diazinon varied from 0.101 to 0.170 ug per bee,

Figure 1. Relationship between diazinon LD 50 and colony
aldrin epoxidase activity. Each point represents LD 50 with
95% Confidence Interval.

$\hat{y} = 0.030 + 0.0028x$
$r^2 = .84$
$P < .001$

LD$_{50}$ (μg bee^{-1})

ALDRIN EPOXIDASE (pmoles min^{-1} midgut^{-1})

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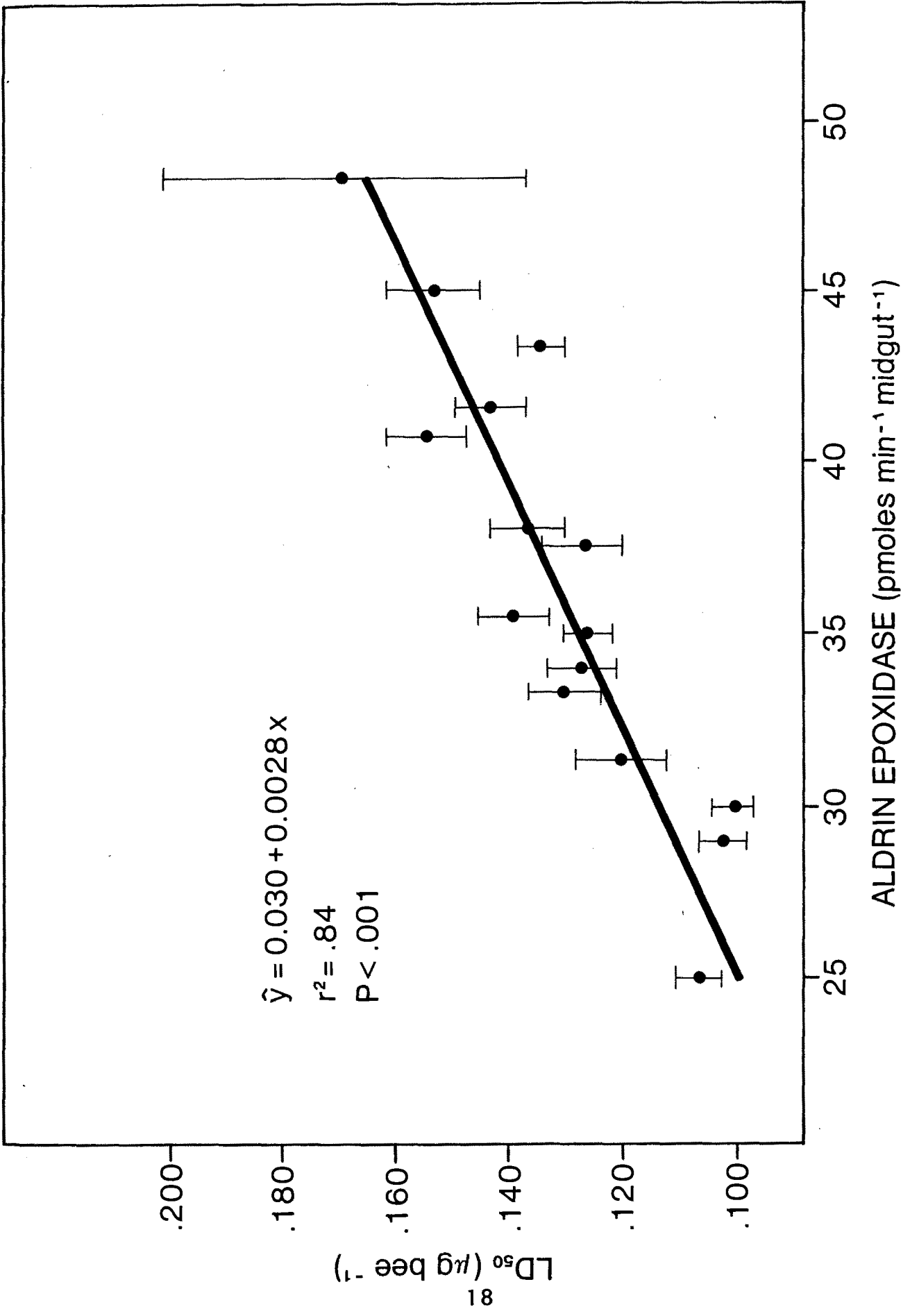
a difference of 68%. Differences of this magnitude in a population of colonies that had not been previously selected for resistance to diazinon suggest that intercolony variation could be exploited as a means of selecting insecticide-resistant honey bees.

The strength of the linear relationship between the two variables was somewhat surprising because aldrin epoxidase is not directly involved in diazinon metabolism. However, my results suggest that the measurement of aldrin epoxidase, one of several cytochrome P_{450} isozymes, gives a good approximation of the activity of diazinon-metabolizing enzymes as well.

The relationship between acute toxicity and mixed-function oxidase levels was investigated by Schonbrod et al. (1968) using preselected strains of houseflies. They concluded that no clear relationship existed between mixed-function oxidase activity and insecticide resistance in this insect. However, my study supports a significant involvement of these enzymes in honey bee resistance to diazinon. The difference between my results and theirs was likely due to my use of nonselected colonies having quantitative differences in enzyme activity rather than strains with known resistance to several compounds (i.e., qualitative differences).

Another interesting feature of my results was the positive slope of the regression line in Fig. I. Colonies with higher levels of enzyme activity were also the most resistant to

diazinon. Diazinon, a phosphorothioate compound, requires metabolic activation for high insecticidal activity (Fig. 2, reaction 1); this conversion of diazinon to diazoxon is achieved through the action of mixed-function oxidases (Wilkinson 1983). Colonies with high monooxygenase activity might be expected to be more at risk from diazinon, but my results indicate that this is not the case.

The finding that colonies are most susceptible to diazinon in the spring has practical implications for beekeeping. The increased susceptibility of overwintered bees may present a danger to these colonies if toxic chemicals are encountered early in the season. However, pesticide use is not widespread at this time of year (at least in western Canada), and foraging workers would not be affected. Risk to the colony would more likely come from insecticide residues in stored pollen, a real danger in many agricultural areas where microencapsulated or dust formulations are used.

Old winter bees have also been reported to be most sensitive to several insecticides by Wahl and Ulm (1983). However, their colonies were heavily infested with *Nosema apis* Zander in the spring, whereas mine were not, having been treated with the antibiotic fumagillin to prevent *Nosema* infection. It is therefore likely that the extreme susceptibility of overwintered colonies that I observed was due to unknown physiological factors associated with the overwintering process.

Figure 2. Mixed-function oxidase-mediated metabolism of diazinon.
Reaction 1: desulphuration (activation). Reactions 2 and 3:
monooxygenation (detoxication).
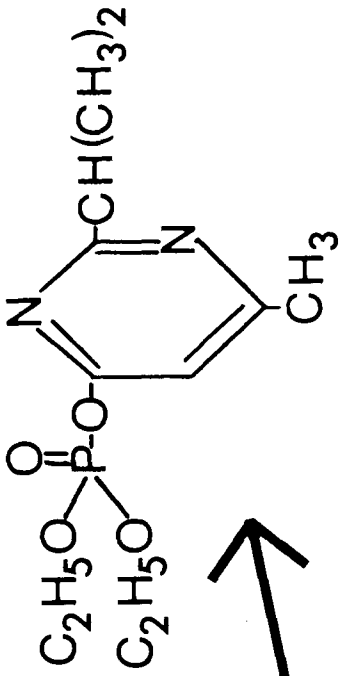
DIAZINON

DIAZOXON

PYRIMIDINOL

22
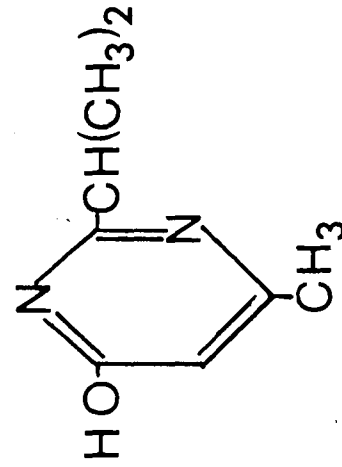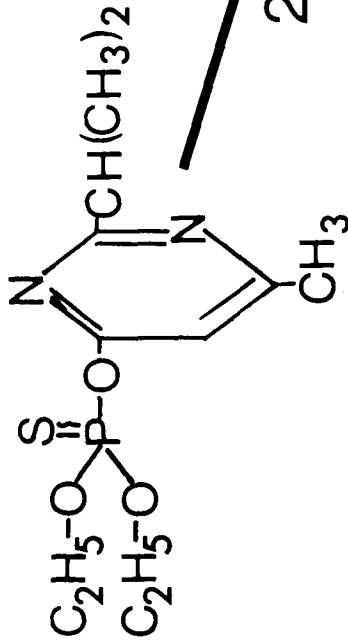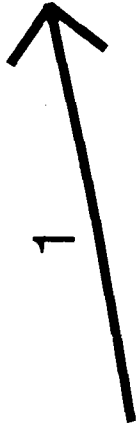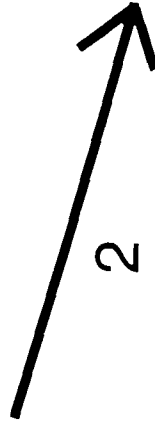
CHAPTER III

DETOXICATION ACTIVITY IN HONEY BEES AND THE TOXICITY OF FOUR INSECTICIDES

The dependence of colony diazinon resistance on mixed-function oxidase activity that was established in Chapter 2 led to the expansion of the study in 1985 and 1986. Many different insecticides are metabolized by the mixed-function oxidase system, and it seemed likely that resistance to other chemicals could be determined by the same mechanism.

In addition to the mixed-function oxidases, other enzyme systems are involved in insecticide detoxication. One of the most important is the glutathione transferase system, which catalyzes the conjugation of glutathione, an intracellular tripeptide (L-y-glutamyl-L-cysteinylglycine), with a wide variety of foreign and endogenous compounds. The general metabolism of glutathione in mammalian tissues is reviewed by Meister and Anderson (1983).

In insects, the end products of glutathione conjugation with foreign compounds are mercapturic acids (Dauterman and Hodgson 1978). Additionally, glutathione conjugation is important for the degredation of intracellular compounds such as juvenile hormone (Hammock 1985). In xenobiotic detoxication, mixed-function oxidases and glutathione transferases often work cooperatively to remove toxic compounds from the organism. Mixed-function oxidase activity results in polar metabolites which are further conjugated with glutathione and excreted.

The objectives of this study were to determine if the relationship found between mixed-function oxidase activity and

diazinon resistance as described in Chapter 2 would be similar for other insecticides, and would extend to the glutathione transferase system as well. Insecticides chosen for study were diazinon, propoxur, carbaryl, and aldrin. Diazinon was of interest for possible glutathione transferase contributions to resistance; these enzymes are involved in diazinon metabolism in both cockroaches and houseflies (Shishido et al. 1972; Yang et al. 1971). Propoxur and carbaryl have been identified as substrates for mixed-function oxidases in other insects (reviewed by Nakatsugawa and Morelli 1976), and show considerable toxicity to honey bees. There is no evidence that either compound is metabolized directly by glutathione transferase, although polar metabolites resulting from mixed-function oxidase activity may be. Aldrin was chosen because it would be expected to exhibit the most straight-forward relationship between epoxidase activity and toxicity. I intended to derive linear regression models from enzyme activity and acute toxicity data that could be used to predict insecticide resistance on the basis of enzyme activity.

Materials and Methods

Chemicals

Sources of chemicals used in this study were: aldrin and dieldrin (analytical grade, >99%), Shell Chemical Co. (New York, N.Y.); diazinon (technical, 98%), Later Chemicals (Richmond, B.C.); carbaryl (technical, >99%), Union Carbide (New York,

N.Y.); propoxur (analytical grade, >99%), Chemagro Corp. (Kansas City, Missouri); and reduced glutathione, Sigma Chemicals (St. Louis, Missouri). Other chemicals were of the highest purity available, and were purchased from commercial suppliers.

Insects, Toxicity Tests, and Enzyme Assays

Adult worker honey bees were obtained from colonies as previously described. Acute toxicity determinations were made on each colony over a 3-week period; enzyme assays for that colony were always conducted near the middle of that period. All colonies had healthy, laying queens for the duration of the experiment.

Toxicity tests were conducted as previously described. Mixed-function oxidase activity was assayed using aldrin as the substrate. For glutathione transferase assays, contents were removed from groups of 20 midguts, tissues were washed, and placed into cold potassium phosphate buffer, pH 6.5. Tissues were homogenized using a motor-driven ground-glass tissue grinder, and centrifuged at 12,000 x g for 15 min ($4^{\circ}C$). The resulting post-mitochondrial supernatant was filtered through glass wool to remove floating lipid and kept on ice for enzyme assays and protein determinations. Five replicates of 20 midguts were used for each test colony.

Glutathione transferase activity was assayed using 1-chloro-2,4-dinitrobenzene as the substrate following the protocol of Yu (1984). The formation of the conjugation product,

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2,4-dinitrophenyl glutathione, was measured by recording the increase in absorbance at 340 nm (22° C) with a Cary 14 spectrophotometer. Each reaction cuvette contained 2 ml post-mitochondrial fraction, 1 ml 15 mM glutathione, and 20 μl 150 mM CDNB in ethanol. Absorbance was recorded for 5 min., and samples were diluted appropriately with buffer to ensure that the increase in absorbance was linear throughout the assay. The protein content of these post-mitochondrial preparations was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Complete assay mixtures without enzymes were used as controls to determine the amount of non-enzymatic conjugation, and all activity data were corrected for this small amount of product formation (ca. 3% of enzymatic activity).

Possible relationships between LD_{50} and enzyme activity for each colony were analyzed using linear regression. The appropriateness of the linear model was investigated by transforming either variable and checking the improvement in r^2 (Gomez and Gomez 1984).

Results

The activity of mixed-function oxidases and glutathione transferases varied significantly from colony to colony (Fig. 3). Activities of the two enzyme systems were significantly correlated with each other (r= 0.90; p< 0.01).

