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THE UNIVERSITY OF ALBERTA

FACTORS INFLUENCING THE SUSCEPTIBILITY OF TWO STRAINS OF *SITOPHILUS GRANARIUS* (L.) TO 1,2-DIBROMOETHANE (EDB)

BY

© CLIFFORD ROY ELLIS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ENTOMOLOGY

EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled FACTORS INFLUENCING THE SUSCEPTIBILITY OF TWO STRAINS OF *SITOPHILUS GRANARIUS* (L.) TO 1,2-DIBROMOETHANE (EDB) submitted by Clifford Roy Ellis in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Supervisor

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External Examiner

Date. September 24, 1970

ABSTRACT

The susceptibility of two strains of *Sitophilus granarius* (L.) to 1, 2-dibromoethane (EDB) is investigated. The LWR strain, selected for 67 generations for resistance to methyl bromide, is 1.9 times more tolerant to EDB than the LWN strain that has no history of fumigation.

The LWR insects are 1.3 times heavier than the LWN strain and approximately 25 per cent of the difference in susceptibility between the LWN and LWR strains is correlated with this difference in weight. A part of the tolerance of the heavier insects is shown to result from weight-dependent respiration and fumigant uptake. There is no significant difference in respiration rate between LWN and LWR insects of the same weight.

Inert storage of EDB in lipids is not a factor in determining strain susceptibility to EDB fumigation and there is no significant difference in the total lipid of LWN and LWR insects of the same weight. LWR insects are more resistant to starvation and survive longer after fumigation with EDB than LWN insects. Lipid is consumed during this interval and its possible importance in endurance and vigor tolerance is duscussed.

True resistance of LWR insects to EDB fumigation is shown by a slower rate of uptake and a faster rate of metabolism of radio-labelled EDB than in LWN insects of the same weight. Vigor tolerance and true resistance of *S. granarius* to EDB fumigation are discussed.

ACKNOWLEDGEMENTS

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I also wish to thank Dr. Y. S. Krishnan and my fellow graduate students for advice and criticism, my wife, Claude, for proof reading the thesis and J. S. Scott for the photographic reduction of the figures.

The insect colonies used in this study were kindly provided by the late Dr. H. A. U. Monro of the Pesticide Research Institute, London, Ontario.

The first year of the project I received a graduate research assistantship for which I am thankful.

AUTOBIOGRAPHICAL SKETCH

I was born on January 7, 1941 in Yarmouth County, Nova Scotia, where I obtained senior matriculation in 1959. I graduated from the two year degree program of the Nova Scotia Agricultural College, Truro, Nova Scotia in 1961 and from Macdonald College of McGill University with a B. Sc. (Agr.) in 1963 and won the Lochhead Memorial Prize in 1963 for achievement in entomology.

Under the supervision of Dr. F. O. Morrison, I obtained an M. Sc. degree in the field of economic entomology from McGill University in 1965.

From 1965 to 1967 I was an extension entomologist with the Nova Scotia Department of Agriculture and Marketing. Responsibilities included control recommendations and extension programs for insect pests of small fruit and vegetable crops.

I enrolled in the Ph. D. program of the Department of Entomology of the University of Alberta in September 1967. Since 1968, I was extension assistant in entomology for this university.

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I. INTRODUCTION

1. LITERATURE REVIEW

Insect resistance to insecticides has been reviewed by Brown (1958, 1961 and 1968), Chapman (1960), Busvine (1960 and 1963), Glass (1960) and many others while the specific case of resistance to insecticidal fumigants was discussed by Lindgren and Vincent (1962) and Monro (1964). The first instance of field resistance of an insect to a fumigant was the resistance in 1916 of the California red scale, *Aonidiella aurantii* (Mask.), to hydrogen cyanide (HCN) (Brown, 1958). Later resistance to HCN developed in two more species of scale insects, *Saissetia oleae* (Bern.) and *Coccus pseudomagnoliarum* (Kuw.) (Quayle, 1943).

Low levels of non-specific resistance have been selected for with fumigants (Monro and Upitis, 1956; Munro, Musgrave, and Upitis, 1961). Page and Lubatti (1963) reviewed the work of Monro and his associates and concluded that the resistant insects were heavier than normal unselected insects, that the slope of the log dosage-probit regression line did not change with increased resistance and that the insects also had increased resistance to fumigants other than those used in selection. They concluded that selection by repeated exposure to methyl bromide "appeared to have been for increased vigor."

The term 'vigor tolerance' as distinct from true resistance originated with the definitions of Hoskins and Gordon (1956). These authors state, "A strain developed by breeding only from the individuals which survive exposure to such diverse stresses as extremes

of temperature, lack of moisture, abnormal food or an injurious chemical will have an altered ability to withstand many kinds of stresses including exposure to chemicals." This change in tolerance, "usually associated with what may be called extra vigor," they called vigor tolerance. Vigor tolerance is characterized by only a 2- to 3-fold increase in the dose lethal to 50 per cent of the test insects (LD50) and decreased heterogeneity. True resistance arises from the breeding of survivors of insecticide treatment that have a greater ability to avoid toxic concentrations of poison at the target by exclusion, metabolism, excretion, etc. Hoskins and Gordon (1956) recognized that no dividing line can be drawn between vigor tolerance and true resistance and that most instances of field resistance are some combination of the two effects. The nature and significance of vigor tolerance are still subject to debate (Grayson and Cochran, 1968).

Very little attention has been given to the study of small differences in susceptibility. O'Brien (1967) mentions the difficulty of finding convincing proof of the causes of susceptibility differences when these are less than four fold. This difficulty largely explains the lack of detailed studies of small susceptibility differences.

The understanding of the tolerance of *Sitophilus granarius* (L.) to methyl bromide and 1, 2-dibromoethane (EDB) is restricted by the limited knowledge of the mode of action and metabolism of these fumigants.

The effect of methyl bromide on enzymes containing SH groups (SH- enzymes) was studied by several authors including Dixon and Needham (1946), Lewis (1948) and Bridges (1955). Winteringham,

Hellyer, and McKay (1958) reviewed these earlier studies and concluded there was no evidence that methyl bromide poisoning was explained by SH-enzyme inhibition or by blocking of coenzyme A. They concluded from their own study with *Musca domestica* (L.) that excessive doses of methyl bromide inhibit triose phosphate dehydrogenase and cause irreversible depletion of *in vivo* muscle ATP. With these excessive doses the insects died without recovering from immobilization. With lower doses, immobilization was temporary and accompanied by a return towards normal levels of thoracic ATP. Insects later died without significant changes in the soluble phosphorus. The mode of action of methyl bromide is probably complex and takes effect through the imbalance of several metabolic processes (Page and Lubatti, 1963).

EDB has also been considered an inhibitor of SH groups (Winteringham and Barnes, 1955). Studies with ^{32}P labelled phosphorylated compounds showed that, unlike methyl bromide, EDB had no effect on tissue ATP (Winteringham and Hellyer, 1954). The mode of action of methyl bromide and EDB are therefore believed to be different.

Methyl bromide and EDB are homologues and one expects some similarity in mode of action and metabolism. Methyl bromide and EDB are similar in their classification as 'chemical poisons' with death due to some biochemical lesion. The higher homologues and the chlorine analogues are less toxic and classified as 'physical poisons' or narcotics (Ferguson and Pirie,1948).

Recently there has been renewed interest in EDB because of its effect on egg production of poultry (Bondi, Olomucki, and Calderon, 1955; Fuller and Morris, 1962; Nachtomi, Alumot, and Bondi, 1968)

and others. Nachtomi, Alumot, and Bondi (1966) studied the metabolism of EDB in rats and found (I) S-(2-hydroxyethyl) cysteine and (II) its N-acetate, N- acetyl - S -(2-hydroxyethyl)- cysteine (a mercapturic acid) and an undetermined metabolite in the urine. The undetermined metabolite was later found to be the S- oxide of (II) namely N- acetyl - S -(2-hydroxyethyl) cysteine S- oxide (Jones and Edwards, 1968; Edwards, Jackson, and Jones, 1970). The initial reaction with EDB is believed to be with reduced glutathione (Thomson, Barnsley, and Young, 1963; Clapp, Kaye, and Young, 1969; Edwards *et al.*, 1970). The production of mercapturic acids by reaction with reduced glutathione followed by hydrolysis and N- acetylation has now been discovered as an important detoxification pathway for a large number of allyl compounds (Clapp *et al.*, 1969). An S- oxide of (I) is produced when (I) is present in large amounts indicating that the formation of the mercapturic acid from (I) is the rate limiting step (Edwards *et al.*, 1970).

2. STATEMENT OF THE PROBLEM

Resistance to insecticides has become a major and widespread problem during the past two decades. Because of the economics of doubling and tripling insecticide doses and the problem of residues, small increases in vigor tolerance can be as important as the more spectacular differences attributed to true resistance. References to vigor tolerance are generally vague but consistently refer to the greater weight of tolerant insects as a contributing factor.

One instance of vigor tolerance is investigated in this study, that of a laboratory selected strain of *Sitophilus granarius* to 1, 2-dibromoethane (EDB). Specific objectives are:

1. to test the hypothesis that a lower respiration rate per mg of tissue and the resulting reduced fumigant uptake of heavier tolerant insects accounts for the differences in susceptibility to EDB between the normal and tolerant strain.
2. to measure total lipid before and after fumigation and to determine if lipid content is a factor determining tolerance to EDB by:
   A. protection by inert storage of the fumigant in lipids
   B. increasing endurance to stress conditions including starvation and fumigation.
3. to compare the uptake and metabolism of ^{14}C labelled EDB in the tolerant and normal strain.

II. GENERAL MATERIALS AND METHODS

1. TEST INSECTS

The test insects were granary weevils, *Sitophilus granarius* (L.). Three strains of this species were obtained from Dr. H. A. U. Monro of the Pesticide Research Institute, London, Ontario. These were: 1. the London Wild Normal strain (LWN), a strain with no history of fumigation; 2. the London Wild Resistant strain (LWR), a strain selected for 67 generations for resistance to methyl bromide fumigation; 3. the Guelph strain (GGA), a strain selected for resistance for 20 generations but with no exposure to fumigants for 44 generations after selection was suspended. The LC50's of these strains were 3.6, 26.0 and 11.0 mg of methyl bromide/liter respectively with a 5 hr exposure at 25 C (H. A. U. Monro, personal communication).

The LWR strain was 5.5 ± 0.2 times more resistant to methyl bromide than the LWN strain and 3.5 ± 0.2 times more resistant to EDB in 1961 (Monro *et al.*, 1961). *Sitophilus granarius* is more susceptible to methyl bromide than to EDB. The LC50 for LWN insects was 2.85 mg of EDB/liter with a 5 hr exposure at 25 C (Monro *et al.*, 1961).

2. REARING AND HANDLING TEST INSECTS

Test insects were reared at 26.5 ± 1.5 C and 60 ± 5 per cent relative humidity. A continuous supply of two-week old weevils was maintained by a rearing program similar to that of Ellis and Morrison (1967).

The culture food was hard wheat that was sieved through 10 mesh screen to remove small grains, steam sterilized at 20 lbs pressure for 15 min, cooled and sealed in air tight containers. The water content of the sterilized wheat was determined and adjusted to 12.6 per cent (Richards, 1947).

One pint Mason jars were used for rearing weevils. The jars contained approximately 400 gm of wheat and were sealed with metal lids with 4.5 cm brass screened holes.

Three hundred two-week old adults of mixed sex were used as breeding stock. These were placed on wheat for one week and then removed with 10- and 40- mesh brass screens which separated the wheat from the weevils and the debris respectively. One or two cultures of each of the three strains were started each week. Adults were used for egg-laying for four weeks and then were replaced by 300 more two-week old weevils of the same strain. Although leaving the cultures of immature weevils undisturbed results in larger weevils (Richards, 1947), cultures were shaken at weekly intervals as this has a beneficial effect on emergence and helps to avoid excessive amounts of carbon dioxide and debris in the cultures (Morrison, 1964). Cultures were also shaken two hours before sieving off the adults as this is a stimulus for emergence. When undisturbed, adult emergence may be considerably delayed (Richards, 1947).

The cultures were sieved after five weeks to remove debris and any early emerged adults. The weevils of the first week of emergence that were of the same strain were combined on 400 grams of prepared wheat. Cultures were discarded after their use for weevil production for one week.

Mite problems in the cultures were kept at a minimum by using sterilized wheat and by oven heating the equipment before using it to start new colonies. An infestation was eliminated by regular screening of the cultures, hand picking breeding stock and by using Kelthane (4, 4'-dichloro-α-trichloromethylbenzhydrol) on the culture shelves (Strong, Pieper and Sbur, 1959). The wire mesh used to ventilate the top of the culture jars was twice as fine as that required to confine the weevils and this kept out stray insects and reduced mite infestation.

3. SEX IDENTIFICATION

Females of *S. granarius* have a longer rostrum than the males (Halstead, 1963) but this character was not used because of difficulties when identifying the sex of both small LWN and large LWR insects. The terminal abdominal segments of the male are more strongly flexed ventrally than in the females (Halstead, 1963) and this character and the more angular tip of the male pygidium are constant in both the LWN and LWR strains (Qureshi, 1963). These characters were used to identify sex when the insects were alive and could not be killed. When in doubt the pygidium was raised and the sex determined on the basis of the last tergite (Richards, 1947). When insects were dead or could be killed the genitalia were removed and examined.

4. FUMIGATION APPARATUS AND PROCEDURE

a. Method I

Fumigation chambers were one quart Mason jars fitted with wire screen cages similar to those used by Ellis and Morrison (1967) to fumigate *S. granarius* with EDB (Fig. 1). Because testing was restricted to *S. granarius*, 20- mesh brass screen was used to construct the exposure cages rather than the 40-mesh screen used by the above authors. The exposure cages were brass screen cylinders 2.5 cm in diameter and 5 cm long soldered under the Mason jar lids. Stainless steel planchets (Planchets Incorporated, Chelsea, Michigan) fitted tightly into the bottom of the screen cylinder. A boiler screw through the planchet provided a handle for pulling the cage bottom out of the screen cylinder.

A 6.3 mm (¼ in) hole through the Mason lid served as a port for placing the insects in the exposure cage in the sealed jar. The hole was sealed with a bolt with a tap washer gasket. The bolt was screwed through the hole into a nut which was soldered on the underside of the Mason lid within the screen cage. At the time of exposure the insects were tipped through the hole in the Mason lid into the exposure cage attached underneath. After exposure, the insects were recovered by removing the steel planchet cage bottom from within the screen cylinder. The inside of the Mason lid, the nut and the part of the screen cylinder soldered to the Mason lid were painted with a polyurethane paint that was resistant to ethylene dibromide (Coggiola

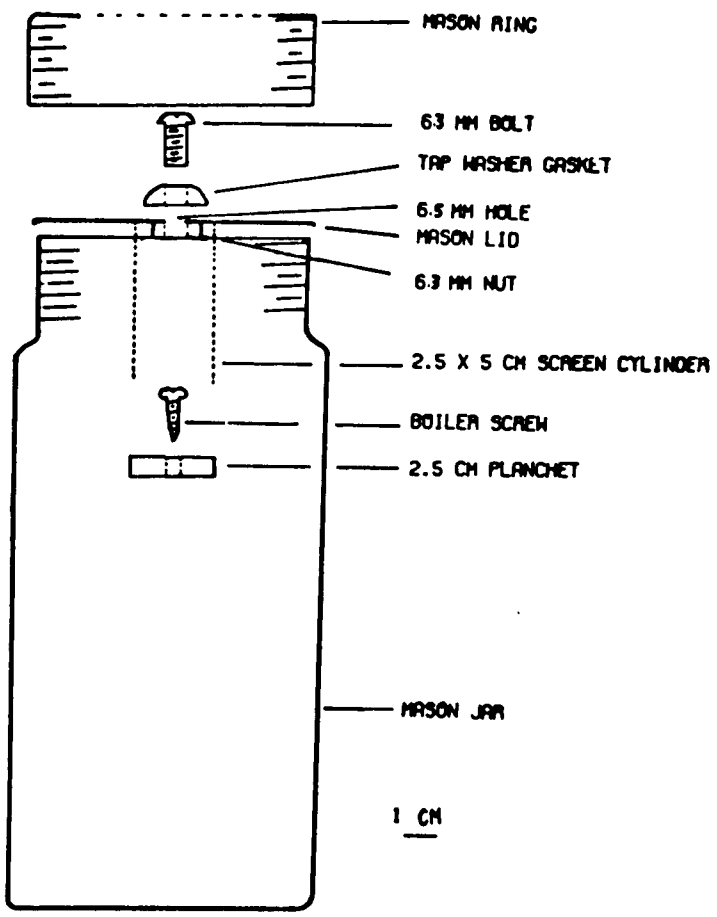

Fig. 1. Expanded view of a Mason jar fumigation chamber

and Huelin, 1964; López D. and Murcio Velasco, 1969).

The fumigant doses were applied with a fine glass capillary tube as described by Ellis and Morrison (1967). The standard procedures of the above authors were also used in fumigating the insects. The pre-exposure and post-exposure cages were screw top vials and 5 dram plastic vials respectively. Ventilation was provided in the pre-exposure vials through a screened hole in the Bakelite cap and in the post-exposure cages by needle-holes in the plastic, snap-on lids. Forty insects were used per chamber.

