THE ROLE OF SELECTED FRASS CHEMICALS AND CUTICULAR LIPID COMPONENTS IN THE ORIENTATION OF CERTAIN LARVAL TENEBRIONIDAE

by

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Abstract

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The larvae of *Tenebrio molitor* Linné and *Alphitobius diaperinus* (Panzer) both aggregated upon substrates treated with aqueous extracts of conspecific larval frass. Lactic acid is a pheromone in the frass of both species. *Alphitobius* larvae were attracted to lactic acid, while lactic acid caused *Tenebrio* larvae to arrest.

Propionic acid is a repellent pheromone present in *Tenebrio* frass, but the lactic acid-induced response is dominant. The role of these chemical factors in population orientation of the larvae of these mealworm species is discussed.

The cuticular lipids of the larvae of both species contained close-range attractants that had a role in aggregate formation. The *Tenebrio* cuticular lipid pheromone is predominately 8,9-pentacosanediol. The *Alphitobius* cuticular pheromone is a mixture of at least two compounds.

The ecological preferences of these larvae suggested that these aggregation pheromones increased the density of individuals per unit volume. This increased density had varying effects on the physiological development of *Tenebrio* individuals.

Résumé

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Les larves de *Tenebrio molitor* Linné et d'*Alphitobius diaperinus* (Panzer) ont formé des agrégations sur des substraits traités avec des extraits aqueux de chiures de larves conspécifiques. L'acide lactique est un phéromone présente dans les chiures des deux espèces. Les larves d'*Alphitobius* étaient attirées par l'acide lactique, alors que les larves de *Tenebrio* ne répondaient à l'acide lactique qu'après son contact.

L'acide propionique est une phéromone répulsive présente dans les chiures de larves de *Tenebrio*, mais la réponse provoquée par l'acide lactique est dominante. On discute du rôle de ces facteurs chimiques dans l'orientation des populations chez les larves de ces deux espèces de ténébrions.

Les lipides cuticulaires des larves des deux espèces contenaient des attractifs de courte portée, qui ont joué un rôle dans la formation de groupes. Chez le *Tenebrio*, la phéromone dominante du lipide cuticulaire est le 8,9-pentacosanédiol. Chez l'*Alphitobius*, la phéromone cuticulaire est un mélange d'au moins deux composés.

Les préférences écologiques de ces larves ont suggéré que ces phéromones d'agrégation augmentaient la densité des individus par unité de volume. Cette augmentation de densité a eu des effets variables sur le développement physiologique des individus de *Tenebrio*.

Suggested short title:

Pheromone-Induced Aggregation In Mealworm Larvae

David K. Weaver

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Project supervision

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Claim to Originality

The following findings comprise the major original contributions to the knowledge of insect physiology, chemical ecology and behavior.

1. The demonstration of conspecific larval frass as an aggregation inducing substance for *Tenebrio molitor* larvae and *Alphitobius diaperinus* larvae.

2. The demonstration of lactic acid as an aggregation pheromone present in the frass of these two species of mealworms.

3. The demonstration of propionic acid as a repellent pheromone present in the larval frass of *Tenebrio molitor*.

4. The demonstration of the lactic acid-induced response dominating that elicited by propionic acid for the larvae of *Tenebrio molitor*.

5. First conclusive evidence that lactic acid stimuli may be received by either contact or olfaction, depending on the insect species.

6. Species recognition pheromones are reported to be perceived by larval Coleoptera for the first time. These cuticular lipid pheromones are stage-specific as well.

7. The cuticular lipids of insect larval exuviae are proposed to have an important role in the chemical ecology of these species.

8. The demonstration of *Tenebrio molitor* and *Alphitobius diaperinus* larval responses to airborne stimuli from cuticular lipids.

9. Two new indices were devised to evaluate the factors contributing to the net spatial displacement of insect responding to close-range olfactory stimuli.

10. The demonstration of 8,9-pentacosanediol as the major pheromone component of larval *T. molitor*. This compound acts as an attractant.

11. The demonstration that the presence of the minor components in the lipid blend of larval *Tenebrio* serves to enhance the overall response to 8,9-pentacosanediol.

12. It is suggested that frass compounds plus cuticular lipid components serve to indicate regions of prior conspecific occurrence.

13. Investigations to evaluate the role of density on development during the entire larval stage of *Tenebrio molitor*.

14. Demonstration of enhancement of early larval growth of *Tenebrio molitor* by the presence of conspecifics.

15. Demonstration of the negative effects of increased density on late instar larvae and pupae of *Tenebrio molitor* as shown by decreasing mean mass and increasing percent mortality.