Figure 3. The relationship between mixed-function oxidase and glutathione transferase activities in seven honey bee colonies.
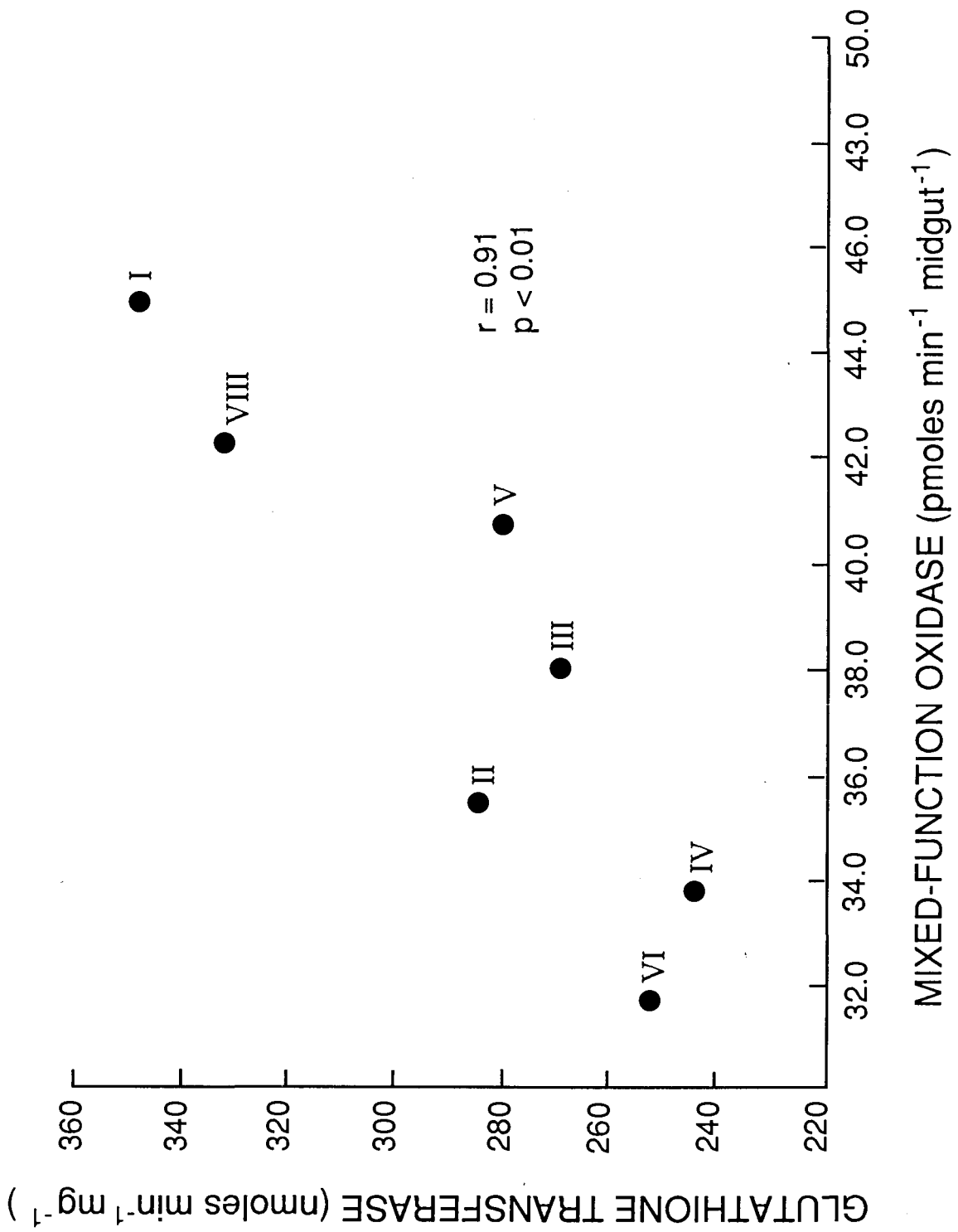
Levels of colony resistance, as indicated by LD_{50} values, were related to mixed-function oxidase and glutathione transferase activities for three of the four insecticides tested. There were significant linear regressions of LD_{50} values for diazinon, propoxur, and aldrin on both enzyme systems (Figs. 4 - 6). These relationships were positive for diazinon and propoxur, and negative for aldrin. No relationship was found between the activity of either enzyme and the acute toxicity of carbaryl (Fig. 7).

Logarithmic transformation of enzyme activity data gave the best linear fit of the data, but improvement in r^2 values were small as compared with untransformed data. In the case of carbaryl transformed data still did not indicate a significant relationship between acute toxicity and enzyme activity ($p > 0.05$). For this reason, only analyses performed on untransformed data are reported in this chapter.

Discussion

These results indicate significant involvement of both mixed-function oxidase and glutathione transferase enzymes in the determination of colony resistance to several insecticides, and represent the first such demonstration of these relationships in previously unselected field populations of insects. They also illustrate a highly significant correlation between the activities of each enzyme system. This is not

Figure 4. (A) Linear regression of diazinon LD 50 on colony aldrin epoxidase activity.

(B) Linear regression of diazinon LD 50 on colony glutathione transferase activity.

Each point represents LD 50 with 95% Confidence Interval.

DIAZINON

A

$\hat{y} = -0.022 + 0.0041x$
$r^2 = 0.74$
$p < 0.01$

LD_{50} (µg/bee)

ALDRIN EPOXIDASE (pmoles min^{-1} midgut^{-1})

B

$\hat{y} = 0.053 + 0.0039x$
$r^2 = 0.61$
$p < 0.05$

LD_{50} (µg/bee)

GLUTATHIONE TRANSFERASE
(nmoles min^{-1} mg^{-1})
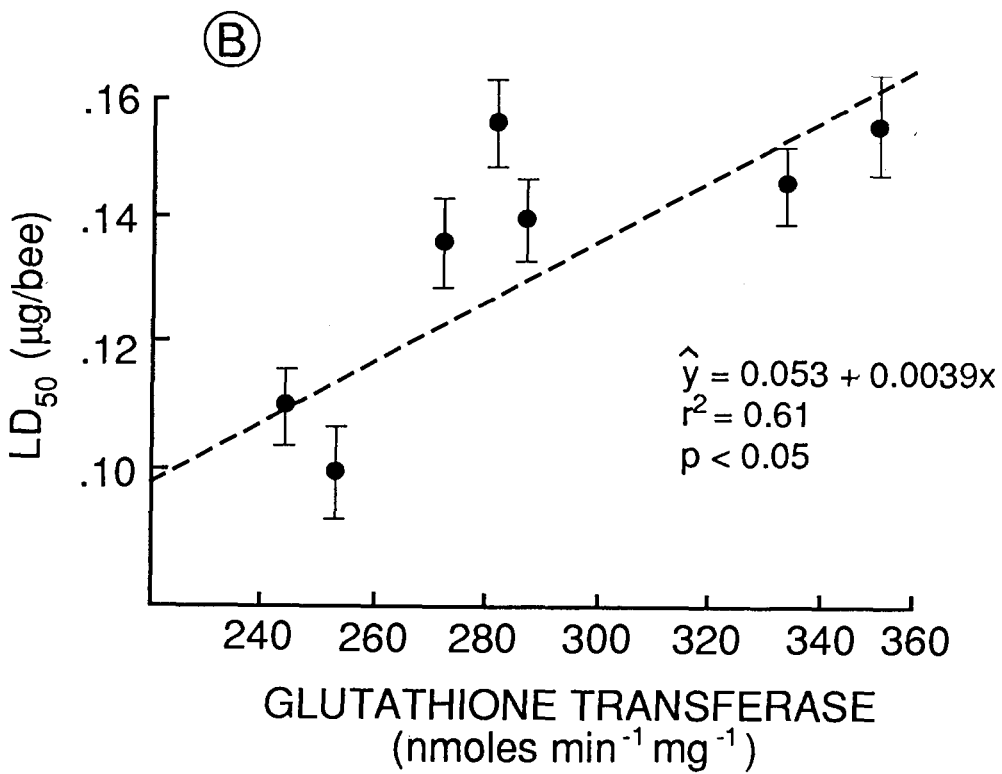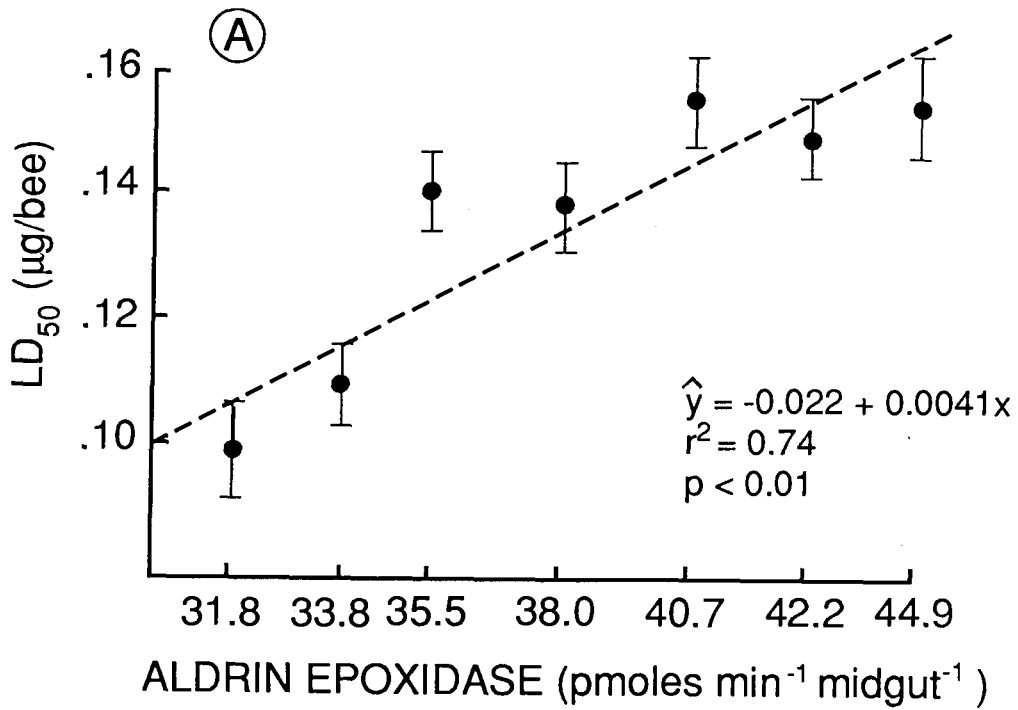
Figure 5. (A) Linear regression of propoxur LD 50 on colony aldrin epoxidase activity.

(B) Linear regression of propoxur LD 50 on colony glutathione transferase activity.

Each point represents LD 50 with 95% Confidence Interval.

PROPOXUR

Ⓐ

LD_{50} (μg/bee)

.08

.07

.06

.05

.04

$\hat{y} = 0.038 + 0.0025x$
$r^2 = 0.69$
$p < 0.05$

31.8 33.8 35.5 38.0 40.7 42.2 44.9

ALDRIN EPOXIDASE (pmoles min^{-1} midgut^{-1})

Ⓑ

LD_{50} (μg/bee)

.08

.07

.06

.05

.04

$\hat{y} = 0.009 + 0.0023x$
$r^2 = 0.51$
$p < 0.05$

240 260 280 300 320 340 360

GLUTATHIONE TRANSFERASE
(nmoles min^{-1} mg^{-1})
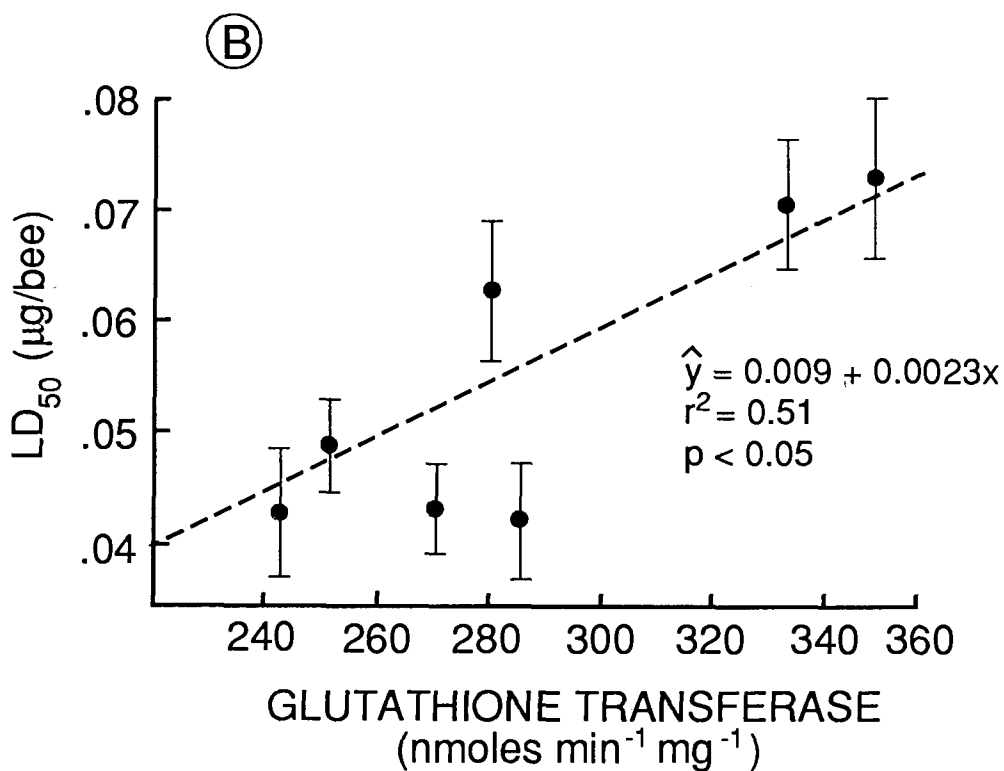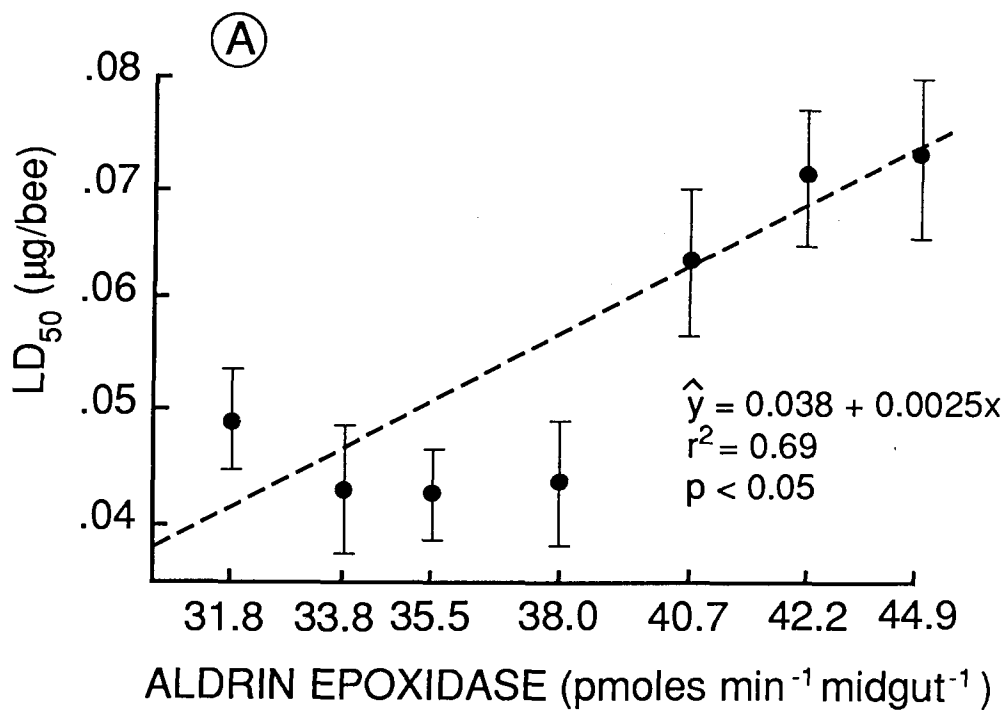
Figure 6. (A) Linear regression of aldrin LD 50 on colony aldrin epoxidase activity.