When this method was used, doses were expressed in mg/liter or mg/chamber.

b. Method II

Florence flasks of 6.5 liter capacity were used in an alternative fumigation apparatus (Fig. 2). These flasks were held in a constant temperature bath with bricks placed on a wooden collar that fitted around the necks of the flasks. The fumigant was placed in the flasks with a micro pipette or micro syringe. One or more 2.5 by 10 cm brass screen cylinders with planchet ends were used in each flask as insect exposure cages. These were suspended by threads that were jammed in the neck of the flasks by the stopper when the flasks were sealed. The rubber stopper was fitted with a glass stopcock for letting off the pressure arising from inserting the stopper in the flasks. A 7 cm Teflon coated magnet was placed in the flasks before they were sealed. A magnetic stirrer placed under the water bath guaranteed

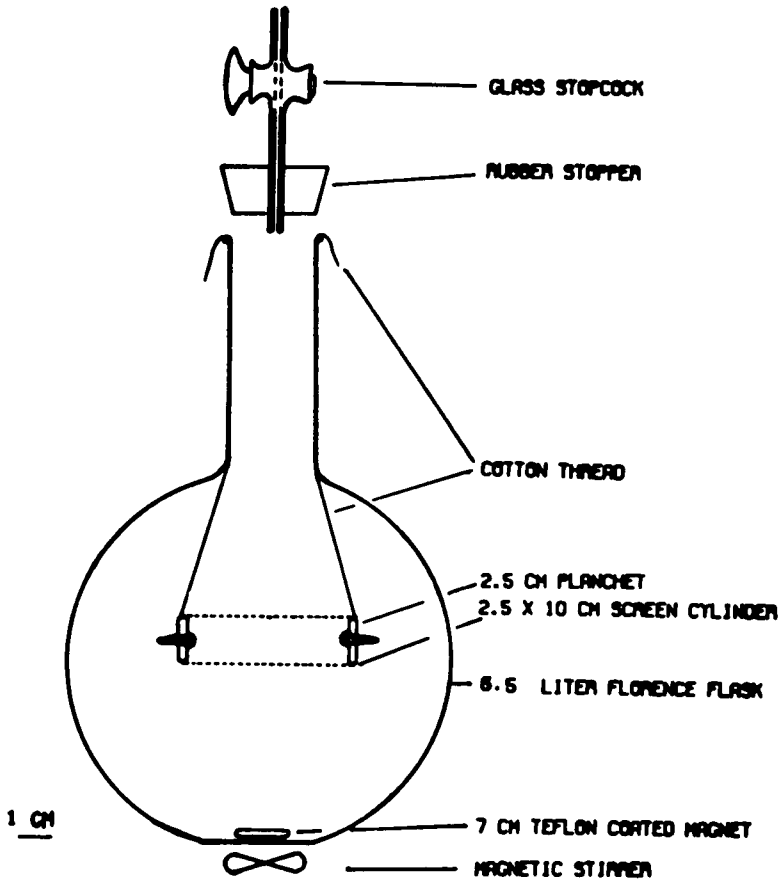

Fig. 2. Fumigation chamber made with a Florence flask

rapid vaporization of the fumigant and its even distribution.

The flasks were placed in the water bath and the stirrer operating within five minutes of applying the fumigant. Vaporization of the fumigant was rapid under these conditions.

These large flasks were especially suited for fumigating large numbers of insects. Because several exposure cages could be suspended in a single flask it also enabled better comparisons between groups of insects. The water bath also improved the reproducibility of fumigant results of different days.

When this method was used, doses were expressed in µliters/liter.

5. ANALYSIS OF DATA

Data were analysed on the University of Alberta, IBM/360 model 67 computer.

Probit analysis was performed using the FORTRAN IV program of Daum and Killcreas (1966) and R. J. Daum (personal communication). I modified this program by using IBM subroutines and by the addition of a subroutine to store and punch probits, log dosages and regression information on computer cards. This permitted drawing graphs with a model 770/663 CalComp plotter with no further card punching or treatment of the data. This same program for the CalComp plotter was flexible enough to enable drawing other graphs as well. This program with a description is now Computer Program No. 5 of the Department of Entomology. A second CalComp plotter program was prepared to draw

labels of various sizes (Department of Entomology, Computer Program No. 4).

Descriptive statistics, correlation, regression etc. were done using APL and University of Alberta programs (Smillie, 1969).

III. THE WEIGHT AND WATER CONTENT OF *SITOPHILUS GRANARIUS*

1. MATERIALS AND METHODS

The mean weight of strains and sexes of *S. granarius* was determined from two-week old adults weighed for lipid determination and for response to fumigation.

The water content was determined in tared aluminium pans containing a short piece of glass rod. Twenty insects were added to each pan and the pan, glass rod and insects weighed. The insects were crushed in the pans with the glass rod which was left in the pan. Samples were dried to constant weight at 100 C. The experiment was replicated three times.

2. RESULTS

a. Weight of strains and sexes

The mean weight and standard deviation of males and females of LWN and LWR *S. granarius* are given in table I.

The pooled data of the three replicates of table I are shown in Fig. 3.

b. Water content

The water content as a percentage of insect wet weight of the LWN, GGA and LWR insects is shown in table II with the mean weights of the three strains.

Table I. Weights of males and females of LWN and LWR *Sitophilus granarius* in mg

	Strains			
	LWN		LWR	
Replication	Males Mean ± SD* (N)**	Females Mean ± SD (N)	Males Mean ± SD (N)	Females Mean ± SD (N)
1	2.70 ± 0.34 (46)	2.55 ± 0.36 (52)	3.73 ± 0.41 (45)	3.41 ± 0.58 (50)
2	2.71 ± 0.34 (47)	2.59 ± 0.28 (49)	3.55 ± 0.35 (46)	3.68 ± 0.51 (50)
3	2.78 ± 0.22 (45)	2.62 ± 0.34 (54)	3.65 ± 0.45 (36)	3.58 ± 0.41 (66)
Pooled	2.73 ± 0.30 (138)	2.59 ± 0.33 (155)	3.64 ± 0.43 (127)	3.56 ± 0.51 (166)

* SD - standard deviation

** (N) - number of insects in sample

Pooled data shown in Fig. 3

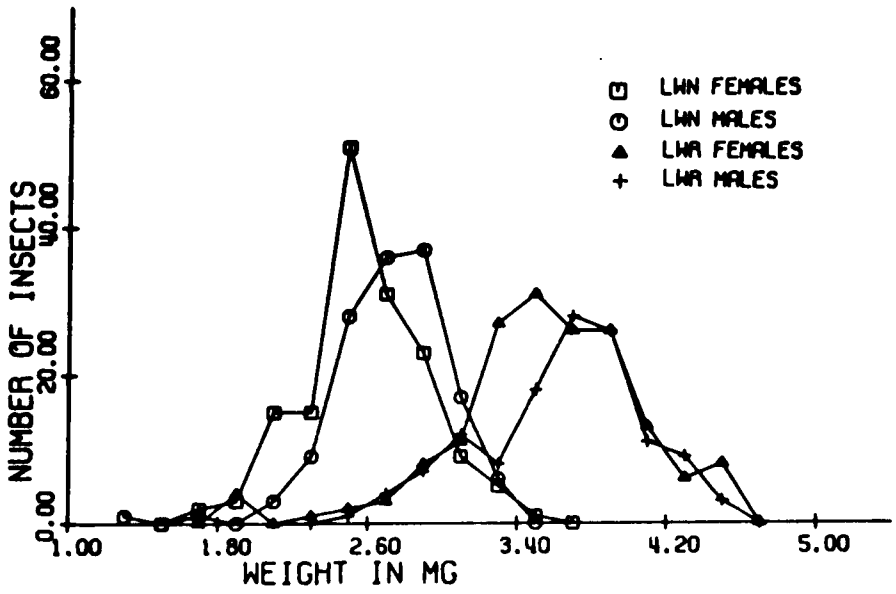

Fig. 3. The weight distribution of females and males of LWN and LWR *Sitophilus granarius*

Means and standard deviations in table I

Table II. Water content as a percentage of insect wet weight of LWN, GGA and LWR *Sitophilus granarius*

Strain	Per cent water Mean ± SD*	Mean wet weight (mg)
LWN	50.12 ± 0.62	2.95
GGA	49.58 ± 0.60	3.62
LWR	48.98 ± 0.82	3.63

* Standard Deviation - based on three samples of 20 insects

3. DISCUSSION

The males of both the LWN and LWR strains are heavier than the females (Fig. 3). Table I shows that the differences in mean weight between the sexes is 0.14 mg for LWN and 0.08 mg for LWR insects. These differences are not significant at the 5 per cent level because of the large standard deviations. Sevintuna and Musgrave (1961) working with the GG strain also noted that males appeared larger than the females but there was no significant difference in weight.

Monro *et al.* (1961) found that the mean weights of the LWN and LWR strains of mixed sexes were 2.87 and 3.56 mg respectively, a difference in weight of 0.69 mg. This difference is less than I found (0.91 mg for males and 0.97 mg for females). The LWR strain was 1.3 times heavier than the LWN strain. Richards (1947) showed that the weight of *S. granarius* varies according to the length of time for adult emergence. In this study, only one week of emergence was used from any culture and this bias in selecting test insects explains the differences between my results and those of Monro *et al.* (1961).

The increase in weight of tolerant strains of *S. granarius* was reported for a Montreal wild strain selected for lindane resistance (Orr, 1958) and for a pyrethrum resistant strain (Lloyd and Parkin, 1963). No decrease in weight was found during six generations of selection with allethrin and piperonyl butoxide (Sevintuna and Musgrave, 1961).

Wigglesworth (1953) gives the water content of *S. granarius* as 46 to 47 per cent. The low water content as compared with other insects he attributes to the high contribution of insect cuticle to total

weight. He also states that water content is influenced to some extent by fat content. Table II shows a trend for the more tolerant insects to have greater mean weight and lower water content. The water contents of table II, however, are not significantly different.

IV. MORTALITY OF *SITOPHILUS GRANARIUS* FOLLOWING FUMIGATION WITH 1, 2-DIBROMOETHANE, EDB

1. MATERIALS AND METHODS

Fumigation method II was used to determine the mortality of the two strains and sexes at several intervals after fumigation. Two exposure cages were used in each flask, one for LWN and one for LWR insects. After fumigation, insects were placed on a wheat diet in post-exposure cages. Within 12 hours after exposure the insects were individually weighed and insects of the same treatment and weight were placed together in post-exposure cages. All the counting and weighing was done in a room at 27 C and 40 per cent relative humidity.

The effect of fumigant dose and post-exposure starvation on the LT50 of EDB fumigated insects was determined by fumigating lots of 60 insects of one strain in quart Mason jar fumigation chambers. These insects were divided into two lots of 30 insects after fumigation and one group was fed wheat grains while the other group received neither food nor water.

2. RESULTS

a. Susceptibility of strains and sexes to EDB

The susceptibility of the LWN and LWR strain was calculated from mortality determinations made 20 days after fumigation with EDB (Fig. 4). At least 30 insects were used to determine mortality at each concentration of fumigant. The LC50's with 95 per cent confidence

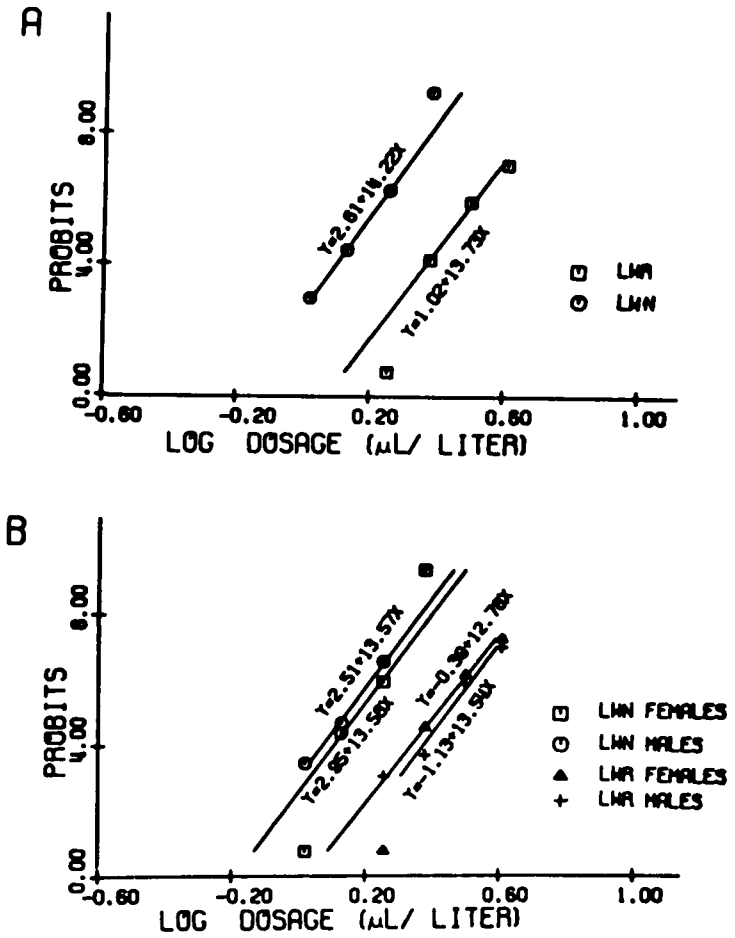

Fig. 4. Log dosage-probit regression lines for *Sitophilus granarius* from mortality determinations 20 days after fumigation with EDB

A. sexes combined; B. sexes treated separately

Data of A from table III

limits for the LWN and LWR strains were 1.46 ± 0.05 and 2.75 ± 0.09 µliters/liter respectively. Under the test conditions the LWR strain was 1.9 times more tolerant than the LWN strain to EDB fumigation.

The susceptibility of the LWN and LWR strains to fumigation by EDB was determined at intervals from 5 to 20 days after fumigation. The regression formulae, LC50's with 95 per cent confidence limits and the susceptibility ratios are given in table III. The susceptibility ratio was calculated by dividing the LC50 of the LWR strain by that of the LWN strain.

Figure 5 shows the LC50 of male and female LWN insects determined from mortality counts made at four times after fumigation with EDB. The best fit to a straight line was obtained when the LC50 in µliters/liter was in logs and the time after fumigation also in logs.

b. Relationship between insect weight and susceptibility to EDB

Insects of mixed sex that were weighed after fumigation with EDB were used to determine the LC50's of several weight classes of LWN and LWR insects. Figure 6 shows the LC50's of four weight classes of LWR insects and two weight classes of LWN insects, the mean LC50 for all insects of each strain and the regression line of best fit for the LC50 as a function of insect weight for LWR insects. The range of weights in each weight class was 0.1 mg on either side of the mean. No regression line was determined for the LWN insects because only two weight classes gave significant log dosage-probit regression.

Table III. The susceptibility of LWN and LWR *Sitophilus granarius* to EDB at six intervals after fumigation

Interval days	Strain*	Regression formula (Y = µliter/liter, X = probits)	LC50 ± 95% confidence limits	Susceptibility ratio (LWR/LWN)
5	LWN	Y = 3.16 + 5.51X	2.16 ± 0.73	
	LWR	NSG**	5.0***	2.3***
7	LWN	Y = 1.83 + 12.56X	1.79 ± 0.07	
	LWR	Y = 1.27 + 6.22X	3.97 ± 0.26	2.21
10	LWN	Y = 2.28 + 12.15X	1.67 ± 0.27	
	LWR	Y = 0.57 + 8.53X	3.30 ± 0.14	1.98
12	LWN	Y = 2.54 + 11.81X	1.62 ± 0.07	
	LWR	Y = 0.01 + 10.38X	3.02 ± 0.11	1.87
15	LWN	Y = 2.63 + 11.74X	1.59 ± 0.06	
	LWR	Y = 0.42 + 11.56X	2.94 ± 0.10	1.85
20	LWN	Y = 2.61 + 14.22X	1.46 ± 0.05	
	LWR	Y =-1.02 + 13.73X	2.75 ± 0.09	1.88

* Sexes not separated ** NSG - non-significant regression *** estimate

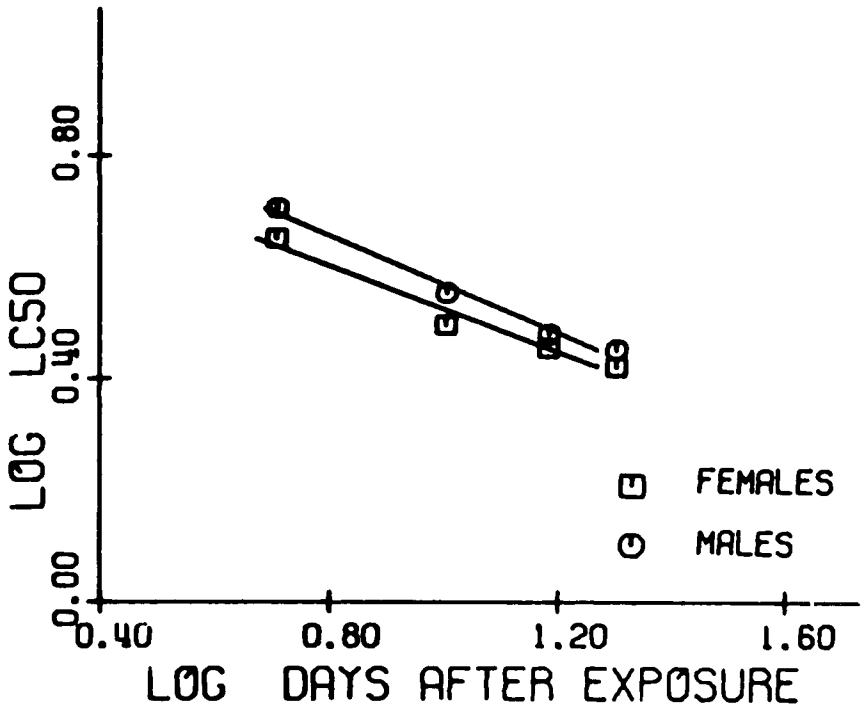

Fig. 5. Regression of log LC50 in µliter/liter on log interval between fumigation with EDB and mortality determinations for males and females of LWN *Sitophilus granarius*

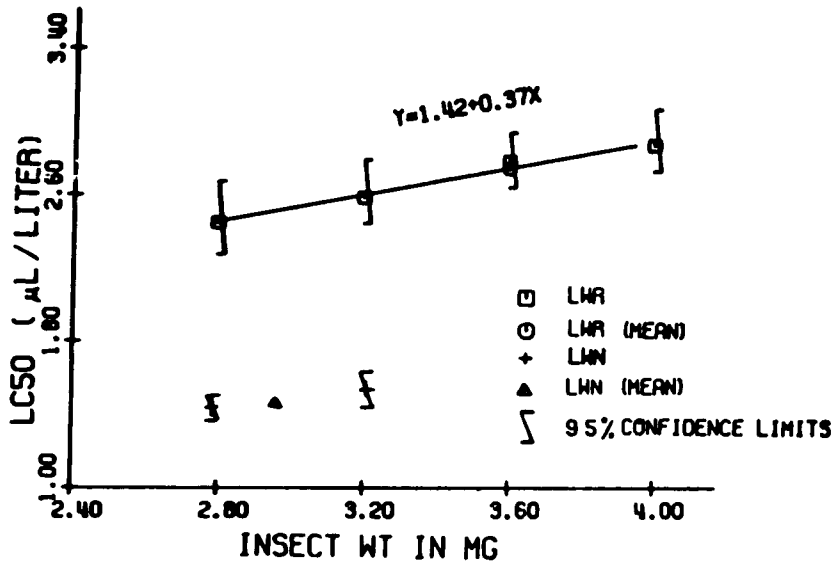

Fig. 6. Susceptibility of LWR *Sitophilus granarius* in µliters/liter as a function of their weight in mg compared with that of LWN insects

c. Interval between fumigation with EDB and mortality

Fumigation method I was used to determine the LC50 of starved and fed insects after treatment with various concentrations of EDB. Figure 7 shows the LT50 in days after fumigation and the dose of EDB in mg/chamber (913 ml) for the LWN and LWR strains fed or starved. The linear regression and correlation analyses of the data of Fig. 7 are shown in table IV.