16. The demonstration of enhanced growth of female *Tenebrio molitor* reared in isolation over that of females reared at higher densities and that of males reared at any density.

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Introduction and Thesis Outline

Need for investigation of the role of larvae-produced semiochemicals in yellow mealworm orientation. The yellow mealworm, *Tenebrio molitor* Linné (Tm), was the most commonly occurring insect in grain elevators, was seventh in occurrence in flour mills, and sixth in occurrence in feed mills in Canada from 1969-1981 (Sinha and Watters 1985). It is generally reported to inhabit decomposing foodstuffs, particularly in darker, cryptic locations occurring in storage facilities (Cotton 1963). Many major stored products pests are known to be long-lived as adults, and many produce aggregation pheromones as adults (Burkholder 1982), while the duration of the larval stage is comparatively short. The larval stage of Tm is comparatively long (cf. my results, Section 7), while the adults are relatively short-lived (Cotton 1927). Therefore, the orientation of individual and subsequently, grouped larval Tm may be influenced by pheromones that indicate environmentally favorable locations and result in aggregation. This duration of the larval stage makes immature individuals appropriate candidates for the production of such semiochemicals, due to the fact that the refugia normally inhabited are conditioned by frass and exuviae accumulated through the long development of multiple generations, and such locations are often favored over unutilized foodstuff nearby. Therefore, conspecific larval frass and conspecific larval epicuticular components were investigated for behavioral activity against larval Tm (Weaver et al. 1989; Section 2: Frass-induced aggregation- *Tenebrio*, Weaver and McFarlane 1989; Section 3: Aggregation behavior- *Tenebrio* larvae, Weaver et al. In Press; Section 4: Frass repellents- *Tenebrio*, Section 6: Cuticular lipids in aggregation). Information derived from such investigations may provide information that helps to explain the apparent tenacity of such slow-developing and conspicuous insects in a relatively unstable environment for long term establishment of a population.

Investigation of the role of larvae-produced semiochemicals in lesser mealworm orientation. The lesser mealworm, *Alphitobius diaperinus* (Panzer) (Ad), is found to inhabit locations that are ecologically similar to those preferred by Tm larvae, but only rarely (Cotton and Wilbur 1974). It is more commonly asssociated with chicken litter and is a serious pest of poultry facilities because it is regarded as a reservoir of avian pathogens (De las Casas et al. 1976). Ad contrasts with Tm in that these larvae are rapidly developing and the adults are comparatively long-lived (Preiss and Davidson 1971, Wilson and Minor 1969). The association of both species with stored products plus the relative chemical and physical similarity between poultry litter and decomposing foodstuffs indicate that environmental preferences are similar in the two species. Therefore, conspecific semiochemical messages might also be used by larval Ad to orient, though this might not be as likely, given the reduced developmental duration and therefore decreased conditioning time. The comparison of the behavioral responses of another, very developmentally distinct (from Tm) larval Tenebrionid to its conspecific larval frass and cuticular components was thus undertaken to determine if larval pheromone production was evident for more than one species of this family (Section 5: Frass-induced aggregation-*Alphitobius*, Section 6: Cuticular lipids in aggregation).

Possible pheromone components of the mealworm frass were investigated selectively. Fermentation products in the frass, specifically lactic acid and the volatile fatty acids, were found to induce behavioral responses in a variety of insect larvae (McFarlane 1984, McFarlane and Alli 1986, McFarlane and Alli 1987, McFarlane et al. 1983, see Section 1: Literature review for discussion). Therefore, a priori justification existed for the quantification of these compounds and for subsequent behavioral analyses. This selective analysis was undertaken for practical reasons, for example, Fuchs et al. (1985) identified 150 compounds, including 57 carboxylic acids, in behaviorally active frass extracts of the German cockroach. Thorough analysis of such a large amount of material is not feasible in a limited time frame, particularly

if one considers that activity may well be linked to specific blends of components. The adult frass was not analyzed for activity because potential larval-larval communication was the objective of the research.

The effect of increased density as a result of aggregation. The aggregation induced by these pheromones would probably increase potential competition, as has been suggested for adult-produced aggregation pheromones in *Oryzaephilus surinamensis* L. by White and Chambers (1989) and makes the potential benefits difficult to address. However, it is possible that increased interaction between young insect larvae can enhance the development of individuals compared to that of an isolated larva, e.g., *Acheta domesticus* L., as described by McFarlane (1962). To evaluate if either competition or enhancement of development occurs as a possible result of association in restricted surroundings, Tm larvae were reared from hatch to adulthood at varying densties. The results of this experiment are presented in Section 7: Effect of density upon larval development.

