THE EFFECTS OF MALATHION ON THE BEHAVIOR AND CHOLINESTERASE ENZYMES OF THE HONEY BEE (*Apis mellifera* L.)

A Thesis

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Denis John Dyer

In Partial Fulfillment of the

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ABSTRACT

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The Effects of Malathion on the Behavior and Cholinesterase Enzymes of the Honey Bee (Apis mellifera L.).

Honey bee samples were initially assayed to determine if cholinesterase enzymes could be measured in a single bee. A sample of bees was assayed with respect to individual body region cholinesterase. Mean values for body region enzyme activity showed that the head had higher measurable cholinesterase enzymes than did the thorax and abdomen. Honey bee heads were then measured for cholinesterase to determine if there was a variation with the age of the bee. Head cholinesterase values for honey bees of four age groups were shown to be statistically of the same population. Attempts to reduce the variation in enzyme levels were made using total head protein and bee brains. However, the results showed that the cholinesterase levels in terms of mU/ml provided the smaller variation.

Preliminary poisoning experiments were conducted using a malathion 50% EC-water mixture applied to the bee between the head and thorax. Cholinesterase levels of treated bees were found to be significantly lower than levels found in untreated bees.

Honey bee head cholinesterase appeared to be affected by the time of year the bee emerged from its cell. It was shown that bees emerging in November had head cholinesterase levels similar to those of summer bees. However, it was observed that these head enzyme levels dropped considerably by the time the bee was 14 days old.

An aerial spray program in Bird's Hill Park (using malathion) in May 1977 was used to test the efficacy of honey bees as environmental

impact monitoring organisms. Honey bees were sampled for malathion residue and cholinesterase. Bees were also released at two distances from hives to ascertain the effects of the poison on the bees' homing abilities. Sampling problems and the possible influence of "seasonal" changes in bee cholinesterase limited any conclusions on this topic, but it was interesting to note that no bees returned to their hives in the treatment areas.

Further tests were conducted to determine the effects of sublethal levels of malathion on the homing abilities of bees. These experiments involved bees of foraging age and the results showed that fewer bees returned to their hive when treated with malathion in sublethal levels.

The behaviors of individual poisoned bees was recorded using glass-walled observation hives. It was found that bees treated with malathion showed five basic stages of poisoning. Bees poisoned with high levels of malathion usually died quickly; however the effects of the poison was seen in a hundredfold mortality in the hive over 24 hours, probably due to contact with the dead bee by other bees. The activity of a poisoned bee was shown to be quite different from its regular hive activities, however "normal" hive behavior returned in sublethally treated bees within one day.

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CHAPTER 1

1.1 Introduction

The widespread aerial applications of pesticides used presently to control insect pest infestations have often destroyed honey bee colonies (Johansen 1977). The problem of finding the means to define the damage to the colonies by such sprays has always been difficult, partly due to a good deal of "after the fact" sampling. The goal of this thesis was to explore different avenues of evaluating the impact of aerial sprays on individual honey bees using bee behavior and cholinesterase inhibition as measuring tools.

Organophosphate pesticides have been used extensively for insect control since the decline in popularity of the organochlorines. Malathion was chosen to be used in this thesis because of its consumer availability and its reputation as one of the least toxic organophosphates on the market. Additionally, it has been generally assumed that malathion is relatively non-toxic to honey bees, so any results gathered from this examination could form the basis for future projections with other pesticides.

Cholinesterase has been deemed the target enzyme for the toxicological effects of organophosphates in insects (O'Brien 1976). This thesis intended to examine the merits of using honey bee cholinesterase as a means of descriminating poisoned from normal bees. The honey bee cholinergic systems was examined initially to determine cholinesterase levels in untreated bees. Subsequently, the cholinesterase levels of malathion-treated bees were measured to observe whether poisoning significantly affects enzyme levels in the bee.

Honey bee colonies were used to monitor the effects of large-scale aerial sprays on non-target organisms. In theory, a honey bee colony should be ideal for this type of examination, due to its compact moveable nature and the homing instinct of its population. Honey bees have been also examined for pesticide-induced influences on their navigational ability. Proper technique development in this direction could evolve into using the hive as an efficient monitoring unit.

Pesticides undoubtedly have exerted some behavioral influences on bees returning to the hive. Honey bees were examined for any pesticide-induced activity changes, both in duties and in relation to next mates. The reaction of the hive bees to the poisoned specimen have been noted to determine the amount and kind of interactions between them; this would show the effects of malathion on the non-target bees involved.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Insect Head Cholinesterase and Organophosphate Poisoning

Cholinesterase analyses of honey bee samples were made on individual bee heads. Comparable analyses of single insect specimens are not available in the literature. All previous work reports measurements on pooled specimens using either the whole organism or parts thereof (Metcalf and March, 1950; Lewis and Fowler, 1956; Mengle and Casida, 1958; Smallman and Fisher, 1958; Stegwee, 1960; Mengle and O'Brien, 1960; Plapp and Bigly, 1961; Brady and Sternburg, 1966; Tripathi and O'Brien, 1973). There are further examples in which insect thoraces (Lewis and Fowler, 1956; Smallman and Fisher, 1958; Stegwee, 1960; Brady and Sternburg, 1966; Tripathi and O'Brien, 1973), whole bodies (van Asperen, 1958; O'Brien, 1961), or combinations of thoraces and abdomens were used (Plapp and Bigly, 1961). Although most research was conducted with insects other than the honey bee (usually the house fly, Musca domestica), the literature indicates that the enzyme is concentrated in the head.

Questions arise in the literature as to whether the insect head is the major poisoning site with organophosphate insecticides. Van Asperen (1958) suggests that low levels of cholinesterase inhibition detected in house fly samples treated with organophosphates may be a misrepresentation of the poisoning sequence, with stronger inhibition of the enzyme occurring at some small but essential part of the nervous system. Stegwee (1960) proposed that house fly cholinesterase may be more inhibited at critical sites in the nervous system than in the head. Plapp and Bigly

(1961) and O'Brien (1961) provide further evidence of "the remarkable recent finding that decapitation has little effect on the LD_{50} for house flies of eight anticholinesterases (Mengle and Casida, 1958) makes it improbable that inhibition of the cholinesterase of the head is important in poisoning". Zettler and Brady (1970) explain their inability to determine the level of cholinesterase inhibition producing death in the red flour beetle as being due to localized inhibition of the enzyme in the peripheral region of the nervous system. Booth and Metcalf (1970) tentatively conclude that they could not histochemically determine any correlation between external poisoning symptoms and overall inhibition of cholinesterase in the brain. They deduce that the peripheral areas of the thoracic ganglion of the CNS (central nervous system) appear to be one of the localized sites of inhibition in the house fly, concluding that the brain is a secondary site of inhibition. Booth and Lee (1971) also find organophosphate-treated house flies experiencing cholinesterase inhibition in the peripheral areas of the thoracic ganglion at knockdown, with no inhibition of brain cholinesterase. It is interesting to note that Booth and Lee (1971) report an eventual complete inhibition of cholinesterase in the central portion of the fly brain with time; however, they also note that crickets poisoned by organophosphates always demonstrate complete inhibition of brain cholinesterase at knockdown. Tripathi and O'Brien (1973) summed it up by stating that (with house flies) "the inhibition of thoracic cholinesterase is far better correlated with the degree of poisoning than is the inhibition of the head cholinesterase". The literature does question the correlation of brain cholinesterase depression with the degree of organophosphate poisoning

in insects other than honey bees.

2.2 Mechanism of Insect Cholinesterase Inhibition

Corbett (1974) reported that cholinesterase (EC 3.1.1.7) is the enzyme responsible for the hydrolysis of acetylcholine in insect nervous systems. A cholinesterase, by definition, is a substance which hydrolyzes acetylcholine into acetate and choline. The reaction is as follows:

$$\underset{\text{acetylcholine}}{CH_3COOCH_2CH_2\overset{+}{N}(CH_3)_3} \xrightarrow{H_2O} \underset{\text{acetate}}{CH_3COO^-} + \underset{\text{choline}}{HOCH_2CH_2\overset{+}{N}(CH_3)_3}$$

Hellenbrand and Krupka (1970) published a report on the mechanism of the hydrolysis of acetycholine by acetylcholinesterase in the housefly, presenting it in the following manner:

$$EH + AcCH \underset{k-1}{\overset{k+1}{\rightleftharpoons}} EH.AcCH \xrightarrow[ChH]{k_2} EAc \xrightarrow[H_2O]{k_3} EH + AcOH$$

where EH is the enzyme, AcCH is the acetylcholine, EH.AcCH is the intermediate reversible complex between enzyme and substrate, EAc is the acetylated enzyme, ChH is choline and AcOH is acetic acid. In the

house fly, the acetylation step (k_2) is the rate determining step.

It is likely that the insect cholinesterase contains two binding sites (Corbett, 1974) as does the vertebrate enzyme (Engelhard et al., 1967), and therefore it should act in a similar manner. Of the two reaction sites, the anionic site (possibly containing a glutamate residue) interacts with the positively charged nitrogen atom of acetylcholine, while the esteratic site (the nucleophilicity of which is enhanced by hydrogen bonding to a neighbouring histidine residue) is responsible for the cleavage of the ester link of acetylcholine (Corbett, 1974). Hellenbrand and Krupka (1970) noted that substrate inhibition in insects differs mechanically from that of vertebrates. They suggested that it involved combination of the substrate with a postulated second anionic site, and blockage of the acetylation (k_2) rather than the deacetylation (k_3) reaction. Organophosphates form a complex with the enzyme at the binding sites and phosphorylate it. The subsequent dephosphorylation reaction, which controls the overall reaction rate, can take a long time (even months) to complete.

The formation of the enzyme-inhibitor complex remained undemonstrated for many years due to the relatively small amounts of the inhibition actually needed for death to occur. According to O'Brien (1976), most of the effective anticholinesterases, when present in concentrations of 10^{-6} M, will satisfactorily inhibit much of the enzyme in the order of 5-50 minutes. Further to this, the recovery of the enzyme is sometimes complicated by the phenomenon of aging, defined as a progressive conversion of the inhibited enzyme to a form which cannot be reactivated by oximes (substances known to reverse the trend towards the formation

of an enzyme-inhibitor complex). O'Brien mentions that the basis of the aging reaction is known to be a dealkylation reaction of the form

$$EOP(O)\ (OR)_2 \longrightarrow EOP(O)\begin{matrix} OR \\ O^- \end{matrix}$$

where E represents the cholinesterase enzyme. This results in the creation of an anionic phosphate group which is insensitive to nucleophilic attack. The rate of aging would invariably depend on the ability of the alkyl substituent to act as an effective "leaving" group. However, the overall result of aging would be to insure that the toxification is irreversible by closing off routes of detoxification taken by oximes or by normal physiological hydrolysis.

2.3 Correlation of Poisoning with Cholinesterase Level Depression

The search for correlations between the amount of pesticide present and the degree of insect cholinesterase inhibition has been inconclusive (Brady and Sternburg, 1967; Zettler and Brady, 1970; O'Brien, 1961; Stegwee, 1960; Mengle and Casida, 1958; Booth and Lee, 1971). Some authors suggest broad correlations between sublethal, LD_{50}, or massive doses of organophosphates and no, partial, or complete inhibition respectively, of the insect cholinesterase (Plapp and Bigly, 1961); however, no data are available correlating pesticide dosage with degree of cholinesterase inhibition. Recent histochemical investigations (Booth and Metcalf, 1970) suggest possible reasons for this dilemma, and these findings will be discussed in relation to some of the old problems.

The standard method of insect cholinesterase analysis is to homogenize whole bodies (or parts thereof), and then to perform measurements on this homogenate (Lewis and Fowler, 1956; van Asperen, 1958; Stegwee, 1960; Brady and Sternburg, 1966; Zettler and Brady, 1970). This form of preparation has inherent problems, one of which would be the inability to detect localized areas of enzyme inhibition. The lack of success in correlating concentrations of organophosphates with degree of insect cholinesterase inhibition has been suggested to be caused by such localized sites of inhibition (Stegwee, 1960; Zettler and Brady, 1970). Histochemical investigation by Booth and Metcalf (1970) has shown this to be the case. Histochemical sections of poisoned house flies gave evidence that the peripheral areas of the thoracic ganglion appear to be an area of localized cholinesterase inhibition. Further to this, Booth and Metcalf found that house fly brain cholinesterase did not play a primary role in organophosphate inhibition, with sections showing the thoracic ganglion to be the main site of attack by such toxicants. Specifically, the region of the non-synaptic perineurium undergoes peripheral inhibition, producing a state which Booth and Metcalf suggest may be responsible for the initial symptoms of poisoning. They postulate that the perineurium, which is involved with active ionic regulation between the nervous system and the blood, is possibly affected in such a way that an upset may occur in the balance of ions necessary for normal nervous activity. Whatever the reason, it is not surprising that the thoracic ganglion, which serves the locomotory apparatus, should be a site of localized inhibition since the initial physical signs of poisoning are related to an upset in locomotory control This,

combined with the secondary importance of brain cholinesterase inhibition in the house fly, could well offer an explanation for the inability to correlate cholinesterase inhibition with concentration of pesticide in much of the previous research on this subject.

It is important to note, however, that the significance of insect brain cholinesterase as a target for organophosphate insecticides varies from species to species. Brain cholinesterase depression may be relatively unimportant in house fly intoxication (Booth and Metcalf, 1970) and yet, be of paramount importance in cricket poisoning (Booth and Lee, 1971). Very little emphasis has been placed on honey bee cholinesterase analysis and details of the intricate poisoning process are not available. An object of this thesis is to determine whether significant depressions of honey bee head cholinesterase could serve as a tool to discriminate poisoned from non-poisoned honey bees.

CHAPTER 3

HONEY BEE CHOLINESTERASE

3.1 Introduction

The approach to investigate the cholinesterase enzymes in the honey bee followed three basic steps: (i) to determine the best area for measurement of this enzyme in the bee and to measure any variation in enzyme levels from bee to bee (ii) to ascertain whether enzyme level variations, if present, could be reduced by dissection of the relevant tissue or by mathematical relation to the amount of protein present, and (iii) to observe whether malathion poisoning could be reflected in depressions in cholinesterase levels. The methods and results of this approach will be described in the following sections.