3. DISCUSSION

Under my test conditions with mortality counts made 20 days after fumigation, the LWR strain was 1.9 times more resistant than the LWN strain (Fig. 4). Table III shows that the susceptibility ratio is dependent on the interval between fumigation with EDB and mortality determination. The LWN insects die sooner than the LWR insects so that mortality determinations made within 12 days after fumigation with EDB gave a misleading tolerance ratio. There was no significant change in the tolerance ratio after 12 days.

Increasing the time interval between fumigation with EDB and mortality determination results in a steeper slope of the log dosage-probit regression lines and narrower confidence limits of the LC50's (table III).

Figure 4B suggests that the LWN females are more tolerant than the LWN males but that LWR males are more tolerant than LWR females. The 95 per cent confidence limits of the LC50's of the sexes overlap however and the LC50's are therefore not significantly different.

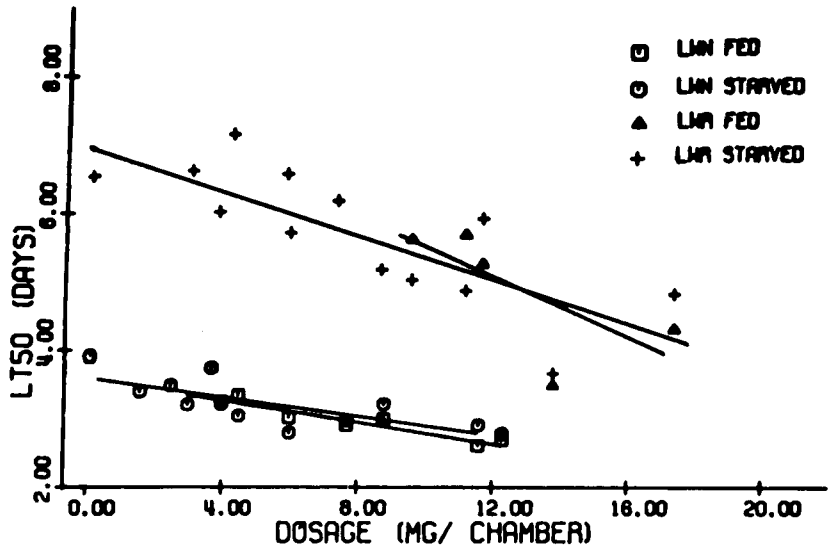

Fig. 7. The LT50 of LWN and LWR *Sitophilus granarius* fed wheat or starved after fumigation with EDB

Linear regression and correlation data are in table IV

Each point based on 40 insects

Table IV. Linear regression and correlation analyses of LT50 in days (Y) on the dosage of EDB in mg/chamber (X) for starved and fed LWN and LWR *Sitophilus granarius*

Strain	Diet	Regression equation	Standard error of slope	Correlation coefficient	*N
LWN	Wheat	Y = 3.59 - 0.08X	0.01	-0.94 (.01)**	7
	Starved	Y = 3.61 - 0.07X	0.02	-0.76 (.01)	12
LWR	Wheat	Y - 7.74 - 0.22X	0.13	-0.70 (not)	5
	Starved	Y = 6.96 - 0.16X	0.04	-0.78 (.01)	13

* number of data points

** level of significance

The regression lines are shown in Fig. 7

Males of *S. granarius* are more tolerant to methyl bromide than females (Brudnaya, Chudinova, and Anoskina, 1966) and male pupae and adults of *Tribolium confusum* Duv. are more tolerant to EDB than females (Loschiavo, 1960). The males of both the LWN and LWR strain of *S. granarius* are heavier than the females (Fig. 3) and this explains the findings of Brudnaya *et al.* (1966) with methyl bromide and my findings with EDB on the LWR strain. The LC50's of males and females of the LWN strain are not significantly different and no conclusions about the importance of sex differences in body weight in determining the relative susceptibility of the sexes to EDB can be made.

The difference in susceptibility of the two sexes of LWN insects is approximately the same at four intervals after fumigation (Fig. 5) and this indicates that the males and females die at approximately the same rate. The greater weight of the males did not significantly influence the survival time after fumigation with EDB.

The greater the weight of the insects the higher the LC50 (Fig. 6). The regression equation of LC50 in μliters/liter on insect weight for LWN and LWR insects were not colinear. The LWR insects were more tolerant than expected on the basis of their greater weight.

The susceptibility ratio for mortality determinations 20 days after fumigation is 1.88 (table III). Substituting the mean weight of the LWR strain into the regression equation for LWR insects, $Y = 1.42 + 0.37X$ (Fig. 6) gives a mean LC50 for the LWR strain of 2.74 μliters/liter as compared to 2.75 ± 0.09 by actual determination. Substituting the mean weight of the LWN strain into the same equation gives 2.40 μliters/liter as compared to 1.46 ± .05 μliters/liter by

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actual determination. Thus, on the basis of the greater weight of the LWR strain, this strain would be 2.74/2.40 or 1.15 times more tolerant than the LWN strain. This represents approximately 25 per cent of the observed difference in susceptibility between these strains. Seventy-five per cent of the strain difference in susceptibility was not correlated with the greater weight of the LWR strain.

It took longer for the LWR insects to die after fumigation with EDB than the LWN insects (Fig. 7). This was true even at doses ten times the LC50 for the strain. Starved and unfumigated control LWN insects had an LT50 of 3.9 days compared to 6.5 days for the LWR insects. The LWR strain was therefore 6.5/3.9 or 1.6 times more tolerant to starvation than the LWN strain. At doses twice the LC50 there was no difference in survival time between fed and starved insects.

The resistance to starvation is undoubtedly a result of the greater size of the LWR insects because Lloyd and Parkin (1963) selected a pyrethrum resistant strain of *S. granarius* that was 1.26 times heavier than the normal non-selected strain and the strain was 2.0 times more resistant to starvation.

There is a significant correlation between the dose of fumigant applied and the time for 50 per cent of the insects to die (table IV). Increasing the dose lowered the LT50.

Because of the correlation between resistance and the length of survival after fumigation, it is very important to hold the test insects for observation until there is no significant mortality due to the fumigant. The change in the susceptibility ratio with time after fumigation (table III) is caused by the LWR strain dying later

than the LWN insects. Whitney and Harein (1959) state that the best suited post-exposure interval depends on the test insect, the fumigant used and the post-exposure treatment and it must be determined by each researcher. This experiment shows that the optimum post-exposure interval changes with strains of the same species.

I found it convenient on the basis of this experiment to use samples of unfumigated but starved insects as controls. Fumigated insects were held for observation until all starved insects died. In no instance was mortality due to fumigant demonstrated after such a post-exposure interval.

The survival time of strains of *S. granarius* was significantly different and may be useful as an index of susceptibility to fumigation with EDB. I found it convenient to verify the strain of *S. granarius* by starving samples of insects and determining the LT50.

Because there is a correlation between insect weight and per cent mortality due to fumigation with EDB (Fig. 6) and because heavier resistant insects survive longer under stress conditions (Fig. 7) it is invalid to use Abbott's formula (Abbott, 1925) to correct for mortality in the controls. Abbott's formula assumes that natural mortality and mortality due to the treatment are independent probabilities (Finney, 1947), an assumption that is not correct in this instance. For this reason,data were rejected when control mortality was greater than 3 per cent.

V. THE RESPIRATION OF *SITOPHILUS GRANARIUS*

1. MATERIALS AND METHODS

a. Gas chromatograph and accessories

The respiration rate of *S. granarius* was studied using a modification of the gas chromatographic technique described by Whitney and Ortman (1962). A Beckman GC4 chromatograph equipped with a single thermoconductivity detector was used to measure either oxygen or carbon dioxide from a gas sample. Jay and Wilson (1960) used a dual column system with a single detector to measure oxygen and carbon dioxide from the same sample. A dual column was not used in this study because of the added expense. Oxygen and carbon dioxide are also best resolved on separate columns, each of which have their own optimum conditions.

Gas samples were measured and passed to the column of the gas chromatograph (GC) with a 15 µliter Beckman gas sampling valve (Fig. 8). Preliminary experiments showed that 1.8 ml of sample was insufficient to flush the sample loop of the valve and leave an uncontaminated sample in the slider portion of the loop. The inlet port of the valve was therefore cut to 2.5 cm in length. With this modification a sample with less than 0.1 per cent contamination with atmospheric oxygen could be obtained up to 5 seconds after injection of the sample into the sampling valve.

The amount of gas was determined by measuring the recorder peak height because gases produce very sharp peaks and peak height is more reliable than peak area in this case (Ball, Harris, and Habgood,

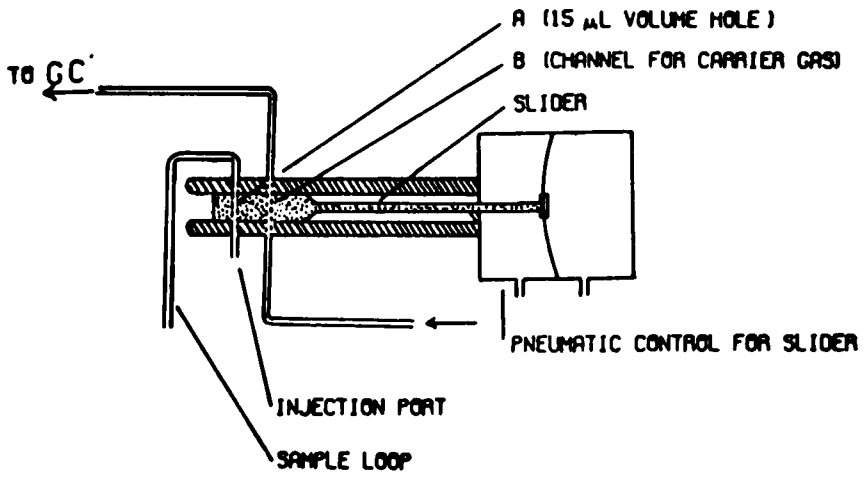

Fig. 8. Gas sampling valve for 15 μliter samples

1968). A zero suppression circuit is indispensable for measuring small changes in the concentration of a gas that is present in a high percentage in the sample as is the case in measuring the oxygen in expired air (Hamilton and Kory, 1960). I used a 1.4 v mercury battery with resistors including a 10 turn 100 units/turn Helipot resistor for this purpose. (This unit was kindly loaned to the author for this study by Mr. R. Brown of Beckman Instruments.) This circuit was connected in series between the recorder leads from the GC so the pen of the recorder was driven down scale by a voltage of from 0 to 800 millivolts as determined by the Helipot resistor.

The zero depression circuit was adjusted by setting the recorder pen to the maximum response (100 units) with the 'zero adjust' of the detector and then applying the necessary voltage with the Helipot control of the depression circuit to set the recorder pen back down scale to zero. This cycle could be repeated to depress the zero to the required 450 recorder units. After adjusting, the depression circuit was turned off and the recorder pen returned to zero with the zero adjust of the detector. The net effect after this adjustment was that the recorder pen was at zero when the depression circuit was turned off but when on, the pen was below the scale and crossed the zero of the recorder paper only with a stimulus equal to 100 recorder units. This circuit enabled using high sensitivity and recording the top of the gas peaks. The unit was turned off between readings to check that the pen had returned to zero. The pen was driven hard down scale except at the moment of the gas peaks but this disadvantage was partly overcome by recording only for five-second intervals when

the oxygen was eluted and loosening the clutch of the recorder to minimize wear.

b. GC columns and operating conditions

Oxygen was resolved on 3.2 mm (1/8 in) outside diameter copper columns. These columns were two meters long and packed with 60-80 mesh, 13X molecular sieve. The carrier gas was helium at a pressure of 2.81 kg/cm^2 (40 psi) and a flow rate of 30 ml/min. The detector temperature was 50 C and the direct current 250 ma. Under these conditions the retention time of oxygen was approximately 50 seconds (Fig. 9C) and a 15 µliter sample of air gave an oxygen peak height of approximately 500 recorder units.

Carbon dioxide was resolved on similar columns one meter long and packed with silica gel. The silica gel was coated with 5 per cent (w/w) Apizone-L stopcock grease to shorten the retention time of the carbon dioxide and to increase the peak height. The carrier gas was helium at the same pressure and flow rate as for oxygen separating columns. The detector temperature was 85 C and the direct current was 275 ma. The retention time was approximately 60 seconds (Fig. 9A).

Slight changes in column pressure and flow rate resulted from using the gas sampling valve. This resulted in an artifact peak at the moment of use of the sampling valve and in a rise of the base line of one recorder unit (Fig. 9B).

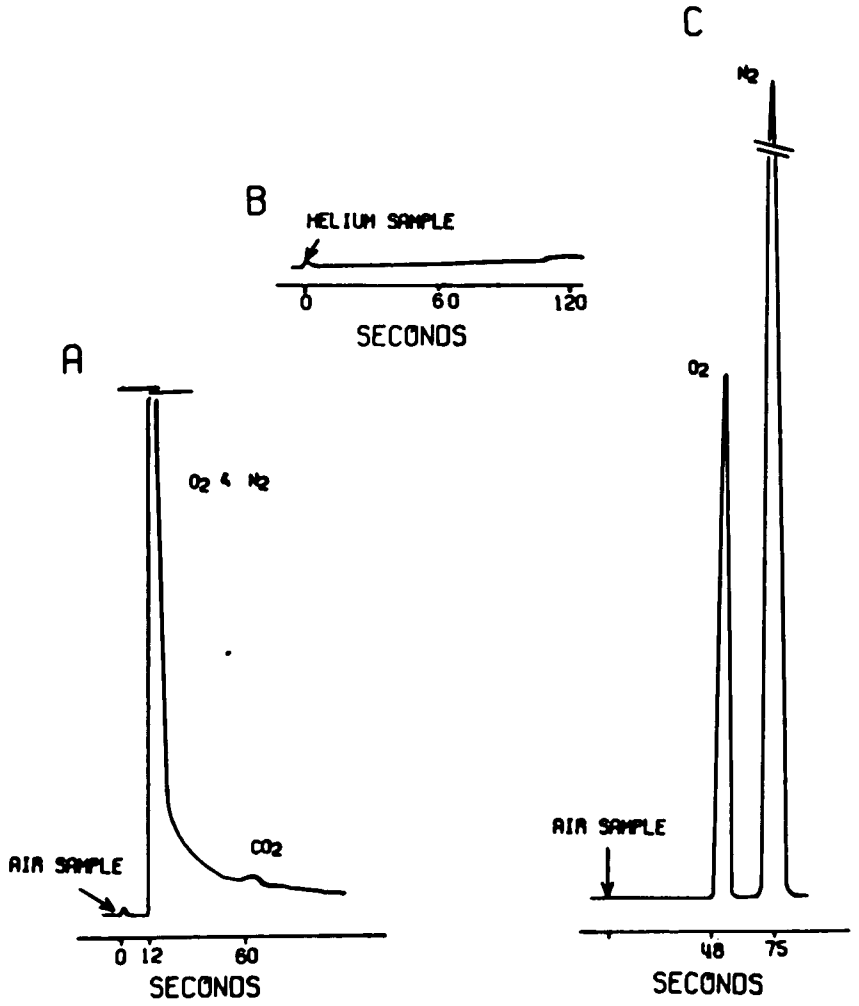

Fig. 9. Resolution and retention times of gas chromatograph columns

A. Separation of carbon dioxide from an air sample

B. Pressure changes only

C. Separation of oxygen and nitrogen from an air sample

A. Silica gel column; B and C. Molecular sieve column

c. Procedure for obtaining gas samples from the insects

In preliminary experiments, test insects were confined in 1.8 cc vials (kind used in positive lock dental syringes) to produce the gas sample (Whitney and Ortman, 1962). These vials were rejected because of the difficulty of keeping them gas-tight, and because 1.8 cc of test gas was insufficient to flush the gas sampling valve of the GC and leave uncontaminated sample for passage to the column.

In all the experiments reported, 5 cc disposable syringes were used. The syringe plungers were removed and the needles imbedded into hard rubber stoppers. The stoppers served to seal the syringe needles and to weight them down in the 27 C water bath. The samples of test insects were placed in the open syringes in the water bath which was then covered with a black plastic film.

After a pre-test period of at least 0.5 hours, the stoppers were removed from the needles, the syringe plungers were inserted and used to fill the syringes with fresh air and the needles resealed with the stoppers. Syringes without insects were the controls.

The test period was from 0.5 to 2 hours depending on the number of insects confined in the syringes. During the test period the syringes containing the insects were kept in the water bath at 27 C in the dark.

After a predetermined test period the rubber stoppers were removed from the syringe needles and the air in the syringes was injected into the port of the gas sampling valve of the GC. By slightly compressing the air in the syringes prior to removing the stoppers,

movement of air was out of the syringes when unstoppered and the contents were left uncontaminated.

d. Recording data

Peak heights were measured to the nearest 0.25 recorder paper divisions and the difference between peak heights for samples with insects and controls without insects was recorded. Because argon is an inert gas there was no need to correct for argon which had the same retention time as oxygen. The difference after substraction represents the oxygen consumed or the carbon dioxide liberated per 15 µliter sample during the test period. The conversion of the peak height differences to µliters of gas at standard temperature and pressure was made from standard curves.

e. Gas standards and standard curves

Gas standards were produced in a 100 ml burette which was modified by breaking off the valve portion and fitting the top with a rubber plunger. The burette was filled with 'heliumated' water. Small amounts of gas were released under the open end of the burette in an heliumated water bath. Volumes were recorded by adjusting the height of the burette in the water bath until the level of the water inside and outside the burette was the same and then reading the volume from the burette scale. Thirty to 40 ml of helium were used and enough oxygen or carbon dioxide to produce the desired concentration.

A small Teflon coated magnet was inserted into the open end

of the burette and the end was sealed with a folded and clamped piece of polyethylene tubing that was slipped over the end of the burette. Tipping the burette end to end caused the magnet to slide back and forth and mixed the gas sample. A 15 cm piece of 4.6 mm diameter polyethylene tubing filled with calcium sulfite was used to dry the sample of gas and pass it to the gas sampling valve. The ends of the above desiccator were fitted with syringe needles that were easily inserted into the polyethylene tubing stoppering the burette and into the port of the sampling valve. The burette was tipped so the water in the burette was at the plunger end and 10-15 ml of gas standard were forced through the desiccator to the sampling valve.