Literature review. A literature review of aspects of the bioecology, physiology and behavior of Tm and Ad that contibute to the objectives of this thesis is given in Section 1: Literature review. More specific discussion of other relevant material is given in the appropriate section.

Thesis format. The present thesis format accepted by the Faculty of Graduate Studies and Research, and the Department of Entomology of McGill University, requires the citation of the following portion of the "Guidelines Concerning Thesis Preparation".

"The inclusion of manuscripts co-authored by the candidate and others is acceptable but the candidate is required to make an explicit statement on who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims, e.g. before the Oral Committee. Since the task of the examiners is made more difficult in

these cases, it is in the candidates interest to make the responsibilities of authors perfectly clear. Candidates following this option must inform the Department before it submits the thesis for review."

I conceived and executed all of the experiments presented in this thesis and wrote the five papers that have been submitted to refereed scientific journals (Section 2-5, Section 7). However, chemical spectra (Section 6) were obtained by Anmar Ali and Dr. T.H. Chan, Department of Chemistry, McGill University, from purified material supplied by me; in all cases I participated in the interpretation of such spectra . Column installation and standards were the gracious contributions of Dr. Inteaz Alli, Department of Food Science and Agricultural Chemistry, when gas chromatographic analysis was performed by me (Section 2 and 4). Enzymatic determination of D- and L-lactic acid (Section 2) was performed by Dr. Inteaz Alli. Gas chromatographic analysis of samples in Section 5 was performed by Dr. Inteaz Alli as well. Mass spectra of benzyl lactate (Section 2) was generated by Michael Evans, Université de Montréal (see "Acknowledgements").

Papers are published in or have been submitted to journals of varied affilation and have been presented in the thesis as conforming to the directives of the CBE Style Manual Committee (1983) and using USA spelling for consistency throughout the entire thesis.

Papers are co-authored by my supervisor Dr. J.E. McFarlane (Sections 2-7), and collaborators Dr I. Alli (Sections 2,4 and 5), Anmar Ali (Section 6) and Dr. T.H. Chan (Section 6), whose guidance and participation in my research have been considerable (see "Acknowledgements").

Section 1: Literature Review

Histories, distributions and status as pests. The yellow mealworm is a cosmopolitan stored products pest (Sinha and Watters 1985) with a long history of synanthropic association (Buckland 1981). Cotton (1927) reports that Tm is never found in the southern regions of the United States and this is subsequently stated to be a preference for a cooler climate in more recent literature (Sinha and Watters 1985). It has been recorded from Fourth Century Roman Settlements in Britain (Robinson 1979). It may have originated from the vast *Urwaldrelikt* Central-European primary forest where it has been found to inhabit rot-holes in deciduous trees (Palm 1959). Tm is often associated with pigeons' nests as well (Brendell 1985). Tm is considered a secondary pest of stored products (Cotton and Wilbur 1974) due to its habit of feeding on decomposing foodstuffs in cryptic locations. It will occasionally forage in the main stored products mass (Cotton 1963), but its presence is more often considered an indication of poor storage sanitation (Sinha and Watters 1985). The conspicuous size of wandering pre-pupal larvae makes them have an effect upon marketability of foodstuffs, by presence rather than damage (Cotton 1963).

The lesser mealworm (Ad) is an occasional stored products pest with a cosmopolitan distribution (Sinha and Watters 1985). Early records of synanthropy and geographical origin of this species are currently lacking, but it has twice been reported from birds' nests in Africa (Britton 1940, Buck 1956). Cotton and Wilbur (1974) classify the type of damage caused in stored products by Ad as secondary because infestations are generally located in identical situations as Tm. Ad is, however, considered a serious pest of poultry houses because it may serve as a reservoir of certain avian pathogens (De las Casas et al. 1972, 1976, Harein et al. 1972). It is also known to tunnel into building insulation and cause costly damage (Vaughan et al. 1984), particularly as late-instar larvae (Geden and Axtell 1987). The larvae and adults of this species are voracious predators and have been reported as feeding upon the flesh of pigeon squabs (Lewis 1958), various reptiles and amphibians (Harris 1966) and dead and moribund chicks and broilers

(Harding and Bissell 1958, Harris 1966). Armitage (1986) noted that the presence of an inverse relationship between the lesser mealworm and house fly populations in Britain indicated that this species might have biocontrol potential. Despins et al. (1988) found a decreased rate of house fly emergence in the presence of Ad under simulated field conditions. The tunneling behavior also has biocontrol implications due to increased aeration of manure pads providing sub-optimal developmental conditions for house flies (Axtell 1986).