3.2 Methods

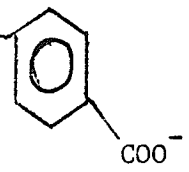
3.2.1 Cholinesterase Analysis

The Ellman method (Ellman et al., 1961) was selected for its ease and simplicity for honey bee cholinesterase analysis. The Ellman method is a photometric method for determining acetylcholinesterase activity based on the absorbance at 405 nm, when thiocholine and dithiobisnitrobenzoate are mixed in solution. The reagents are commercially available from Boehringer Mannheim Corporation and they consist of reagent 1, a mixture of 50mM phosphate buffer (pH 7.2) and 0.25mM dithiobisnitro benzene acid, and reagent 2, 0.156M acetylthiocholine iodide. The colorimetric reaction is produced by pipetting 300µl of reagent 1 into a cuvette, followed by the addition of 10µl of samples containing

cholinesterase enzymes and 10µl of reagent 2 in consecutive order. The rate limiting step is hydrolysis of acetylthiocholine to thiocholine and acetate catalyzed by cholinesterase. The Ellman method requires measurement of the rate of thiocholine production. According to Ellman, this is accomplished by the continuous reaction of the thiol with 5,5'-dithiobis-2-nitrobenzoate ion as follows:

$$H_2O + (CH_3)_3\overset{+}{N}CH_2CH_2SCOCH_3 \xrightarrow{\text{cholinesterase}} (CH_3)_3\overset{+}{N}CH_2CH_2S^- + CH_3COO^- + 2H^+$$

$$(CH_3)_3\overset{+}{N}CH_2CH_2S^- + RSSR \qquad ((CH_3)_3\overset{+}{N}CH_2CH_2SSR + R\bar{S}$$

where R = 

The rate of production of 5-thio-2-nitro-benzoic acid (demonstrated visibly as the rate of color change to yellow) by the reaction of thiocholine and dithiobisnitrobenzoate has been shown to be sufficiently rapid so as not to be rate limiting in the measurement of the cholinesterase, and the concentrations involved do not inhibit enzymic hydrolysis (Ellman et al., 1961).

The procedure for cholinesterase analysis was as follows: immediately upon sampling, the honey bees were frozen on dry ice until return to the laboratory where they were transferred to a deep freeze until assayed. Bees to be analyzed were removed from cold storage and decapitated; each head was then placed in 1.0 millilitres of a 0.25M sucrose solution. These heads were homogenized individually at full speed with a Polytron tissue homogenizer for a period of 15 seconds.

The homogenized samples were allowed to stand for 2 hours at 4°C and then centrifuged at 7000xg for 10 minutes. Subsequently, 150µl of the supernatant was removed and analyzed for "soluble" cholinesterase.

Assays were conducted in duplicate on a Zeiss PMQ II spectrophotometer using prepared reagents from Boehringer Mannheim Corporation based upon the procedure of Ellman et al. (1961). Measurements were made using 10.0µl of sample and amounts of reagents 1 and 2 indicated previously, at 25°C. Soluble bee head cholinesterase values (expressed in milliUnits/ml) were obtained from recorder charts printing the changes in optical density at 405 nm as a function of time (with a correction for non-enzymatic substrate hydrolysis). The 10.0µl sample volume chosen was sufficient to yield activities consistently in the range measured with reagents and equipment used. The extraction time of 2 hours, plus the extraction temperature (4°C), and the use of 0.25M sucrose as a solvent are taken from a procedure used by Nelson et al. (1973) for cholinesterase analysis of honey bee drones and workers.

3.2.2 Protein Analysis

The honey bee cholinesterase activity was also expressed in terms of milli Units/mg bee head protein: protein in bee head extracts was measured by the Lowry method (Lowry et al., 1951) as modified by Miller (1959) for use with large numbers. The analytical procedure involved the addition of the Folin-Ciocalteau phenol reagent to the copper-treated protein sample, producing a colour change from yellow to blue. As the accompanying reaction neared completion the protein composition was determined through comparison of solution absorbance values (in nanometers) with values for a previously-established protein standard curve.

Several reagents were used in the protein assay. The first reagent, Reagent A, consisted of a mixture of 2% sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH) in proportions indicated by Lowry (1951). To 50 ml of Reagent A was added 1.0 ml of Reagent B (0.5% cupric sulphate $CuSO_4 \cdot 5H_2O$ in 1% sodium or potassium tartrate), to produce Lowry Protein Reagent C. To 500µl of Reagent C (mixed within one hour of analysis) was added 10µl of honey bee head extract (prepared in the manner described for cholinesterase analysis - 3.1). Ten minutes later, 50µl of the Folin phenol reagent, diluted 1:3 with water (Miller, 1959), was added to the copper-protein solution. With the elapse of another ten-minute period, the solution was measured for absorbance at 600 nm with a Zeiss PMQ II spectrophotometer.

The prescribed Lowry protein assay was modified briefly for the honey bee analyses. The suggested 30-minute reaction time for a 30µl sample was reduced to 10 minutes using a 10µl sample. A plot of reaction rate versus time showed the reaction for the smaller sample and time period to be 91.4% complete, using the larger sample and longer time period as the 100% standard.

3.2.3 Marking Procedure

The necessity for identifying different age groups of honey bees demanded a reliable means of marking the bees. To fulfill this need, frames of emerging brood were taken from the hive, swept clean of adult bees and incubated at 30°C (± 2°C) with 30-60% relative humidity for a period of 18-24 hours. The frames with newly emerged bees were taken from the incubator, and the bees were marked on the dorsal thorax with model airplane dope (paint). The dope was delivered by a 1-3 cc twenty-

two and one-half guage needle syringe, with the needle point filed square to facilitate application. Care was taken to prevent the dope from seeping into the wing bases and membranous areas of the neck, or from covering portions of the wings and the head. The marked bees were introduced into the hive using smoke to disguise the paint odour, thus enhancing acceptance. The ages of the marked bees were determined by using different colors for different marking dates, and by referring to this color code when a particular age was needed.

3.2.4 Pesticide Dilution Procedure

The malathion dilution procedure was based on the need for a control which would cause minimal short-term disturbances in honey bee behavior when applied to the body. Consequently, water was chosen as the control. Malathion, being only slightly miscible in water, did form an even emulsion with water that could be applied via syringe. The final concentration of malathion delivered was determined by the volume of water mised with the malathion 50% EC stock solution. A 2.0µl micropipette was used to deliver either the malathion-water emulsion or the water control to the test honey bees.

3.3 Results and Discussion

3.3.1 Honey Bee Cholinesterase Distribution

Cholinesterase distribution in the honey bee was determined from assays conducted using one day old bees sampled 8 November, 1977. Enzyme assays were made on the three general body regions; the head, thorax and abdomen. Measurements of these three regions should produce a composite cholinesterase value very close to the total enzyme level

of the honey bee in question.

The experimental results demonstrate that the head contains more than half the measurable soluble honey bee cholinesterase. An unpaired "t" test showed that there was a significant difference between the mean cholinesterase values for the head and thorax ($t = 15.9$, $P<0.001$) and for the head and abdomen ($t = 17.6$, $P<0.001$) as well as a significant difference between thorax and abdomen mean values ($t = 4.8$, $P<0.001$), as shown in Table 3.1.

The data for honey bee body region cholinesterase may have been influenced by a seasonal variation in the enzyme distribution; the time of year does seem to affect the amount of measurable soluble cholinesterase in the bee. However, it should be noted that this effect exerts its greatest influence sometime after the bee is one day old (see Appendix H). Previous preliminary work on honey bees of various ages sampled during June did produce levels similar to the distribution of cholinesterase in these one day old bees.

3.3.2 Honey Bee Brain Cholinesterase

Since the head contains the highest activity of honey bee cholinesterase, it seemed logical to examine the enzyme content of isolated bee brains. Honey bees were taken from cold storage and dissected under a binocular microscope. Dissections were completed within 90 seconds at room temperature. Bee brain samples were placed in small test tubes sealed with Parafilm and then transferred to a deep freeze until assay. Samples to be measured for cholinesterase were transferred to 6 x 50 mm test tubes and homogenized in 150µl of a 0.25M sucrose solution by a ground glass rod tissue grinder, under cold room conditions ($4^{\circ}C$). The

Table 3.1 Body region cholinesterase distribution in one day old honey bees sampled 8 November, 1977

	Soluble cholinesterase (mU/ml)		
Sample	Head	Thorax	Abdomen
1	56.08	18.34	9.99
2	57.12	19.69	15.15
3	52.29	20.03	14.40
4	56.08	19.58	14.83
5	58.18	17.52	11.86
6	66.96	15.04	15.48
7	60.14	19.35	11.03
8	44.39	20.61	16.68
Mean	56.4	18.8	13.7

Table 3.2 Mean brain cholinesterase values of honey bee samples taken during 1975

Sample	Sample number	Mean soluble brain cholinesterase (mU/ml)
May 27-29	30	60.1
June 16-18	30	102.5

homogenate was allowed to stand for two hours and was then centrifuged for 5 minutes at 7000xg. Soluble brain cholinesterase was measured in duplicate as described in Section 3.2.1.

Bee brain cholinesterase assays were conducted on bee samples obtained between 27 May and 18 June, 1975. The results listed in Appendix A were grouped according to month, and tested using an unpaired "t" test. These groups, as shown in Table 3.2, were observed to be separate populations ($t = 5.2$, $P<0.01$), an indication of the error involved in the elaborate brain preparation technique, or of a seasonally-related change in bee brain cholinesterase levels. This question could be further clarified if the ages of the bees were known. However, the wide variation in brain cholinesterase levels led to the investigation of the whole head as a more rapid means of determining honey bee cholinesterase levels, with more reproducible results. Hence, further research on brain cholinesterase was discontinued in favour of whole head enzyme assay.

3.3.3 Method Development for Cholinesterase Analysis

Two areas of the honey bee cholinesterase measurement technique were examined more closely to determine the degree to which they contributed to technique error.

The variation of cholinesterase measurement values within a particular sample may serve as an indication of the reproducibility of the analytical procedure. With reference to this, six replicates were taken for cholinesterase assay from a homogenized one day old bee head sample on 8 November, 1977. These replicates showed a mean of 51.8 (mU/ml) with a range of 49.3-54.5 as shown in Table 3.3. The standard

Table 3.3 Cholinesterase assay replicates measured using the head of a one day old honey bee

Replicate	Soluble head cholinesterase
1	51.3
2	54.5
3	52.8
4	49.3
5	51.3
6	51.7
Mean	51.8
Standard deviation	1.76

deviation of 1.76 gives a coefficient of variance of only 3%. This value includes pipetting errors and judgement errors from lines drawn on readout charts, as well as errors arising from actual replicate variation and mechanical fault.

The stability of the honey bee cholinesterase enzyme under cold storage required closer examination. Previous work on cholinesterase levels in pigeons allowed to decay at room temperature for 12 days (Bunyan et al., 1968) showed very little difference in brain enzyme values when compared to control specimens. Dead honey bees sampled in front of the hives as reported by Lockhart et al. (1978) generally did not demonstrate significant decreases in cholinesterase levels from healthy bee values. To examine this question, a sample of 14 day old honey bees was frozen on 2 August, 1977 and held in storage for a period of 48 days. The results of this storage test produced a mean value of 65.8 m U/ml with a range of 30.2-88.2 m U/ml (Appendix F). This was later compared with the 67.8 m U/ml value for a population cross-section of 177 bees in Section 3.3.4 and was not found to differ significantly ($t = 0.487$, $P>0.5$). Consequently, cold storage appeared to be a good means of storing honey bee samples.

3.3.4 Honey Bee Cholinesterase Variation with Age

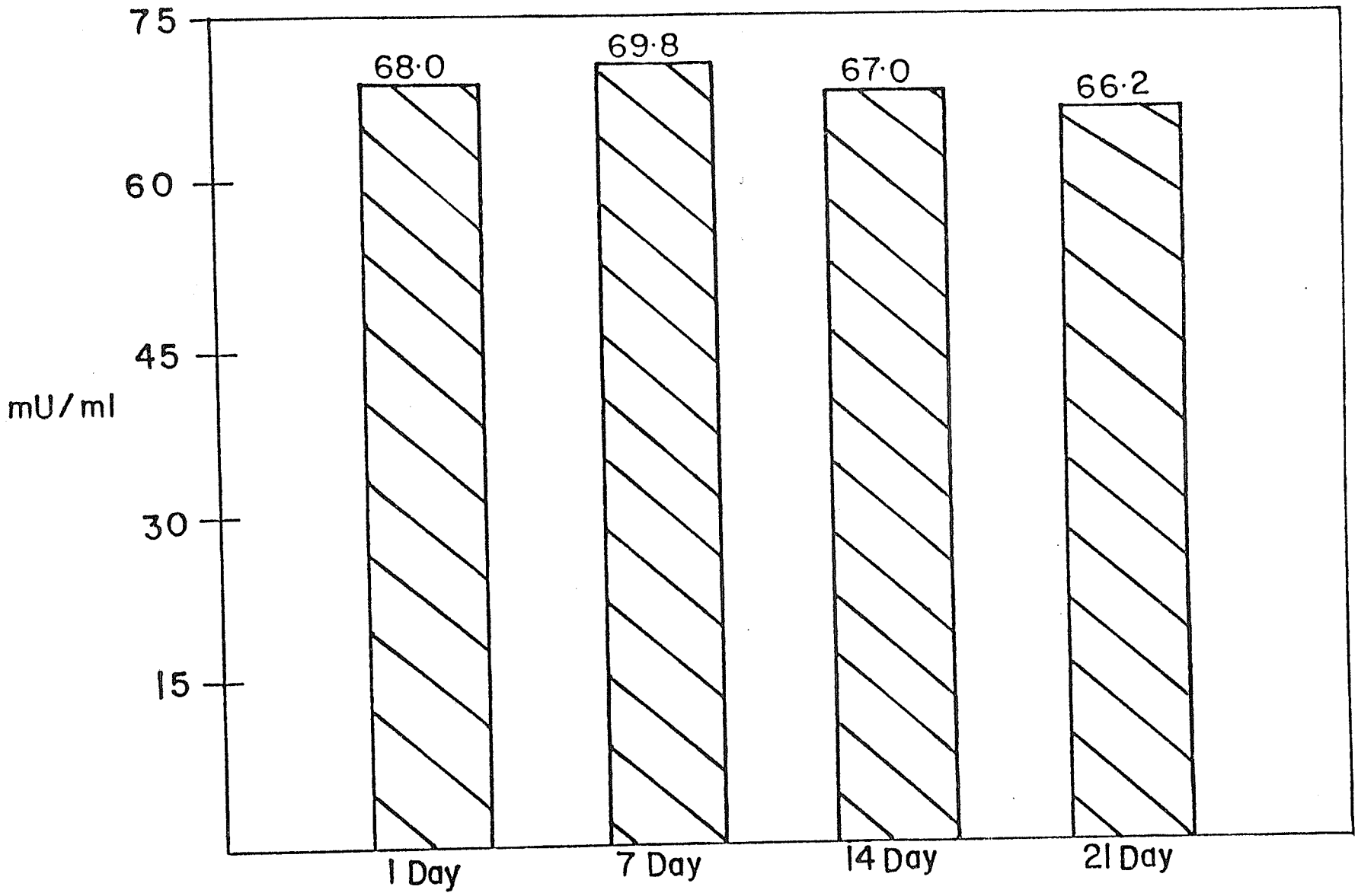
Senescence, with its corresponding physiological changes, might be a potential source of variation in the overall assessment of honey bee cholinesterase levels. Rockstein (1950) reported such a variation in the honey bee, although he found this change to be independent of the general degeneration of brain and nervous tissue which occurs with age. He reported a significant increase in the cholinesterase activity of

the brain during the first week after emergence, followed by a levelling off which remained undiminished into extreme old age. Since Rockstein's methods employed the pooling of honey bee brains and the use of a different analytical procedure (Warburg), it was necessary to establish a set of cholinesterase values based on individual bee head samples with the Ellman (1961) method for the purposes of this thesis.