The temperature and barometric pressure were recorded and the amount of oxygen or carbon dioxide in 15 µliters converted to standard temperature and pressure. Samples of pure helium were the controls and gave the amount of contamination of the gas standards with gases dissolved in the water bath or from mixing of the sample with atmospheric air when it was injected into the gas sampling valve. The amount of contamination was substracted from all the other gas standards before correcting to standard temperature and pressure. Figure 10 shows a typical standard curve for converting peak height differences to µliters of oxygen at standard temperature and pressure. After correcting for contamination the line of best fit passed through zero and was linear throughout the range of concentrations used. The sensitivity of the GC was tested every day by analyzing several samples of a single gas mixture. This enabled making minor corrections in the oxygen calibration curve for treatment of the data of a particular day.

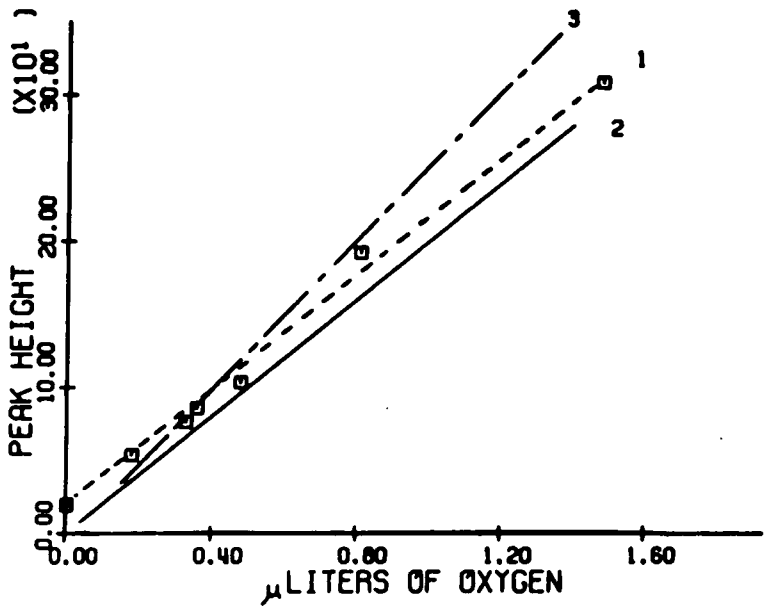

Fig. 10. A standard curve for conversion of recorder peak height differences to μliters of oxygen

1. 15 μliter sample at 22 C and 692 mm of Hg and not corrected for contamination with oxygen from the air;

2. line 1 corrected for 0.05 μliters of oxygen contamination in producing the gas mixture; 3. line 2 corrected to volume at standard temperature and pressure

2. RESULTS

a. Respiration rate of strains and sexes

The oxygen consumed or carbon dioxide given off/mg of tissue/min was expressed as a function of the weight of the test insects. Table V shows linear regression and correlation analyses of gas exchange as a function of insect weight for LWR and LWN strain males. This data is represented graphically in Fig. 11.

Table VI shows the linear regression and correlation analyses of gas exchange as a function of insect weight for males and females of the LWN strain. The regression lines for oxygen consumption of males and females are shown in Fig. 12.

b. Respiration rate after fumigation with EDB

Groups of five females of the same weight class of the LWN and LWR strains that were fumigated with EDB by method I were used to measure the carbon dioxide given off two hours after fumigation. Figure 13 shows the relationship between fumigant dose and respiration rate. No food was provided during the fumigation period, the two hour pre-test period or during the measurement of respiration. The groups of five females varied in total weight by less than 0.6 mg and this difference was corrected for by using the regression equations of table V.

The oxygen consumption of LWR insects fumigated with 4.7 mg of EDB/liter by method I was compared with that of non-fumigated and fed controls and non-fumigated starved controls (Fig. 14).

Table V. Oxygen consumption of males of LWN and LWR *Sitophilus granarius* in µg/mg tissue/min (Y) as a function of their mg weight (X)

Strain	Regression equation	Standard error of slope	Correlation coefficient	N*
LWN	Y = 0.15 - 0.03X	.004	-0.96(.01)**	8
LWR	Y = 0.12 - 0.02X	.008	-0.71(.05)	8

* number of data points ** level of significance

The regression lines are shown in Fig. 11

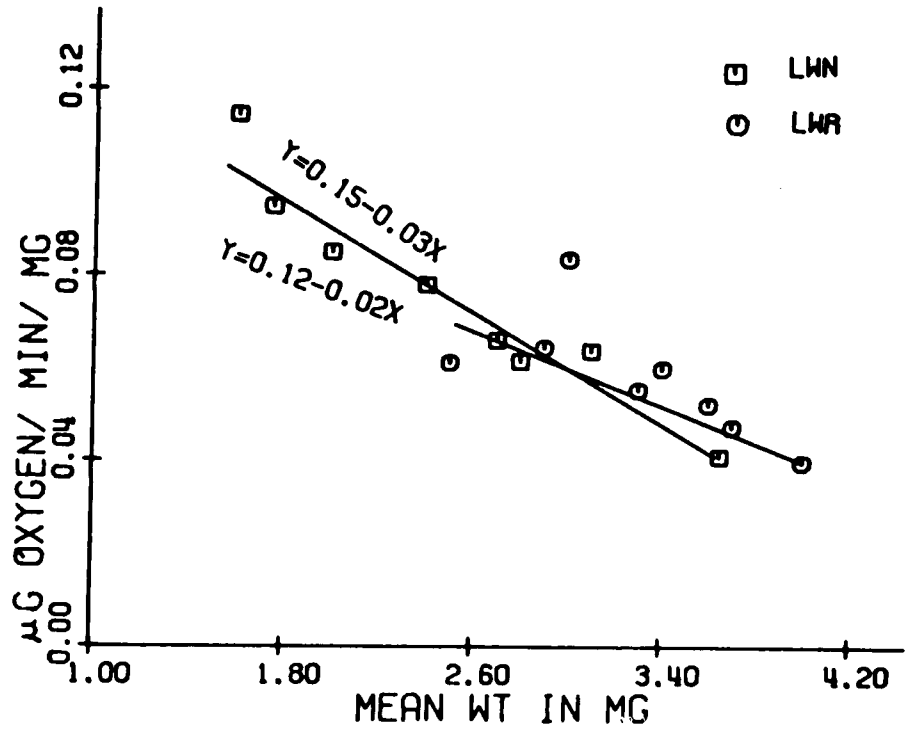

Fig. 11. The oxygen consumption of males of LWN and LWR *Sitophilus granarius* as a function of their weight

Linear regression and correlation analyses are in table V

Table VI. Gas exchange of males and females of LWN *Sitophilus granarius* in µg/mg tissue/min (Y) as a function of their mg weight (X)

Gas	Sex	Regression equation	Standard error of slope	Correlation coefficient	N*
***				**	
CO_2	F	Y = 0.118 - .023X	.004	-0.97(.05)	4
	M	NSG		-0.05(not)	4
	M & F	Y = 0.087 - .012X	.007	-0.58(not)	8
***					
O_2	F	Y = 0.097 - .014X	.005	-0.88(.05)	4
	M	Y = 0.072 - .007X	.005	-0.69(not)	4
	M & F	Y = 0.087 - .012X	.004	-0.78(.01)	8
****					
O_2	F	Y = 0.11 - .02X	.007	-0.78(.05)	7
	M	Y = 0.143 - .03X	.013	-0.72(.05)	7

* number of data points ** level of significance

*** 5 insects/test sample; duration of test 30 min

**** 1 insect/test sample; duration of test 2 hr

Regression lines for oxygen are shown in Fig 12

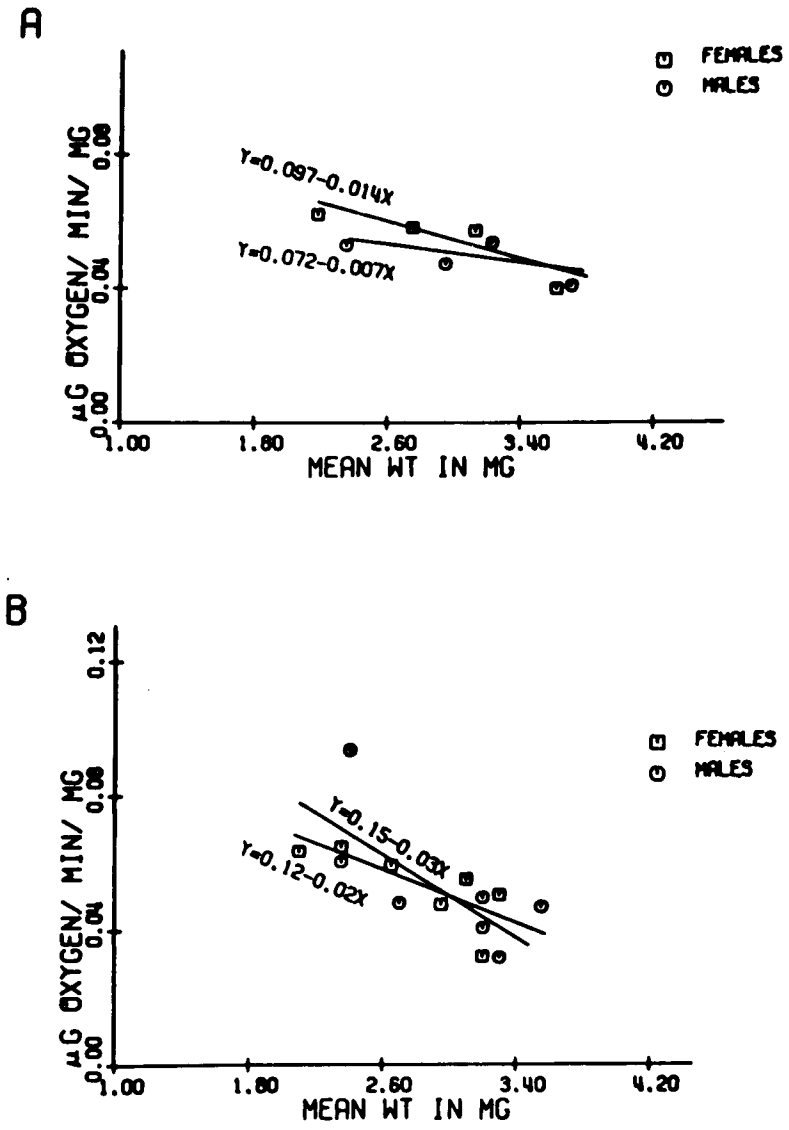

Fig. 12. The oxygen consumption of males and females of LWN *Sitophilus granarius* as a function of their weight

A. 5 insects/sample; B. 1 insect/sample

Linear regression and correlation analyses are in table VI

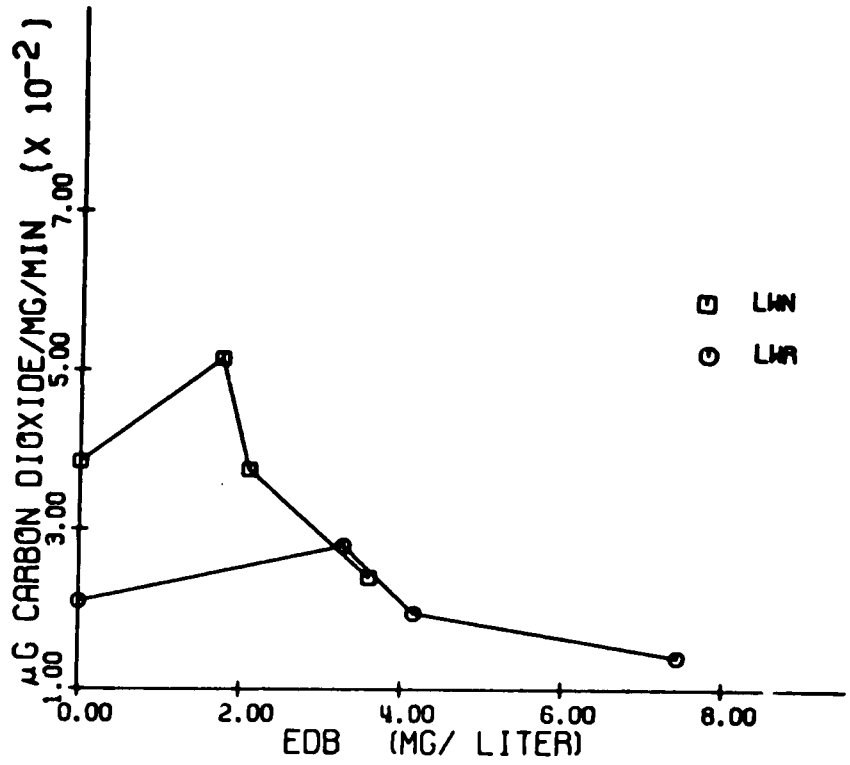

Fig. 13. Micrograms of carbon dioxide liberated from females of LWN and LWR *Sitophilus granarius* two hours after fumigation with EDB

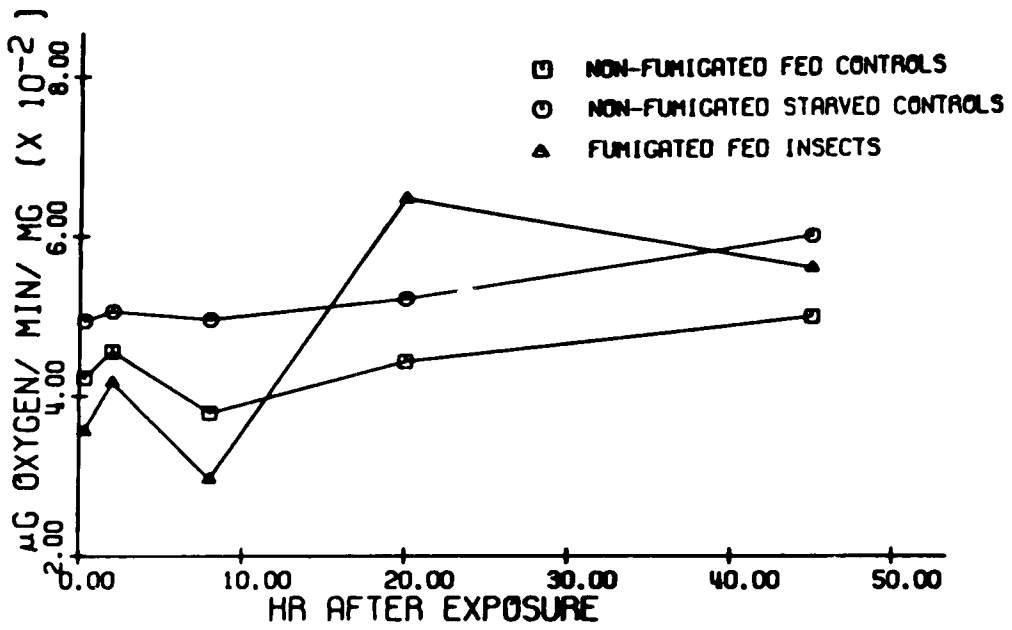

Fig. 14. Oxygen consumption of LWR *Sitophilus granarius* fumigated with 4.7 mg/liter of EDB

In a second experiment, LWN strain insects were fumigated with 3.6 mg of EDB/liter and LWR insects with 7.5 mg of EDB/liter (approximate LC50's). After four and five days the insects were classified into the following symptom classes; 0- unfumigated controls, 1- fumigated but no symptoms of poisoning, 2- fumigated and walking with difficulty, 3- fumigated and unable to walk. Groups of five insects were used to measure carbon dioxide production after four days and another group the oxygen consumed after five days. The micrograms of gas exchanged/insect/min and the micrograms of gas exchanged/mg tissue/min are shown in Figs. 15 and 16 respectively. Insects were released after the measurement of respiration on day four and were reclassified on day five to avoid bias in the symptom classes. Because the oxygen and carbon dioxide measurements were made on different days, the respiratory quotients were too variable to be meaningful.

3. DISCUSSION

Williams, Williams, and DeWitt (1965) reported a 19.3 per cent difference in the oxygen consumption of two subspecies of the desert ant, *Pogonomyrmex barbatus* (F. Sm.). *Pogonomyrmex barbatus barbatus* had an oxygen consumption of 0.413 ± 0.012 grams/hour whereas *P. b. rugosus* Emery had an oxygen consumption of 0.346 ± 0.010 grams/hour. Shibazaki and Itô (1969) showed a difference between the volume of carbon dioxide produced/mg of live body weight/hour of green and black larvae of the armyworm, *Pseudaletia (Leucania) separata* (Wlk.). The importance of respiration rate in determining fumigant uptake has been discussed by Cotton (1932) and others. Because differences in

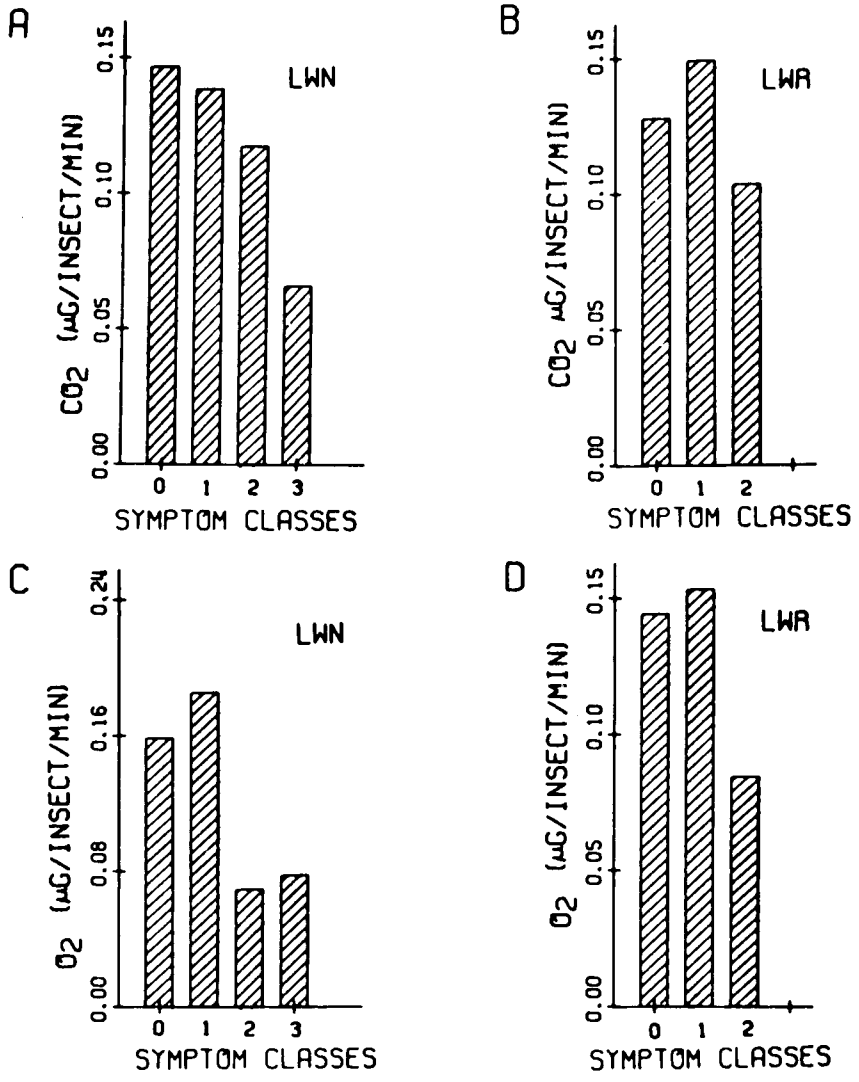

Fig. 15. Gas exchange in µg/insect/min of insects fumigated with EDB

A & B-carbon dioxide produced four days after exposure;