Descriptions. Tm is classified as follows: Coleoptera (order); Polyphaga (suborder); Tenebrionoidea (superfamily); Tenebrionidae (family); Tenebrioninae (subfamily); Tenebrionini (tribe). This is according to the most current classification by Arnett (1983). It was first described by Linné (1758). The synonym for the species given by Arnett (1983) is *Tenebrio laticollis* (Stephens). The known common name of this species is the yellow mealworm. Descriptions of the egg, larva, pupa and adult are given in Sinha and Watters (1985), among others. External morphological differences between the sexes are given in Bhattacharya et al. (1970).

Ad is classified as follows: Coleoptera (order); Polyphaga (suborder); Tenebrionoidea (superfamily); Tenebrionidae (family); Tenebrioninae (subfamily) Ulomini (tribe). This is according to the most current classification by Arnett (1983). It was first described by Panzer (1797), who placed it in the genus *Tenebrio*; it was subsequently placed in the genus *Alphitobius* erected by Stephens (1832). The synonymy of the species given by Arnett (1983) is as follows: *Alphitobius mauritanicus* (Curtis); *Alphitobius opatroides* (Brullé). The known common name of this species is the lesser mealworm. Descriptions of the egg, larva, pupa and adult are given in Wilson and Miner (1969). External morphological differences between the sexes are given in Hewlett (1958) and Barke and Davis (1967).

Bio-ecologies and physiologies. The developmental biology of Tm was well described by Arendsen Hein in Europe (1920) and by Cotton in the United States (1927). These are not described here, because the ob-

servations of Mellanby and French (1958) and Murray (1968), who found that these earlier authors had considered rearing conditions optimal without the supply of free water from which the larvae drink. This consumption of water was found to enhance larval development in two strains of Tm (Urs and Hopkins 1973). The data from their fast-growing strain reared under optimal conditions are briefly given here: The average pre-imaginal period was 99.2 days (range 79.0-125.3) with first pupation occurring as early as 64 days (cf. my results, Section 7) and the pupal period averaged 7.7 days in duration. Adult longevity was found to be enhanced to an average of 105.2 days. The number of moults averaged 12.2 (range 11-14) during the larval stage, which is much less than the 17-19 (range 9-20) moults reported for half the specimens reared by Cotton (1927). First mention of this high number of moults was made by Riley (1883). Cotton and St. George (1929) found the fecund period of female Tm to range from 21 to 67 days and the average fecundity was 276 eggs per female.

Tm larvae delay their pupation in response to a variety of stimuli. Tschinkel and Willson (1971) report that contact between individuals or in part, with lengths of chain equivalent to large larvae in size, delays pupation significantly. Tyschenko and Sheyk Ba (1986) report that the interaction of temperature and photoperiod have strong effects. The shifting of daylength from ten to twelve hours causes a shift from significant delay in pupation to a stimulation of pupation at 25^{0}C. The development of larvae is sensitive to changes in photoperiod during the entire larval stage. Photoperiod was also found to have a role in cold-hardiness of Tm larvae. Exposure to short-day photoperiod decreases the point of haemolymph supercooling and lowers lethal temperature (Patterson and Duman 1978). Early mention of the dessication tolerance of the larvae of this species was made by Riley (1883), and this has been noted by other authors (Buxton 1930, Mellanby 1932). They found that the larvae can survive long periods of 0% relative humidity, though water losses due occur under these conditions. Under conditions of high humidity (90% r.h.) these larvae are able to absorb water

through their cuticle (Buxton 1930, Mellanby 1932). These larvae have been found to utilize metabolic water to supplement the absence of available environmental water (Leclerq 1948). This preference for water has been correlated in hygrotaxis for larval (Cotton and St. George 1929) and adult Tm (Gunn and Pielou 1940).

Ad development has been studied at various temperatures (Wilson and Miner 1969) and data from the optimal temperature of 32°C are briefly given here: The pre-imaginal period was 45.6 days, the number of larval moults varied from 6-8, though this could increase with decreasing temperature, as could the duration of the pupal period which averaged 5.9 days at optimal temperature. Females were found to lay an average of 5.5 eggs per day under optimal conditions. Preiss and Davidson (1971) found that adult female Ad would oviposit for most of their life span, which was greater than 400 days for 59 of 94 beetles in their study. This corroborates the earlier work of Wilson and Miner (1969). The average pre-oviposition period in Ad was found to be 10-12 days (Lancaster and Simco 1967, Preiss and Davidson 1971).

The role of environmental factors in the development of Ad is not well known. The wandering and tunneling behavior of late-instar larvae has been found to be maximal at pre-dawn scotophase and enhanced by increasing density (Geden and Axtell 1987).

Observations on mixed cultures of Tm and Ad found that Ad, an active predator, preyed upon Tm until the larger competitor was eradicated (Harris 1966).