(B) Linear regression of aldrin LD 50 on colony glutathione transferase activity.

Each point represents LD 50 with 95% Confidence Interval.

(A) ALDRIN

$\hat{y} = 0.39 - 0.0060x$
$r^2 = 0.91$
$p < 0.01$

ALDRIN EPOXIDASE (pmoles min^{-1} midgut^{-1})

(B)

$\hat{y} = 0.28 - 0.0057x$
$r^2 = 0.73$
$p < 0.05$

GLUTATHIONE TRANSFERASE
(nmoles min^{-1}mg^{-1})
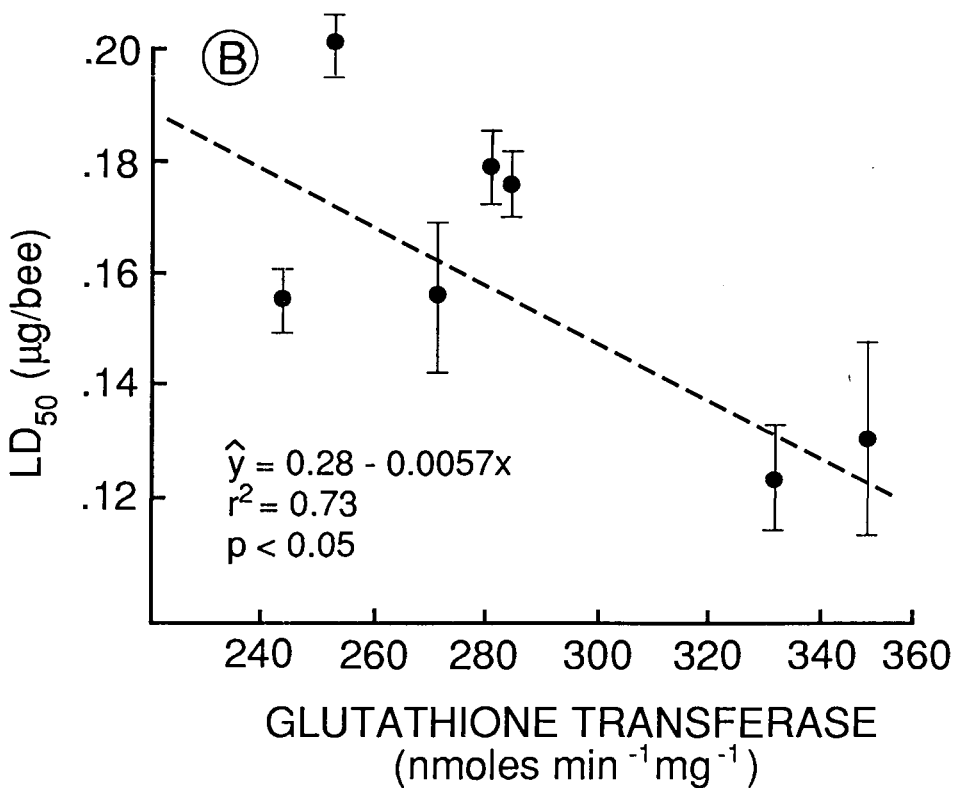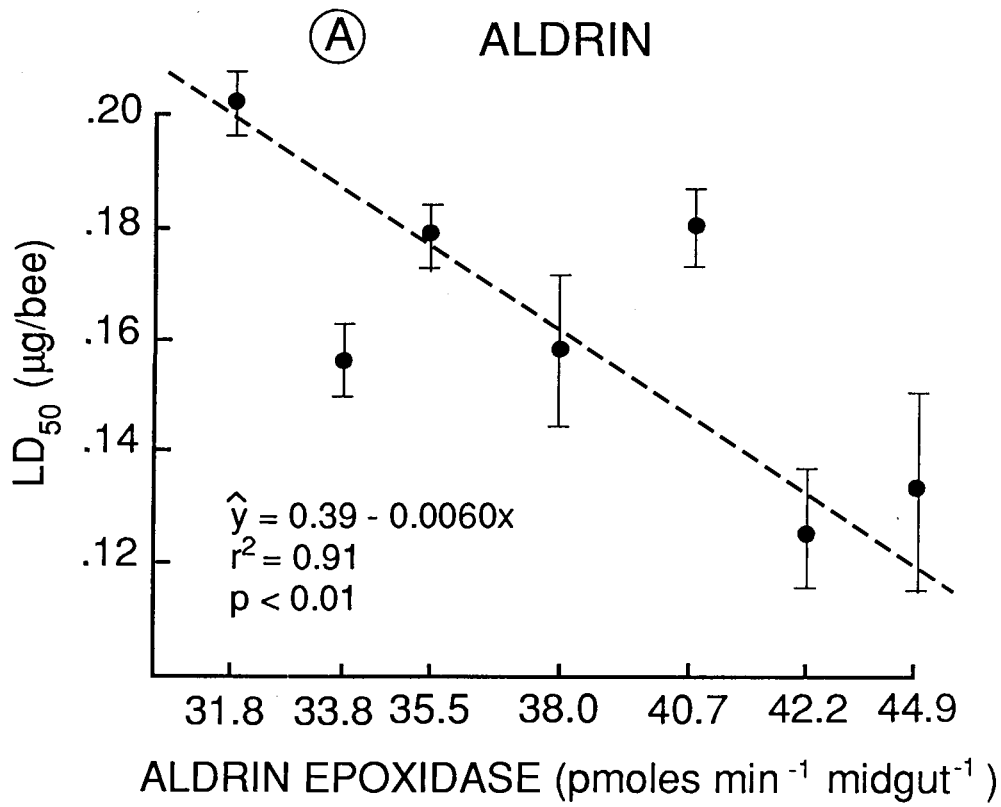
Figure 7. (A) Carbaryl LD 50 plotted against colony mixed-function oxidase activity.

(B) Carbaryl LD 50 plotted against colony glutathione transferase activity.

Each point represents LD 50 with 95% Confidence Interval.

CARBARYL
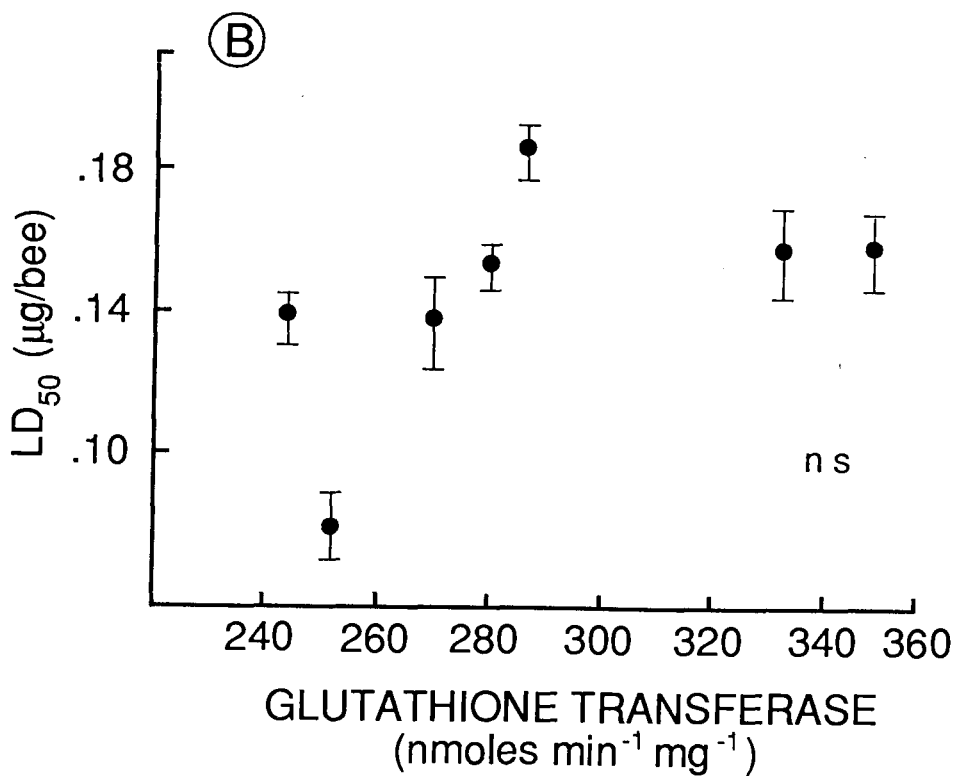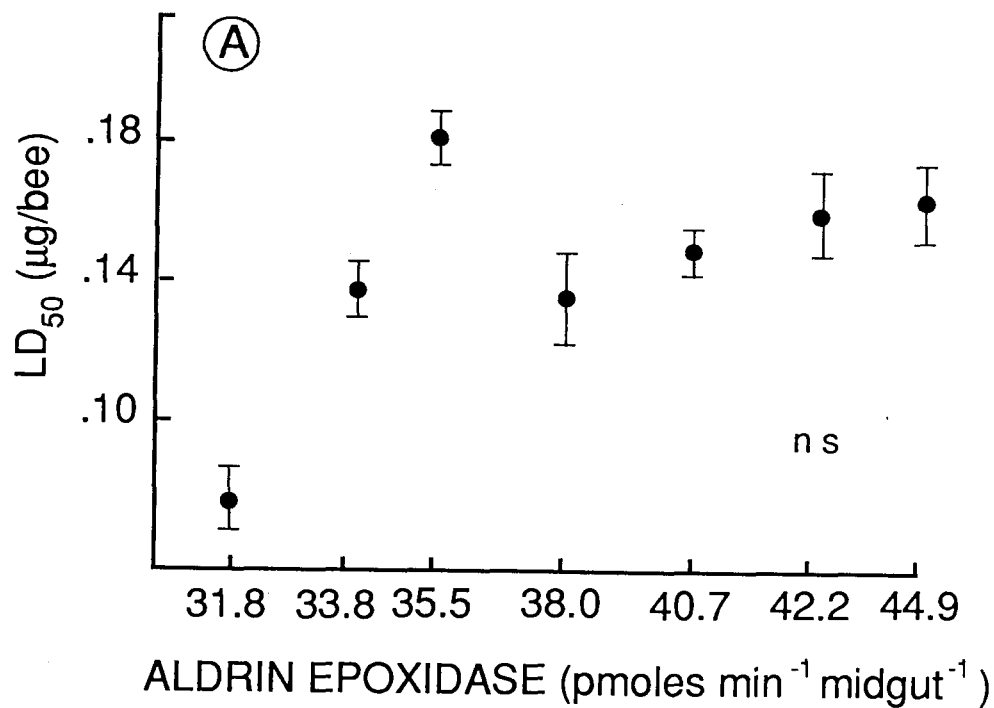
surprising, considering the cooperative nature of these detoxification enzymes; selection for high activity of one enzyme would be expected to result in high levels of the other.

The significant regression of diazinon LD_{50} on enzyme activity agree with the results reported in Chapter 2. Diazinon metabolism, some aspects of which are outlined in Fig. 2, consists of a balance between activation (reaction I) and detoxication (reactions 2 and 3) (Ahmad & Forgash 1975). The positive slope of the regression line indicates that detoxification reactions, whether mixed-function oxidase mediated, glutathione transferase linked, or otherwise must be proceeding at a net rate faster than metabolic activation.

The metabolism of propoxur, a carbamate insecticide, has likewise been shown to be mediated by mixed-function oxidase enzymes in house flies (Shrivastava et al. 1969). My findings support the involvement of these enzymes in propoxur metabolism in honey bees as well.

My results also make a strong point against using relationships obtained for one specific chemical to predict possible relationships for other untested compounds. This is best illustrated by the results for aldrin. Linear regression of aldrin LD_{50} on mixed-function oxidase activity indicates the involvement of these enzymes in aldrin resistance, but the negative slope of the regression line reflects an inverse relationship between mixed-function oxidase activity and aldrin

resistance, in contrast to the positive relationship found for diazinon and propoxur.

Aldrin is an example of an insecticide that is activated by a mixed-function oxidase reaction, but is not subsequently detoxified (Fig. 8). Dieldrin, the 6,7-epoxide product of the reaction, is of higher toxicity to honey bees (Atkins et al. 1976) but is further metabolized very slowly, at least in other insects and mammals (Brooks et al. 1970; Matthews et al. 1971). Therefore, colonies with efficient mixed-function oxidase systems convert aldrin to dieldrin quickly, and are significantly less resistant to aldrin than are colonies where this conversion takes place more rapidly.

The significant regression of aldrin LD_{50} on glutathione transferase activity is not surprising, considering the high degree of correlation between the two enzyme systems. Aldrin and dieldrin, however, are not metabolized by glutathione transferases in other species, and these enzymes are likely not involved in honey bees as well.