C & D-oxygen consumed five days after exposure

Symptom classes: 0- non-fumigated controls, 1- fumigated but no symptoms, 2- fumigated walking with difficulty, 3- fumigated unable to walk

Above graphs are from data in the appendix, table 1

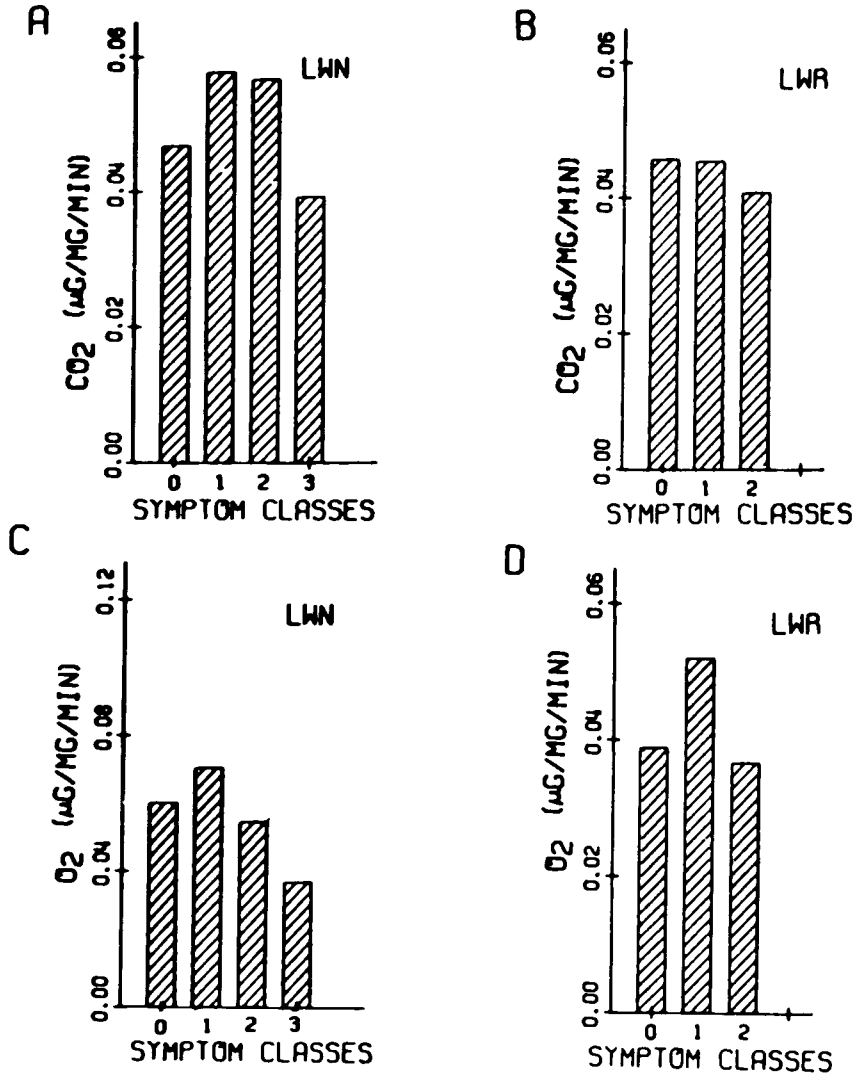

Fig. 16. Gas exchange in µg/mg tissue/min of insects fumigated with EDB

A & B- carbon dioxide produced four days after exposure;

C & D- oxygen consumed five days after exposure

Symptom classes: 0- non-fumigated controls, 1- fumigated but no symptoms, 2- fumigated walking with difficulty, 3- fumigated unable to walk

Above graphs are from data in the appendix, table 1

respiration rate between subspecies and physiological forms are well established as is the importance of respiration rate in determining fumigant uptake, one might expect the LWR strain to have a different respiration rate than the LWN strain. Table V and Fig. 11 show that the LWR strain does have a lower respiration rate than the LWN strain. By determining the gas exchange of insects of several weights and expressing gas exchange as a function of insect weight (Fig. 11) it was seen that the lower oxygen consumption/mg of tissue of the LWR insects was entirely a result of their greater weight. The regression equations for micrograms of oxygen consumed/mg/min as a function of insect weight for LWN and LWR insects were not statistically different.

Williams *et al.* (1965) and Shibazaki and Itô (1969) and others who have reported within-species differences in respiration rate did not report the mean weight of the insects under comparison. Williams *et al.* (1965) did not give the weight of the test insects at all and the difference they observed may have been entirely due to a difference in subspecies weight.

The metabolic rate generally decreases progressively with increasing body weight (Hoar, 1966). Weight-independent respiration has however been reported for dragonfly larvae tested at 27 and 34 C by Petitpren and Knight (1970) who also reviewed literature on weight-independent respiration in some fresh water snails, a vertically migrating crustacean, a terrestrial isopod, and a stonefly. Establishing that metabolism in *S. granarius* at 27 C is weight-dependent is important in determining the possible importance of insect size in influencing fumigant uptake.

Table VI and Fig. 12 show no significant difference in oxygen consumption of males and females. These data also confirms weight-dependent respiration in *S. granarius*.

Richards (1969) observed a 3- to 5-fold increase in the oxygen consumption of the American cockroach, *Periplaneta americana* (L.), just prior to death by starvation. Mortality of *S. granarius* in my experiments was highest in the lighter weevils of each strain. Richard's observations may be the explanation for the greater than expected oxygen consumption of some insects of lower than mean weights (Figs. 11 and 12).

Vincent and Lindgren (1965) state that carbon dioxide production is depressed after exposure to methyl bromide and EDB and that this depression is dose dependent. Figure 13 shows that two hours after fumigation with EDB the carbon dioxide produced by *S. granarius* was stimulated by sublethal doses and depressed by higher ones. Bang and Telford (1966) observed that the respiration rate of *Tribolium castaneum* (Herbst) increased from below normal to normal and later to above normal levels after fumigation. They attributed this to recovery and later payment of an oxygen debt that was incurred when respiration was lowered due to the effect of the fumigant. Figure 13 confirms that the observations of Bang and Telford with *T. castaneum* are valid for *S. granarius*, in that, two hours after fumigation with EDB the respiration rate was normal or above normal when low doses of fumigant were used. Higher doses resulted in a progressively lower respiration rate after two hours.

LWR insects fumigated with 4.7 mg of EDB/liter had a reduced

respiration rate as compared to non-fumigated controls but the respiration rate of the fumigated insects was greater than that of the controls 20 hours after fumigation (Fig. 14). When 3.6 mg of EDB/liter were used the respiration rate was greater than that of non-fumigated controls 2 hours after fumigation (Fig. 13). This shows that the length of time after fumigation before recovery of the respiration rate is dependent on the dose received.

The respiration rate of fumigated insects remained at or above the level for non-fumigated controls until symptoms of poisoning occurred (Figs. 15 and 16). The more severe the symptoms the greater the decrease in respiration rate, whether this was expressed as micrograms/mg/min or as micrograms/insect/min.

VI. LIPID CONTENT OF FUMIGATED AND CONTROL INSECTS

1. INTRODUCTION

The solubility of chlorinated hydrocarbons in lipids has led to the hypothesis that inert storage in lipids is a protective mechanism against these insecticides. This subject was discussed by Hoskins and Gordon (1956) and reviewed by Fast (1964) and Moriarty (1968). The strongest evidence for a correlation between lipid content and susceptibility has been compiled for tolerance to DDT by the American cockroach (Munson, 1953b; Munson and Gottlieb, 1953; Munson, Padilla, and Weissmann, 1954; Lofgren and Cutkomp, 1956), by some strains of houseflies (Wiesmann and Reiff, 1956; Ascher and Neri, 1961), and by the alfalfa weevil (Bennett and Thomas, 1963; and 1964). A correlation between resistance to a chlorinated hydrocarbon and lipid content has been found by many others including Vinson (1967), Bacon, Riley, and Zweig (1964) and Reiser *et al.* (1953). There have, however, been many instances where lipid content has not been a factor in susceptibility (Cox and Bowman, 1955; Perrott, Shorland, and Czochanska, 1965; Brazzel *et al.*, 1957; Fast and Brown, 1962; Fast, 1964).

Apparently no one has studied the relationship between lipid content and susceptibility to fumigants although a significant amount of volatile fumigant can be extracted from fumigated foodstuffs after fumigation (Heuser and Scudamore, 1967 and 1968).

Sun (1947) reared *T. confusum* Duv. at three temperatures and found that the susceptibility to carbon disulfide was greatest at the highest rearing temperature. This may have been due to a greater

proportion of unsaturated fatty acids as found in other insects (Munson, 1953a; Fast, 1964; Moore *et al.*, 1967).

Distinct from inert storage of poison in the lipid is the effect of lipid on the vigor and endurance of the insect to the stress conditions of poisoning. Fast (1964) stressed that many experiments with lipids and insect susceptibility have not distinguished between these two effects. The effect of insect nutrition on resistance to insecticides was reviewed by Gordon (1961).

The object of this study of the lipids of *S. granarius* is to measure the total lipid of the tolerant and normal strain and to compare the metabolism of lipids under the stress conditions of starvation and fumigation with EDB.

2. MATERIALS AND METHODS

Insects were sexed, counted, weighed and frozen for temporary storage. Guss (1969) observed lipolysis in stored corn rootworm eggs after two weeks storage at -20 to -40 C and for this reason samples of insects were extracted for lipids within 12 hours after freezing.

Two methods of extraction of lipids were used. The procedure of Folch, Lees, and Sloane Stanley (1957) was modified for small samples by use of a Sorval, high speed, micro-homogenizer and by vacuum-filtering the homogenate directly into calibrated, glass stoppered centrifuge tubes. Homogenization was for 15 minutes in 20 volumes of a chloroform:methanol mixture (2:1v/v). The residue was rinsed and extracted three times in small volumes of the same extraction mixture. Each rinse was for 2 minutes. The filtered homogenate

was separated in the centrifuge tubes by the addition of 0.2 volumes of 0.90 per cent sodium chloride solution followed by centrifuging. The upper methanol:water:salt layer was removed with Pasteur pipettes. The lower phase was then evaporated to dryness and redissolved in 20 ml of chloroform. The total lipid was determined by evaporating aliquots to dryness in a vacuum oven at 40 C in tared aluminium foil pans. Because of the small amount of extract, over one-half of the sample was used to measure the total lipid and there was insufficient sample left to measure the neutral lipids and phospholipids.

The other extraction method was with petroleum ether in a Soxhlet apparatus. Insect samples were crushed in a folded piece of aluminium foil that had previously been extracted with petroleum ether. Both the crushed insects and the aluminium foil were placed in the extraction thimble of the Soxhlet apparatus and extracted with petroleum ether for 18 hours. This petroleum ether was then evaporated and the lipids redissolved in 10 ml of petroleum ether. The total lipid was determined from aliquots as described for the first method of extraction.

The fumigated insects received a dose of 1.5 µliters/liter (approximate LC50) and were lipid-extracted 10 days later. Each regression formula was determined from insects of the same colony, strain, age and sex.

3. RESULTS

a. Lipid content of control insects

Groups of five insects of the same weight, strain and sex were analyzed for total lipid by the method of Folch, Lees, and Sloane Stanley (1957). The results of linear regression and correlation analyses of mg of lipid/insect on the weight of the insects in mg is shown in table VII. The regression lines for the pooled data of table VII are shown in Fig. 17. The mean weights of the strains and sexes with the lipid content and per cent lipid of wet weight as calculated from the regression equations of table VII are shown in table VIII.

Petroleum ether extractions with a Soxhlet apparatus were compared with chloroform : methanol extractions (table IX).

b. Lipid content of treated insects

The lipid content of starved and of fumigated insects was determined. The starved insects were given water from wet cloth. In addition to the water the fed insects were given grains of wheat that were changed every day to prevent molds. Both the starved, fed, and fumigated fed insects were kept at 100 per cent relative humidity and 27 C for 10 days prior to analysis of lipids. The linear regression and correlation analyses of mg lipid/insect (Y) on insect weight in mg (X) are given in table X. The regression lines are shown in Fig. 18.

Table VII. Linear regression and correlation analyses of total extracted lipid in mg (Y) of male and female LWN and LWR *Sitophilus granarius* as a function of their mg weight (X)

Strain	Rep	Sex	Regression equation	Standard error of slope	Correlation coefficient	N*
					**	
LWR	1	F	Y = -0.015 + 0.075X	0.033	0.92(not)	3
		M	Y = -0.211 + 0.134X	0.040	0.96(.05)	3
	2	F	Y = -0.740 + 0.288X	0.064	0.95(.05)	4
		M	Y = -0.367 + 0.188X	0.018	0.98(.01)	5
	3	F	Y = -0.429 + 0.211X	0.016	0.99(.01)	5
		M	Y = -0.106 + 0.104X	0.004	0.99(.01)	5
	Pooled	F	Y = -0.419 + 0.203X	0.034	0.89(.01)	12
		M	Y = -0.189 + 0.132X	0.021	0.89(.01)	13
LWN	1	F	Y = -0.185 + 0.136X	0.059	0.92(not)	3
		M	Y = -0.104 + 0.050X	0.028	0.88(not)	3
	2	F	Y = -0.476 + 0.262X	0.106	0.87(not)	4
		M	Y = -0.128 + 0.119X	0.010	0.99(.01)	4
	Pooled	F	Y = -0.422 + 0.234X	0.077	0.80(.01)	7
		M	Y = -0.005 + 0.082X	0.030	0.77(.01)	7

* number of data points (5 insects/point) ** significance level

The regression lines for the pooled data are shown in Fig. 17

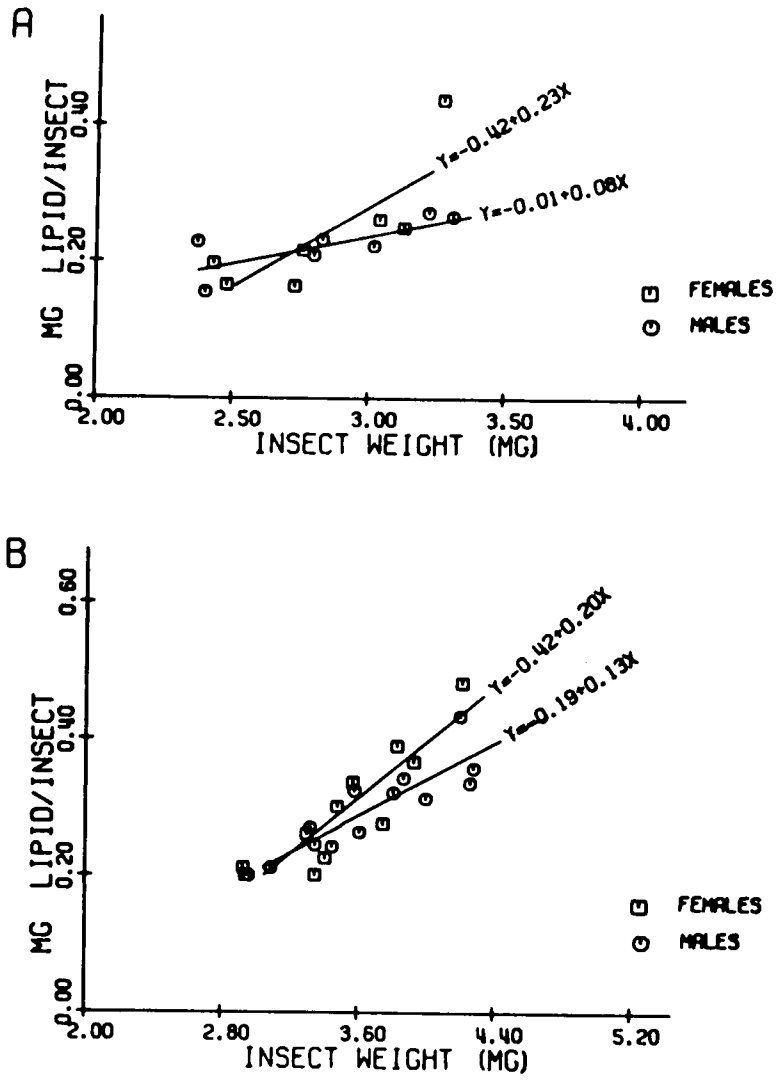

Fig. 17. Total chloroform: methanol extracted lipid of male and female *Sitophilus granarius* as a function of their weight

A- LWN strain; B- LWR strain

Linear regression and correlation analyses are in table VII

Table VIII. Lipid content of male and female LWN and LWR *Sitophilus granarius* of mean weight

Strain	Sex	*Mean weight (mg)	**Lipid content (mg/insect)	% Lipid of wet weight
LWN	F	2.59	0.184	7.10
	M	2.73	0.219	8.02
LWR	F	3.56	0.304	8.54
	M	3.64	0.291	7.99

* mean weights from table I

** lipid content calculated from the regression equations of Fig. 17

Table IX. A comparison between Soxhlet extractions with petroleum ether and chloroform:methanol extractions of lipids of male and female LWR *Sitophilus granarius*

Sex	Extraction method	Regression equation Y = mg of lipid/insect; X = mg weight	Standard error of slope	Correlation coefficient	N*
F	Soxhlet	Y = -0.325 + 0.175X	0.075	0.80(not)**	5
	Ch:Met	Y = -0.429 + 0.211X	0.016	0.99(.01)	5
M	Soxhlet	Y = 0.033 + 0.109X	0.002	0.99(.01)	4
	Ch:Met	Y = -0.106 + 0.104X	0.004	0.99(.01)	5

* number of data points (5 insects/point)

** significance level

Ch:Met data from table VII rep 3

Table X. Linear regression and correlation analyses of extracted lipid in mg (Y) of control insects and insects 10 days after fumigation with EDB as a function of mg weight (X)