Pheromones. Pheromones have been described as "chemical substances that are released by an individual of a species and convey information to one or more receiving individuals of the same species" (Katz and Shorey 1979, adapted from Karlson and Luscher 1959). This is a broad definition of pheromones and this has been subsequently been modified (Nordlund 1981), but the earlier definition is favored here because the verb "secretion" used by Nordlund (1981) may imply a mechanism that may not include excretion of such compounds in the frass.

Sex pheromones are thus described as "a chemical or mixture of chemicals that is secreted to the outside by an individual and triggers an immediate response of sexual behavior in the receiving individual" and are generally "secreted by females and act as odorous stimuli for males", though there are many secreted by males that stimulate females (Tamaki 1985). The sex pheromone of Tm was identified by Tanaka et al. (1986). This compound is produced by female Tm only, and is 4-methyl-1-nonanol. Many early studies (Tschinkel et al. 1967, Happ 1969, Happ and Wheeler 1969, August 1971) reported multiple sex pheromones in this species and were confounded by the complexity of the response. The other active component isolated was a combination of cuticular components of both sexes which released copulatory behavior in males (Tanaka et al. 1986, Tanaka et al. 1988). It is of interest that a cuticular component is implicated to have behavioral activity in adult Tm (cf. with my results, Section 6: Cuticular lipids in aggregation). Wilson and Miner (1969) reported that adults of both sexes of Ad had large fleshy scent glands that caused sexual excitement in both sexes when they were punctured and the fluid inside was released. A thorough recent review of sex pheromones in insects is given in Tamaki (1985).

Aggregation pheromones are "substances produced by members of either or both sexes, that induce members of both sexes to aggregate" (Borden, 1985). Borden (1985) gives a thorough review of the aggregation pheromones of major pest species known up until that time, with an emphasis on Scolytid pests of conifers . Recently, Oehlschlager et al. (1988) reviewed the literature on the aggregation pheromones of a variety of stored products Cucujidae. Burkholder and Ma (1985) reviewed the literature on the Bostrichidae, Curculionidae, and Tenebrionidae and discussed their potential in pest monitoring and control. These works emphasize adult-produced and received pheromones. Many insect larvae also respond to pheromones contained in the frass of adults or to pheromone-marked substrates that have been conditioned in conspecific cultures. McFarlane et al. (1983) found that propionic acid was an aggregant for larval *Acheta domesticus* that was deposited upon papers as

they were conditioned in conspecific cultures. Butyric acid was found to function in a similar manner for the larvae of the American cockroach on papers conditioned in *Periplaneta americana* cultures (McFarlane and Alli 1987). Lactic acid is an aggregative component of papers conditioned in *Blattella germanica* cultures and presented to one to two week old larvae (McFarlane and Alli 1986), however various repellent components are also present upon these conditioned papers (McFarlane 1984) that must be masked when lactic acid is present (cf. my results, Section 2: Frass-induced aggregation- *Tenebrio*, Section 4: Frass repellents- *Tenebrio*, Section 5: Frass-induced aggregation- *Alphitobius*). The volatile fatty acids are present in the frass of other insect species, as well (McFarlane and Alli 1985, Odelson and Breznak 1983).

Epideictic pheromones have been defined by Prokopy (1981) as compounds that stimulate dispersal from a resource once an optimal density is reached. These compounds have been demonstrated to play a role in the orientation of several species of stored products insect larvae. Larvae of *E. kuehniella* release a mandibular gland secretion when they are in contact with each other that deters adult oviposition if it is present in sufficient quantity. Smaller amounts stimulate oviposition (Corbet 1971, 1973). Larvae of *Trogoderma granarium* aggregate upon wheat grains that have been fed upon by conspecific larvae (Finger et al. 1965), but high concentrations appear to inhibit further feeding (Tannert and Hien 1976). The repellency of higher concentrations of lactic acid reported by McFarlane and Alli (1985) may be evidence of epideictic activity (cf. my results, Section 2: Frass-induced aggregation- *Tenebrio*, Section 4: Frass repellents- *Tenebrio*, Section 5: Frass-induced aggregation- *Alphitobius*).

The terminology used in this thesis to describe the behaviors elicited by these compounds, in particular, the terms arrest and attract, are derived from Dethier et al. 1960 and Kennedy 1977a, 1977b, 1978). Briefly, these terms were proposed to represent the types function of chemicals by the behavior elicited by them. Attractants were considered to be volatile substances that result in directed movements

toward their point of volatilization. Arrestants were known as compounds that caused movement away from the source of the stimulus after an insect contacted the stimulus directly (Dethier et al. 1960). Many volatile substances are known to cause arrestment in either space or time by decreasing net velocity or increasing the rate of turning (Kennedy 1978). In this study, first choices will be used to determine if a compound is attractive or not, other parameters collected during assay may be used to determine whether or not olfactory arrestment has occurred. Fortunately, a compound which causes spatial displacement of individuals either by remaining or by leaving after contact still fit the original classification of arrestant and dispersant, if there is no evidence of response to volatile stimuli (Dethier et al. 1960).