The absence of a significant regression of carbaryl LD_{50} on mixed-function oxidase activity was unexpected, as carbaryl is a well-known substrate for these enzymes (Oonnithan and Casida 1968). It seems doubtful that mixed-function oxidases are not involved in carbaryl metabolism in honey bees; rather, it is likely that the measurement of aldrin epoxidase does not accurately reflect aryl hydroxylase activity. The occurence of

Figure 8. Mixed-function oxidase-catalyzed epoxidation of aldrin.

DIELDRIN

ALDRIN

42
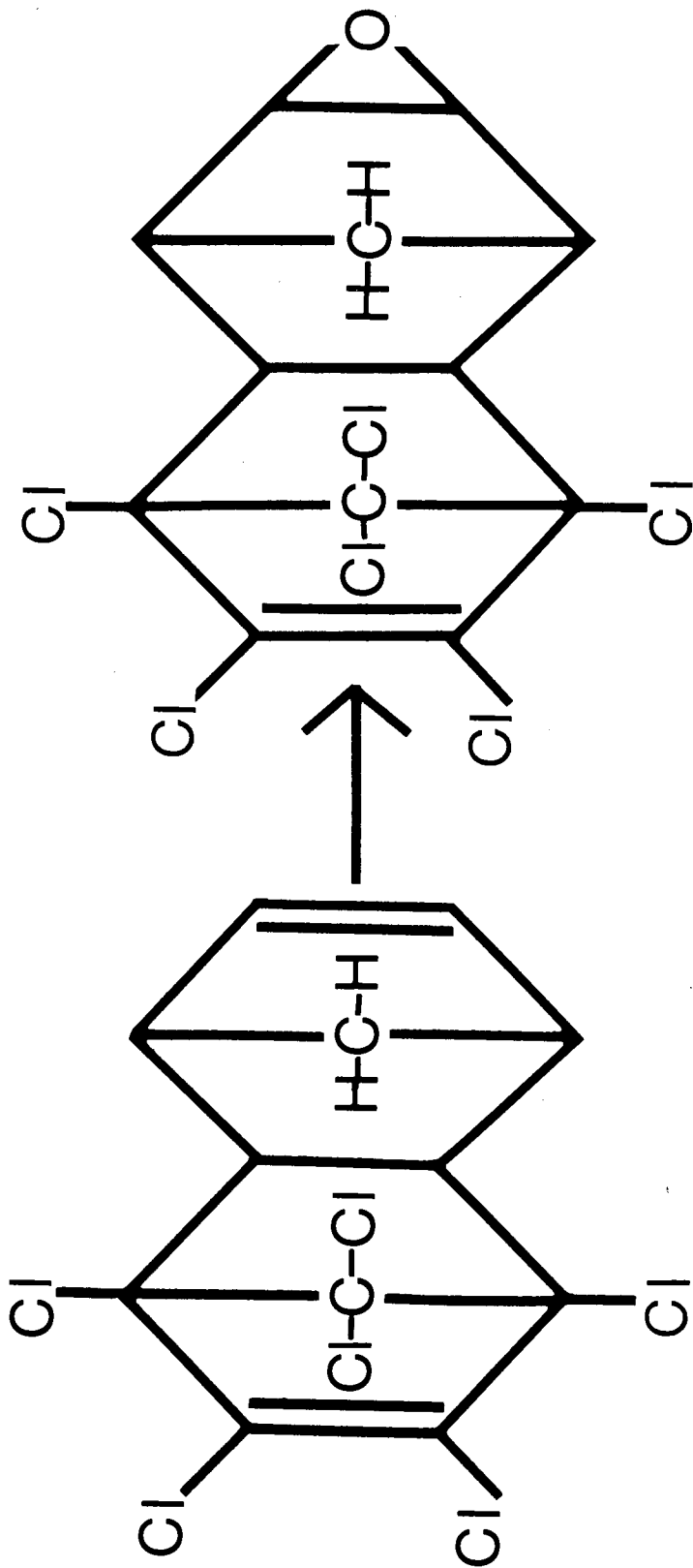
multiple forms of these enzymes has been well documented, and several isozymes are likely present in honey bees. It is possible that the low carbaryl synergist ratios observed by Metcalf et al. (1966) are the result of inactive aryl hydroxylase. However, biphenyl hydroxylase activity was clearly demonstrated by Yu et al. (1984) in honey bee midguts, and the reasons for the lack of dependence of carbaryl toxicity on mixed-function oxidase activity in my study are not clear. The use of insecticide synergists such as piperonyl butoxide may be useful in answering these questions.

Several conclusions can be drawn from the findings presented here. Firstly, the variation in detoxifying enzyme activity that is present in honey bee colonies is a significant factor in determining colony resistance to several insecticides. It is possible to derive regression models that can be used to predict resistance based on detoxifying enzyme activity.

Secondly, the nature of the relationship between toxicant and enzyme system is specific to the toxicant itself. Each insecticide undergoes specific metabolism and must be evaluated independently to determine how its toxicity is affected by enzyme levels. The example of aldrin, for which the relationship between colony resistance and enzyme activity is the opposite of that for diazinon and propoxur, best illustrates this phenomenon.

Thirdly, the strong correlation between colony mixed-function oxidase and glutathione transferase activities makes it unnecessary to include both enzymes in regression analysis for predictive purposes. The r^2 values obtained are somewhat higher when mixed-function oxidase activity is used as the independent variable, and measurements of this system would be preferred. However, glutathione transferase activity is also a good predictor of colony resistance, and the decision regarding which enzyme to assay should be made on the basis of the analytical equipment at hand.

CHAPTER IV

DETOXIFYING ENZYME ACTIVITIES IN WORKER HONEY BEES: AN ADAPTATION FOR FORAGING IN CONTAMINATED ECOSYSTEMS

Honey bees are eusocial insects with a well-developed caste structure. In addition to the familiar physical castes (queen, worker, and drone), temporal worker castes also exist within the colony. Under normal circumstances, worker bees perform in-hive duties such as feeding brood, cleaning cells, and storing food when they are young, and perform foraging tasks outside the colony when they are older, generally beginning at 18 to 25 days of age (reviewed by Winston 1987). Once a worker begins to forage, she normally does not return to inside duty but remains a forager for the remainder of her lifetime.

This flexible schedule of age polyethism presents different demands to workers of different ages, and physiological specialization for certain tasks has been well documented. Worker bees involved in brood care have large and active hypopharyngeal glands (Brouwers 1982; Fluri et al. 1982), and development and resorption of wax glands is related to comb building activities (Boehm 1965). Foraging bees undergo several physiological changes consistent with a shift from a relatively confined existence within the hive to an actively flying lifestyle in the field, including increased glycogen storage and higher oxygen consumption (Harrison 1986).

The transition from the hive environment to the field presents other challenges to these organisms in addition to the increased physiological demands of flight. One such challenge is the presence of environmental toxins, both man-made in the case of pesticides and natural in the case of plant allelochemicals

in toxic nectar. The ability of bees to withstand exposure to such contaminants may be a critical factor in determining colony foraging performance, particularly in agricultural ecosystems where pesticide use is high.

There are several mechanisms by which insects can withstand environmental contamination, and metabolic detoxication of pesticides and allelochemicals has been shown to play an important role in the biology of a number of insect species. The development of resistance to pesticides often has a metabolic basis (Plapp 1976); allelochemical metabolism has been implicated as a limiting factor in establishing the breadth of the host range of herbivorous insects (Krieger et al. 1971).

Honey bees have been shown to have active detoxifying enzyme systems, and worker bee detoxication activity may play a major role in determining the efficiency of colony foraging in contaminated environments. There would therefore be selective pressure to increase the activity of enzyme systems involved in detoxication in foraging worker bees.

In order to test the prediction that detoxifying enzymes will increase in activity as workers age, I assayed the mixed-function oxidase and glutathione transferase systems throughout the adult lifespan of worker honey bees. I assayed total detoxication capacity as well as specific enzyme activity; total capacity is dependent on enzyme concentration, while specific activity reflects the activity of the enzymes when

corrected for protein content.

Materials and methods

All worker honey bees used in these experiments were taken from a single queenright colony maintained at Simon Fraser University. Frames of sealed brood were removed from the colony and workers were allowed to emerge overnight in a laboratory incubator (34°C.). They were marked on the dorsal surface of the thorax with a spot of enamel paint and returned to the parent colony. At regular intervals, every 3 days for mixed-function oxidase and every 7 days for glutathione transferase assays, cohorts of marked workers were removed from the colony and assayed for detoxifying enzyme activity. Newly-emerged workers were assayed within 12 hours of emergence, and were not marked with paint. Worker bees of foraging age (21 days and older) were collected at the hive entrance as they returned to the colony; younger hive bees were collected from the brood nest area.

For glutathione transferase assays, groups of 10 midguts with contents removed were dissected into ice-cold potassium phosphate buffer, pH 6.5. Tissues were homogenized using a motor-driven ground-glass tissue grinder, and centrifuged at 12,000g for 15 minutes (4°C). The resulting post-mitochondrial supernatant was filtered through glass wool and kept on ice for enzyme assays and protein determinations. Four replicates of 10 midguts were used for each age group.

Glutathione transferase activity was assayed using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate as described previously. The protein content of these post-mitochondrial preparations was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Mixed-function oxidase activity was determined using aldrin as the test substrate. Intact midguts, including contents, were assayed individually for aldrin epoxidase activity; 10 midguts were used for each age group. Tissues were dissected, blotted dry, and 10 ug of aldrin in 2.0 ul methyl cellosolve was applied evenly over the midgut surface using a micropipette. Each midgut was then incubated for 10 minutes ($40°$ C) in 1.0 ml of 0.1 M potassium phosphate buffer, pH 7.4.

Reactions were stopped by the addition of 1.0 ml acetone, followed by further addition of 1.0 ml isooctane. The final mixture was spun in a vortex mixer for 1 minute, the phases allowed to separate, and the isooctane layer drawn off and kept in teflon-capped vials at $-20°C$ until analysis using electron-capture gas chromatography. The efficiency of dieldrin extraction using this method was 71.5 $\pm$ 1.5% ($\overline{x}$ $\pm$ SE of 5 replicates).

Protein content data were subjected to linear regression analysis using worker age as the independent variable. Enzyme activity data were correlated with worker age. Data from newly-emerged workers were not included in the correlation and

regression analyses because of the obvious difference in the physiological state of newly-emerged individuals.

Results

The post-mitochondrial protein content of honey bee midguts decreased in a linear fashion from the time workers were 7 days old until the end of their life ($p < 0.01$; Fig. 9). The regression equation obtained from these data was used to predict the protein content on days when only intact midguts were used for mixed-function oxidase assays. Changes in post-mitochondrial protein content were assumed to reflect changes in both the soluble and microsomal fractions of midgut tissues.

There was a significant correlation between worker age and glutathione transferase activity. When expressed as nmoles CDNB conjugated/minute/midgut, glutathione transferase activity was negatively correlated with worker age ($r = -0.63$; $p < 0.05$; Fig. 10a). However, when the specific activity of the glutathione transferase enzymes was analyzed (specific activity= activity/mg protein [Lehninger 1975]), that activity was found to increase as the bees aged and began to forage ($r = 0.81$; $p < 0.01$; Fig. 10b).

The relationship between mixed-function oxidase activity and worker age followed the same pattern as glutathione transferase. Enzyme activity on a *per bee* basis declined with age ($r = -0.73$; $p < 0.05$; Fig. 11a), but specific activity increased ($r = 0.91$; $p <$

Figure 9. The relationship between midgut post-mitochondrial protein content and the age of worker honey bees. Each point represents $\bar{x} \pm$ SE of 4 replicates, 10 bees per replicate. N.E. = newly emerged.

$\hat{y} = 0.413 - 0.004x$
$r^2 = 0.86$
$p < .001$

AGE (DAYS)

PROTEIN CONCENTRATION
(mg/midgut)

5 2
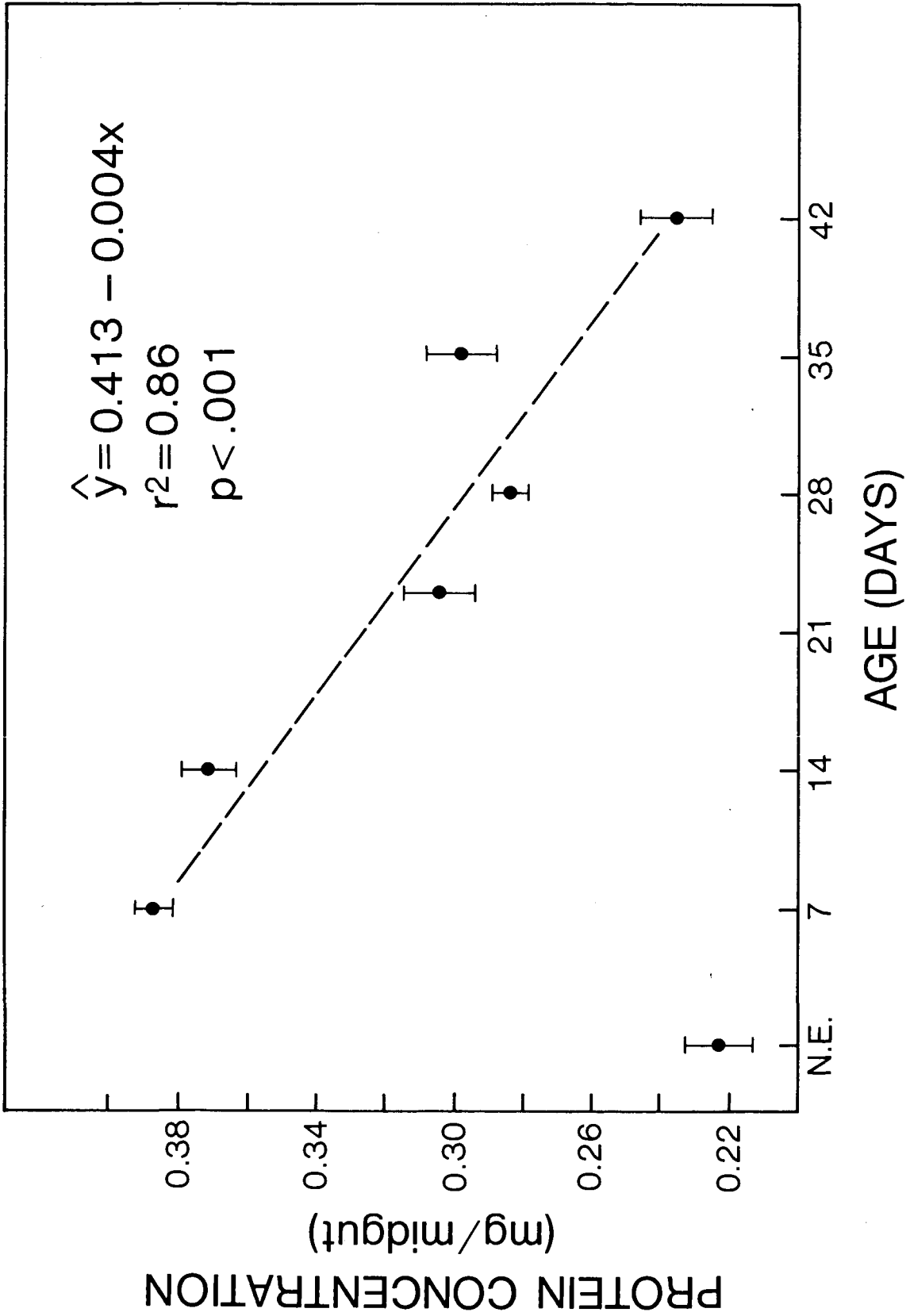
0.01; Fig. 11b).