Honey bees, taken from queen-right colonies maintained at the University of Manitoba apiary, were marked upon emergence (see Section 3.2.3) to establish an age sequence of worker bees. Between 23 June and 20 July, 1976, bees of four age groups (1 day, 7 days, 14 days, 21 days) were randomly sampled for analysis. These bees were frozen and assayed for cholinesterase as recorded in Section 3.2.1.

The study on cholinesterase variation with age involved 179 individual honey bees from the four age groups, with a minimum number of 36 samples in each group. The mean values for the 1 day, 7 day, 14 day and 21 day groupings were 68.0, 69.8, 67.0 and 66.2 - mU/ml soluble bee head cholinesterase, respectively (see Appendices B, C, D, E and Figure 3.1). The four age groups were shown statistically to belong to the same population ($P>0.05$), using an unpaired "t" test. The significant initial rise in brain enzyme levels fro bees under 7 days reported by Rockstein (1950) was not evident; this was perhaps due to the different assay technique used and the measurement of individual versus group samples. Values for the cholinesterase levels of the four age groups differed from the overall mean value of 67.8 mU.ml (standard deviation 9.45) by no more than 2.9% in the greatest instance.

Figure 3.1. Honey bee cholinesterase activity measured with honey bees of four age groups sampled between 23 June and 20 July, 1976.


75
60
45
30
15
mU/ml
68·0
69·8
67·0
66·2
1 Day
7 Day
14 Day
21 Day

3.3.5 Preliminary Poisoning Experiments

The next step after establishing mean bee head cholinesterase values was to determine whether this particular analytical method would be sensitive enough to detect organophosphate-influenced changes in the enzyme levels. The objective was to detect large fluctuations in cholinesterase levels, rather than small increments of elevation and depression.

The literature abounds with examples of the inability to correlate the amount of organophosphate applied with a corresponding cholinesterase level (eg. Zettler and Brady, 1970; Booth and Metcalf, 1970; Brady and Sternburg, 1967; O'Brien, 1961). However, it is true that the depression of these enzyme levels is an indication of organophosphate poisoning (O'Brien, 1967). Consequently, the following tests were done to determine if large levels of cholinesterase depression could be measured using the analytical techniques in Section 3.2.1.

A sample of 21 day old honey bees, taken from colonies maintained at the University of Manitoba apiary, were treated with malathion 50% EC in varying concentrations on 28 July, 1976. The malathion was diluted with water to ratios of 1:1, 1:5, 1:50, 1:500 (see Section 3.2.4), and applied at the rate of 2.0 µl per bee to the intersegmental area between head and thorax. The bees were frozen 30 minutes after treatment and held in cold storage until assayed.

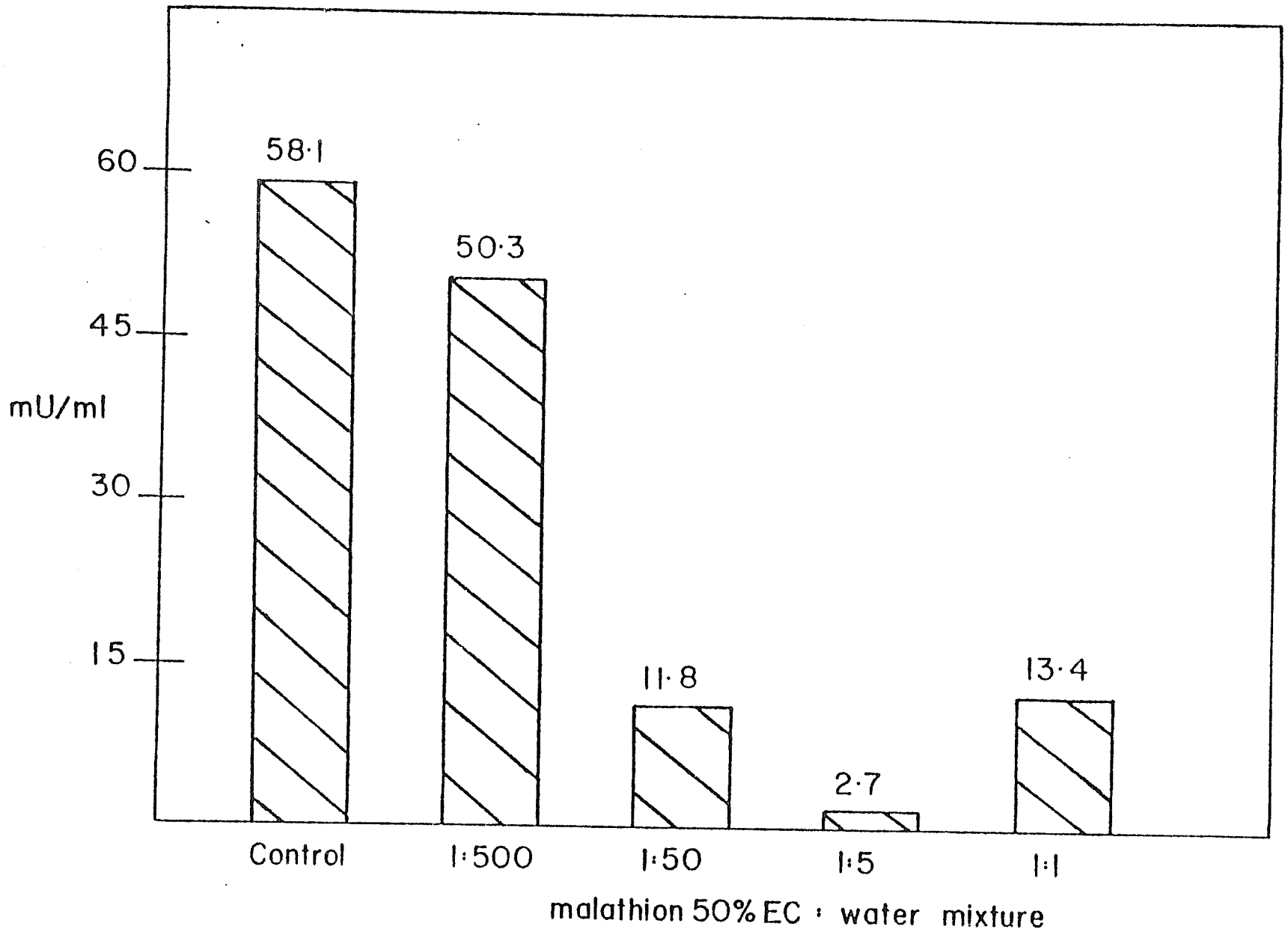
The results showed that there was a very obvious difference in mean cholinesterase values between the untreated bees and three of the treated groups (see Appendix G). Bees treated with malathion-water mixtures of 1:1, 1:5 and 1:50 showed mean enzyme levels 20.3%, 4.6% and 23.1%

respectively, of the mean enzyme levels for untreated bees. In addition, these three treatment groups were shown statistically (using an unpaired "t" test) to belong to a population different from the control group ($P<0.001$). The 1:500 malathion-water treatment group was not shown to be statistically different from the control group ($P>0.05$), although the former showed a decrease in the mean cholinesterase level. Both the control and 1:50 malathion-water groups were, however, shown to be statistically different from the 67.8 m U/ml meal value for honey bees $\geq$ 21 days old stated in Section 3.3.4. This difference may be due, in part, to a seasonal variation described further in Section 3.11. Overall, the experiment does show that large organophosphate-induced depressions in honey bee head cholinesterase can be detected using the method of analysis recorded in Section 3.2.1.

Although a dose-effect relationship between the concentration of pesticide and a corresponding degree of cholinesterase inhibition has not been determined, it is generally accepted that organophosphates act to produce a sudden decline in enzyme levels. The results of this experiment indicate that this sudden decline takes place somewhere between the dosages of 1:500 and 1:50 malathion-water mixture, applied intersegmentally between head and thorax (see Figure 3.2).

Van Asperen (1958) argued for "protection" techniques when homogenizing organophosphate-treated insects for cholinesterase analysis. The protection technique consisted of homogenizing the insect (in this case, the house fly) in an acetylcholine solution to prevent free organophosphate present in the tissues from introducing an analytical error by binding with the free cholinesterase. The procedure followed in this

Figure 3.2. Mean soluble honey bee head cholinesterase values measured in bees treated with 2.0 µl of a malathion 50% EC:water dilution, in proportions indicated.


60
45
30
15
mU/ml
58·1
50·3
11·8
2·7
13·4
Control
1:500
1:50
1:5
1:1
malathion 50% EC : water mixture

thesis did not involve the use of any protection technique because the concern was not to relate increments of cholinesterase depression to concentrations of insecticide applied. The question in this thesis is whether an acutely poisoned bee can be detected using cholinesterase levels as a measure. Enzyme values were used merely to decide the presence or absence of poisoning by organophosphate insecticides.

3.3.6 Honey Bee Head Protein Analysis

The original purpose for the analysis of honey bee head protein was statistical in nature. The intention was to provide an additional parameter of reference for the enzyme assay, such that the cholinesterase would be measured in terms of milliUnits (of activity) per milligram of bee head protein. It was thought that a reference point dependent on the internal tissues of the head would prove useful in reducing the standard deviation of individual cholinesterase values from mean levels.

Calculated values taken from protein and cholinesterase analyses show that protein measurements do not statistically improve the standard deviations of the honey bee head cholinesterase levels. More than 170 honey bees of four different age groups (1 day, 7 days, 14 days and 21 days old) were measured for cholinesterase and protein levels further to the tests stated in Section 3.3.4. Table 3.4 illustrates that the coefficient of variance (a percentage assessment of standard deviation) for the cholinesterase levels (13.9%) is just over half the 26.9% figure for protein levels. Dividing the individual cholinesterases by the protein counterparts to translate in terms of "mU/mg" results in an even higher coefficient of variance (32.9%). Further research conducted

Table 3.4 A comparison of values of honey bee head protein levels taken from bees sampled during June - July, 1976

	Sample size	$\bar{X}$	Standard deviation	Coefficient of variance
Cholinesterase	179	67.84 mU/ml	9.45	13.9%
Protein	177	0.847 mg/ml	0.228	26.9%
Cholinesterase ÷ protein	175	175 mU/mg	28.2	32.9%

on the protein assay itself (Appendix I), shows that the protein sample variation for one bee is over twice that of the cholinesterase sample variation for that same bee, even though both assays were taken from the one preparation (see Sections 3.2.1 and 3.2.2). The large standard deviations are likely related to hypopharyngeal and mandibular glands contained in the head, which may be in various stages of development of disuse from one bee to the next. Clearly this would indicate that the measurement of protein levels, in conjunction with bee head cholinesterase, produces a greater standard deviation from the mean than the cholinesterase levels alone, and that the expression of enzyme activity on a milliUnits (of activity) per bee head basis would be a less variable alternative.

3.3.7 Seasonal Changes in Cholinesterase Levels

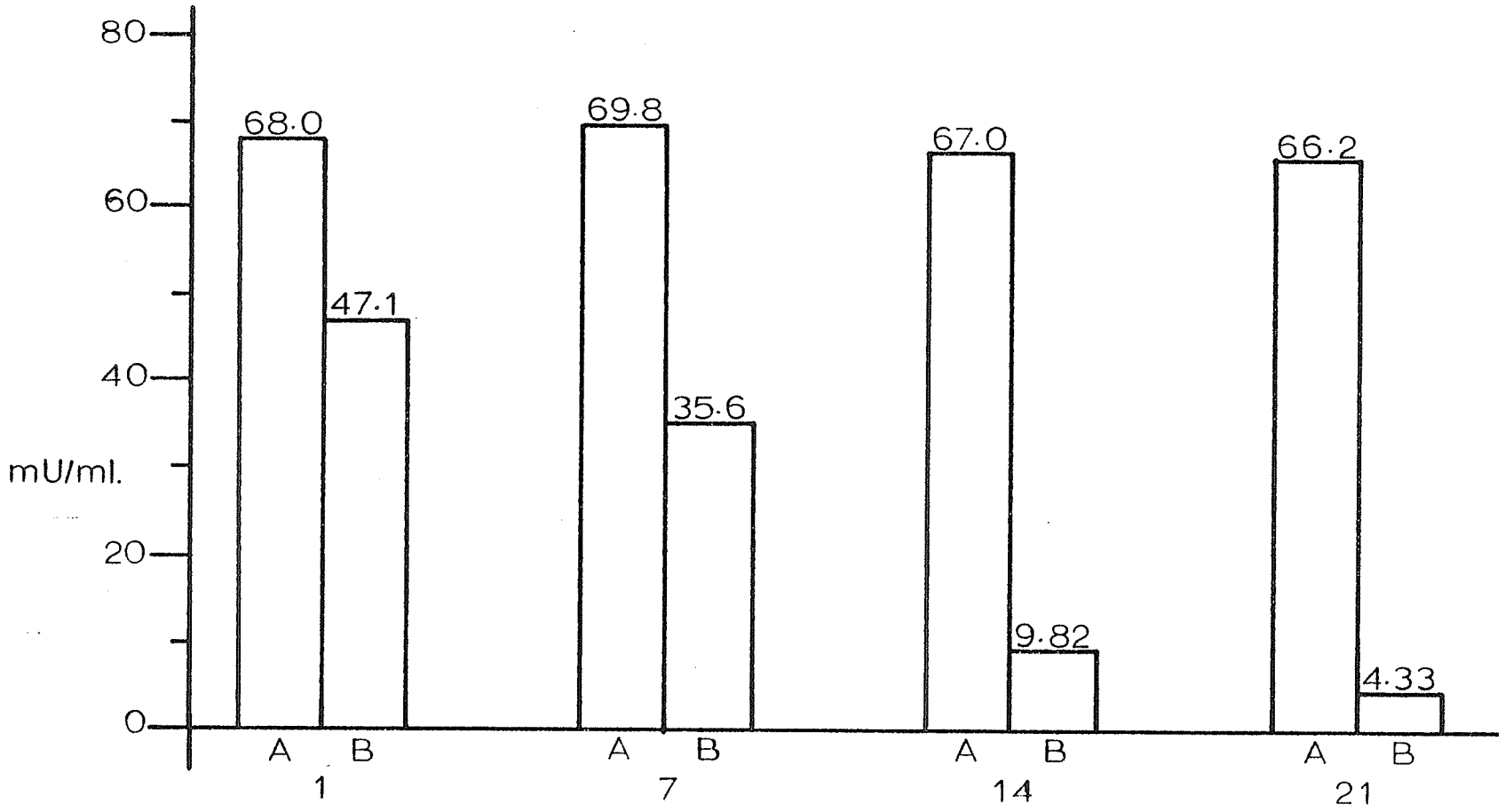
There is little information about seasonal changes in insect cholinesterase levels in the literature. The one reference located (Baust, 1972) involved the testing of two species of beetles which had been acclimatized to two temperature ranges (15-20^{o}C and above 0^{o}C). Baust found that the beetles, held in the higher temperature environment, demonstrated temperature optima for activity approaching ambient exposure, whereas those at low temperatures showed no such temperature optima for activity. His conclusion was that the cholinesterase systems for these beetles appeared independent of environment temperatures. It is important for an overwintering insect such as the honey bee to possess a cholinergic system which will function independent of the temperature: a test of this hypothesis can be made quite easily by measuring the cholinesterase of honey bees taken from the winter cluster.