Cuticular lipids. Cuticular lipids are known to be the sex pheromones of a variety of Diptera (see Blomquist and Dillwith 1985, Lockey 1988 for reviews) as well as the German cockroach (Jurenka et al. 1989) and the rove beetle *Aleochara curtula* (Goeze), (Peschke and Metzler 1987). Components of the cuticle of both sexes are copulation release pheromones of *Callosobruchus chinensis* L. (Tanaka et al. 1981, Tanaka et al. 1988) and *Tenebrio molitor* (Tanaka et al. 1988). Cuticular lipid compounds have been evalauated in preliminary bioassay as species recognition cues in two species of *Reticulitermes* (Howard et al. 1982). In addition, Norris (1970), found that conspecific contact and extracts of cuticular lipids were factors in the aggregation response of ovipositing female *Schistocerca gregaria*. The cuticular lipids of adult Tm have been described by Lockey (1978) and the lipid components of the larval exuviae of Tm have been analyzed by Bursell and Clements (1967). The cuticular lipids of adult Ad have been investigated by Lockey (1979). The cuticular lipid study upon the exuviae of *Tenebrio* larvae indicated that > 55% of the larval lipid was 8,9-pentacosanediol(Bursell and Clements 1967). This is a very high proportion of the total lipid. The cuticular lipids of larval Ad have not been studied. The adult *Tenbrio molitor* have no 8,9-pentacosanediol present in their epicuticle (Lockey 1978).

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Connecting Statement I

The larvae of Tm are frequently associated with cryptic regions in storage facilities, where they inhabit decomposing foodstuffs and other litter. The frequency of reported infestation and similarity in reported locations of infestation suggest that semiochemicals might influence these groups of insects. The frass, particularly that of the long-developing larvae, represents a significant proportion of the litter inhabited and might be a potential source of pheromone, as it is for other aggregating Coleoptera. Fermentation products, particularly lactic acid and the volatile fatty acids, have been identified as aggregants for other insect larvae (see Introduction and Literature review). The role of fermentation products in the frass of larval Tm as potential pheromones for conspecific larvae was thus investigated (Section 2: Frass-induced aggregation).

Section 2: Frass-Induced Aggregation- *Tenebrio*

Title

AGGREGATION IN YELLOW MEALWORMS, *Tenebrio molitor* L. (COLEOPTERA: TENEBRIONIDAE) LARVAE: I. INDIVIDUAL AND GROUP ATTRACTION TO FRASS AND ISOLATION OF AN AGGREGANT.

Authors: Weaver, D.K., J.E. McFarlane and I. Alli. 1989.
J. Chem. Ecol. 15: 1605-1615.

ABSTRACT Late instar larval *Tenebrio molitor* L. were found to be attracted to aqueous extracts of conspecific larval frass. The attraction was evident at both the individual and group level. The attraction of larval groups to frass indicated the possibility of an aggregation pheromone which would be chemically distinct in the mealworm environment. Chemical analysis of short carbon chain acids present in both the mealworm frass and the diet indicated that lactic acid was present in the mealworm frass only. Acetic acid was identified in both the diet and the larval frass. Larvae aggregated on filter papers treated with aqueous frass extracts which had been dried and also on those freshly wetted. The larvae also aggregated on dried or freshly wetted papers treated with lactic acid, but failed to aggregate on freshly wetted papers or dried papers treated with acetic acid. The role of excreted lactic acid as a discriminant of already infested and, therefore, safer environmental regions is discussed.

Introduction

The yellow mealworm, *Tenebrio molitor* L. is a cosmopolitan stored-products pest of economic importance with infestations generally originating in damp, dark places where cereals may be decaying (Cotton 1963). The presence of the conspicuous larvae in a stored-products mass is an indication of poor storage sanitation (Sinha and Watters, 1985). Pheromone communication among *Tenebrio* adults has been studied (Tschinkel et al. 1967, Happ 1969, Tanaka et al. 1986). McFarlane and Alli (1986) have shown DL-lactic acid to be an excreted aggregant of larval *Blattella germanica*. In this article aggregation of larval *Tenebrio molitor* on frass treated substrates is evaluated. Chemical analysis of diet and frass for short carbon chain acids indicated candidate chemicals which may be used by yellow mealworms to analyze substrates for suitability for aggregate formation. These were tested in a behavioral bioassay. The interaction between water and pheromone found in lactic acid-induced larval aggregation in the German cockroach (McFarlane and Alli 1986) was also evaluated for *Tenebrio*. This present article is the first report of a larval aggregation pheromone in *Tenebrio*. This pheromone might also display epideictic activity (Wynn-Edwards 1962, Prokopy 1981).