Newly-emerged worker bees had lower enzyme activities than at any other time of their life. Protein content was also much reduced. Clearly, the physiological state of newly-emerged workers was qualitatively different from that of older workers; three days of maturation resulted in dramatic differences in mixed-function oxidase activity (Fig. 11a), while at seven days the midgut protein content and the glutathione transferase activity were much higher as well (Figs. 9 and 10a).

Discussion

These findings support my prediction of increased specific activity of detoxifying enzymes in older worker bees (summarized in Fig. 12), and suggest a biochemical adaptation for foraging in contaminated ecosystems. The percentage change in midgut protein content, which amounts to a decrease of 40% throughout the life of the worker bee, was partially balanced by a 26% increase in glutathione transferase specific activity and a 33% increase in mixed-function oxidase specific activity. Although detoxication activity on a *per bee* basis was lower in older workers, the conservation of detoxifying enzymes as reflected by an increase in specific activity compensated for most of the protein loss.

It is apparent that the dramatic loss of protein content in these insects is a major factor that puts them at risk from

Figure 10. (A) The relationship between glutathione transferase activity, expressed on a per bee basis, and the age of worker honey bees.

(B) The relationship between glutathione transferase specific activity and the age of worker honey bees.

Each point represents $\bar{x} \pm$ SE of 4 replicates, 10 bees per replicate. N.E. = newly emerged.

Figure 11. (A) The relationship between mixed-function oxidase activity, expressed on a $\underline{per}$ $\underline{bee}$ basis, and the age of worker honey bees.

(B) The relationship between mixed-function oxidase specific activity and the age of worker honey bees.

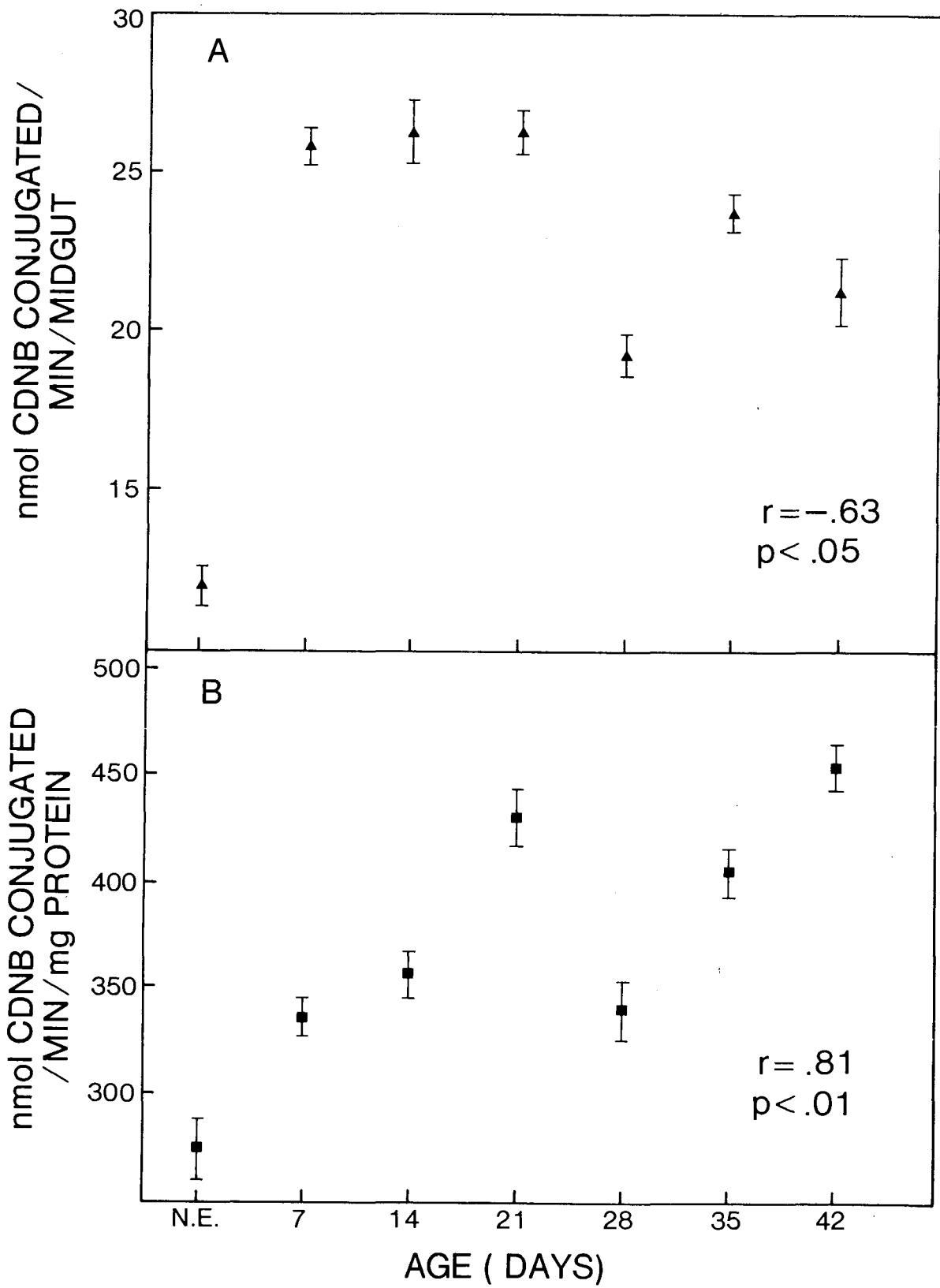Each point represents $\bar{x} \pm$ SE of 10 individuals.

N.E. = newly emerged.

57
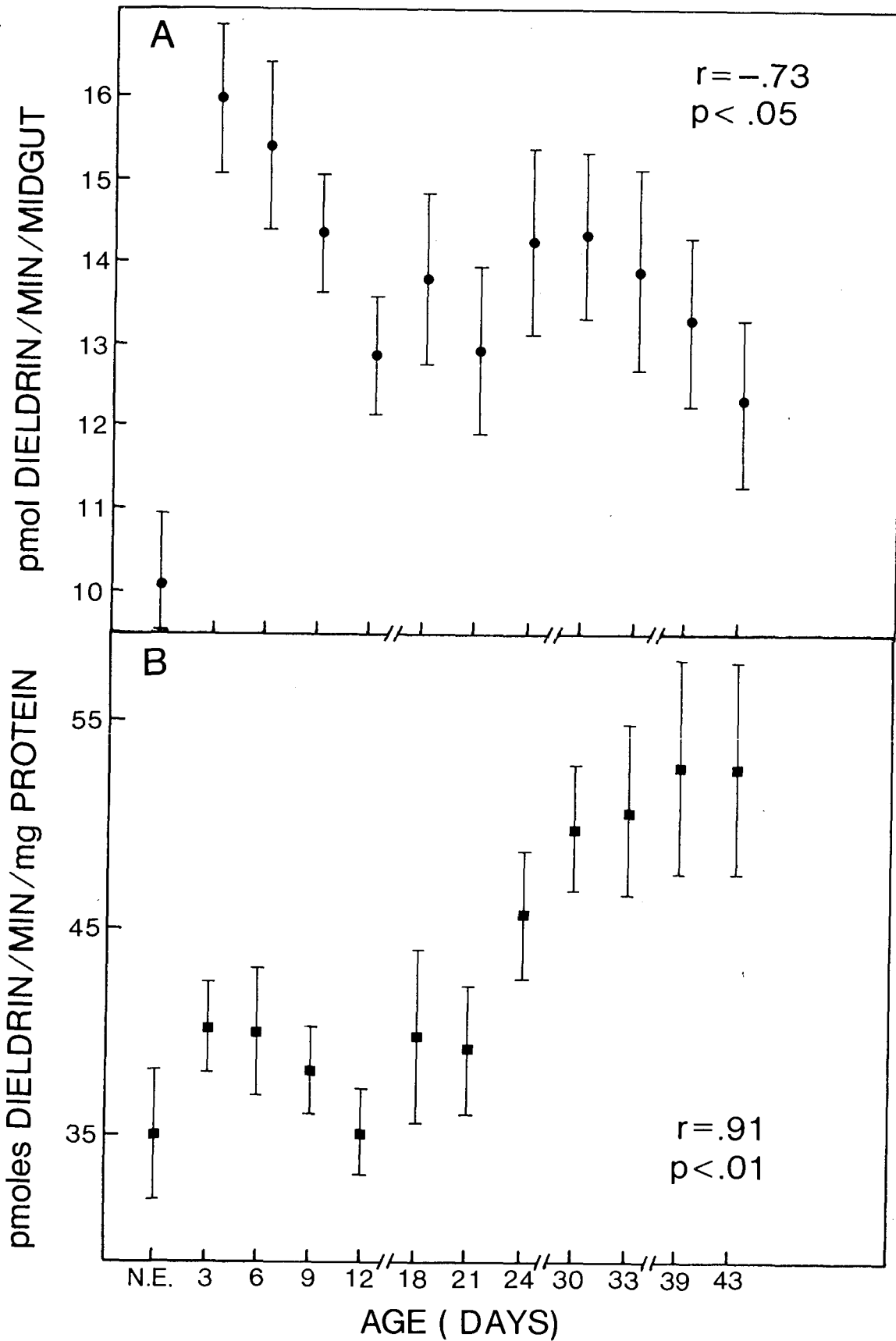
environmental contaminants, and is no doubt part of the reason why honey bees show marked sensitivity to many insecticides relative to other insects. The adaptive significance of the loss of 40% of the midgut protein content is unclear. Harrison (1986) suggested that shrinkage of gut tissue in foraging bees makes room for increased nectar storage in the crop, and serves to maximize foraging loads. However, Schmid-Hempel et al. (1985) clearly demonstrated that foragers often do not fill their crop even in non-depleting nectar resources, and argued that this serves to decrease the metabolic costs of food transport. Loss of protein content may therefore not assist the insect in maximizing foraging efficiency. It is more probable that the protein loss is associated with the physiological demands of flight, since honey bees have a fixed amount of flight capacity which may be determined by the activity of enzymes involved in carbohydrate metabolism (Neukirch 1982).

The large decrease in protein concentration in older workers is similar to the findings of Porter and Jorgensen (1981), who hypothesized that the harvester ant, *Pogonomyrmex owyheei*, has evolved a "disposable" caste of short-lived workers. Interestingly, these ant workers lose approximately 40% of their dry weight during their lifetime, the same value obtained by Harrison (1986) for dry weight measurements in honey bees and the same percentage decrease in protein content I report here.

It is somewhat surprising that younger bees also experience protein loss while still in the colony. The loss of protein is a

Figure 12. Summary of percentage changes in protein content
and enzyme activity as a function of worker age.

GLUTATHIONE TRANSFERASE

MIXED-FUNCTION OXIDASE

Δ ACTIVITY/mg +26%

Δ ACTIVITY/BEE −19%

Δ PROTEIN −40%

Δ ACTIVITY/mg +33%

Δ ACTIVITY/BEE −23%

Δ PROTEIN −40%

PERCENT

100

90

80

70

60

7   14   21   28   35   42

7   14   21   28   35   42

AGE (DAYS)