The possibility of a seasonal change in honey bee cholinesterase was brought to light through the analysis of a series of samples from the Bird's Hill Park field experiments. Initially, the decrease in cholinesterase was thought to be the result of some procedural error. To examine this further, newly emerged bees were marked (see Section 3.2.3) with the intention of measuring the enzyme levels at intervals during a three week period in November, 1977. The hive containing these bees was surrounded by 0.3 meters of snow, and the honey bees were in a winter cluster on the frames. Bees were sampled at periods of 1 day, 7 days and 21 days from the 8 November starting date, and immediately frozen for analysis. The cholinesterase assay was conducted as per Section 3.1.

The results show a drop in cholinesterase levels during the following three week period (see Appendix H). Figure 3.3 shows the decline in mean head cholinesterase levels from 47.1 mU/ml in the 1 day old bees to a mean of 4.3 mU/ml in the 21 day old bees. Thus, there appears to be a positive trend towards the reduction of cholinesterase with age. Compared with mean head cholinesterase values for honey bees of the same ages sampled during June-July, the difference becomes more apparent. Using an unpaired "t" test, all summer values were shown to be statistically different from the November honey bee levels ($P<0.001$). Clearly, there is some physiological mechanism in the bee which acts to alter head cholinesterase levels (assayed at $25^{\circ}C$) with respect to the seasons of the year.

It is conceivable that the reduction in honey bee head cholinesterase found in November samples may be indicative of a redistribution of the enzyme within the bee, rather than its complete disappearance. To examine

Figure 3.3. Comparison of honey bee head cholinesterase levels between bees sampled June-July 1976 and November 1977.



A = June/July sample

B = November sample

Age of Bees (days)

this possibility, honey bees 1 day, 14 days and 21 days old (7 day old bees were unavailable) were sampled in November 1977 for cholinesterase levels in their heads, thoraces and abdomens. The results show the dramatic drop in head enzyme levels experienced from the 1 day old bee to the 14 day old ones (Table 3.5). However, the values also demonstrate a decrease in thoracic and abdominal enzyme levels. Figure 3.3 shows the relationship between the three ages, showing an interesting significant recovery of thoracic cholinesterase in the 21 day old bees to 21.30 mU/ml, which surpasses the 18.77 mU/ml figure for 1 day old bees.

There is a very obvious difference between the relatively low mean cholinesterase value for the 1 day old bees sampled in November versus the 1 day old bees sampled in June. One might postulate that this discrepancy could lie in some qualitative seasonal difference in food fed to the immature stages. Only a detailed diet examination could supply answers to this query.

The question of redistribution within the honey bee cholinergic system was prompted by the comments of Lewis and Fowler (1956) who suggested that acetylcholine may be redistributed via the haemolymph and reconcentrated in the hind-gut by the action of the malpighian tubules (in house flies). Cholinesterase assays, conducted in the honey bee body regions (Table 3.5), do not show any redistribution of cholinesterase, but rather suggest a re-synthesis of the same. The drop in head cholinesterase with time after emergence is quite dramatic, and may represent an adaptive advantage with respect to activity. Honey bees are subjected to a greater number of stimuli from the environment and within the hive during the summer than in the winter, and it is plausible

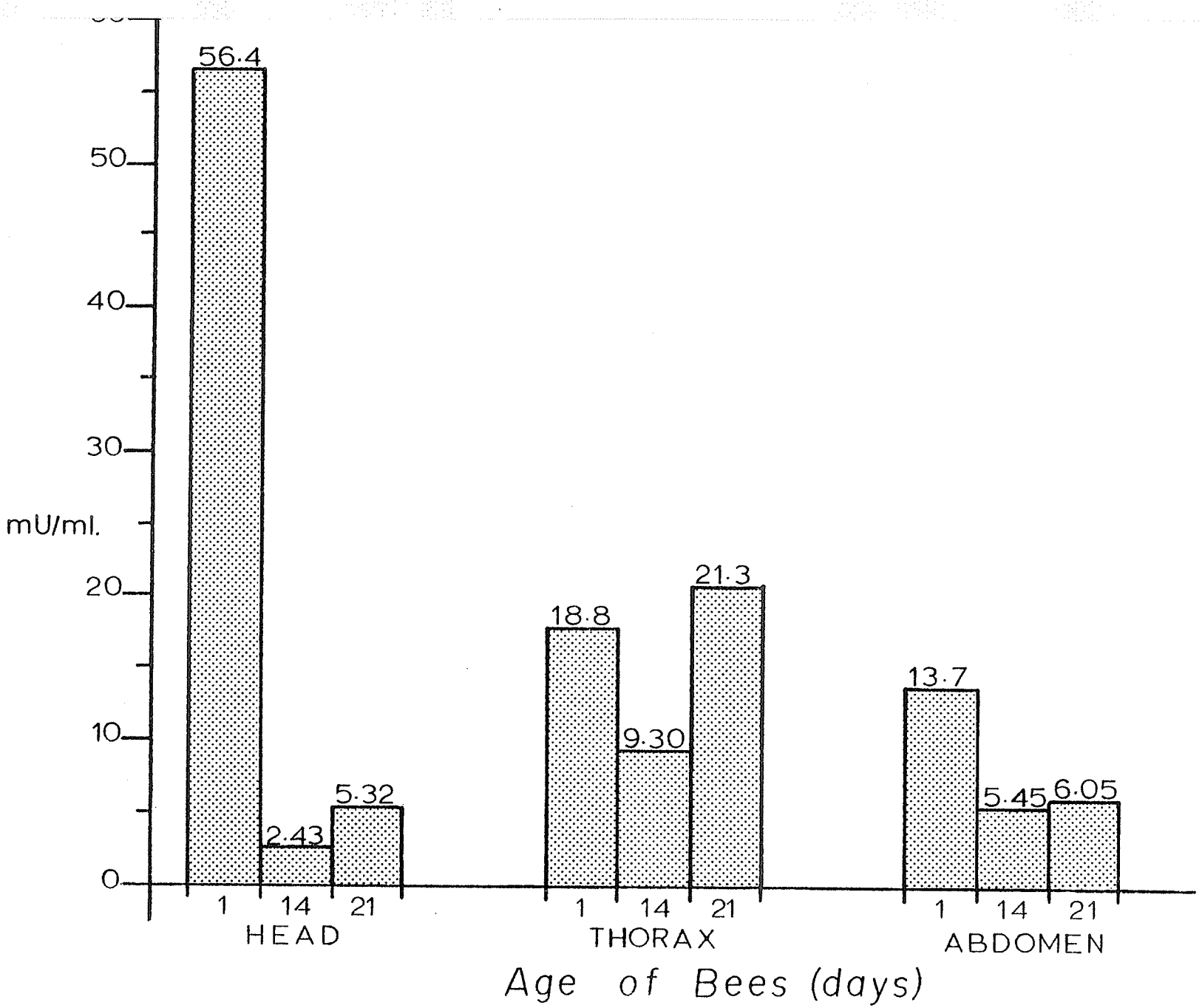
Table 3.5 Honey bee body region cholinesterase levels measured from bees sampled November, 1977

	Sample	Sample size	Mean soluble cholinesterase (mU/ml)	Standard deviation
Head	1 day old bee	8	56.41	6.44
	14 day old bee	6	2.43	2.40
	21 day old bee	6	5.32	8.25
Thorax	1 day old bee	8	18.77	1.79
	14 day old bee	6	9.3	2.60
	21 day old bee	6	21.3	2.92
Abdomen	1 day old bee	8	13.68	2.40
	14 day old bee	6	5.45	1.46
	21 day old bee	6	6.05	3.48

that this change in neural activity is reflected in cholinesterase levels. However, Figure 3.4 demonstrates that thoracic cholinesterase levels of 14 day old bees fall to half the 1 day old values, but then rise again in 21 day old bees to slightly surpass the initial levels. This phenomenon may be of adaptive significance in relation to heat production in the cluster. It is generally accepted that honey bees maintain cluster temperature during the winter months through heat generated by the thoracic muscles, and this activity would likely be reflected in increased neural activity. Hence, the levels of thoracic cholinesterase increase accordingly, while the enzyme levels in the head and abdomen remain low due to the lack of demand on these regions.

Overall, it would appear that the honey bee cholinesterase levels may be demand dependent, accommodating the variation in physical activity from winter to summer with corresponding decreases and increases in the bee enzyme. This would coincide well with what Lindauer (1952) termed as the ability of the individual bee to respond to the varying needs of the colony brought about by any capricious environmental changes.

Figure 3.4. Honey bee body region cholinesterase measured from three age groups of bees sampled November 1977.


56.4
50
40
30
mU/ml.
20
10
0
2.43
5.32
18.8
9.30
21.3
13.7
5.45
6.05
1
14
21
HEAD
THORAX
ABDOMEN
Age of Bees (days)

CHAPTER 4

BIRD'S HILL PARK FIELD EXPERIMENT

4.1 Introduction

In Manitoba most wide-scale aerial pesticide applications to combat forest pest infestations are carried out in May. Thus, the field test was organized around a mid-May spray date. Permission was obtained from the Manitoba Parks branch to conduct a field trial at Bird's Hill Park, located 16 kilometers north of Winnipeg, Manitoba. The spray program was instigated to counteract a forest tent caterpillar infestation, using malathion as a contact insecticide.

4.2 Methods

Packaged honey bees were hived into the sixteen field trial colonies on 26 April, 1977, and allowed to settle down for one week before the addition of marked bees. Frames of capped brood, taken from overwintered colonies at the University of Manitoba apiary, were incubated overnight on 2 May and newly emerged bees were marked with a colour the following morning (see Section 3.2.3). The bees were introduced to the field trial colonies with smoke to disguise unfamiliar odours and thus enhance acceptance. The marked bees were distributed, 160 per hive, with acceptance being very good in all cases.

The cages designed to contain the honey bees durin the field exposure required the following: firstly, the mesh had to be small enough to preclude the escape of the bees and yet large enough not to impair exposure to airborne pesticide; secondly, the cage had to remain compact enough to facilitate storage within the colony. A 7.6 cm x 5.1 cm x 2.5 cm - 2.5 mm mesh wire screen cage was designed to fulfill

both requirements. The mesh size gave maximum exposure with no losses of bees, while the dimensions enabled it to be stored between hive frames, or under the top lid. These cages were designed with a small cork entrance on a 2.5 cm face for easy sampling of bees, with a 7.6 cm x 5.1 cm wire-fastened foldout side to give a large release surface. The cages were suspended at a fixed level of 1.0 m from the ground during exposure.

The field trial experiment was designed to examine three facets of the effects of malathion poisoning on honey bees. The first and more traditional test involved measuring the mortality of the malathion-exposed honey bee samples. The second set of observations were behavioral in nature, testing the ability of sublethally poisoned bees to return to the home hive. The measurement of honey bee head cholinesterase, in conjunction with the previous two factors, constituted the third area of examination.

The field test was designed to examine the parameters of distance, height, cover and age in relation to the exposure time. The height of the suspended sampling cage was fixed at 1.0m and the age of the bees at 14 days old. Three sampling periods were planned, one hour, two hours and three hours post-spray, with samples placed in equal numbers under tree canopy cover and in an open field position. The location of the sampling cages from the home hives was fixed to the 7-12m range for all time periods. A second sampling distance of 23m was added for the first time period only. Figure 4.1 gives a flow

Figure 4.1. A flow chart representation of the honey bee poisoning monitoring scheme implemented at Bird's Hill Park, May 1977.