The objectives of the following experiments were to evaluate a broad range of concentrations of pheromone for behavioral activity and to observe the nature of the response of the insect to these chemicals during a limited period of presentation in a behavioral bioassay. The possible role of this pheromone in population orientation is discussed.

Materials and Methods

Rearing- Yellow mealworms, *Tenebrio molitor* L., were raised on a diet of wheat bran, whole wheat flour and brewers yeast (50:45:5 w/w) at 25±1^0 C and 55±5% relative humidity and a photoperiodic regime of 14:10 light-dark. The density of all stages per filled 4.55 l glass culture jar was not allowed to exceed 250 individuals.

Collection of frass and diet- Larvae (>100 mg) were removed from the culture and individually placed in 20 ml glass jars for 24 hours. Insects were then removed and the collected frass was immediately frozen at $^-40^0$ C. Frass from any moulting larvae was discarded to avoid contamination by the cuticle. Freshly prepared diet was also frozen at $^-40^0$ C.

Methods of acid determination in frass and diet- Frass (380 mg) was ground with a mortar and pestle and placed in a 40 ml centrifuge tube. A quantity (20 ml) of de-ionized distilled water was added to the test tube and the mixture was agitated (1 min) with a vortex mixer and allowed to stand 2 min. This agitation procedure was repeated 3 times. The resulting mixture was centrifuged (200 X *g*, 10 min). The supernatant was filtered into a volumetric flask (25 ml). The same procedure was followed for the diet sample (500 mg).

For acid determination by gas chromatographic analysis, the acids were converted to benzyl ester derivatives (Jones and Kay 1976). Briefly, tetrabutylammonium salts of the acids were prepared by titration of the aqueous acid solution to pH 8 with tetrabutylammonium hydroxide. The aqueous component was evaporated and the salts were solvated in acetone. Benzylation was acheived by adding a slight excess of the amount of benzyl bromide required by reaction stoichiometry to the acetone solution of the salts.

The conditions of gas chromatographic analysis were as follows: Varian gas chromatograph Model 3700 equipped with a flame ionization detector; DB 17 (Chromatographic Specialities Ltd., Brockville, Ontario, Canada) capillary column (30 m X 0.25 mm ID); helium carrier gas (flow

rate = 1 cm^3/min; makeup gas flow rate = 30 cm^3/min); temperature programming: initial temperature = 70°C, initial hold = 12 min, program rate = 4°C/min, final temperature = 180°C, final hold = 1 min. The benzyl derivatives of the acids in the samples were identified by reference to standard preparations of the benzyl derivatives of the benzyl derivatives of the acids. Peak areas were calculated by means of a HP 3390A Reporting Integrator (Hewlett-Packard Co., Avondale, Pennsylvania).

The identity of the peaks of the benzyl derivatives was confirmed by mass spectrometry using a Kratos MS 50 TCTA mass spectrometer coupled to a Kratos DS 55 data system (Kratos, Manchester, England); conditions of mass spectrometry were: electron impact source, run at 79 eV; source temperature = 200°C.

Frass extracts were analyzed for D-lactic and L-lactic acids using the enzymatic assay method of the Sigma Chemical Company, 1984, (Sigma Diagnostics, pyruvate/lactate; quantitative enzymatic determination in whole blood at 340 X 10^{-6}m; procedure No. 826-Uv, St. Louis, Missouri).

Bioassay arena- The arena used was similar to that of McFarlane et al. (1983). Experiments were conducted at room temperature and in arenas kept in darkness. Two 14 X 3.8 cm strips of Whatman No.1 filter paper folded three times were placed on edge and evenly spaced in a 19.2 cm diam. glass culture dish. Test papers were maintained upright by clamping the upper edges of the test with two clamp-style hairpins suspended from a copper wire extending across the arena and attached to either side by clothespins.