60
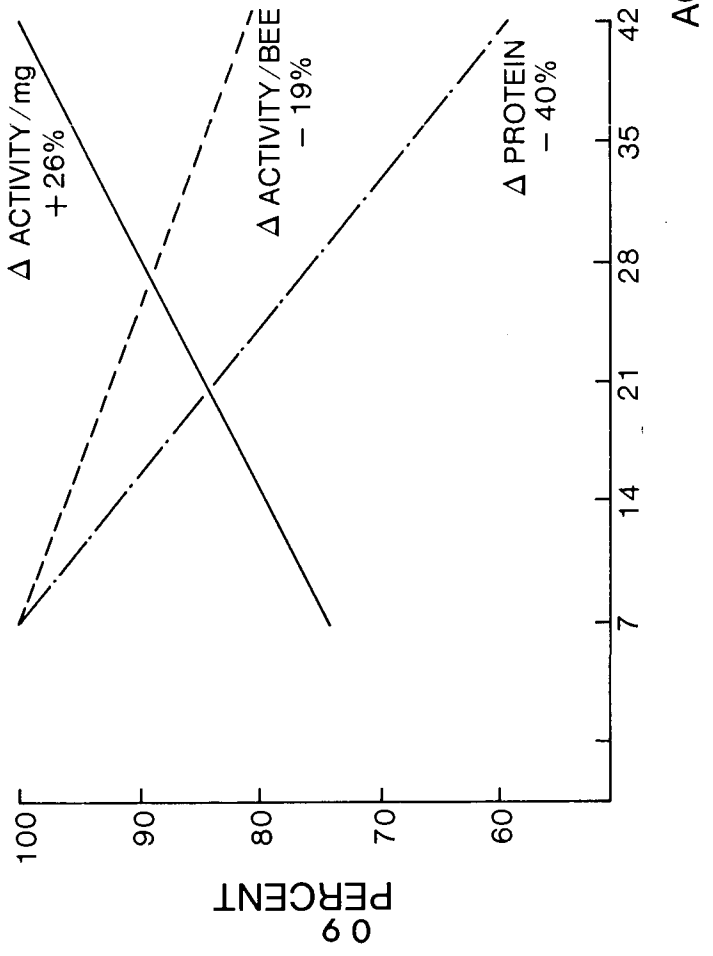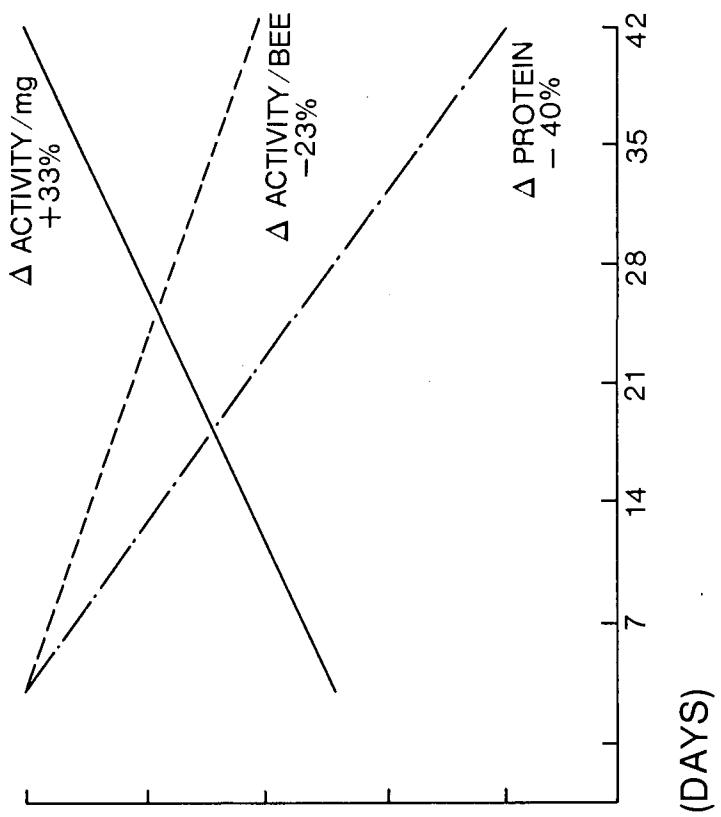
linear function of age throughout the bee's lifetime, and does not occur only in foragers. It seems likely that the metabolic demands of brood rearing result in an overall decrease in protein content; Crailsheim (1985) showed that the incorporation of leucine into haemolymph protein also decreases in a linear fashion in young hive bees, in agreement with my results.

Newly-emerged bees have lower levels of midgut protein than at any other time of their lives and this, combined with low specific activities of detoxifying enzymes, makes these insects particularly vulnerable to any type of environmental toxin. This lack of detoxication capacity poses a considerable risk to the colony, since the death of a newly-emerged worker deprives the colony of the benefits from a lifetime of work. Young workers have greater potential value to the colony than older workers; this is thought to be the reason why hazardous foraging tasks have evolved to take place at the end of a worker's life rather than at the beginning (Jeanne 1986; Kolmes 1985). This is why microencapsulated insecticides have such a devastating effect on honey bee colonies when brought back to the hive with collected pollen (Johansen 1977), largely due to the resultant death of young workers.

The finding that the specific activities of glutathione transferases and mixed-function oxidases increase in older bees suggests a biochemical adaptation by these insects to the demands of an uncertain and changing environment. Being able to compensate for a dramatic loss of protein may enable foraging

bees to better exploit resources in areas where environmental contamination is present, which may be extremely important in achieving optimum foraging efficiency in these insects.

The mechanisms of these changes in enzyme activity are unknown. Older bees may selectively degrade and metabolize some proteins to a larger extent than others, thereby conserving "more important" cellular materials. Alternatively, there may be changes in the qualitative nature of detoxifying enzymes in aging bees, altering the complement of isozymes and increasing specific activity. Evidence for such a mechanism in the cockroach, *Diploptera punctata*, has been presented by Feyereisen and Farnsworth (1985). The answer to the question of how honey bees adjust the activity of detoxifying enzymes would require a detailed study of metabolic regulation in this social insect.

It is also possible that the increase in specific activity in foraging workers is due to induction by environmental contaminants. The induction of detoxifying enzymes in insects is a widespread and well known phenomenon (reviewed by Terriere 1984), and serves an obvious adaptive function by increasing metabolic capacity. Pesticides and plant allelochemicals have been shown to induce detoxifying enzymes (Brattsten *et al*. 1977; Terriere and Yu 1974), and both have probably exerted selective pressure on honey bees. Resistance to chemical pesticides in insect pest species has been a serious problem in agricultural pest management, and pesticides, despite their relatively recent existence, are likely a major selective force on honey bees. As

well, toxic nectars can be widespread in nature (Baker 1978), and Rhoades and Bergdahl (1981) suggest that they function to manipulate the behaviour of pollinators by excluding nectar thieves and less specialized Lepidopterans. They go on to speculate that bees must have developed some form of tolerance to these toxic nectars, and my results provide the first evidence in support of this hypothesis.

The increased specific activity of detoxifying enzymes in foraging worker honey bees is another example of physiological and biochemical specialization of these insects for the demands of a particular task. It seems probable that adaptation of this type plays a major role in the success of insect societies exhibiting temporal division of labour.

CHAPTER V

BEHAVIOURAL STATUS AND DETOXICATION ACTIVITY ARE RELATED IN

WORKER HONEY BEES

One of the major questions remaining from the experiments in Chapter IV was whether changes in enzyme activity, while appearing adaptive, were related to foraging activities or simply to developmental processes that are independent of behavioural maturation. As has been pointed out by Lewontin (1978) and Gould and Lewontin (1979), adaptive explanations for observed characteristics should be entertained only after more parsimonious explanations, such as developmental processes, have been ruled out.

The objective of the experiment described in this Chapter was to determine if changes in detoxication activity are dependent upon worker age or worker behaviour. This was a collaborative project with Dr. G.E. Robinson of The Ohio State University that involved subjecting a colony of honey bees to conditions that affect temporal polyethism, including the ages at which nursing and foraging occur. An association between worker occupation and glutathione transferase activity, regardless of age, would support the hypothesis of biochemical adaptation for foraging. An association between worker age and enzyme activity independent of behavioural status would indicate that increased enzyme activity and foraging are not related.

Preliminary experiments with frozen midgut tissue indicated a complete absence of mixed-function oxidase activity in intact midguts. Since the bees to be analyzed had to be shipped on dry ice from Ohio, only glutathione transferase activity was assayed in this experiment.

Materials and Methods

A colony of honey bees was established in an apiary at The Ohio State University with 2000 one-day-old workers, a queen, one comb containing unsealed brood, one comb of honey and pollen, and one empty comb. Bees were obtained from combs of sealed brood taken from one colony and placed in a 33°C incubator. Each bee was marked on the thorax with a paint dot to ensure that all workers sampled were residents of the experimental colony.

As expected (Ribbands 1952), division of labour occurred in the experimental colony within a few days despite the abnormal age structure. Some young bees displayed typical nursing behaviour, while other young workers foraged prematurely. New bees were prevented from emerging by the removal of all combs containing developing pupae; a few weeks later there were overaged nurses, and foragers of normal ages. Nurses and foragers were identified according to established criteria (Robinson 1987). Fifty nurses and fifty foragers were collected when they were 7, 14, and 21 days old and stored at -70°C until assayed for enzyme activity and protein content. All bees were frozen for the same amount of time (60 days) to ensure that sample activity was not affected by variable length of freezing. Groups of ten midguts were dissected, gut contents removed, and tissue washed in 0.15 M KCl. Tissue samples were homogenized in 0.15 M potassium phosphate buffer, pH 6.5, centrifuged at

12,000g for 15 min., and filtered through glass wool. The resulting post-mitochondrial supernatant was kept on ice and used immediately for enzyme assays and protein determinations.

Glutathione transferase activity was assayed with 1-chloro-2,4-dinitrobenzene as the substrate as previously described. Reactions were continued for 5 min. at $22^{\circ}C$, and enzyme preparations were diluted with buffer to ensure linear increase in product formation. Protein content was assayed (Bradford 1976) using bovine serum albumin as the standard. Four or five replicates of 10 midguts were assayed for each test group. The effect of age on enzyme activity and protein content was assessed with one-way analysis of variance for both foragers and nurses. Differences between behavioural groups at each age were analyzed with t-tests.

Results

There was a significant ($p<0.001$) decrease in protein content in both nurses and foragers between 7 and 21 days of age (Fig. 13a). Glutathione transferase activity did not change with age in nurse bees ($p> 0.05$). In contrast, there was a significant ($p <0.01$) age effect on glutathione transferase activity in foragers, with 21-day-old bees showing the highest specific activity (Fig. 13b). Comparisons between nurses and foragers revealed highly significant ($p< 0.001$) differences in protein content at all three ages (Fig. 13a). There was no

67

Figure 13. The effect of age and behavioural status on:
(A) midgut post-mitochondrial protein content; and
(B) glutathione transferase activity in adult worker
honey bees. $\overline{X} \pm$ SE of 4-5 replicates, 10 bees per replicate.
Open bars represent nurse bees; solid bars, foragers.
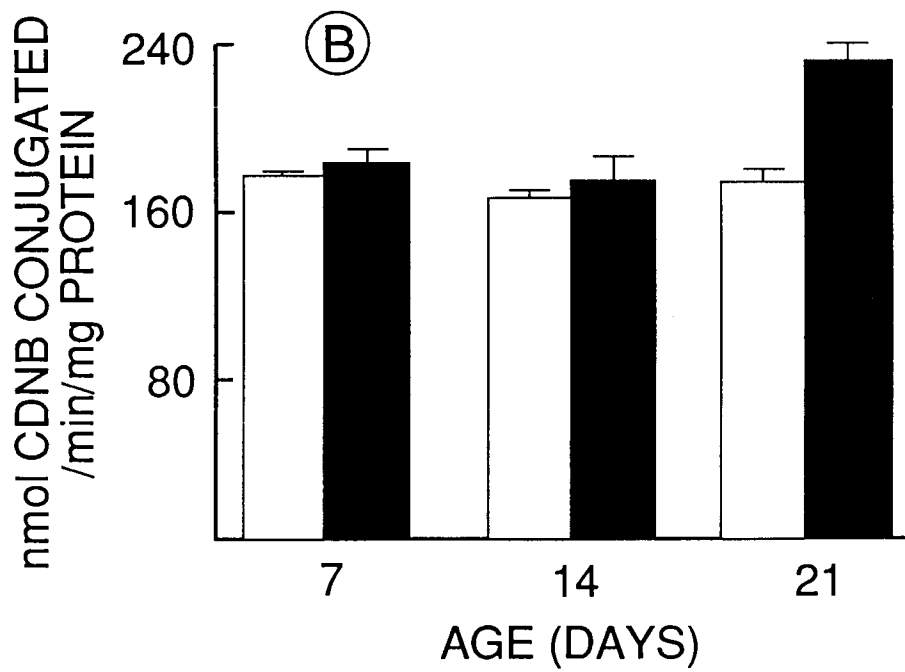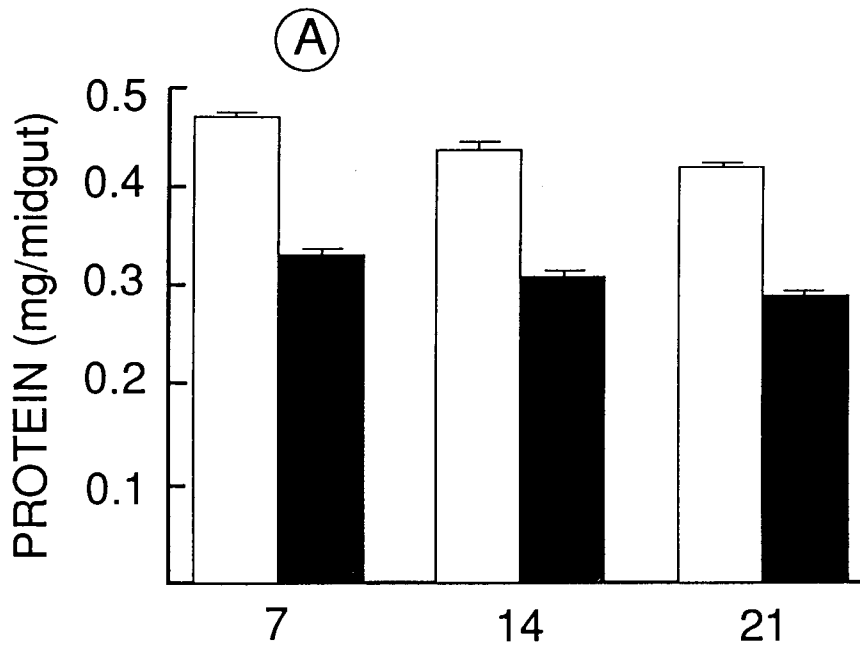
difference in enzyme activity between nurses and foragers aged 7 and 14 days. At 21 days foraging workers had significantly higher enzyme activity than did nurse bees ($p < 0.001$) (Fig. 13b).

Freezing and/or shipping resulted in an approximate 50% decrease in glutathione transferase activity from values reported for unfrozen, fresh workers (Yu et al. 1984). However, as each sample was handled identically, this comparative analysis should be sufficient to address the experimental objective.

Discussion

Although there was an age-related decrease in general midgut protein in nurse bees, the dramatic differences between foragers and nurse bees at all ages supports the hypothesis that protein loss is associated with flight activity. Increases in enzyme activity are not a direct consequence of the loss of midgut protein because there were significant differences in protein associated with age in both nurses and foragers, but an accompanying increase in enzyme activity only for 21-day-old foragers.

Behavioural status influenced glutathione transferase activity in worker honey bees. Enzyme activity in nurse bees did not increase despite increasing worker age. In contrast, enzyme activity was elevated in foragers, but only at 21 days of age.

These results suggest that changes in the activity of detoxifying enzymes may be influenced by both age and behaviour. Changes may occur only after a certain age, under the stimulus of field-related duties. Alternatively, there may be a quantitative relationship between foraging behaviour and enzyme activity; a certain amount of foraging may be necessary to induce changes in enzyme activity. This change may be due to the direct effects of foraging behaviour on worker bee physiology, or a consequence of enzyme induction due to exposure to environmental contaminants. My failure to detect changes in glutathione transferase activity in 7 and 14-day-old foragers may thus be a consequence of sampling bees that began foraging recently, which is likely because there were few individuals observed foraging from this small colony on any given day.