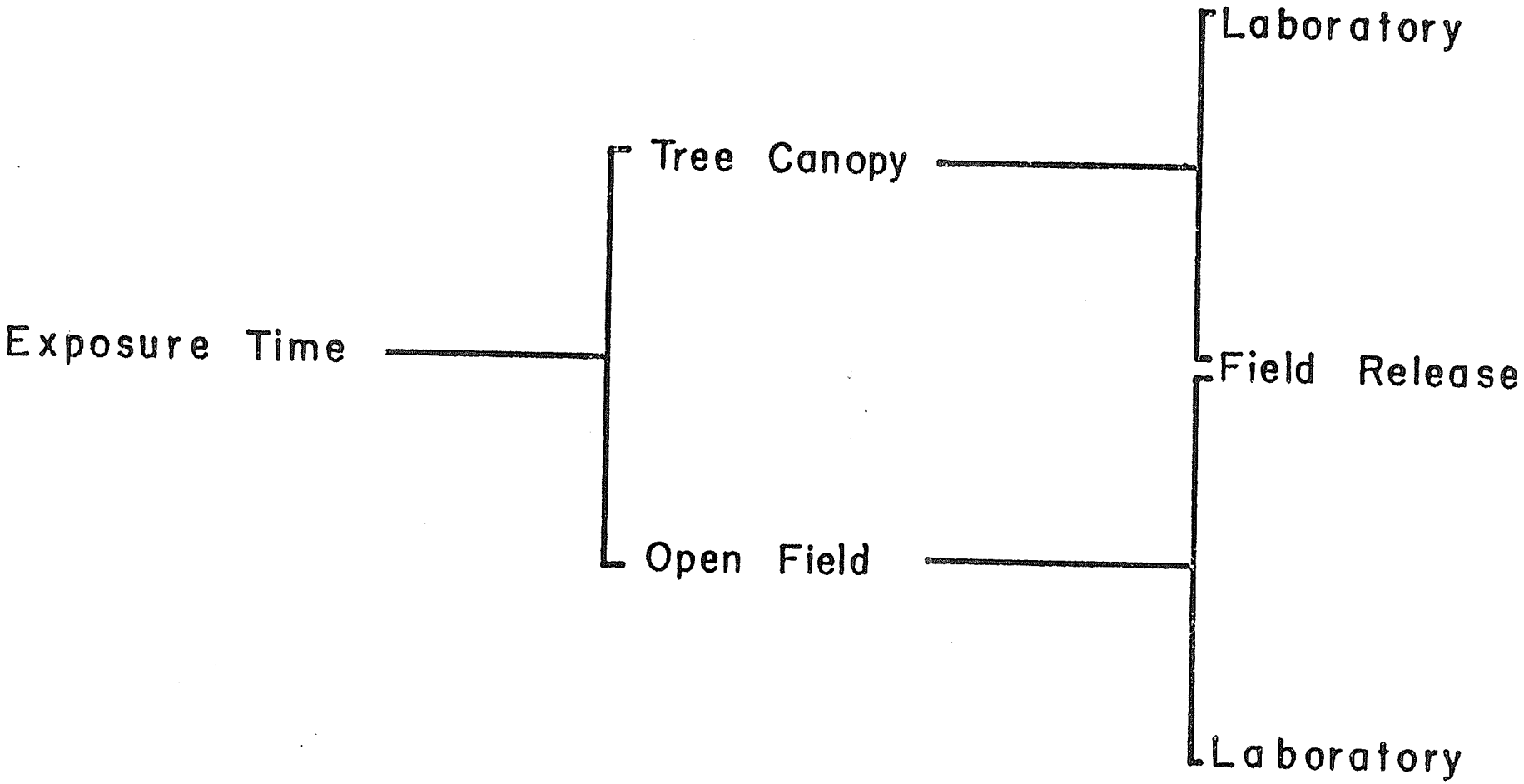

Exposure Time
Tree Canopy
Open Field
Laboratory
Field Release
Laboratory

chart representation of the design.

Two sites were chosen in Bird's Hill Park to accommodate the size of the experiment, one site for a malathion-exposure test, and a second for a control. The sites were separated by a distance of 3 km. The hives were moved into the park on 10 May, 1977 to allow the bees to acclimatize to the new surroundings before the projected 17 May spray date. Both sites were sufficiently similar so that the hives could be arranged in open fields away from mature tree and bush cover, yet still meeting the minimum demands of tree canopy for the assorted sampling posts located under such cover. At each site, hives were positioned with entrances facing south, east and west and with a distance of over 5.0m between any two hives. The procedure for caging the bees involved removing marked bees from the frames using tweezers and catching the bee by the hind pair of legs to avoid injuring it. In anticipation of an early morning spray, the bees were caged on the evening of 16 May, and stored overnight between the hive frames of brood.

The aerial malathion application planned for 17 May, 1977 was postponed due to windy weather conditions. The spray actually commenced at 1000h 18 May, and everything was readied for the first sampling period at 1115h. The caged bees were placed in position in both the control and exposure sites at 1015h, with three people manning both sites. Those in the malathion-exposure site wore coveralls, gloves, goggles and masks fitted with filters designed to remove airborne malathion, in order to minimize contact with the pesticide. The treatment site was sprayed at 1115h on 18 May with malathion 50% EC at a rate of 5 oz a.i./acre as part of a selective aerial spray program in

the park. Conventional boom and nozzle spray equipment delivered the malathion mixture in the volume of one gallon of solution per acre.

The sampling method proceeded as follows: Each of a total of 14 sampling posts contained two cages, one with bees to freeze for cholinesterase analysis and a second to release for a navigation study. The 14 sampling posts were divided equally between the control and malathion exposure sites. During each of the three time periods, caged bees were sampled under tree canopy cover and open field situations. Three additional samples were taken of unmarked foragers exhibiting erratic and lethargic behavior at the hive entrances. All stored samples were immediately frozen on dry ice ($-70^{o}C$) to ensure preservation for cholinesterase and residue analysis.

4.3 Results and Discussion

4.3.1 Cholinesterase and Residue Analyses

The field samples were individually analyzed for soluble bee head cholinesterase by the method described in Section 3.2.1. Malathion residues were measured in pooled samples of bee thoraces and abdomens from each sampling post using the gas chromatographic procedure of Grift and Lockhart (1974) except that the column was 1.5% OV-101 on Chromosorb G H/P, 100/120. An alkali flame ionization detector was used on a Varian model 2100 gas chromatograph to give malathion detection limits of 0.01 ppm on a one gram sample of bees. Recoveries of internal standards added ranged from 87 - 100% (using fish liver tissue).

The results for the cholinesterase and malathion residue analyses of the honey bee samples are shown in Table 4.1. The equally low head

for bees sampled during the Bird's Hill Park aerial malathion spray 18 May, 1977.

Post-Spray Time (hr.)	Site Conditions	Sample Site	Sampling	Post	Name Code Abbreviation	Mean Soluble Head Cholinesterase (mU/m)	Malathion Residue Analysis (g/g
1:00	Malatnion Exposure (M.Ex.)	9	Tree canopy cover	(7-12 m)	BHP-1	5.47	7.1
1:00	M.Ex.	9	Open field	(7-12 m)	BHP-2	9.30	4.4
1:00	M.Ex.	1	Open field	(23 m)	BHP-3	9.10	5.2
2:00	M.Ex.	9	Tree canopy cover	(7-12 m)	BHP-4	1.95	4.9
2:00	M.Ex.	9	Open field	(7-12 m)	BHP-5	4.20	3.1
3:00	M.Ex.	9	Tree canopy cover	(7-12 m)	BHP-6	9.40	3.0
3:00	M.Ex.	9	Open field	(7-12 m)	BHP-7	17.30	0.61
0.40	M.Ex.	8	Within 2 m of hive entrance		BHP-15	9.20	11.0
0.50	M.Ex.	6	Within 2 m of hive entrance		BHP-16	4.90	4.1
1:30	M.Ex.	7	Within 2 m of hive entrance		BHP-17	14.80	0.03
1:00	Control (C)	9	Tree canopy cover	(7-12 m)	BHP-8	13.29	0.12
1:00	C	9	Open field	(7-12 m)	BHP-9	5.98	<0.04
1:00	C	9	Open field	(23 m)	BHP-10	7.38	0.04
2:00	C	9	Tree canopy cover	(7-12 m)	BHP-11	3.76	<0.04
2:00	C	9	Open field	(7-12 m)	BHP-12	18.33	<0.04
3:00	C	3	Tree canopy cover	(7-12 m)	BHP-13	3.13	0.07
3:00	C	9	Open field	(7-12 m)	BHP-14	9.18	<0.03

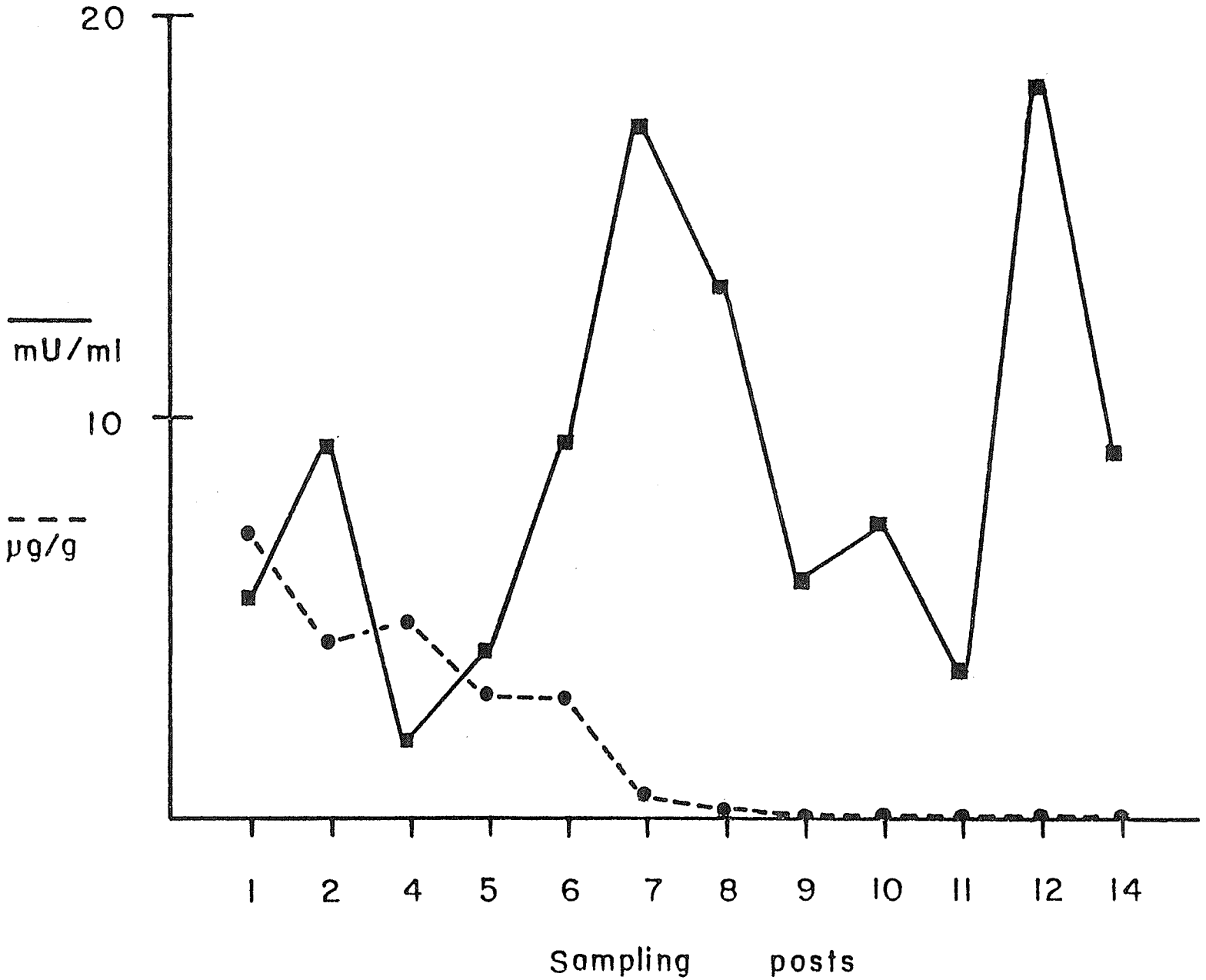
cholinesterase values for the control group and the malathion-exposure group makes it extremely difficult to statistically distinguish between the two samples. Table 4.2 gives the statistically different populations ($P<0.05$) among the sample groups, not only showing eleven instances of significance between control and malathion-exposure groups, but also considerable differences within the two groups. The obvious conclusion one can draw from these results would be that the control and malathion-exposure cholinesterase levels cannot be clearly distinguished as two separate populations. The information obtained in Section 3.8 established that the cholinesterase levels of healthy bees sampled during June and July 1976 measured at 67.8 mU/ml, a full five times higher than the mean value for the Bird's Hill Park control sample. These results suggest that there was contamination in the control site. However, on-site inspection proved that the pungent malathion odour was readily detectable for three hours post-spray at the exposure site, yet was totally absent within 1.5 km of the control area. The results of the malathion residue analysis (Table 4.1) support this initial observation. A composite graph of cholinesterase and residue levels at each individual sampling post demonstrates that the head enzyme values of the control area samples were not influenced by organophosphate exposure (Figure 4.2).

The low values for control area honey bee cholinesterase remains a mystery. These could not be directly related to procedural errors and were not apparently influenced by test area contamination. A more comprehensive year round examination of the whole honey bee cholinergic system is needed to provide the necessary insight into this question.

Table 4.2: Sampling groups found to be statistically different populations ($P < 0.05$), using an unpaired "t" test (including BHP-3 and BHP-13 due to small sample size)

Comparison within the Malathion exposure site sampling groups	Probability	Comparison within the control site sampling groups	Probability	Comparison between exposure and control site sampling groups	Probability
BHp-1 vs. BHP-7	< 0.05	BHP-8 vs. BHP-11	< 0.025	BHP-2 vs. BHP-11	< 0.025
BHP-2 vs. BHP-4	< 0.025	BHP-11 vs. BHP-12	< 0.05	BHP-4 vs. BHP-8	< 0.025
BHP-2 vs. BHP-5	< 0.05	BHP-11 vs. BHP-15	< 0.05	BHP-4 vs. BHP-12	< 0.025
BHP-2 vs. BHP-7	< 0.01			BHP-4 vs. BHP-15	< 0.05
BHP-4 vs. BHP-6	< 0.005			BHP-5 vs. BHP-8	< 0.05
BHP-4 vs. BHP-7	< 0.001			BHP-6 vs. BHP-11	< 0.001
BHP-5 vs. BHP-6	< 0.005			BHP-7 vs. BHP-9	< 0.001
BHP-5 vs. BHP-7	< 0.001			BHP-7 vs. BHP-10	< 0.001
BHP-6 vs. BHP-7	< 0.005			BHP-7 vs. BHP-11	< 0.001
				BHP-7 vs. BHP-14	< 0.05
				BHP-7 vs. BHP-15	< 0.025

Figure 4.2. Honey bee head cholinesterase levels measured from twelve sampling posts versus the concentration of malathion residue found in the thoraces and abdomens of these bees. All values were taken from bees sampled during an aerial malathion application at Bird's Hill Park, 18 May, 1977.


20
10
mU/ml
µg/g
1
2
4
5
6
7
8
9
10
11
12
14
Sampling posts

This phenomenon may be of adaptive significance in relation to heat production in the cluster. It is generally accepted that honey bees maintain cluster temperature during the winter months through heat generated by the thoracic muscles, and this activity would likely be reflected in increased neural activity. Hence, the levels of thoracic cholinesterase increase accordingly, while the enzyme levels in the head and abdomen remain low due to the lack of demand on these regions.

Overall, it would appear that the honey bee cholinesterase levels may be demand dependent, accommodating the variation in physical activity from winter to summer with corresponding decreases and increases in the bee enzyme. This would coincide well with what Lindauer (1952) termed as the ability of the individual bee to respond to the varying needs of the colony brought about by any capricious enivronmental changes.