Strain	Treatment*	Regression equation	Standard error of slope	Correlation coefficient	N**
LWN	Control I	Y = -0.078 + 0.093X	0.035	0.88(.05)***	4
	Control II	Y = -0.057 + 0.034X	0.006	0.96(.05)	3
	Fumigated A	Y = 0.202 + 0.032X	0.50	0.54(not)	3
	Fumigated B	Y = 0.061 + 0.037X	0.020	0.80(not)	4
LWR	Control I	Y = 0.027 + 0.050X	0.038	0.60(not)	5
	Control II	Y = -0.139 + 0.082X	0.022	0.90(.05)	5

* Control I - unfumigated fed Control II - unfumigated fed water
Fumigated A - insects able to walk
Fumigated B - insects unable to walk

** number of data points (5 insects/point) *** significance level

The regression lines are shown in Fig. 18

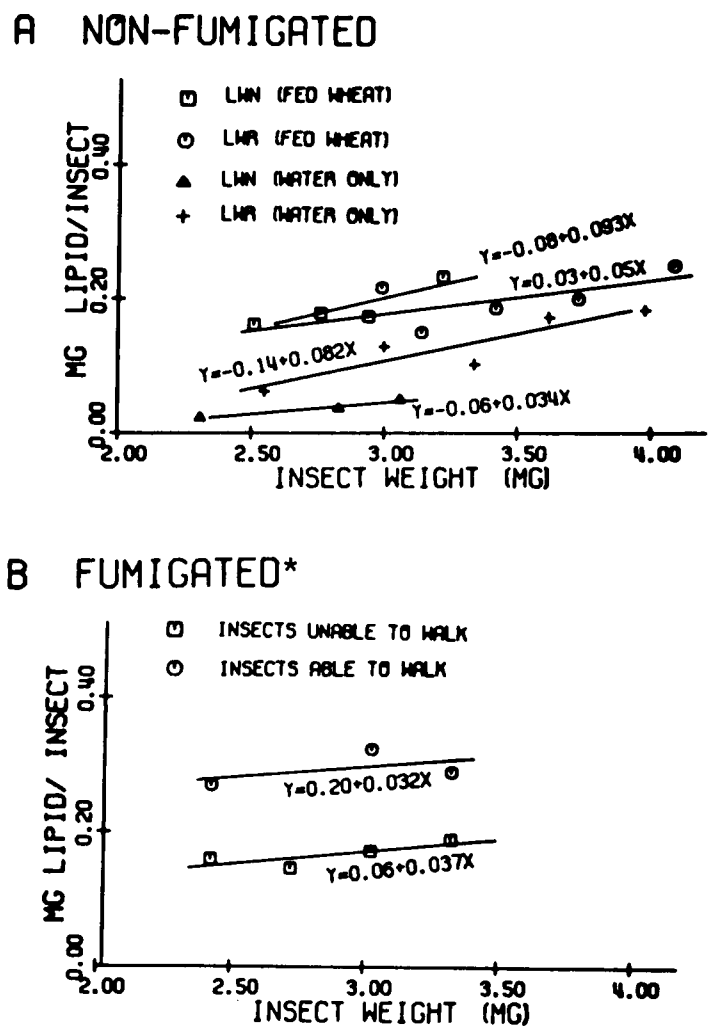

Fig. 18. Total lipid of EDB fumigated and control *Sitophilus granarius* as a function of their weight

Linear regression and correlation analyses are shown in table X

* LWN strain

4. DISCUSSION

As expected the heavier insects had more total lipid (table VII, Fig. 18). The correlation coefficients of table VII were significant in all but two instances and in these cases the correlation coefficient was high but the number of points was less than four. The slope and the standard error of the slope of the three replicates of LWR strain and two replicates of the LWN strain (table VII) show that the slopes of the regression lines for the two sexes are significantly different. In four of the five replicates the slope plus and minus one standard error of the slope for the females did not overlap with the slope and one standard error of the males. The increase in lipid per increase in weight was greatest for females. Mortality was greater in insects of lightest weight and lipid use by dying insects may partly account for the non-significant difference in lipid content of light weight insects.

The LWR strain had more lipid/insect than the LWN strain but this was a function of their greater weight. The per cent lipid of wet weight was not significantly different (table VIII).

The relationship between insect weight and extractable lipid was the same whether obtained by chloroform:methanol extraction or by Soxhlet extraction with petroleum ether (table IX). The slopes of the regression lines were not significantly different although a significantly greater amount of lipid was indicated by the Soxhlet extractions. This difference is expected because of differences in the properties of the extracting solvents and because some oxidation

occurs in the Soxhlet apparatus.

The regression formulae of table X and Fig. 18 show that the reduction of lipid due to starvation and due to immobilization by fumigant are comparable. Only insects that were dying (i.e. unable to walk) showed a significant reduction in total lipid. There is no indication that fumigation with EDB prevents lipid metabolism. Lipids may be a factor in the greater tolerance to EDB by heavier insects.

There was more lipid left in the starved LWR insects than in the starved LWN insects after 10 days (Fig. 18). The amount of lipid explains the longer survival time of LWR insects before starvation or death due to EDB fumigation.

VII. UPTAKE AND METABOLISM OF ^{14}C LABELLED EDB

1. MATERIALS AND METHODS

a. Fumigation

1, 2-dibromoethane ^{14}C (U) of specific activity 9.7 mCi/mM was obtained from Amersham/Searle Corporation, Don Mills. Ont.

Fumigation of the insects was in a modified 300 ml Erlenmeyer flask fitted with a side arm (Fig. 19). This fumigation chamber was clamped in a plastic water bath. The exposure cages were 4.5 cm by 1 cm brass screen cylinders sealed with small squares of aluminium foil.

A Teflon coated magnet was placed in the fumigation chamber and the test insects were sealed in the exposure cages. Eight exposure cages were then suspended in the flask by cotton threads wedged between the neck of the flask and the rubber stopper. The stopper was lightly lubricated with silicone stopcock grease to ensure a tight seal. The fumigant was then frozen into one end of the shipping ampoule by immersing it in liquid nitrogen. The end of the ampoule was removed and the ampoule was placed in the side arm of the fumigation apparatus. The flask was placed in the water bath at 27 C and the magnetic stirrer started. The stirrer was operating within 2 minutes of opening the fumigant ampoule.

For the first fumigation the apparatus with the insects was assembled except for the side arm and placed in a small chamber at 27 C and 50 per cent relative humidity for one-half hour prior to attaching

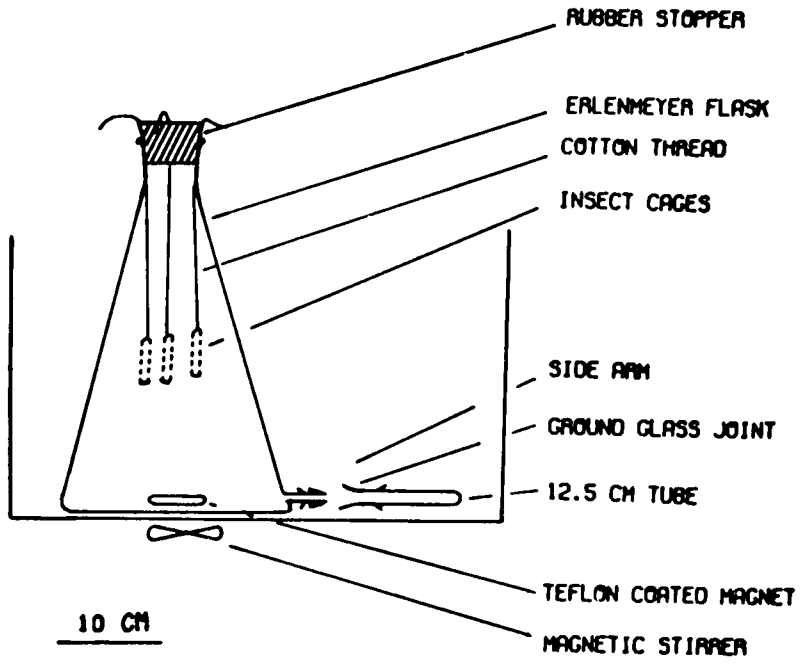

Fig. 19. Fumigation chamber for exposing *Sitophilus granarius* to ^{14}C labelled EDB

the side arm with the fumigant. At the end of the fumigation a part of the fumigant and the water vapor were recovered by freezing them in the side arm by immersion in liquid nitrogen. In the second fumigation the recovered fumigant and water vapor were reused by attaching the side arm. The relative humidity during the second fumigation was therefore unknown. With active insect stages and a 5 hour exposure period, relative humidity does not have a significant effect on insect mortality (Sun, 1947).

The advantages of the apparatus were: 1. rapid vaporization of the fumigant with stirring, 2. partial recovery of the fumigant after exposure by immersion of the side arm in liquid nitrogen and removing and stoppering the side arm, 3. removing samples of gas from the chamber with a syringe inserted through the stopper enabled determination of the vapor concentration by scintillation counting and adjusting the exposure time accordingly, 4. placing the side arm on the side of the flask rather than on the bottom allowed use of a magnetic stirrer and avoided heavy fumigant vapors settling into it.

b. Post-exposure treatment

Insects and approximately 3.5 gm of wheat were placed in the post-exposure vials in a desiccator containing a saturated solution of calcium nitrate (relative humidity approximately 55.8 per cent). The desiccator was placed in a water bath at 27 C to control the temperature. The desiccator was opened once daily to remove insects, to check mortality and to change the wheat.

c. Preparation of samples for radioassay

Two methods of sample preparation were used. Insects were crushed in scintillation vials with a glass rod which was washed off into the vials with 2 ml of petroleum ether. The vials were sealed for 24 hours and then 10 ml of scintillation mix were added and the samples placed in the dark for 24 hours before counting the disintegrations/minute (DPM). This method of sample preparation extracted the unchanged fumigant.

In the second method, after crushing the insects the glass rod was washed off into the vial with 1 ml of 1 N sodium hydroxide. The vials were then sealed and left overnight. The sealed vials were then heated to 80 C for 45 minutes in a water bath. After cooling, three drops of 30 per cent hydrogen peroxide were added to bleach the sample. The oxygen from the hydrogen peroxide was removed by ultrasonic vibration. Two ml of Bio-Solve 2 (Beckman tissue solubilizer) were added and the sample gently swirled and then 10 ml of scintillation mix were added. Samples were left overnight in the dark before counting. This method solubilized the tissue and enabled counting total ^{14}C in the insects. This method of preparation was also used after extraction with petroleum ether to determine the portion of ^{14}C that was not ether extractable and by inference not present as EDB.

In both methods the scintillation mix was Beckman Fluoralloy containing 8.0 gm of butyl PBD (2-(4'-t-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxdiazole) and 0.5 gm of PBBO (2-(4'-biphenyl)-6-phenyl-benzoxazole)/liter of toluene.

d. Radioassay

Radioactivity was measured by a Picker Nuclear, Liquimat 200 liquid scintillation counter which was connected to a digital PDP-8/L computer and teletype terminal. The ^{14}C spectrum was obtained by adjusting 'window settings' to count the disintegrations of particular energies. The 'B'channel was selected to include the whole spectrum except a small portion in the very low energy range where machine background was high. The 'A' channel was selected to include approximately the lower one-third of the spectrum. The actual settings were: A.15-340, B.15-650. The sample counting efficiency was determined by the channel ratio method (Bush, 1963).

2. RESULTS

a. Fumigant uptake during exposure

Lots of three insects of four weight classes of LWN and LWR insects were prepared for determination of total radioactivity by sodium hydroxide digestion one hour after fumigation. The experiment was replicated three times, twice with females and once with males. Preparation of the samples for digestion took one hour and the mean time between the end of fumigation and sealing of the samples in sodium hydroxide was 66, 76 and 85 minutes respectively for the three replicates. Replicate two was the samples of males. The DPM/insect (Y) was regressed on the insect weight (X) (table XI). The linear regression of DPM/insect and DPM/mg of tissue on the weight of the LWN and LWR insects are shown in figs. 20 and 21.

Table XI. Linear regression and correlation analyses of DPM/insect (Y) 1.5 hr after fumigation with labelled EDB as a function of their mg weight (X)

Rep.	Strain	Sex*	Regression equation	Standard error of slope	Correlation coefficient	N**
1	LWN	F	Y = 4406 + 4149X	853	0.96(.05)***	4
	LWR	F	Y = 7848 + 2613X	2525	0.59(not)	4
3	LWN	F	Y = - 479 + 6726X	787	0.99(.01)	4
	LWR	F	Y = 10140 + 2321X	1140	0.82(not)	4
2	LWN	M	Y = -5006 + 8095X	1627	0.96(.05)	4
	LWR	M	Y = -6826 + 6547X	990	0.98(.05)	4
avg F						
1&3	LWN	F	Y = 1963 + 5456X	773	0.98(.05)	4
	LWR	F	Y = 8993 + 2467X	1759	0.70(not)	4
avg M&F						
1,2&3	LWN	M&F	Y = -360 + 6335X	971	0.98(.05)	4
	LWR	M&F	Y = 3720 + 3827X	1221	0.91(not)	4

*F- females; M- males ** N- number of data points (3 insects/point)

*** significance level

Data from appendix, table 2

Regression lines shown in Fig. 20

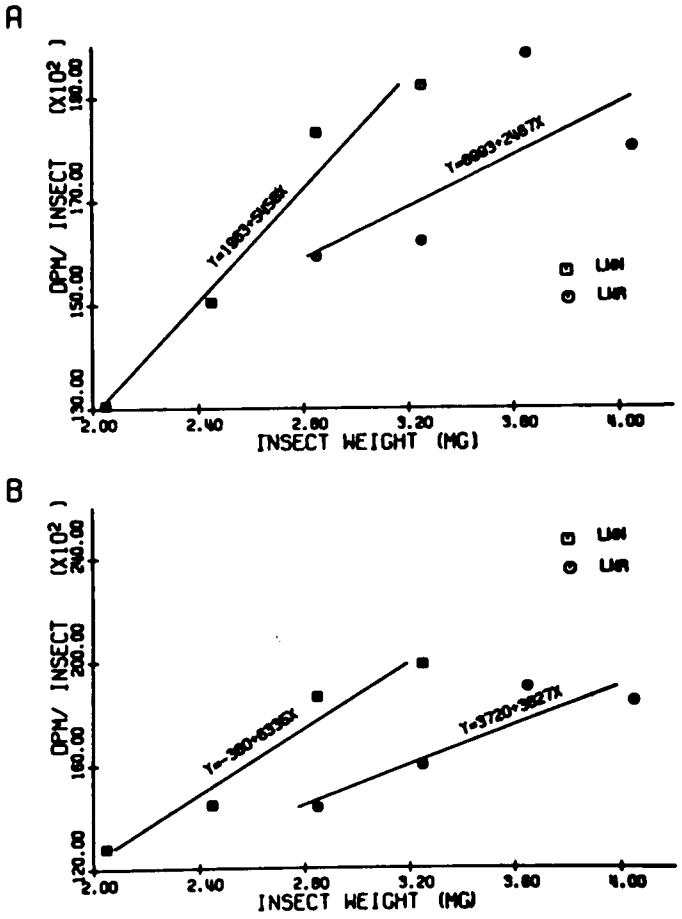

Fig. 20. DPM of ^{14}C/*Sitophilus granarius* adults as a function of their weight

A- average females; B- average males and females

Linear regression and correlation analyses are shown in table XI.

Calculated from the data of the appendix, table 2

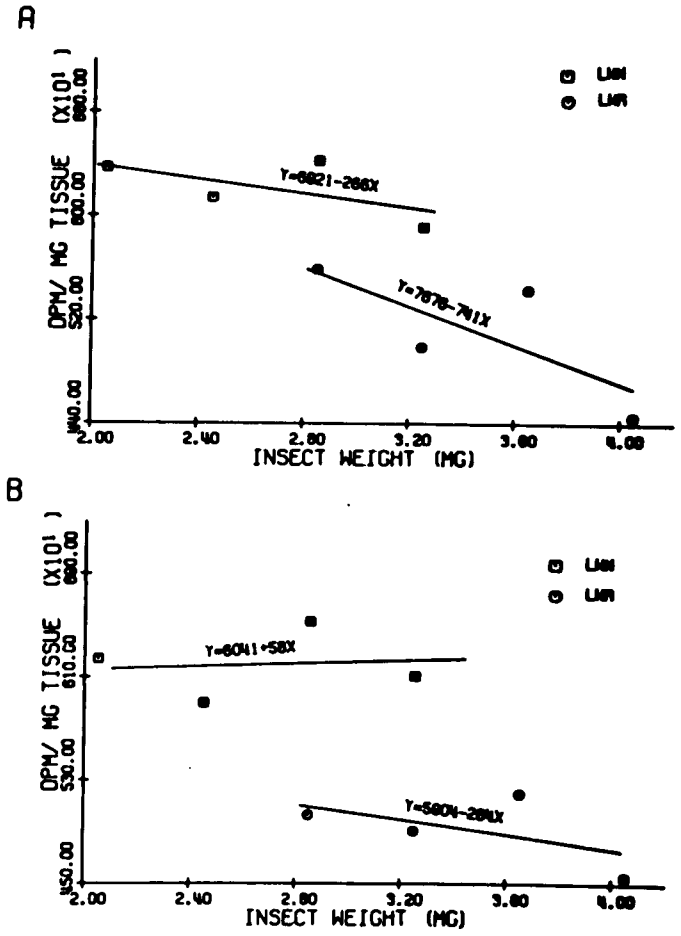

Fig. 21. DPM of ^{14}C/mg of tissue of *Sitophilus granarius* as a function of their weight

A- average females; B- average males and females

Calculated from the data of the appendix, table 2

b. Petroleum ether extractable ^{14}C

In a preliminary test, four lots, each of 10 insects of mixed sex were washed 1.5 hours after fumigation in 1 ml of petroleum ether. The weight of each of the test insects was 2.6 to 2.8 mg and the washing period was 1 minute. The insects were removed after washing and the petroleum ether was prepared for radioassay (table XII).

In a second experiment, lots of five insects were crushed in the vials and extracted with petroleum ether for 24 hours as described in the materials and methods.

Regression and correlation analyses were done on the log DPM/insect (Y) versus the log of the time after fumigation (X) (table XIII).