If increases in detoxication activity are due solely to enzyme induction, the reported differences between foragers and nurses may be a consequence of differential exposure to toxicants rather than biochemical specialization for foraging. Measurements of enzyme activity in bees that have foraged for specific amounts of time, and comparative analyses of the enzyme induction capabilities of nurse bees and foragers, are needed to further elucidate the relationship between foraging behaviour and detoxication enzymes.

CHAPTER VI

DETOXICATION ACTIVITY IN CROSS-FOSTERED WORKER HONEY BEES

The question of how environmental factors influence the expression of genetic traits is central to many fields of biology. The interaction of genotype and environment to produce the phenotypic characteristics that are the targets of natural selection is, however, difficult to study in any sort of ecologically relevant manner. This is largely due to the fact that, once one leaves the confines of the laboratory, the variable nature of the environment and the movement of organisms through that environment makes it difficult to quantify environmental factors and relate them to a specific group of individuals. An experimental system suitable for addressing these types of questions requires test environments that differ in some quantifiable way, and a source of related individuals that could be introduced into those environments and then assayed for their response to them.

The honey bee, with its unique social structure, provides such an experimental system. A worker bee, during that phase of its life when it is performing in-hive duties, is naturally confined to the environment within the colony. Worker bees are usually plentiful, so it is relatively easy to obtain sufficient numbers of individuals to introduce into colonies that differ in some quantifiable aspect. It therefore becomes possible to relate phenotypic characteristics in groups of workers to environmental parameters, and to do this in an ecologically relevent situation.

The objective of this experiment was to assess the influence of environmental factors on the activity of mixed-function oxidase and glutathione transferase enzymes. A cross-fostering methodology whereby workers from one parental colony are introduced into a series of foster hives was chosen because it is ideally suited to studying the relative importance of genetic and environmental influences. Cross-fostering studies have been useful in the assessment of racial differences in foraging age and longevity (Winston and Katz 1981,1982).

Materials and Methods

Frames of sealed brood were removed from a single parent colony and allowed to emerge in a laboratory incubator. These bees were marked with paint and introduced into nine foster colonies founded from swarms 1-2 months prior to the start of the experiment. One cohort was reintroduced into the parent colony. One hundred marked workers were cross-fostered in this fashion every 2-3 days beginning June 3, 1987 and continuing until June 26.

Foster colonies were chosen to cover a wide range of colony populations. Populations were determined at the time of worker introduction by using a plexiglass grid to measure the percentage of frame coverage by adult workers, and then deriving the number of adults per frame using the method of Burgett and Burikam (1985). The area of sealed brood in each colony was also

measured, and converted to the number of worker cells using a value of 4 cells/cm² (Dadant 1975). The numbers of adult workers and potential worker cells were summed, and this value was used as the colony population at the time of worker introduction.

Marked workers were removed from all colonies 21 days after introduction. Population measurements were repeated at this time, and the average between populations at introduction and removal was used as the colony population level in subsequent analyses. The ratio of brood area to adult population was calculated for each colony from the population measurements. This cross-fostering protocol, along with the population measurements, is shown in Figure 14.

Workers recovered from the test colonies were assayed for mixed-function oxidase activity, glutathione transferase activity, and protein content as described previously. For mixed-function oxidase assays, 10 individuals from each cohort were assayed; for glutathione transferase, 4 replicates of 10 midguts were used. Enzyme activity data were correlated with total colony population and the ratio of brood/adults.

Results

Recovery of marked workers from foster colonies is indicated in Table 3. Two colonies had a greatly reduced rate of recovery (<40%); for all others the recovery was between 68% and 88%. Cross-fostered workers from colonies with low recovery were

75

Figure 14. Populations of parental and test colonies used for cross-fostering experiments.

PARENTAL

29.8
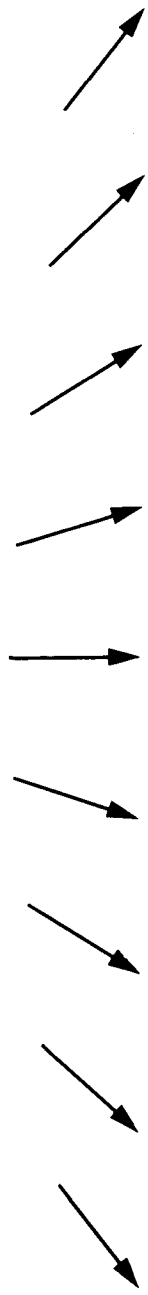
2.9   7.6   8.9   12.6   26.6   28.6   29.2   37.5   38.5

POPULATION (10^3)

Table 3. Numbers recovered from foster colonies 21
days after the introduction of 100 marked workers.

Colony Number	Recovered
Parental	71
668	71
670	32
665	88
667	79
658	82
660	73
664	68
673	37
663	75

assumed to have been foraging, and were not included in the subsequent correlation analyses.

There were significant negative correlations between colony population and the activities of mixed-function oxidase and glutathione transferase (Figs. 15 and 16); colonies with low populations had high enzyme levels. Enzyme activity levels in the parental colony were intermediate, and workers reintroduced into the parent hive did not differ from many of the cross-fostered cohorts. Enzyme activity was positively correlated with the ratio of sealed brood to adult worker population (Figs. 17 and 18).

Discussion

The determination of detoxifying enzyme activity in worker honey bees appears to have a significant environmental component. The negative relationship between enzyme activity and colony population suggests that bees in less populous colonies may be under greater pressure from environmental contamination than workers in larger colonies. The "dilution" effect of large population size would serve to reduce exposure of individual workers to given quantities of toxicant. Therefore, workers in small colonies may come into contact with proportionally greater amounts of toxins, hence their need for a more efficient detoxication system.

Figure 15. Correlation of colony population and mixed-function
oxidase activity in cross-fostered cohorts. Each point represents
$\bar{x} \pm$ SE of 10 individuals.

Figure 16. Correlation of colony population and glutathione
transferase activity in cross-fostered cohorts. Each point
represents $\bar{x} \pm$ SE of 4 replicates, 10 bees per replicate.

83

Figure 17. Correlation of sealed brood/adult bees and the mixed-function oxidase activity in cross-fostered cohorts. Each point represents $\bar{x} \pm$ SE of 10 individuals.
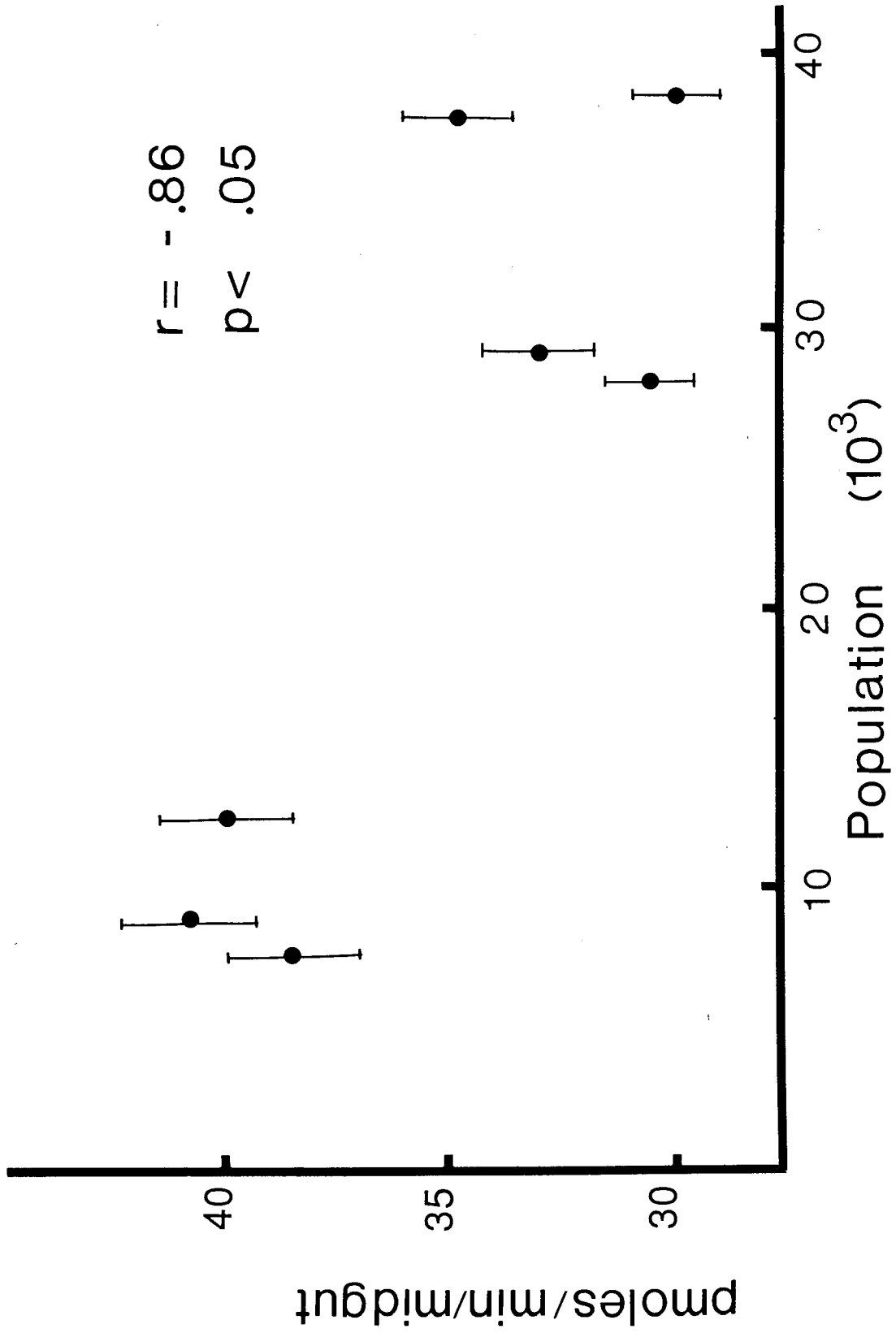
Figure 18. Correlation of sealed brood/adult bees and the
glutathione transferase activity in cross-fostered cohorts.
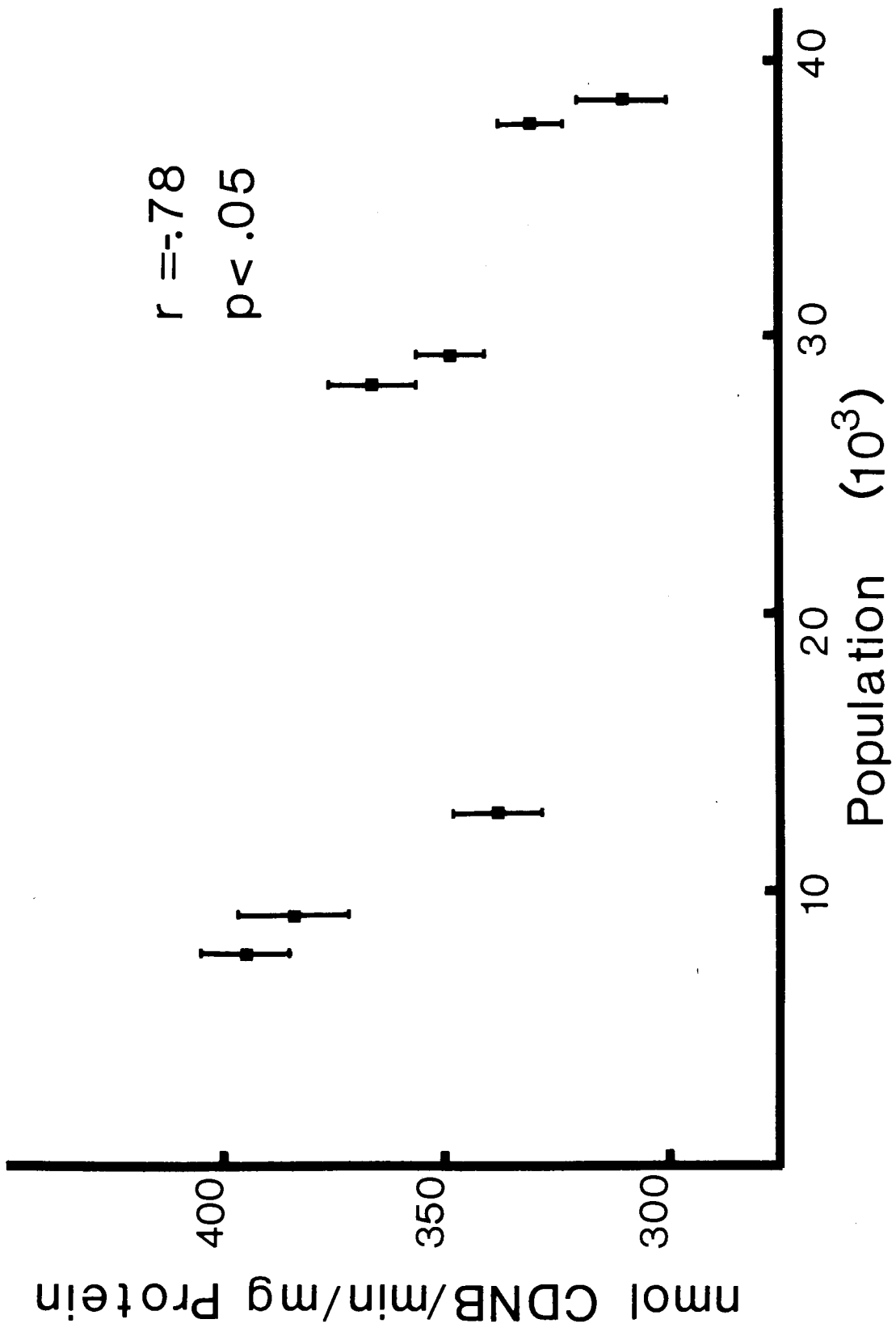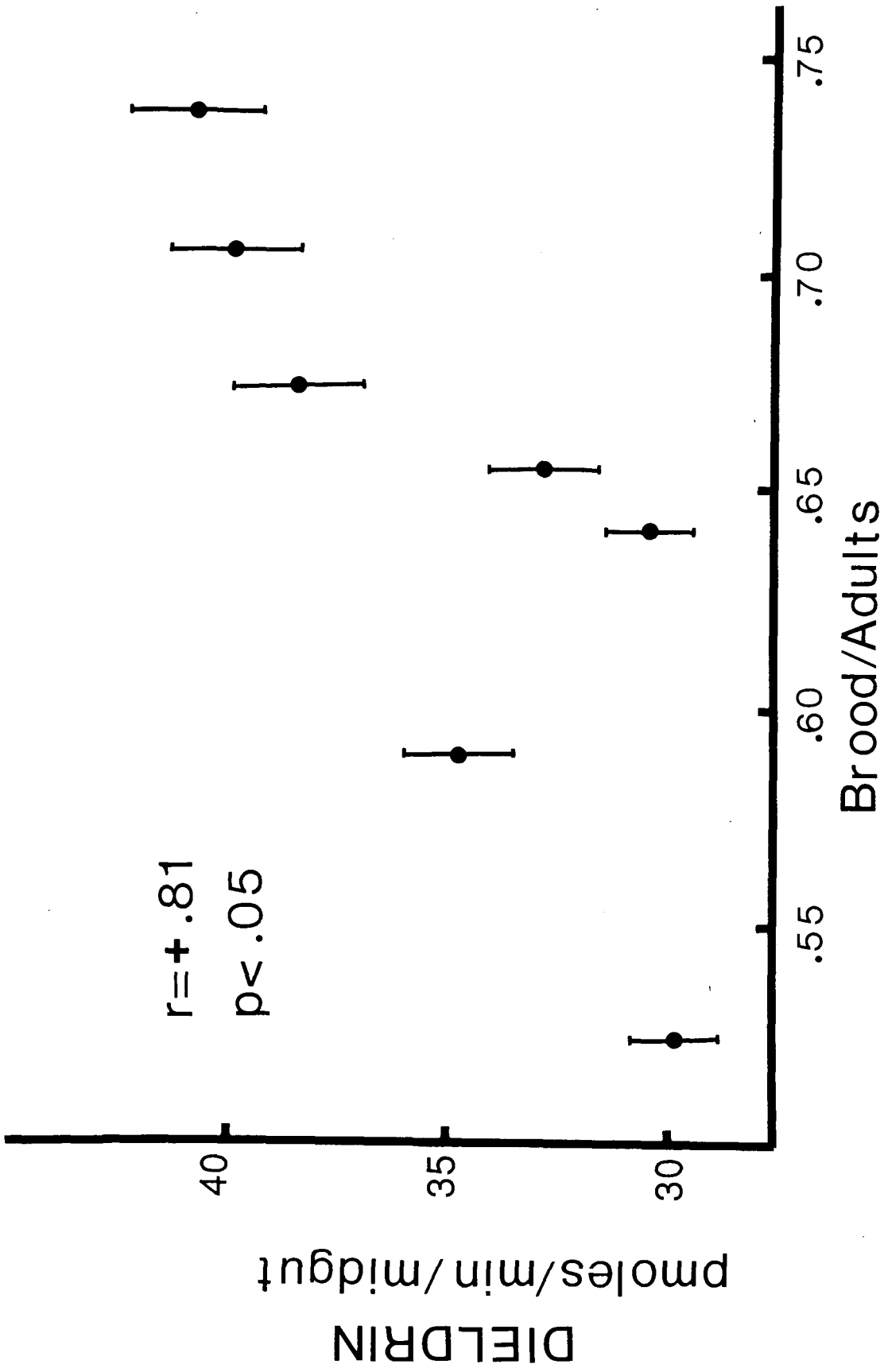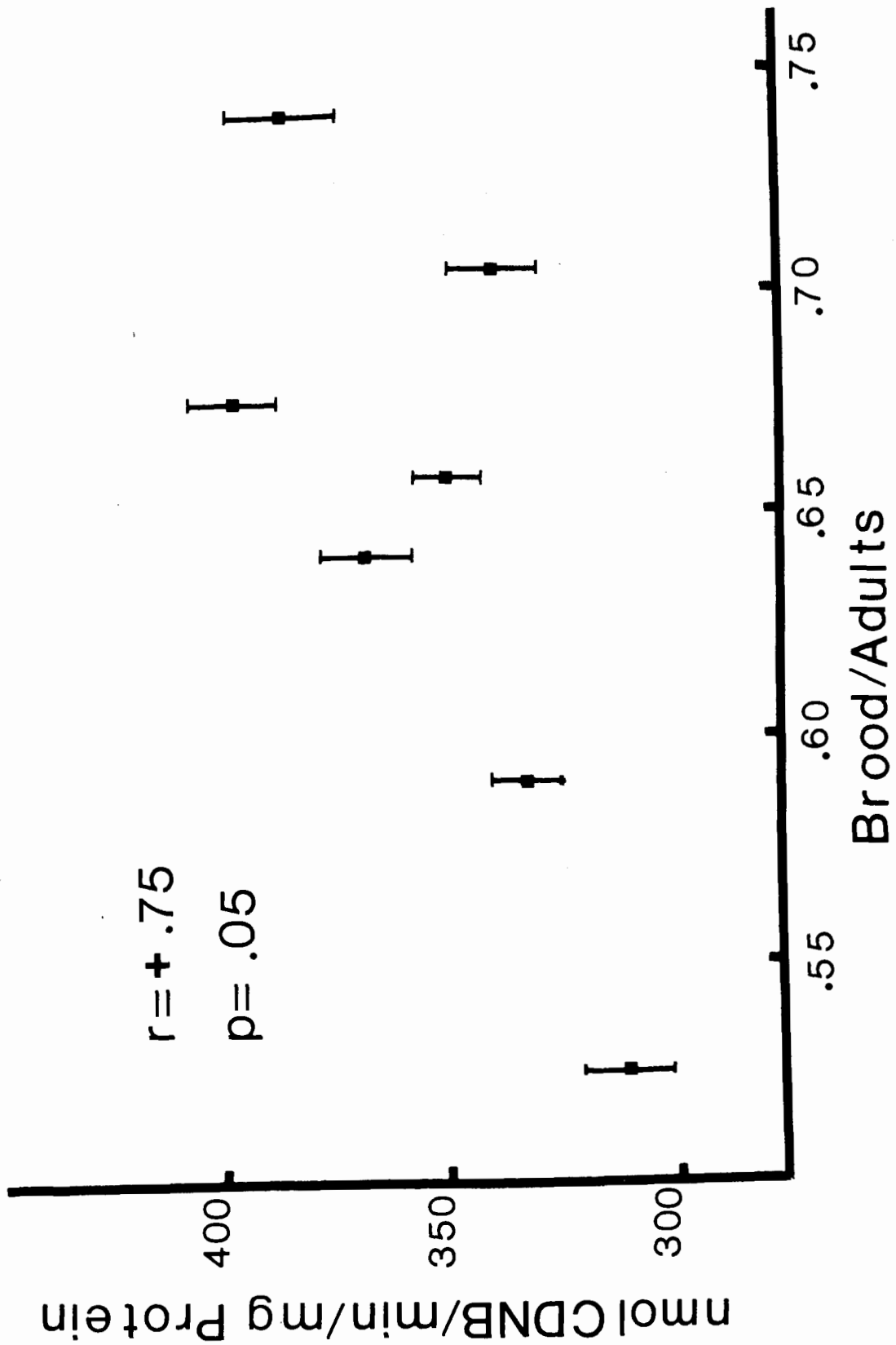Each point represents $\bar{x}$ ± SE of 4 replicates, 10 bees per
replicate.