4.3.2 Release Experiment

The results of this release experiment were as follows: in five of the seven exposure site sampling posts described in Section 4.2, all caged bees died within 60 minutes post-spray (with malathion 50% EC sprayed at a rate of 5 oz a.i./acre by conventional boom and nozzle spray equipment). In a sixth cage, located in an open field position, one honey bee survived for 120 minutes after exposure (all other bees from the same cage had died within 60 minutes). This bee was released, but it did not return to the home hive within the next 60 minutes. The seventh exposure cage, held under tree canopy cover, had eight bees remaining alive after 180 minutes. Six of these could fly. However, they did not return to the hive within 60 minutes of release. Observations of bee activity around exposure area hive entrances during the

180 minute post-spray period showed that a large number of bees exhibited tremors. Many were unable to stand, and flight patterns were haphazard, with some bees colliding with fixed objects. The survival of 13 bees from the sampling cages, out of a total of just under 160 is an indication of the impact of the malathion spray on the foraging population.

Unfortunately, a proper release experiment was not conducted in the control area. Only three of the seven sampling posts were recovered through a misunderstanding with the samplers; the experiment required six volunteers to aid in the sampling and it was physically impossible for the author to supervise both control and exposure sampling sites at the same time. Table 4.3 indicates that the recovery of bees at the three remaining sampling posts was 50% or less and it could not be determined objectively how much this may have been influenced by the inexperience of the samplers. Returns at both control and exposure sites may have also been affected by the inclement weather induced 24 hour overstay of release bees in the wire cages, although experiments conducted later in the home yard suggested this may not have affected the homing abilities as much as originally thought.

Table 4.3. Honey bee release experiments conducted during the aerial application of malathion in Bird's Hill Park, May, 1977

Experiment	Date	Bee age (days)	Treatment	Time during which bees returned to hive	Total bees returned	Total number returning
Bird's Hill Park	18/05/77	14				
60 min.			Control site	60 min.	4	2
120 min.			Control site	"	8	4
180 min.			Control site	"	8	3

CHAPTER 5

HONEY BEE RELEASE EXPERIMENT

5.1 Introduction

It has been known for a very long time that the survival of a honey bee colony rests with its ability to collect food from the environment. This ability is inseparably tied to the navigation skills of the honey bee and the aim of this experiment was to determine the effects of bee exposure to malathion on such skills. If successful, this simple measurement could provide clues to another possible facet of influence of organophosphates on the life of the bee.

5.2 Methods

The release tests were conducted as follows: honey bees of the selected age group were collected in 7.6 cm x 5.1 cm x 2.5 cm cages (2.5 mm mesh wire screening) and chilled to inactivity (about 5 minutes at $4^{o}C$). The bees were then treated with 2.0µl of the malathion solution, applied by micropipette to the intersegmental area between head and thorax. The bees were immediately double-marked with paint on the abdomen for identification and returned to the cages for a one-hour recovery period. One hour later, the caged bees were placed on site and released at a constant height and distance from the home hive (1.0 m high x 5.0 m from the hive entrance).

Returning bees were visually identified at the hive entrance and this information was recorded in reference to time elapsed since the release of the bees.

This experiment was conducted in good flying weather (minimum temperature of 18.5°C with light breezes).

5.3 Results and Discussion

The results of the home yard release experiments, held at the University of Manitoba apiary during the summers of 1977 and 1978 are recorded in Table 5.1. There is a slight difference between control and malathion-exposure groups for the 1977 release experiments, perhaps due to small sample size. Accordingly, the sample size was increased during the 1978 release experiments and the results (Table 5.1) show proportionately higher returns in control samples versus those treated with malathion. All home year experiments were conducted using water as a control and a 1:500 malathion-water mixture as the exposure dosage, both delivered at the rate of 2.0 µl per bee.

Statistical relevance is related to sample size and a major drawback of the home yard release experiments was the limitation on numbers the technique imposed. Trial and error indicated that 60 honey bees would be the maximum release sample per sampling period. The sublethal dose was chosen to explore a range which allowed exposed bees to home, yet to demonstrate some impairment of the homing ability. No mortality was ever found with bees treated with the 1:500 malathion-water emulsion, yet disorientation was evident. This kind of behavior could well be related to the Bird's Hill Park situation in which malathion was sprayed by aircraft on site. A honey bee flying through air containing malathion droplets could conceivably encounter the same impairment of the homing ability found in topically treated samples.

Table 5.1. Honey bee release experiments conducted during 1977 and 1978

Experiment	Date	Bee age (days)	Treatment	Time during which bees returned to hive	Total bees returned	Total number returning
Home Yard						
Trial 1	26/07/77	14	Water control	20 min.	5	3
Trial 2			Water control		5	3
Home Yard	02/08/77	14			5	5
Trial 1			Water control	20 min.	5	3
Trial 3			" "	"	6	4
Trial 4			1:500 malathion	"	5	2
Trial 5			Water exposure	"	6	3
Home Yard	09/08/77	14				
Trial 1			Water control	60 min.	18	12
Trial 2			1:500 malathion -Water exposure	"	15	8
Home Yard	18/08/77		Pollen gatherers			
Trial 1			Water control	81 min.	12	11
Trial 2			1:500 malathion -Water exposure	"	13	9
Home Yard	22/08/77		Pollen gatherers			
Trial 1			Water control	60 min.	18	12
Trial 2			1:500 malathion -Water exposure	"	15	8
Home Yard	09/08/78	28				
Trial 1			Water control	60 min.	16	13
Trial 2			1:500 malathion	"	11	6
Trial 3			-Water exposure	"	15	7
Trial 4			" "	"	15	8
Home Yard	10/08/78	28				
Trial 1			Water control	60 min.	15	11
Trial 2			1:500 malathion	"	16	9
Trial 3			-Water	"	15	6
			exposure	"	13	5
Home Yard	11/08/78	28				
Trial 1			Water control	60 min.	13	11
Trial 2			1:500 malathion	"	14	7
Trial 3			-Water exposure	"	11	6
Home Yard	12/08/78	28				
Trial 1			Water exposure	60 min.	12	9
Trial 2			1:500 malathion	"	13	6
Trial 3			-Water	"	14	6
Trial 4			exposure	"	13	7

CHAPTER 6

OBSERVATION HIVE BEHAVIORAL EXPERIMENT

6.1 Introduction

The literature contains little or no data relating to the behavioral changes which honey bees undergo within their hive when poisoned with malathion, nor about the behavior of hive bees toward bees poisoned with this insecticide. The research contained within this Section attempts to provide information about these two aspects of bee behavior.

6.2 Methods

Behavioral studies were undertaken using four single-frame glass-walled observation hives placed 1.0m above the ground; these allowed one to observe the bees under natural conditions. Drifting and loss of bees was minimized by arranging the observation hives 3.0m apart and facing the entrances in different directions.

Each hive contained about 600 worker bees, a few drones, one mated queen, brood of mixed ages and stores of pollen and honey. Newly emerged bees were marked with paint and introduced into the observation hives at various intervals to provide for a sequence of aged bees (see Section 3.2.3).

The method for poisoning bees was as follows: a manually controlled microapplicator, with a 2.0µl micropipette, was used to deliver the 50% EC malathion-water solution between the head and thorax of each experimental bee. This area was chosen for its permeability and its location between the well-innervated head and thorax. The malathion-water solutions were prepared as per Section 3.2.4, using water as a diluant.

Control bees treated with water only or with nothing.

In order to measure behavioral changes due to poisoning we must have a dependable means of recording bee behavior. Thus, the behavior of the bees was measured both in relation to the kind of activity undertaken as well as the duration of the activity. This was accomplished by patient observation of the bees' behavior along with the use of a tape recorder, prepared charts showing possible behavioral patterns and a stopwatch. Observations included the behavior of both the poisoned bees as well as the responses of the hive bees to the poisoned bees and were usually carried out between 0900h and 1600h.

It is not difficult to follow an individual bee in a glass-walled observation hive provided that the bee has been clearly marked with paint on its thorax or abdomen. Occasionally problems occurred either when a bee passed beneath a cluster of bees or when it moved along the top or bottom of the frame.

Several experiments were conducted as outlined below.

An initial test was done to determine if different concentrations of malathion applied to bees results in death at different rates. Malathion was mixed with water in the volume relation of 1:1, 1:50, 1:125, 1:250 and 3.5:500 to give the treatment concentrations. A total of 10 bees (ages 7, 9, 10, 11, 19 days) were treated witn 2.0µl of the treatment mixture. The bees were observed to determine any behavior peculiar to the poisoning process.

The "normal" behavior of the hive bees (i.e. untreated bees) was determined by observing marked bees of six different ages (i.e. 8, 10, 11, 13, 15 and 16 days) for a total of 668.62 min. The bees' activities

were summarized under various categories (see Table 6.1).

Another test involved treating bees of various ages (i.e. 7, 9, 10, 11, 14, 15, 18 and 19 days) with 2.0µl of the malathion-water mixture previously mentioned (i.e. 1:1, 1:50, 1:125, 1:250 and 3.5:500). Their subsequent behavior was then summarized under several activity categories (see Table 6.2).

To determine if a sublethally poisoned bee will exhibit behavioral changes over a period of 3 days a bee 7 days of age was treated with 2.0µl of a 1:500 malathion-water mixture and its activity (along with that of a water-treated control bee) was observed for two days post-treatment.

Finally, to determine if the grooming of a poisoned bee by other hive bees magnifies the impact of a pesticide the following test was done. A 9 day old bee was administered 2.0µl of a 1:1 malathion-water mixture (see above) and introduced to an observation hive where its, and the other bees, activities were monitored.

6.3 Results and Discussion

The results are shown in Figures 6.1 and Tables 6.1 to 6.4.

Organophosphate poisoning in the honey bee, as with any living creature, is usually accompanied by symptoms of systemic distress. Tremors are seen as a general body response to organophosphate intoxication, and malathion does produce this response in honey bees. There are, however, certain other physical manifestations of the internal poisoning process quite predictably present in lethally treated bees, as borne out by my observations. These are (i) the loss of integrated

Figure 6.1. Malathion-water ratio used in the 2.0 µl sample versus the time taken to produce death in the honey bee.

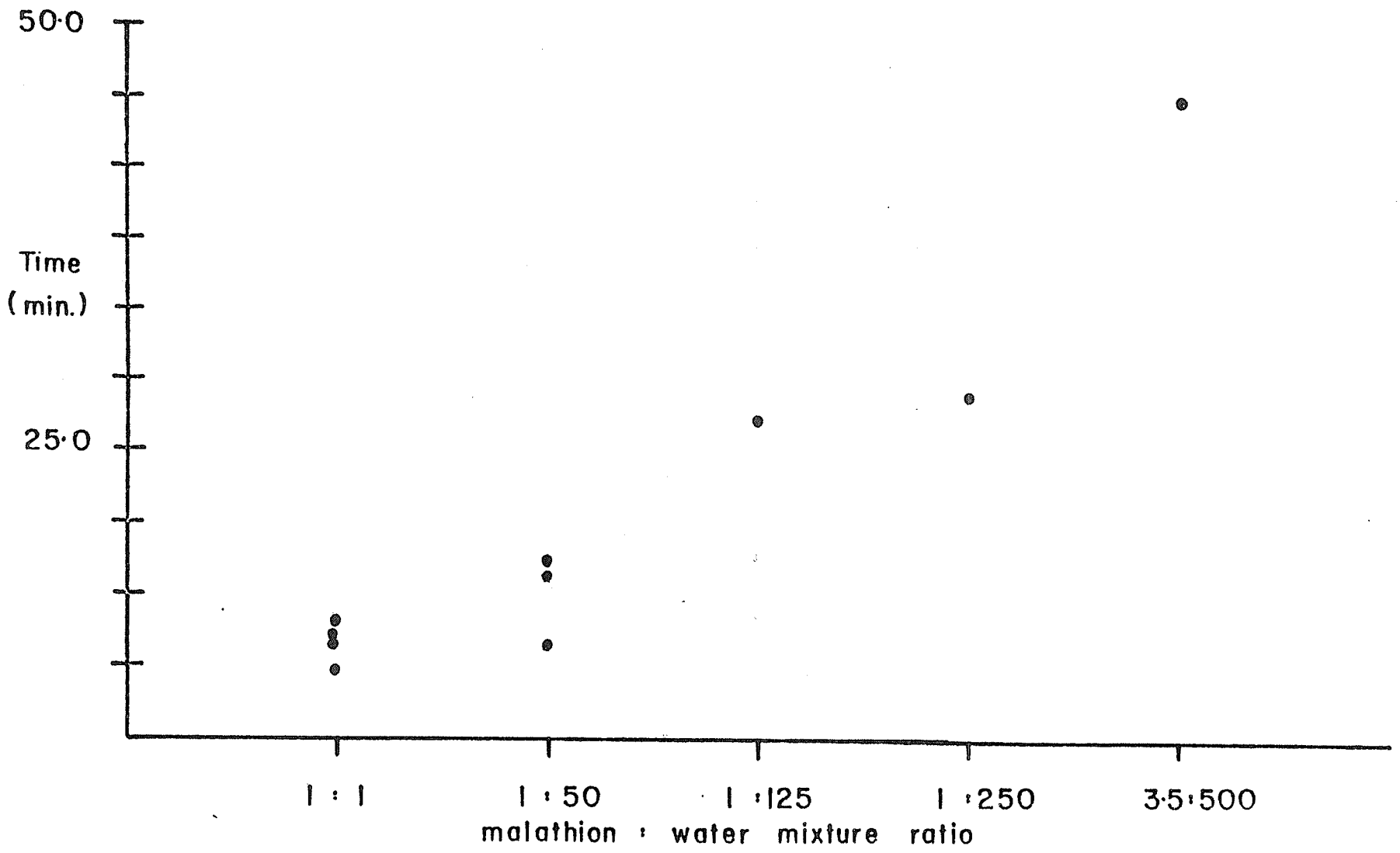

50·0
Time
(min.)
25·0
1 : 1
1 : 50
1 : 125
1 : 250
3·5 : 500
malathion : water mixture ratio

Table 6.1 Hive activity of individual honey bees observed through a glass-walled single-frame observation hive, under no-treatment conditions

Age of bees (days)	Wandering and standing	Head and body in empty cell	Head and body in honey cell	Head and body in brood cell	Head and body in pollen cell	Self-grooming	Grooming dance	Trophylaxis	Queen attendance	Wax working	Hive guarding	Tremors	Attended by 1 or 2 bees	Attended by 3 or 4 bees	Attended by more than 4 bees	Vigorous leg movement	Front leg movement only	Self-fanning	Hive bee fanning	Time
8, 10, 11	430.48*	8.00	40.13	7.08	0.07	6.48	0.20	3.49	2.47	-	0.92	-	-	-	-	-	-	-	-	499.32
	86.37%	1.60%	8.05%	1.42%		1.30%		0.70%	0.50%											
13, 15, 16	117.04	8.34	12.94	15.91	0.01	5.19	0.42	1.95	0.44	7.06	-	-	-	-	-	-	-	-	-	169.30
	69.13	4.95	7.64	9.40	-	3.07	-	1.15	-	4.17										
Totals	547.52	16.34	53.07	22.99	0.08	11.67	0.62	5.44	2.91	7.06	0.92	-	-	-	-	-	-	-	-	668.62
	82.00	2.45	7.95	3.44		1.75		0.81	0.44	1.06										