In a third experiment, the petroleum ether extracted ^{14}C of groups of three males of different strains and weight classes was determined after 2 days and this was related to the lipid content of the insects (table XIV).

c. Loss of ^{14}C with time after fumigation with EDB

The total ^{14}C activity was determined on samples of three insects by digesting them with sodium hydroxide. The DPM/insect of samples of three different insect weights, two strains and five intervals after fumigation are shown in Figs. 22 and 23. The DPM/insect was found to decrease with time according to log-linear regression. The results of regression and correlation analyses of log DPM/insect on the first two days after fumigation are shown in table XV. The

Table XII. Carbon-14 extracted with a 1 min wash in 1 ml of petroleum ether 1.5 hr after fumigation with labelled EDB

Strain	DPM ± SD Rep 1	Rep 2	Average
LWN	9681 ± 50 *	11340 ± 54	10510
LWR	5041 ± 37	4588 ± 35	4814

* 10 insects of mixed sex/sample; insect weight 2.6-2.8 mg

Table XIII. Linear regression and correlation analyses of log DPM of ^{14}C/insect (Y) on log time after fumigation with labelled EDB (X)

Strain	Sex*	Weight class(mg)	Regression equation	Standard error of slope	Correlation coefficient	Number of points
LWR	F	2.6-2.8	Y = 3.12 - 0.666X	0.029	-0.998(.01)**	4
		3.0-3.2	Y = 3.08 - 0.655X	0.073	-0.988(.01)	4
		> 3.2-3.4	Y = 3.03 - 0.565X	0.048	-0.993(.01)	4
		3.6-3.8	Y = 3.02 - 0.688X	0.032	-0.998(.01)	4
	M	3.0-3.2	Y = 3.14 - 0.867X	0.075	-0.992(.01)	4
LWN	F	2.2-2.4	Y = 3.16 - 0.629X	0.133	-0.958(.05)	4
		2.6-2.8	***			
		3.0-3.2	***			
	M	2.6-2.8	Y = 3.22 - 0.870X	0.008	-1.000(.01)	4

* F- females; M- males ** level of significance *** insufficient data

The data is from the appendix, table 3

Table XIV. DPM of ^{14}C/insect extracted with petroleum ether 2 days after fumigation with labelled EDB compared with total DPM/insect and total lipid

Strain	Weight class (mg)	DPM/insect petroleum ether extracted after 2 days	Total DPM/insect 0.125 days after fumigation	Per cent of ^{14}C from ether extract after 2 days	Expected* lipid/ insect (mg)
LWN	2.0-2.1	1183 ± 17	12798 ± 59	9.24	0.1631
	2.4-2.5	1351 ± 21	14450 ±101	9.35	0.1959
	2.8 - 2.9	**	18610 ± 78		0.2287
	3.2 - 3.3	1751 ± 37	19858 ± 75	8.82	0.2615
LWR	2.8 - 2.9	634 ± 13	14338 ± 55	4.42	0.1872
	3.2 - 3.3	622 ± 11	15951 ± 82	3.90	0.2400
	3.6 - 3.7	738 ± 16	18971 ± 80	3.89	0.2928
	4.0 - 4.1	749 ± 22	18434 ± 87	4.06	0.3451

* from the regression equations of table VII

** value was discarded because it was less than 1/3 other values for the strain

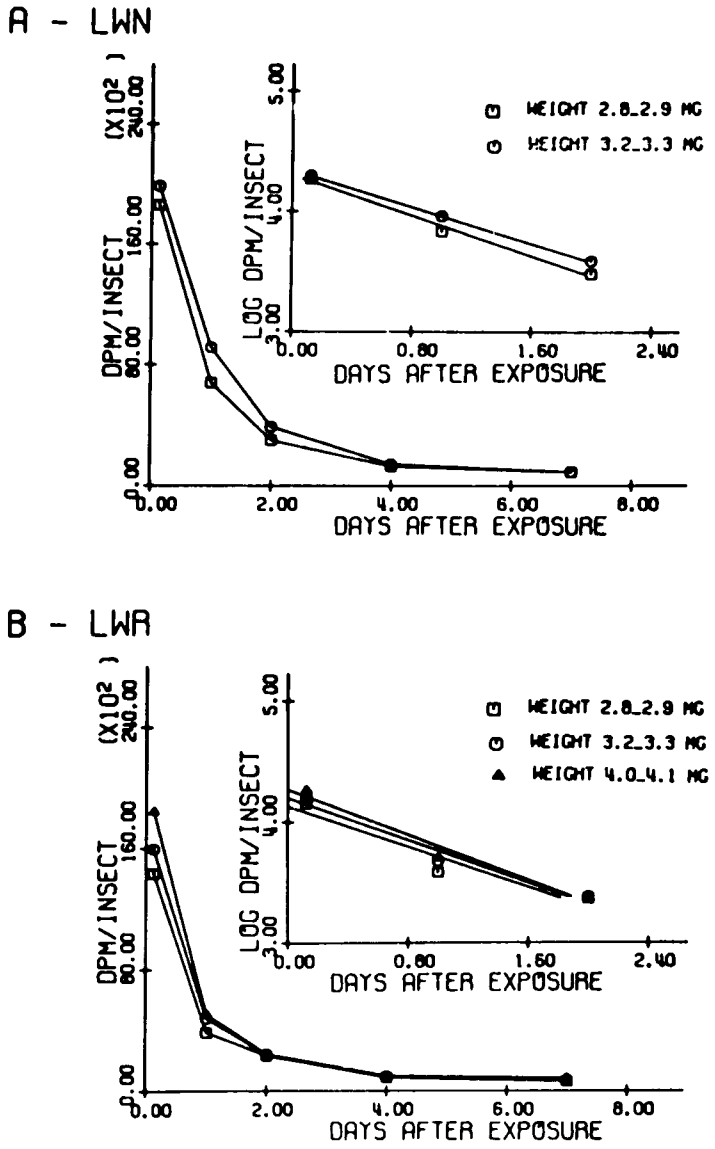

Fig. 22. Decrease with time of the total DPM/LWN and LWR *Sitophilus granarius* adults of various weights

Data from appendix, table 4

Linear regression and correlation analyses for the log inserts are shown in table XV

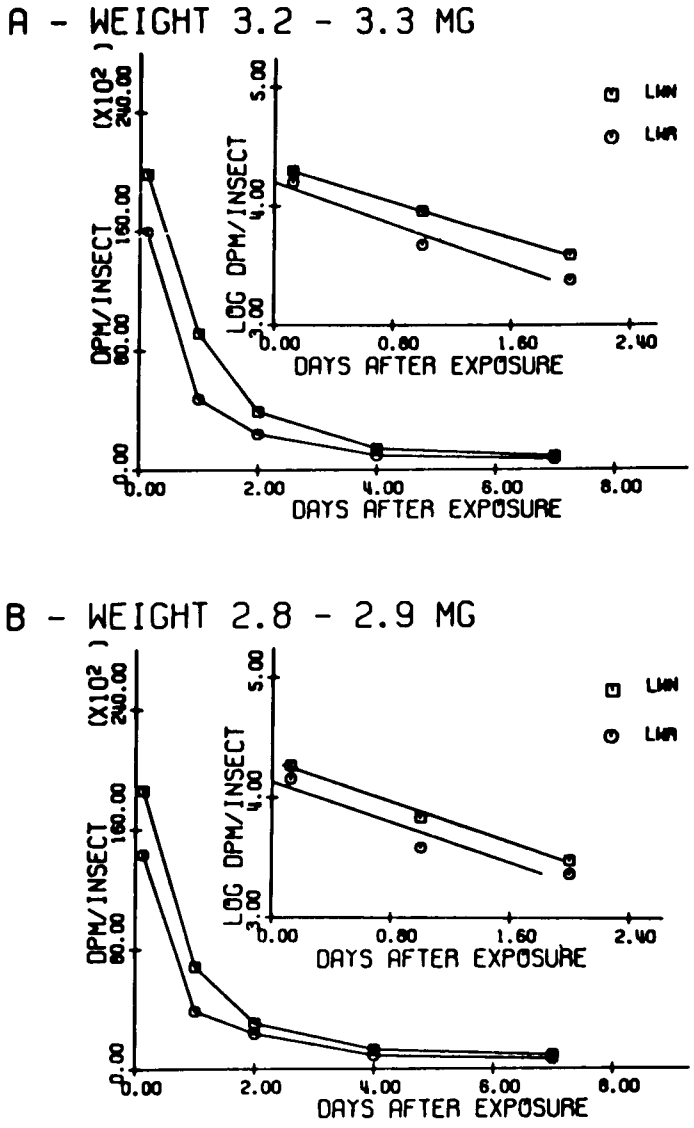

Fig. 23. Decrease with time of the total DPM/LWN and LWR *Sitophilus granarius* adults of the same weight

Data from appendix, table 4

Linear regression and correlation analyses for the log inserts are shown in table XV

Table XV. Linear regression and correlation analyses of log DPM obtained by NaOH digestion/insect of lots of three insects three intervals after fumigation with labelled EDB

Strain	Weight (mg)	Regression equation*	Standard error of slope	Correlation coefficient	Number of points
LWN	2.8 - 2.9	Y = 4.30 - 0.42X	0.041	-0.995(not)**	3
	3.2 - 3.3	Y = 4.34 - 0.38X	0.004	-0.997(.05)	3
LWR	2.8 - 2.9	Y = 4.13 - 0.42X	0.125	-0.957(not)	3
	3.2 - 3.3	Y = 4.20 - 0.44X	0.086	-0.981(not)	3
	4.0 - 4.1	Y = 4.27 - 0.47X	0.094	-0.980(not)	3

* Y = log DPM/insect; X = days after exposure

** significance level

The data is from appendix, table 5

The regression lines are shown in the log inserts of Figs. 22 and 23

results of table XV are shown in the log inserts of Figs. 22 and 23. Figure 22 compares the loss of radioactivity of insects of the same strain but different weights and Fig. 23 compares the loss of radioactivity of insects of different strains but the same weight.

In a second experiment, sodium hydroxide digestion was performed on the residue of five insects that remained after extraction for 24 hours with petroleum ether. This measured the ^{14}C other than that present as unchanged EDB. Four weight classes of LWR females and one weight class of each of LWR males and females and LWN males were digested. Samples were prepared after 0.125, 2, 4, 7, and 10 days (Fig. 24). The results of regression and correlation analyses on log DPM/insect on interval after fumigation are shown in table XVI and in the log insert of Fig. 24.

d. Carbon-14 in feces

The feces and debris of LWN and LWR *Sitophilus granarius* were collected four intervals after fumigation of the insects with labelled EDB. These samples of feces and debris were prepared for scintillation counting by sodium hydroxide digestion and solubilization as described for insect tissues. There was difficulty in obtaining a large enough sample for analysis so the feces and debris of all the insects of the same strain were combined in a single sample. Insufficient material was obtained the day following fumigation to permit an analysis. The lack of material was probably the effect of the starvation period prior to and during the fumigation and partial immobilization of the insects immediately after exposure. No attempt

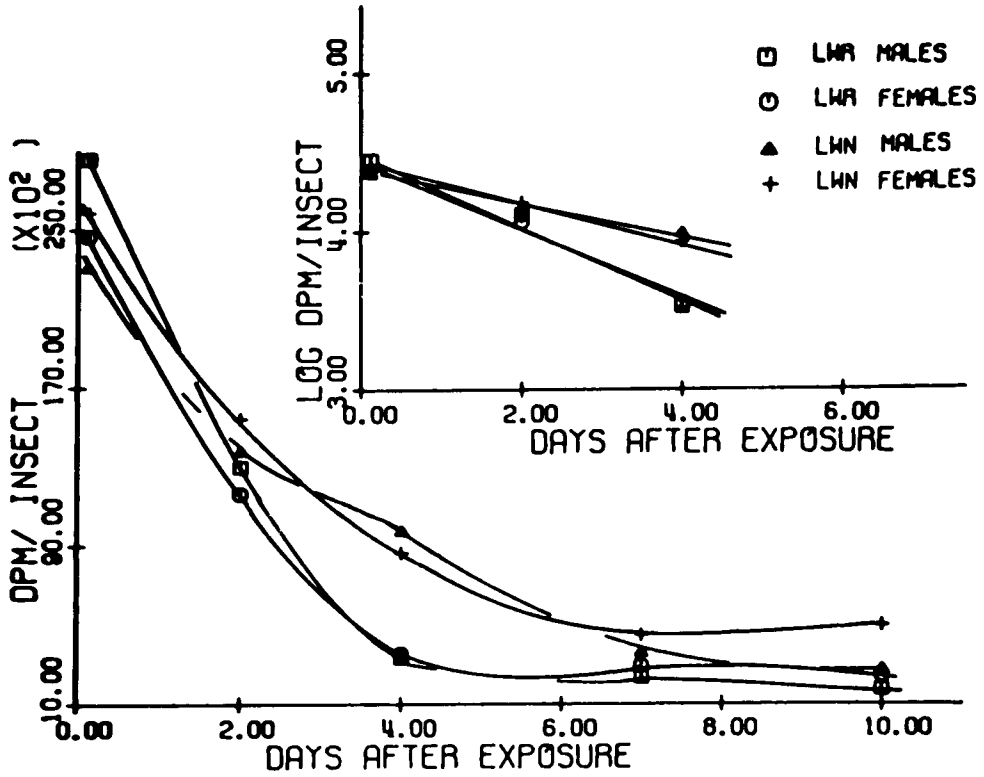

Fig. 24. **Decrease with time of DPM of ^{14}C/NaOH digested LWN and LWR** *Sitophilus granarius*

Insects were previously extracted with petroleum ether for 24 hours

Data from appendix, table 6

Linear regression and correlation analyses for the log insert are shown in table XVI

Table XVI. Linear regression and correlation analyses of log DPM/insect (Y) at three intervals after fumigation with labelled EDB (X)

Strain	Sex	Weight class	Regression equation	Standard error of slope	Correlation coefficient	N*
LWR	F	2.6-2.8	Y = 4.45 - 0.23X	0.01	-1.00(.01)**	3
		3.0-3.2	Y = 4.51 - 0.23X	0.01	-1.00(.01)	3
		> 3.2-3.4	Y = 4.45 - 0.25X	0.03	-0.99(.05)	3
		3.6-3.8	Y = 4.57 - 0.25X	0.07	-0.96(not)	3
	Pooled F		Y = 4.52 - 0.24X	0.03	-0.99(.05)	3
	M		Y = 4.45 - 0.22X	0.02	-0.99(.05)	3
LWN	F	2.2-2.4	Y = 4.36 - 0.10X	0.01	-0.99(.05)	3
	M	2.6-2.8	Y = 4.43 - 0.12X	0.01	-1.00(.01)	3

* number of data points (5 insects/point) ** significance level

The data is from appendix, table 6

The regression lines are shown in the log insert of Fig. 24

was made to separate the feces and debris resulting from the feeding of the insects. The DPM/mg of material are shown in table XVII.

3. DISCUSSION

The fumigant uptake of both strains of insects is directly related to the weight of the insects (Fig. 20). On a DPM/mg basis the heavier insects received a smaller dose of EDB in their tissues (Fig. 21). A part of the observed resistance of the LWR strain is due to the greater weight of this strain. Fumigant uptake parallels oxygen consumption in that the heavier the insect the smaller the amount of fumigant per mg of tissue. The data are variable as is seen from the correlation coefficients of table XI. Some of the correlations are not significant but this is because of the low number of degrees of freedom. This variation is expected because of the influence of the insect activity and respiration rate on fumigant uptake. The variation may be controlled by determining the amount of ^{14}C at several intervals after fumigation and then fitting a regression line for amount of ^{14}C as a function of the time after fumigation (Fig. 22). Such regression lines are less influenced by variations of individual observations and enable projection of the line to predict the amount of fumigant immediately after fumigation. Figure 22 shows that the heavier the insects the greater the fumigant uptake.

Figure 20 shows a second component of resistance. The LWR insects took up less fumigant than LWN insects of the same weight. This difference may arise from a difference in the activity and respiration

Table XVII. DPM of ^{14}C/mg of LWN and LWR *Sitophilus granarius* feces and debris collected at four intervals after fumigation with labelled EDB

Days after fumigation	LWN Mean ± SD	LWR Mean ± SD
2	47813 ± 75	25505 ± 92
4	10238 ± 16	5226 ± 24
5	1514 ± 23	811 ± 29
7	910 ± 6	709 ± 12

rate between the two strains during exposure to the fumigant. EDB has a depressing effect on respiration and it is possible that EDB resistant insects respond by reducing their respiration rate faster or to a lower concentration of poison. Protective stupefaction by HCN has been observed for a number of insects including the California red scale and this was at first believed responsible for the resistance of this insect but this has been found not to be the case (Lindgren, 1938; Yust, 1952; Yust and Shelden, 1952).

The slopes of the regression lines of Figs. 20 and 21 are not significantly different as seen from the variation in the slopes of table XI and the large standard errors of the slopes.

The regression equations of Fig. 21A were used to estimate the relative importance of the insect weight and differences in strains in influencing fumigant uptake. The mean weight of the insects (table I) were used in the regression equations of Fig. 21A. Solving the regression equation for LWR females ($Y = 7676 - 741X$) with $X = 3.56$ gives 5035 DPM/mg of tissue and the equation for LWN females ($Y = 6921 - 266X$) with $X = 2.59$ gives 6232 DPM/mg of tissue. This is a difference of 1197 DPM. LWN insects weighing 3.56 mg would have 5999 DPM/mg of tissue according to the regression formula for LWN insects. Thus approximately 233/1197 or 20 per cent of the observed difference in fumigant uptake may be attributed to the increased weight of the LWR strain and the remaining 80 per cent is attributed to a strain difference in response to the fumigant. The LWN strain received 6232/5035 or a 1.2 times greater dose of EDB in their tissues than the LWR strain (based on mean weights of the strains).

Table XII shows that LWN insects had approximately twice as much ether extractable ^{14}C as LWR insects. This experiment was designed to measure the surface absorbed fumigant. Because the insects of both strains were approximately the same weight one would expect the amount of fumigant on their surfaces to be approximately the same. The fact that twice as much fumigant was extracted from the LWN insects as from the LWR insects in one minute shows that fumigant was extracted from the interior of the insects as well as the surface. The greater amount of ether extractable ^{14}C in the LWN strain agrees with the results of sodium hydroxide digestions made after fumigation.

Extractions made with petroleum ether at various intervals after fumigation with labelled fumigant showed that the loss of ^{14}C was according to a log-log function with time (table XIII). The slopes of the regression lines are not different except for the differences between the sexes (males -.867 and -.870 as compared to -.666, -.655, -.688 and -.629 for females). Males converted the ^{14}C labelled EDB to some non-petroleum ether extractable material faster than females. The heavier than average females had more lipid than males (Fig. 17) and one explanation is that more of the EDB was present in the lipids of females and thus not metabolized.

Table XIV shows 8-9 per cent of the total dose received by the LWN strain was still present in an ether extractable form after 2 days whereas only 3-4 per cent was ether extractable in the LWR strain after 2 days. One expects a higher percentage of ether extractable ^{14}C in LWR insects if lipid content and the solubility of EDB in lipids were factors in resistance. The low percentage of petroleum ether

extractable ^{14}C in the LWR strain demonstrates biochemical resistance and that the LWR strain was able to get rid of the ^{14}C labelled EDB at a faster rate than the LWN strain. This test for retention of labelled EDB in the lipids of the two strains is therefore invalid and no conclusions can be reached.