r=+.75
p=.05

nmol CDNB/min/mg Protein

400
350
300

.55
.60
.65
.70
.75

Brood/Adults

87

This interpretation is supported by the positive relationship between enzyme activity and the proportion of brood in the colony. As expected, smaller colonies had a higher ratio of brood/adults than did more populous hives (Free and Racey 1968); workers in these small colonies expend proportionally more energy caring for brood than workers from larger hives. Physiological changes associated with brood rearing are well-documented (Brouwers 1982; Fluri et al. 1982), and workers engaging in these tasks to a greater extent may require a greater degree of physiological specialization. One such change may be increased levels of detoxication capability. Young workers (<21 days, as were the bees in this experiment) are responsible for the processing of food brought to the colony by foragers (Winston 1987), and the detoxication of xenobiotics entering the colony may be an important aspect of young worker function. In colonies expending a large amount of energy rearing large quantities of brood, the detoxication of xenobiotics before feeding to the brood could be vital to colony survival.

Workers reintroduced into the parental colony have enzyme levels no different from those introduced into foster colonies of comparable size. Colony population appears to be an overriding factor in the determination of enzyme activity, and different genetic backgrounds are not likely to be as important as overall colony strength. In fact, colony strength as represented by population is closely related to genetic factors, as is any quantitative colony trait. It is possible that

colonies of similar population in this study are more alike genetically that colonies with substantially different populations, and would therefore be similar in enzyme levels.

The elevated enzyme activities in two colonies from which the 21 day old workers were likely foraging are consistent with the changes described in Chapter 4, providing further evidence that behavioural status, particularly foraging, is a major factor determining the activity of detoxifying enzymes. It should be noted that activity differences between foraging and non-foraging test colonies are much larger than differences between non-foraging colonies alone. Glutathione transferase activity in the two foraging cross-fostered cohorts was 495 and 519 nmoles of chlorodinitrobenzene (CDNB) conjugated/min/mg of protein; the mean activity in non-foraging cohorts was 356 nmoles/ min/mg (range 331-383). The average increase was 42% in the foraging cohorts; similar increases were observed for mixed-function oxidase activity. These changes appear to represent a qualitative shift in enzymatic detoxication activity rather than the more subtle changes observed in nurse bees as a function of brood-rearing activity.

One of the colonies where foraging was occurring had gone queenless, which explains the small size of the adult population and the lack of sealed brood. With no brood to stimulate nursing behaviour, workers in this colony likely began to forage earlier than normal. Alternatively, some physiological change associated with queenlessness may have triggered these changes in enzyme

levels. The other "foraging" colony was queenright, so the reasons for early foraging are unclear. Winston and Katz (1982) demonstrated that workers cross-fostered into colonies of different racial background began to forage at the same time as other workers in the foster hive. In other words, the colony environment determined when foraging would begin, and it is possible that the environment in this colony promoted early onset of this task.

These experiments demonstrate that enzyme activity levels have a large environmental component, but do not compare workers from the foster hives with the cross-fostered cohorts to establish whether workers put into "high" detoxifying colonies have higher enzyme levels than workers cross-fostered into less active hives. This type of study is needed to better understand the role of colony environment in the determination of detoxication capacity in these insects.

CHAPTER VII

CONCLUSIONS

The overall objective of this work was to investigate factors that contribute to insecticide resistance in honey bees, particularly biochemical and physiological aspects. I will summarize my results in this concluding chapter.

Expanding earlier work by H.S. Tahori *et al*. (1969), I was able to establish relationships, in the form of linear regression models, between levels of mixed-function oxidase and glutathione transferase enzymes and colony levels of insecticide resistance. These results provide information on the mechanisms behind variation in resistance levels in honey bee colonies, and are applicable to populations of other insects as well. To my knowledge, this is the first demonstration of the relationship between resistance and enzyme activity in previously unselected field populations.

These findings may benefit research into the genetic manipulation of other beneficial insects, particularly biological control agents such as hymenopterous parasitoids. Attempts have been made to select resistant field populations of such insects (Rosenheim and Hoy 1986), and to increase these resistance levels through selective breeding programs. However, the determination of "resistance" is often subjective, and requires large numbers of test individuals for bioassay purposes. It may be possible, with some modification of the techniques used in my studies, to assess populations suitable for genetic manipulation using enzyme assays. These procedures would require fewer test individuals, and would provide an

accurate and objective assessment of the potential for resistance development.

Having established that resistance levels are associated with the activity of detoxifying enzymes, I then wanted to assess factors that could influence enzyme activity. I chose to study the effect of age because of the influence of temporal division of labour on worker bee behaviour and physiology. My finding that honey bees are able to compensate for the loss of midgut protein by increasing the specific activity of detoxifying enzymes suggests metabolic regulation of this protein loss, and represents a possible adaptation of these insects for foraging in areas of environmental contamination. This interpretation is reinforced by my subsequent experiments demonstrating that adaptive changes in enzyme activity are related to worker behaviour and not merely to the process of aging.

It could be argued that foraging workers carrying toxic material are dangerous to the colony, and that it is better for colony survival to have them die in the field. However, I would argue that some tolerance to toxins in the foraging force is essential for adequate resource exploitation, and increasing the activity of "defensive" enzymes allows foragers to continue to work in areas where food sources are rich, but some level of contamination is present. Also, the detoxication capacity *per bee* is highest in the hive bees that must process incoming nectar and pollen (Figs. 9a and 10a). Thus, the colony maintains

its defence against poisoning while being able to exploit food resources efficiently.

In the context of selection for resistant strains of bees, my results indicate that some thought must be given to how and when samples should be taken from breeding colonies. It is important to take a large enough sample to include a random mix of ages, and to take this from the same location within the hive each time. The top super of a two- or three-super colony would be best, as the age distribution would be less likely to be skewed towards old or young workers.

Chapter VI examined the effects of colony environment, as reflected by worker population, on the activity of detoxifying enzyme systems. This study indicates that enzyme activity levels have an environmental component that may be related to functions such as brood rearing. Activity levels therefore reflect a plasticity of response that is beneficial for adapting to changing environmental conditions. This type of plasticity in behavioural responses, and its evolutionary significance, has also been described by Crozier and Page (1984), Frumhoff and Baker (1988), Kolmes et al. (1988), and Robinson and Page (1988).

However, the very fact that this plasticity exists makes it difficult to predict the outcome of selection for increased detoxication. The variation described in Chapter VI indicates that these enzyme levels are influenced by many factors, and

their heritability may be too low to be useful for selection for pesticide resistance. In addition, it is almost certain that resistance of this type is based on polygenic inheritance (Tahori *et al.*, 1969; Malaspina and Stort, 1983). The contribution of detoxifying enzymes will therefore only be a part of the resistance development in honey bee populations.

It is unclear at this time whether polygenic resistance in beneficial insects like the honey bee has any practical benefits in the field. My feeling is that any increase in resistance that will enable bees to function better in areas of environmental contamination are worth selecting for, providing they don't come at the expense of other desirable traits. Until actual selection experiments are done and the progeny assessed, pesticide-resistant honey bees will remain an attractive but untested component in the successful integration of insect pollination and chemical pest control.

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