*Minutes

Table 6.2. Hive activity emphasis of individual honey bees observed through a glass-walled single-frame observation hive, under malathion treatment conditions *

Ages of bees (days)	Wandering and standing	Head and/or body in empty cell	Head and/or body in honey cell	Head and/or body in brood cell	Head and/or body in pollen cell	Self-grooming	Grooming dance	Trophylaxis	Queen attendance	Wax working	Hive guarding	Tremors	Attended by 1 or 2 bees	Attended by 3 or 4 bees	Attended by more than 4 bees	Vigorous leg movement	Front leg movement only	Self-fanning	Hive bee fanning	
7,9,10,11	29.74**	0.07	-	-	-	25.79	0.59	1.79	-	-	-	1.38	7.82	5.10	5.35	8.95	5.60	1.23	0.03	93.64
	31.97%					27.54%		1.91%				1.47%	8.35%	5.45%	5.71%	9.56%	5.98%	1.31%		
14,15,18,19	52.50	0.04	0.06	0.09	0.01	85.05	2.58	2.22	-	-	-	1.25	8.95	4.70	5.10	2.20	1.40	-	-	166.18
	31.59					51.20	1.55	1.54				0.75	5.39	2.83	3.07	1.32	0.84			
Totals	82.44	0.11	0.06	0.09	0.01	110.87	3.17	4.01	-	-	-	2.63	16.77	9.80	10.45	11.15	7.00	1.23	0.03	
	31.73					42.67	1.22	1.54				1.01	6.45	3.77	4.02	4.29	2.69	0.41		

*Malathion-water mixtures of 1:1, 1:50, 1:125, 1:250, 3.5:500 (times combined).

**Minutes.

Table 6.3. Hive activity emphasis of a malathion-treated* bee versus a water-treated control at random intervals between 09:00 and 16:00 hours post-treatment period.

		Wandering and standing	Head and/or body in empty cell	Head and/or body in honey cell	Head and/or body in brood cell	Head and/or body in pollen cell	Self-grooming	Grooming dance	Trophylaxis	Queen attendance	Wax working	Hive guarding	Tremors	Attended by 1 or 2 bees	Attended by 3 or 4 bees	Attended by more than 4 bees	Vigorous leg movement	Front leg movement only	Self-fanning	Hive bee fanning	
DAY 1	Malathion-Treated Bee (A)	36.47**	0.04	0.06	-	0.01	29.87	0.10	0.31	-	-	-	1.15	6.50	3.50	4.00	-	-	-	-	82.01
		44.5%					36.4%						1.4%	7.9%	4.3%	4.9%					
	Water-Treated Bee (B)	19.36	0.25	-	0.02	-	3.97	-	0.39	0.02	-	-	-	-	-	-	-	-	-	-	24.01
		80.6%	1.0%				16.5%		1.6%												
DAY 2	(A)	17.77	-	2.69	0.12	-	1.87	-	0.04	-	-	-	-	-	-	-	-	-	-	-	22.49
		79.0%		12.0%			8.3%														
	(B)	8.87	-	-	-	-	-		0.03	=	15.99	-	-	-	-	-	-	-	-	-	24.89
		35.6%									64.2%										
DAY 3	(A)	9.31	0.02	6.23	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15.58
		60.0%		39.9%																	
	(B)	15.91	0.08	0.06	-	-	1.20	-	0.02	-	-	-	-	-	-	-	-	-	-	-	17.27
		92.1%					6.9%														

*Malathion-water mixture of 1:500.

**Minutes.

Table 6.4. Relationship between symptoms appearing in lethally poisoned bees, and the time of occurrence of these symptoms with varying concentrations of malathion.

Date	01/09/77	01/09/77	02/09/77	08/08/77	11/08/77	11/08/77	11/08/77	01/09/77	30/08/77	29/08/77
Bee age (days)	9	9	10	18	11	11	11	9	7	19
Malathion:water mixture	1:1	1:1	1:1	1:1	1:50	1:50	1:50	1:125	1:250	3.5:500
Loss of integrated motor control	-	5.75*	2.15	4.25	3.80	-	6.10	-	15.60	22.65
Abdomen curls under	7.30	6.35	1.75	3.85	5.65	6.70	5.50	10.50	17.55	19.65
Paralysis of middle leg pair	7.30	6.35	2.15	3.85	9.10 ;	10.85	9.85	19.30	18.50	27.80
Final knockdown	7.30	6.35	2.15	4.80	10.00	12.00	10.05	17.30	17.55	33.05
Paralysis of hind leg pair	8.05	7.30	4.75	-	10.00	12.00	10.05	20.80	19.80	34.20
Paralysis of front leg pair	8.05	7.30 :	4.75	6.54	11.45	12.50	10.75	22.25	24.00	45.00

*Minutes

motor control, (ii) the tucking under of the abdomen, (iii) the paralysis of the middle leg pair, (iv) the final knockdown (i.e. the point after which the bee cannot rise, (v) the paralysis of the hind leg pair and (vi) the paralysis of the front leg pair. The first symptom, the loss of integrated motor control, is often not discernible without careful scrutiny. In a moving bee it is, translated as an awkward, unsure movement. The second symptom, the tucking under of the abdomen, marks the point of "no return" for a poisoned bee, meaning no bee exhibiting this symptom ever survived the poisoning. This symptom usually occurs before or at the time of the final knockdown, depending on the concentration of malathion applied, and always preceded other symptoms of paralysis. The paralysis of the middle pair of legs usually happened just before or after the final knockdown and always before the paralysis of the other leg pairs. The symptom of hind leg pair paralysis is the next physical manifestation of poisoning, followed by the paralysis of the front leg pair. This latter symptom is usually indicative of total body paralysis, which always resulted in death for the bees observed. Consequently, it is taken as the time of death of the honey bee.

The symptoms presented in the previous paragraph are listed roughly in the order of occurrence, although several may appear at once depending on the concentration of malathion used. Table 6.4 demonstrates the kind of time relationship between the symptoms that can occur and the concentrations used. The measurements for a 1:1 malathion-water application tend to show the abdomen tucking under, the middle legs undergoing paralysis and the final knockdown all occurring at once, whereas a 3.5:500 dilution shows a spread of 13-14 minutes between these symptoms.

A bee may undergo several apparent knockdowns, recovering to climb the comb once more; hence, the term final knockdown must be used to designate the point of no recovery of co-ordinated motor facilities. The amount of pesticide applied usually determined if the honey bee would undergo one fatal knockdown, or several knockdown recoveries before demonstrating the inability to recover. The general trend seen in Table 6.4 and supported by all observations indicates that there is an order in the sequence of manifestations of physical distress in a malathion-treated bee, and that the swiftness with which these symptoms appear depend on the concentration of pesticide applied. Perhaps this poisoning sequence is indicative of the progress of the neurophysiological poisoning process.

In the malathion-water mixture trials the lowest mixture resulting in the death of bees occurred with a malathion-water mixture of 3.5:500 (0.014µl of malathion 50% EC in a 2.0µl dilution). No mortalities were ever recorded for a 1:500 mixture (0.004µl of malathion 50% EC in a 2.0µl dilution), indicating that the toxicological threshold of malathion producing death in the honey bee (using the application techniques outlined in Section 3.2.4) lies between these two concentrations.

The observations relating to untreated bees showed that 82.0% of the bees' time was spent wandering or standing on the frame. There was also a shift with age towards cleaning empty cells and building new ones. Slight increases in trophylaxis and self-grooming also appeared with age.

During the 259.82 min. that malathion poisoned bees were observed, only 31.7% of the time was spent in wandering or standing in the frame.

The poisoned bees spent the greatest amount of time (42.7%) in self-grooming. A small, but higher amount of time was spent in trophylaxis and grooming dances among the poisoned bees compared to the non-poisoned ones. Of interest is that poisoned bees submitted for cleaning by other bees for 14.2% of the observation period. Water-treated control bees do not undergo the series of behavioral changes observed in the malathion treated bees. However, three other categories involved loss of motor control; the poisoned bees underwent body tremors for 1.0% of the total time, vigorous erratic leg tremors for 4.3% of the 259.82 minute total and vigorous movement of the front legs for only 2.7% of the total period measured. Two additional categories recorded miscellaneous instances of fanning by the poisoned bee and fanning by other hive bees directed towards the poisoned bee.

A general examination of the effect of age on the reaction of the bee to malathion produced differences in time spent on certain activities, but the main activities still occurred within the same categories for both age groups. Divided into a younger group (i.e. 7, 9, 10, 11 days) versus an older group (i.e. 14, 15, 18, 19 days) it can be seen that both groups spent a similar amount of time (32.0% versus 31.0%) wandering and standing in the frame (see Table 6.2). The two groups differ markedly, however, in the amount of time spent in self-grooming (27.5% versus 51.2%). There was also a difference in time spent submitting to be cleaned by other hive bees, between the two groups, with the younger bees engaging in this behavior 19.5% of the time compared to 11.3% for the older group.

The activities (see Table 6.3) of a sublethally poisoned bee and

a water-treated control bee follow. On day 1 (treatment day), the malathion-treated bee spent 44.5% of its time wandering and standing in the frame versus 80.6% of the time for the control bee. The malathion-treated bee was involved in self-grooming over twice the amount of time spent by the control bee (36.4% versus 16.5%). However, on day 2 and day 3 the malathion-treated bee returned to "normal" hive duties, with a minimum amount of activity spent on self-grooming and with no attention (versus 17.1% of the time on day 1) being paid by other hive bees. The control bee was also engaged in "normal" hive activities.

The reaction of hive bees to a lethally poisoned bee was as follows. The 9 day old bee treated with the 1:1 malathion-water mixture was introduced to the observation hive and died 7.30 min. later; at that point another hive bee attempted to remove it from the hive for 4.30 min. Several other hive bees also attempted to remove the bee and each of these bees engaged in trophylaxis with other bees after failing in the attempt. One such bee was marked (19.70 minutes after the death of the originally treated bee) while it attempted for a period of one minute to remove the bee. Sixty-seven minutes later that same marked bee was exhibiting symptoms of severe malathion poisoning, with tremors, loss of motor control and tucking under of the abdomen. In another instance, a bee, which had engaged in trophylaxis for 4 seconds with the then-living original poisoned bee, showed signs of tremors and erratic behavior 13.5 minutes later and died 22.5 minutes after the initial contact. The overall impact on the hive was reflected in the mortalities 55.0 minutes after the death of the original malathion-treated bee; in the test hive there were 78 newly killed bees, versus no mortalities in a

control hive. This experiment was a replicate of a preliminary experiment done on 1 September 1977, which produced a similar number of kills ($\geq$ 120). The practical implications of this experiment may be questioned due to the relatively high pesticide concentration (1.0 µl of malathion 50% EC actually applied in the 2.0 µl dilution). Nevertheless, the principle remains that returning pesticide-exposed forager bees are very capable of inadvertently contaminating other hive bees through social interaction.

CHAPTER 7

SUMMARY AND CONCLUSION

7.1 Honey Bee Cholinesterase

The investigation of honey bee cholinesterase provides some very interesting information concerning the distribution and importance of this enzyme in the bee. It was found that the majority of the detectable total body cholinesterase of a honey bee newly emerged from its cell was contained in the head. The maintenance of this high head enzyme level seems to depend on the month of the year the honey bee emerges. In months other than the June-July period, honey bee head cholinesterase was discovered to decrease in abundance with age, reaching a level of 25% or less of emergence values by age 21 days. Honey bee thorax enzyme levels were found to be measurably higher than abdomen levels, with both body regions containing an average combined value of 37% of the honey bee body total, on emergence. The emergence levels for these two regions were seen to decline with age in November sampled bees, although not as markedly as the head enzyme level drop. At age 21 days, the thorax cholinesterase values for the November samples was noted to return to emergence levels, perhaps as an adaptive response to the need for cluster warmth, by increasing thoracic muscle activity.

Honey bee brains were measured for cholinesterase as an alternative to bee head cholinesterase analysis. The brain cholinesterase assay was discontinued due to the need for a simple, swift means of analysis, features not present due to the time-consuming brain dissections. Honey bee head protein was measured along with head cholinesterase in an effort

to reduce the standard deviation of the enzyme analysis, but the results demonstrated that the protein measurement was not effective in this respect and the cholinesterase remained to be expressed in milli Units of soluble head cholinesterase activity per milliliter of bee head homogenate. Bees sampled during the June-July period were found to maintain emergence head cholinesterase levels for the four age periods measured up to and including 21 day old bees. Preliminary poisoning experiments conducted on bees from the same period show that malathion intoxication can be demonstrated analytically as a depression in honey bee head cholinesterase. This depression is related to the amount of malathion applied.

7.2 Bird's Hill Park Field Experiment

The Bird's Hill Park field experiment demonstrates that honey bee colonies can be adapted as a measuring tool in aerial pesticide applications. A combination of honey bee head cholinesterase measurements and residue analysis on caged bees taken from a control and an exposure area could provide evidence for or against organophosphate intoxication of the bees, be it accidental or otherwise. This type of measurement could prove invaluable in assessing the impact of organophosphate sprays (or other insecticide sprays) on non-target organisms, a tool which is much in need during widescale aerial pesticide applications.

Further research on this subject should include behavioral studies. The topic of honey bee drift resulting from disorienting influences of pesticides may easily be assessed using hives arranged one meter apart in straight rows in both control and exposure areas. A suggestion of

three replicates per treatment should secure enough data to comment on any difference in drifting trends between control and exposure areas. The behavior of hive guard bees should be examined, particularly in response to incoming poisoned bees. Using the experimental techniques as a monitoring tool only, the whole operation could be reduced to three or four hives per site. Considering the honey bee colony as a portable biological unit containing creatures with instinctive homing natures, one can envision a multitude of research possibilities, with implications overlapping into many other fields of knowledge.