The linear regression of log DPM, obtained by sodium hydroxide digestion, on interval after fumigation was correlated less than -.96 in all instances when only the first three days after exposure were considered (table XV). The reason correlation was not significant was because of only one degree of freedom. The intercepts of the regression lines changed according to the weight of the test insects but the slopes were not significantly different.

There was a greater difference between the DPM/insect of insects of the same weight but different strains than there was between insects of the same strain but different weights (Figs. 22 and 23). Insect strain was therefore more important than insect weight in determining the uptake of EDB.

When the insects were extracted with petroleum ether prior to sodium hydroxide digestion the results were quite different. The slopes of the regression lines for LWR insects was approximately twice as steep as for LWN insects (Fig. 24). Table XVI shows that the various weights of LWR females did not have a significantly different slope. Although previous data showed that the dose received by the LWN strain was greater than that received in the tissues of the LWR strain, the LWR strain had about the same amount in their tissues that was not petroleum ether extractable (i.e. metabolites or bound). This

data shows that the LWR strain converted the ^{14}C labelled EDB to a non-petroleum ether extractable material more efficiently than the LWN strain and demonstrates that part of the difference in tolerance between the two strains is biochemical resistance. The reason a difference in slopes was not observed when total DPM/insect was examined was probably because the amount of unchanged fumigant in the insects masked the difference.

Large amounts of ^{14}C appeared in the feces and debris (table XVII). The difficulty of obtaining more than two samples of feces for each strain during the first 4 days after fumigation when the levels of activity in the feces were changing rapidly made it impossible to compare ^{14}C excretion in LWN and LWR insects. Table XVII indicates that the LWN strain had greater amounts of ^{14}C in the feces but this strain also had a higher concentration of ^{14}C in its tissues.

VIII. GENERAL DISCUSSION AND CONCLUSIONS

The strain difference in susceptibility under investigation was only approximately one-half that expected when the project was undertaken. After 40 generations of selection for tolerance to methyl bromide, the LWR strain was 5.5 ± 0.2 times more tolerant to methyl bromide than the LWN strain and 3.0 ± 0.2 times more tolerant to EDB (Monro *et al.*, 1961). Ellis (1965) found after the same number of generations of selection that the LWR strain was only 1.5 times more tolerant to EDB than the LWN strain. In this study after 67 generations of selection the LWR strain was 1.9 times more tolerant.

This study is similar to that of Ellis (1965) in that selection with methyl bromide was suspended during the study. The differences in tolerance are not explained on the basis of reversion towards wild type tolerance levels in the absence of selection pressure because Monro (1964) suspended selection of a Montreal wild strain of *S. granarius* after 14 generations and found no reversion of the tolerance during the next 25 generations. Monro (1964) interpreted this to mean that the strain was isogenic for tolerance to methyl bromide and that all the genetic factors for susceptibility to methyl bromide fumigation had been eliminated and the factors for tolerance assembled. The strain could not have been isogenic for tolerance because the continuation of selection with methyl bromide increased the tolerance of the Montreal wild strain to methyl bromide (Monro, 1964) and continuing the selection of the LWR strain from generation 40 to 67 increased the tolerance from 6-fold to 7-fold (H. A. U. Monro, personal communication).

The stability of tolerance does suggest that some loci were fixed for tolerance.

The stability of tolerance in the absence of selection may be due to the integration of factors for tolerance into the residual genotype (Keiding, 1967). The longer the selection is continued the more the genotype of the insect is modified for compatibility between the factors for tolerance and the rest of the genetic material. With 67 generations of selection it is also possible that genes for tolerance have become linked with genes governing vital processes resulting in the stability of tolerance in the population. The resistance of the California red scale to HCN was also stable through 5 generations in the absence of fumigation while at the same time fumigation was increasing the resistance (Lindgren and Vincent, 1962).

Ellis (1965) found by feeding sugar solutions after fumigation with EDB that the susceptibility ratio for strains of *S. granarius* was influenced by post-exposure diet. Table III of this study shows that the susceptibility ratio is influenced by the interval between fumigation with EDB and mortality determination. Richards (1947) found that the weight of *S. granarius* varied according to the length of time for emergence. In this study only one week of emergence was used from each colony and the difference in weight between LWN and LWR insects was different than that of Monro *et al.*,(1961). This was undoubtedly also a reason why the strain difference in susceptibility was less than that found by Monro and his colleagues.

I conclude that no significant changes in tolerance occurred during this study and that the differences in susceptibility ratios

are due to bias in selecting test insects and also due to different exposure and post-exposure conditions. Monro *et al.* (1961) fumigated at 25 C and 70 per cent relative humidity whereas in this study the temperature was 26.7 C and the relative humidity was 50 per cent.

The hypothesis was tested that a lower respiration rate/mg of tissue accounts for a part of the tolerance of the heavier LWR strain. Fumigations showed that the weight of the test insects and their susceptibility were significantly correlated. Approximately 25 per cent of the observed difference in susceptibility between the two strains was correlated with the 1.3 times greater weight of the LWR strain insects. Lloyd and Parkin(1963) reported that less than one-twentieth of the observed resistance of a pyrethrum resistant strain of *S. granarius* was due to the greater weight of that strain. The resistance however was 34 fold. I found that the respiration rate/mg of tissue was negatively correlated with insect weight as was the fumigant uptake.

Experiments with labelled EDB showed that 2 hours after fumigation the LWN insects contained 1.2 times more fumigant/mg of tissue than the LWR insects. As expected, the lighter insects contained more fumigant/mg of tissue however only 20 per cent of the greater concentration of tissue ^{14}C was explained by the smaller weight of the LWN insects. The most significant factor determining fumigant uptake was a strain difference in uptake that was not correlated with insect weight. Insect size is therefore a significant but small factor determining fumigant uptake by *S. granarius*. Other undetermined factors must be correlated with insect weight to account for the 25

per cent of tolerance correlated with the weight increase of the LWR strain as determined by actual fumigation.

In defining vigor tolerance (Hoskins and Gordon, 1956) failed to specify whether referring to tolerance to poison in the insects environment or tolerance to concentrations in the insects' tissues. It is assumed that concentrations in the tissues (LD50) was meant. In this connection, the literature regarding vigor tolerance of *S. granarius* to methyl bromide and EDB is confused by the predominent use of the term 'LD50' when a lethal concentration of vapors in the insects' environment is meant (LC50) (Monro *et al.*, 1961; Page and Lubatti, 1963; Ellis, 1965).

When speaking of LC50's it is understandable why larger strains of insects can survive greater concentrations of a variety of fumigants. Larger insects have a greater ratio of metabolically active tissue compared to the cuticle surface area where absorption occurs. They also generally have a lower respiration rate/mg of tissue than lighter insects of the same species and would be expected to take up less fumigant through the trachae. This protective mechanism is distinct from vigor tolerance as defined by Hoskins and Gordon (1956). Apparently no one else has distinguished between the physical effect of insect size on fumigant uptake and the tolerance or endurance of greater concentrations of poison in the tissues. Because a principal criterion for vigor tolerance is a greater weight of the tolerant strain, I think a part of many of the differences in susceptibility that have been attributed to vigor tolerance is due to reduced fumigant uptake as shown in this study with *Sitophilus granarius*.

The term vigor tolerance has been criticized by Crow (1960) and Busvine (1963) as being incompatible with natural conditions of selection as it implies that selection with one particular insecticide has rapidly improved tolerance beyond the balance obtained with eons of natural selection. The criticisms of this term have been largely semantical and the existence of a 'selection syndrome' has been generally accepted (Georghiou, 1965).

The LWN and LWR strains did not have a significantly different lipid content when insects of the same weight were compared. The weight of lipid/insect increased as expected with the weight of the insect. Any effect of greater lipid/insect on susceptibility to EDB would be correlated with insect weight as described above.

The study with ^{14}C labelled EDB however showed no relationship between weight of insects and percentage of the total ^{14}C that was ether extractable after 2 days. There was a significant difference in the per cent of ether extractable ^{14}C in the two strains. This was due to a difference in rate of metabolism in the two strains resulting in the LWR strain eliminating the ^{14}C faster than the LWN strain. The greater lipid content of LWR insects and the solubility of EDB in lipids were not shown to determine strain susceptibility to EDB. Females, however, eliminated the ^{14}C at a slower rate than males and this may have been due to more EDB in the lipids.

Although EDB depressed the respiration rate of fumigated insects during exposure, the respiration rate recovered to normal levels after the insects were removed to fresh air and the respiration rate did not decrease again until the onset of immobility prior to death. Lipid

reserves of fumigated insects were depleted at approximately the same rate as in starved insects. There was no indication that EDB poisoning interfered with lipid metabolism.

LWN insects die sooner than LWR insects when starved or when fumigated with EDB. The length of time for mortality due to fumigation or starvation to occur depends on the post-exposure treatment or the conditions during starvation. The susceptibility factor is also dependent on the post-exposure conditions. These facts suggest that energy reserves are important in tolerance to EDB.

A pyrethrum resistant strain of *S. granarius* was 1.26 times heavier than the non-selected strain and was 2.0 times more tolerant to starvation, 1.2 times more tolerant to heat and 1.8 times more tolerant to desiccation (Lloyd and Parkin, 1963). Probably, as these authors suggest, the tolerance to these stress conditions was a result of the greater weight. The effect of greater tolerance or endurance of heavier LWR strain insects on their survival after fumigation could be difficult to measure.

Because under the conditions of this study only a 1.9 fold difference in strain susceptibility was found and because the strain difference in fumigant uptake was highly significant as was the strain difference in metabolism of the EDB, it would be very difficult to conclusively demonstrate energy reserves and greater endurance of stress conditions as factors in the strain susceptibility to EDB poisoning and the subject was not further pursued. This could be one of the factors responsible for the 25 per cent of the strain difference in susceptibility that is correlated with insect weight.

There is therefore circumstantial evidence that some of the tolerance of the heavier LWR insects resulted from vigor tolerance as defined by Hoskins and Gordon (1956).

Studies with ^{14}C labelled EDB showed that LWN and LWR insects of the same weight take up significantly different amounts of fumigant. No significant difference in the respiration rate of the two strains was found.

EDB has a depressing effect on respiration rate during fumigation and my hypothesis is that the resistant insects respond by reducing their activity and respiration rate faster or to a lower concentration of EDB. An alternative hypothesis is that EDB penetrates the two strains at different rates due to differences in the cuticle or trachae. This is a subject that requires further research.

Experiments with ^{14}C labelled EDB showed excretion of ^{14}C in feces and a different rate of metabolism between the two strains. The LWR strain converted the EDB to some non-petroleum ether extractable material faster than the LWN strain and the amount of recoverable ^{14}C decreased faster. This indicates true resistance as defined by Hoskins and Gordon (1956).

EDB is a homologue of methyl bromide so one expects similarities in metabolism and some cross resistance. The fact that there is biochemical resistance in the LWR strain to EDB indicates biochemical resistance to methyl bromide as well. One would expect biochemical resistance against methyl bromide to be more important than against EDB because it was the fumigant used in selecting the resistance. The possibility of biochemical differences in metabolism of methyl

bromide between LWN and LWR *S. granarius* has not been investigated and is an area where further research is needed. A study of methyl bromide resistance could be coupled with identifying labelled cysteine conjugates and mercapturic acids in the feces and determining their per cent abundance in the two strains.

Monro *et al.* (1961) found that besides a 5.5 fold tolerance to methyl bromide and a 3 fold tolerance to EDB, the LWR strain had a 2.0 fold tolerance for HCN, a 4.7 fold tolerance for acrylonitrile, a 4.8 fold tolerance to ethylene oxide and a 5.9 fold tolerance to phosphine. The tolerance of the LWR strain to phosphine and acrylonitrile was thus greater than its tolerance to the methyl bromide analogue, EDB. Biochemical cross resistance is not probable for these fumigants.

The HCN resistant California red scales were also more resistant to methyl bromide, ethylene oxide and hydrogen sulfide (Quayle, 1938; Yust and Shelden, 1952). A strain of *S. granarius* that was 34 times more resistant to pyrethrum was 15 times more tolerant to DDT, 5.7 times more resistant to malathion, 5.5 times more resistant to lindane and 3.1 times more resistant to carbaryl but had no resistance to methyl bromide (Lloyd and Parkin, 1963).

The tolerance of the LWR strain to fumigants unrelated to methyl bromide was an important factor in categorizing the strain differences in susceptibility as vigor tolerance and was a reason for expecting animal size and physical factors determining fumigant uptake to be important in tolerance.

The biochemical resistance of the LWR strain to EDB and the indicated resistance to methyl bromide cannot explain the greater

tolerance of this strain for phosphine, acrylonitrile and HCN. The resistance of the LWR strain to each of the fumigants may be different in some respects and in order to interpret the tolerance to these fumigants further studies with labelled fumigants are necessary.

From this study with *S. granarius*, general caution in the use of the term 'vigor tolerance' was indicated. I suggest the use of this term only as an epitome for multifactorial, non-specific, low-order susceptibility differences.

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X. APPENDIX

Table 1. Gas exchange of *Sitophilus granarius* after fumigation with EDB

Strain (symptom)*	µg/insect/min (X 10^{-2}) O_2	µg/insect/min (X 10^{-2}) CO_2	µg/mg/min (X 10^{-2}) O_2	µg/mg/min (X 10^{-2}) CO_2
LWN(0)	12.67	15.26	5.51	5.06
	19.06	14.09	6.53	4.31
(1)	21.96	13.62	8.07	5.58
	15.08	14.09	6.03	5.97
(2)	6.89	11.74	5.46	5.66
(3)	7.72	6.58	3.68	3.92
LWR(0)	13.76	12.68	3.66	4.12
	15.08	12.91	4.12	5.02
(1)	18.58	14.09	6.19	3.46
	12.06	14.09	4.22	4.63
(2)	8.45	10.33	3.67	4.07

* symptom classes: 0- not fumigated, 1- fumigated no symptoms, 2- fumigated walking with difficulty, 3- fumigated unable to walk

Carbon dioxide measured 4 days after fumigation; oxygen 5 days after fumigation; 5 insects/sample

Table 2. Total DPM of lots of three insects of four weight classes of LWN and LWR *Sitophilus granarius* 1.5 hr after fumigation with labelled EDB

Strain	Weight (mg)	DPM ± SD Rep 1 (F)*	Rep 3 (F)	Rep 2 (M)	Avg(F)	Avg(M&F)
LWN	2.0-2.1	39022 ± 178	39338 ± 71	36820 ± 145	39180	38393
	2.4-2.5	42026 ± 149	48283 ± 304	39740 ± 204	45154	43350
	2.8-2.9	51205 ± 235	58767 ± 201	57519 ± 121	54986	55830
	3.2-3.3	53557 ± 138	62893 ± 119	63272 ± 225	57725	59574
LWR	2.0-2.9	46480 ± 164	49118 ± 121	33448 ± 122	47799	43015
	3.2-3.3	44345 ± 208	53018 ± 247	46195 ± 135	48682	47852
	3.6-3.7	59734 ± 241	59305 ± 227	51699 ± 149	59520	56913
	4.0-4.1	51803 ± 123	56304 ± 260	57802 ± 257	54053	55303

* F- females; M- males

Samples prepared by sodium hydroxide digestion

Table 3. DPM of ^{14}C/insect extracted with petroleum ether for 24 hours

Days after exposure	LWR strain					LWN strain			
	F* 2.6-2.8	F 3.0-3.2	F 3.2-3.4	F 3.6-3.8	M** 3.0-3.2	F 2.2-2.4	F 2.6-2.8	F 3.0-3.2	M 2.6-2.8
.125	5481	4919	3346	4473	9137	5201	11768	11949	10182
2	754	625	851	654	604	843	770	***	938
4	526	617	430	366	396	971	***	***	489
7	390	323	355	303	317	307	***	***	309
10	46	35	38	13	***	39	***	***	100

* F- females; ** M- males; ***- missing data

5 insects/sample

Table 4. Total DPM of ^{14}C/female insect of four weight classes of LWN and LWR *Sitophilus granarius* at five intervals after fumigation with labelled EDB

Days after exposure	DPM/insect ± SD							
	LWN strain				LWR strain			
	* 2.0-2.1	2.4-2.5	2.8-2.9	3.2-3.3	2.8-2.9	3.2-3.3	3.6-3.7	4.0-4.1
.125	12798±59	14450±101	18610±78	19858±75	14338±55	15951±82	18971±80	18434±87
1	**	**	6826±37	9154±51	3837±32	4767±48	**	5018±41
2	**	**	3030±49	3894±20	2329±15	2393±32	**	2416±30
4	**	918± 19	1287±33	1425±26	878±16	952±22	1010±21	994±31
7	**	692± 15	924±14	930±17	633±10	691±17	723±18	832±24

* weight classes in mg ** missing data

samples prepared by sodium hydroxide digestion

3 insects/sample

Table 5. Total DPM of ^{14}C/mg tissue of females of four weight classes of LWN and LWR *Sitophilus granarius* at five intervals after fumigation with labelled EDB

	DPM/mg tissue ± SD							
Days after exposure	LWN strain					LWR strain		
	* 2.0-2.1	2.4-2.5	2.8-2.9	3.2-3.3	2.8-2.9	3.2-3.3	3.6-3.7	4.0-4.1
.125	6243	5898	6530	6110	5031	4908	5198	4552
1	**	**	2395	2817	1346	1467	**	1239
2	**	**	1063	1198	817	736	**	596
4	**	375	452	438	308	293	277	245
7	**	282	324	286	222	213	198	205

* weight classes in mg ** missing data

samples prepared by sodium hydroxide digestion

3 insects/sample

calculated from the data of appendix, table 4

Table 6. DPM of ^{14}C/insect of samples of five insects previously extracted with petroleum ether for 24 hours

Days after exposure	LWR * F 2.6-2.8	F 3.0-3.2	F 3.2-3.4	F 3.6-3.8	F Mean	M 3.0-3.2	LWN F 2.2-2.4	M 2.6-2.8
0.125	25777±22	29374±23	29816±23	29357±23	28581±1881 **	24655±22	23037±37	25889±23
2	10755±16	11466±16	12858±17	16818±17	12974±2707	11622±17	13744±22	15446±18
4	3426±12	3682±12	3170±12	3086±12	3341±269	3548±13	9685±16	8627±15
7	1997±11	1990±11	2271±11	3199±12	2364±495	2925±12	3552±12	4487±13
10	1573±20	1979±18	1630± 9	1868±25	1763±193	2442±34	1704±12	4976±18

* F- females; M- males

** this column of SDs is for the four samples of females; the others refer to counting statistics of one sample

samples prepared by sodium hydroxide digestion