7.3 Honey Bee Release Experiments

The design of the release program was to determine the concentrations of malathion which would allow the bees to fly, yet still permit the pesticide to exert some toxicological influence. This sublethal range was found to lie between 0.004-0.014 µl of malathion 50% EC delivered topically in a 2.0 µl water dilution to the membranous area between the head and thorax. Honey bees treated with sublethal dosages in this manner did not return to the home hive as consistently as the control treated bees. This was evident even in the case of pollen gatherers which would have been well oriented to the home hive. Malathion, in sublethal dosages, appears to cause immediate disorientation in the honey bee, affecting homing abilities for short periods of time, possibly even resulting in drifting in some cases. On site fogging of malathion at a rate of 5 oz a.i./acre, aerially applied, results in the death of most directly exposed honey bees, within one hour. Honey bees released after the aerial application of malathion

did not return to the home hive within 60 minutes.

7.4 Honey Bee Behavior

The investigation into alterations in honey bee behavior induced by contact with malathion, produced some interesting results. The honey bees were shown to shift activity to self-grooming from other hive activities immediately after application of the pesticide. Several uncommon pesticide-related behavioral changes also occurred. A lethally poisoned bee was seen to undergo predictable symptoms of poisoning; loss of integrated motor control, tucking under of the abdomen, paralysis of the middle leg pair, final knockdown, paralysis of the hind leg pair and paralysis of the front leg pair. The symptoms always occurred in this order, with the length of time between the appearance of successive symptoms related to the amount of pesticide used. A very interesting discovery to come from this work was the observation of other hive bees intent on cleaning the poisoned bee. The poisoned honey bee was always seen to submit to be cleaned for several minutes at a time, an action which was not the habit of control bees. When the tendency of hive bees to clean a poisoned bee was routinely tested by treating a single bee with 1.0 µl of malathion 50% EC combined with 1.0 µl of water, the observed impact on the hive was startling and unexpected: although the poisoned bee was cleaned by fewer than twenty bees before death, within fifty-five minutes post-mortem over seventy other bees died with obvious organophosphate poisoning symptoms. The eighteen hour total rose to one hundred and fourteen deaths, excluding the original poisoned bee. The implications of findings such as these are far reaching, when

considered in the context of an aerial spray program.

Research into the effects of pesticide poisoning on the honey bee colony behavior demands further investigation. The influence of various kinds of pesticides on the bees can be documented in relation to one another using the techniques described in this thesis as a guide. This kind of investigation can go beyond the general kills-versus-no kills measurement that is traditionally used to measure honey bee response to a pesticide, and delve more intensely into a major area of indirect influence, the workings of the hive itself.

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A P P E N D I X

Appendix A. Honey bee brain cholinesterase levels measured from individual samples taken during late May and mid-June, 1975

Sample period	Brain sample	Soluble brain cholinesterase (mU/ml)	Sample period	Brain sample	Soluble brain cholinesterase (mU/ml)
May 27	1	45.8	May 29	1	101
I)	2	59.2	II)	2	92.5
	3	81.8		3	72.7
12:40 hr	4	64.9	10:30 hr	4	44.6
	5	58.0		5	85.3
May 27	1	55.0	June 16	1	88.8
II)	2	28.6	I)	2	128
	3	30.6		3	155
12:50 hr	4	54.3	11:00 hr	4	93.2
	5	21.0		5	41.3
May 28	1	36.0	June 16	1	106
I)	2	51.3	II)	2	106
	3	30.7		3	119
13:00 hr	4	35.7	11:10 hr	4	113
	5	42.1		5	56.0
May 28	1	78.7	June 16	1	109
II)	2	47.0	III)	2	96.0
	3	55.1		3	113
13:15 hr	4	78.0	12:00 hr	4	73.0
	5	32.8		5	64.9
May 29	1	77.1	June 18	1	116
I)	2	97.6		2	126
	3	57.5		3	128
10:15 hr	4	102	12:00 hr	4	153
	5	86.5		5	64.6

Appendix B. Head cholinesterase values for one day old honey bees sampled during June-July 1976

Sample	Soluble head cholinesterase (mU/ml)	Sample	Soluble head cholinesterase (mU/ml)
1	65.5	28	69.4
2	65.5	29	67.8
3	65.5	30	67.8
4	77.3	31	77.3
5	71.7	32	73.9
6	62.2	33	73.9
7	68.9	34	81.2
8	69.4	35	67.2
9	69.4	36	73.1
10	67.2	37	63.3
11	49.8	38	62.4
12	71.7	39	68.5
13	86.2	40	60.2
14	70.0	41	60.2
15	71.7	42	69.0
16	59.4	43	61.1
17	59.9	44	65.6
18	60.5	45	69.8
19	76.2	46	62.2
20	39.2	47	70.3
21	67.8	48	66.1
22	76.7	49	63.1
23	77.3	50	73.2
24	76.7	51	69.0
25	85.7	52	66.8
26	61.6	53	58.9
27	80.6	54	56.9

Appendix C. Head cholinesterase values for seven day old honey bees sampled during June-July 1976

Sample	Soluble head cholinesterase (mU/ml)	Sample	Soluble head cholinesterase (mU/ml)
1	45.4	23	79.2
2	73.4	24	62.3
3	47.0	25	76.4
4	62.9	26	77.6
5	63.8	27	79.9
6	59.2	28	80.2
7	63.8	29	84.2
8	60.1	30	76.1
9	80.7	31	76.1
10	61.4	32	77.3
11	74.3	33	65.5
12	76.3	34	83.2
13	74.0	35	80.8
14	58.2	36	59.9
15	54.8	37	74.6
16	74.9	38	73.1
17	73.5	39	48.3
18	69.8	40	59.9
19	71.1	41	70.6
20	54.4	42	81.5
21	83.5	43	71.4
22	82.2	44	68.9

Appendix D. Head cholinesterase values for fourteen day old honey bees sampled during June-July 1976

Sample	Soluble head cholinesterase (mU/ml)	Sample	Soluble head cholinesterase (mU/ml)
1	70.2	24	67.2
2	74.5	25	63.6
3	74.8	26	62.9
4	76.8	27	59.2
5	55.7	28	48.3
6	85.4	29	65.0
7	71.5	30	65.3
8	69.7	31	49.6
9	68.9	32	54.8
10	78.5	33	62.4
11	70.5	34	79.5
12	56.1	35	64.9
13	63.3	36	77.6
14	51.3	37	66.8
15	63.6	38	68.9
16	56.3	39	59.1
17	46.6	40	65.1
18	70.3	41	95.4
19	63.6	42	89.3
20	70.9	43	79.8
21	72.5	44	81.4
22	63.8	45	68.1
23	47.3		

Appendix E. Head cholinesterase values for twenty-one day old honey bees sampled during June-July 1976

Sample	Soluble head cholinesterase (mU/ml)	Sample	Soluble head cholinesterase (mU/ml)
1	78.2	19	64.4
2	57.9	20	58.3
3	72.1	21	79.8
4	75.8	22	64.0
5	61.2	23	76.1
6	60.5	24	63.5
7	64.4	25	54.1
8	54.9	26	63.5
9	66.9	27	66.0
10	66.4	28	64.4
11	64.7	29	64.9
12	65.6	30	64.4
13	73.8	31	58.5
14	73.8	32	67.1
15	66.6	33	66.6
16	48.2	34	76.7
17	68.4	35	71.6
18	85.0	36	55.1

Appendix F. Cholinesterase values of fourteen day old honey bees frozen for a 48 day period before assay

Sample	Soluble head cholinesterase (mU/ml)
1	64.73
2	65.53
3	88.19
4	73.39
5	67.20
6	80.74
7	63.46
8	30.18
9	83.24
10	44.61
11	49.51
12	76.34
13	36.47
14	69.92
15	71.98
16	68.46
17	83.87
$\bar{x}$	65.8
Standard deviation	16.7

Appendix G. Head cholinesterase levels of 21 day old honey bees treated with malathion 50% EC

Sample	Malathion:water concentration	Soluble head cholinesterase (mU/ml)
1	Control (water only)	76.35
2	" " "	52.46
3	" " "	53.27
4	" " "	55.35
5	" " "	53.27
1	1:500	50.09
2	"	55.35
3	"	44.42
4	"	51.26
5	"	45.12
6	"	55.77
1	1:50	22.06
2	"	17.27
3	"	16.37
4	"	2.74
5	"	7.80
6	"	4.24
1	1:5	0.07
2	"	2.74
3	"	6.77
4	"	0.66
5	"	0.66
6	"	0.36
1	1:1	11.03
2	"	18.64
3	"	18.30
4	"	12.10
5	"	8.83
6	"	11.89

Appendix H. Soluble head cholinesterase levels of honey bees of different ages sampled during November 1977

Sample	Soluble head cholinesterase (mU/ml)	Sample	Soluble head cholinesterase (mU/ml)
1 day		14 day	
1	49.73	1	13.89
2	46.67	2	16.93
3	52.77	3	20.54
4	40.84	4	8.16
5	42.44	5	13.47
6	42.93	6	8.27
7	41.32	7	0
8	52.19	8	2.95
9	46.67	9	5.84
10	46.67	10	9.08
11	50.47	11	0
12	51.99	12	18.72
Mean	47.06	Mean	9.82
SD	4.40	SD	6.98
7 day		21 day	
1	30.95	1	6.22
2	51.80	2	0.39
3	35.12	3	0
4	39.74	4	7.22
5	29.36	5	1.37
6	33.28	6	4.43
7	24.81	7	4.53
8	45.97	8	5.12
9	32.17	9	7.12
10	26.55	10	4.53
11	19.74	11	5.92
12	57.88	12	5.12
Mean	35.61	Mean	4.33
SD	11.33	SD	2.46

Appendix I. Soluble protein and cholinesterase assay replicates measured using the head of a one day old honey bee

Replication	Soluble head cholinesterase (mU/ml)	Soluble head protein (mg/ml)	Cholinesterase ÷ protein (mU/mg)
1	51.27	0.56	91.6
2	54.54	0.59	92.4
3	52.78	0.61	86.5
4	49.25	0.58	84.9
5	51.27	0.57	89.9
6	51.67	0.50	103.3
Mean	51.8	0.57	91.4
SD	1.76	0.04	6.5
CV	3%	7%	7%

Appendix J. Statistical comparison of head cholinesterase levels for four honey bee age groups using an unpaired "t" test

Age of honey bee samples	Sample size	$\bar{x}$	Standard deviation	t	p
1 day	54	68.0	8.23		
				0.933	<0.4
7 day	44	69.8	10.41		
1 day	54	68.0	8.23		
				0.490	>0.5
14 day	45	67.0	10.86		
1 day	54	68.0	8.23		
				1.03	<0.4
21 day	36	66.2	7.88		
7 day	44	69.8	10.41		
				1.23	<0.4
14 day	45	67.0	10.86		
7 day	44	69.8	10.41		
				1.75	<0.1
21 day	36	66.2	7.88		
14 day	45	67.0	10.86		
				0.390	>0.5
21 day	36	66.2	7.88		

Appendix K. Soluble bee head cholinesterase levels taken from bees sampled May 18, 1977, during an aerial malathion application at Bird's Hill Park (BHP) with the adjacent number indicating the sampling site

Sample		Soluble head cholinesterase (mU/ml)
BHP-1:	1	1.02
	2	1.66
	3	2.16
	4	1.32
	5	0*
	6	0*
	7	41.89
	8	0*
Mean		5.47
Standard deviation		13.7
BHP-2:	1	10.40
	2	1.67
	3	14.92
	4	1.18
	5	4.53
	6	8.65
	7	20.00
	8	11.59
	9	10.81
Mean		9.3
Standard deviation		6.1
BHP-3:	1	9.10
BHP-4:	1	0*
	2	0*
	3	0.03
	4	0*
	5	0*
	6	17.52
	7	0*
	8	0*
	9	0*
Mean		1.95
Standard deviation		5.84
BHP-5:	1	8.78
	2	7.75
	3	1.27
	4	1.12
	5	3.15
	6	8.16
	7	4.30
	8	0*
	9	2.90
Mean		4.20
Standard deviation		3.3

Continued .....

Appendix K (Continued)

Sample		Soluble head cholinesterase (mU/ml)
BHP-6:	1	11.74
	2	7.64
	3	14.06
	4	11.22
	5	7.64
	6	11.53
	7	8.04
	8	6.73
	9	6.13
Mean		9.40
Standard deviation		2.8

Sample		Soluble head cholinesterase (mU/ml)
BHP-8:	1	3.65
	2	6.98
	3	3.90
	4	28.68
	5	18.79
	6	16.40
	7	31.83
	8	5.03
	9	4.38
Mean		13.29
Standard deviation		11.1

BHP-7:	1	16.95
	2	19.87
	3	26.92
	4	18.89
	5	15.39
	6	14.57
	7	14.95
	8	16.84
	9	11.50
Mean		17.30
Standard deviation		4.4

BHP-9:	1	12.82
	2	4.39
	3	5.39
	4	15.03
	5	5.98
	6	3.40
	7	3.40
	8	2.42
	9	0.95
Mean		5.98
Standard deviation		4.8

Continued .....

Appendix K (Continued)

Sample	Soluble head cholinesterase (mU/ml)
BHP-10: 1	4.24
2	6.23
3	2.81
4	6.50
5	15.65
6	5.01
7	7.00
8	4.02
9	8.00
Mean	6.61
Standard deviation	3.8

Sample	Soluble head cholinesterase (mU/ml)
BHP-11: 1	4.28
2	10.47
3	1.51
4	5.08
5	2.99
6	1.90
7	2.99
8	1.11
9	3.48
Mean	3.76
Standard deviation	2.8

Sample	Soluble head cholinesterase (mU/ml)
BHP-12: 1	14.16
2	16.22
3	57.08
4	3.68
5	5.28
6	25.76
7	10.36
8	15.78
9	16.66
Mean	18.33
Standard deviation	18.3

Sample	Soluble head cholinesterase (mU/ml)
BHP-13: 1	5.19
2	1.58
3	2.62
Mean	3.13
Standard deviation	1.9

Continued .....

Appendix K (Continued)

Sample	Soluble head cholinesterase (mU/ml)
BHP-14: 1	5.48
2	7.28
3	2.32
4	1.44
5	1.00
6	2.22
7	20.54
8	24.56
9	17.74
Mean	9.18
Standard deviation	9.2
BHP-15: 1	12.24
2	4.51
3	9.31
4	10.53
5	23.55
6	2.12
7	4.74
8	6.88
Mean	9.20
Standard deviation	6.7
BHP-16: 1	5.91
2	4.42
3	4.42
4	4.62
5	5.91
6	4.12
Mean	4.90
Standard deviation	0.80
BHP-17: 1	8.81
2	9.11
3	4.62
4	32.85
5	7.40
6	35.88
7	4.91
Mean	14.80
Standard deviation	13.5

*Null values indicate that the enzyme levels measured equaled the lower limits of detectability of the technique.