Testing for frass attraction- Frozen frass was ground with a mortar and pestle and added to 5 ml of de-ionized, distilled water. The resulting mixture was agitated in a vortex mixer for 2 min and immediately subjected to Buchner filtration through a Whatman No. 1 filter disc. An aliquot (0.68 ml) of the filtered liquid was immediately applied to a test strip. A control strip was wetted with de-ionized, distilled water. Other frass test strips were similarly prepared and allowed to air dry 1.5 hrs prior to testing. A yellow mealworm (140-160mg) was introduced into the centre of the arena to test for in-

dividual attraction; twenty such larvae were used to test group responses. The filter papers were examined at 0.5 hr intervals after the initiation of the trial. The criteria for aggregation/attraction included larvae in full contact with the test strip or those insects contacting the test strip with their antennae, mouthparts or legs, but not actually upon the paper. The dominant response of tested mealworms was to come to rest on or in contact with a test strip. Each trial was terminated after 5 hours. All group trials were replicated 18 times.

Testing for lactic and acetic acid attraction- Analytical grade DL-Lactic acid (Fisher Scientific Company, Montreal, Canada) and Analytical grade acetic acid (Anachemia Limited, Montreal, Canada) were tested by making serial dilutions (10^{-1}M - 10^{-6}M) in de-ionized distilled water and treating a filter paper strip with 0.68 ml of solution. Dried and wetted test papers were prepared and tested using the method described in the frass aggregation bioassay.

Trials to evaluate larval exploration and arrest- To determine if either lactic acid or frass were responsible for either larval attraction or full arrest, groups of larvae and individual larvae were subjected to the described bioassay and directly observed. Data collected were the choice of the first two insects in each group trial and whether these first two responding larvae remained on the test strip for at least 300 seconds. Replication was conducted as soon as possible after the previous trial was completed ($\leq$12/day), 36 trials in total. Individual larvae were observed to determine the significance of their initial choice and tendency to remain on the chosen substrate for at least 300 seconds. This was undertaken to avoid bias possible by scrutinizing data collected at preset times only and also to determine if, in the grouped trials, later larval response is biased by the presence of those first responding individuals.

Statistical analysis- Statistical comparisons between the various test stimuli for grouped responses were based on analysis of variance using Tukey's HSD test on mean aggregation indices. Aggregation indices (A.I.) were calculated by subtracting the number of larvae on the con-

trol paper from that on the treated paper and dividing by the total number on the two papers. Index values may range from -1 (complete repellency) to +1 (all responding individuals on the treatment paper). A value of -0.2 corresponds to 50% more insects on the control paper and +0.33 corresponds to twice as many insects on the treatment as were on the control paper (Roth and Cohen 1973). Indices were based on eight observations per replicate with eighteen replicates being conducted per test chemical. Indices were analyzed by t-test for significant differences from a value of zero (SAS Institute 1988), which would represent identical numbers on treatment and control papers. Chi-square analyses were used to test for significant differences in control vs. control trials (groups and individuals) and all treatment vs. control individual trials. Chi-square analyses were also used to determine if the choices of the first two responding larvae were significantly different in group treatment vs. control tests, and to determine if there was a difference in the tendency to arrest of insects responding to either treatment or control papers during their initial exploration of the arena (Sokal and Rohlf 1981).

Results

Analyses of frass indicated lactic acid at 0.24 ± 0.01g/ 100g frass and acetic acid at 0.25 ± 0.01g/ 100g frass. However, analyses of the diet indicated that although acetic acid was present (0.023 ± 0.004g/ 100g diet), lactic acid is not present in the mealworm diet. The lactic acid isomeric analysis gave the following results: 0.098 % D- lactic acid and 0.087% L- lactic acid per frass sample (frass was 0.185% lactic acid). Dry weight determination indicates fresh frass has a 10.2% moisture content and freshly prepared diet has a 9.8% moisture content. Figure 1 gives representative gas chromatograms of frass and diet samples.

All tested concentrations of frass resulted in aggregation of the larvae with the highest numbers responding to the highest concentration. Aggregation always occurred on papers treated with frass extracts

whether they were presented wet or dry (Table 1). Papers treated with 0.68 ml (the minimum amount of liquid required to fully wet the filter paper) of 10^{-1} M lactic acid, both freshly wetted and dried, were found to be repellent. Papers treated with 0.68 ml of 10^{-3}M lactic acid and dried were most attractive, with 10^{-2}M freshly wetted and 10^{-4}M wetted and dried lactic acid being slightly less attractive. Aliquots of lactic acid at concentrations below 10^{-4}M showed no effect (Table 2). In all trials DL-lactic acid was used to simulate the naturally occurring equilibrium between D-lactic acid and L-lactic acid.

Acetic acid was found to have no effect on the larvae at any concentration, regardless of the method of presentation (Table 3).

All control vs. control trials indicated no significant differences in preference for substrate (data not shown, no significant difference). Geomagnetic bias is unlikely, therefore.

Figure 2 illustrates the insect response to dried papers treated with 0.68 ml of the highest concentration of aqueous frass during the aggregation bioassay. Figure 3 illustrates the insect response to dried papers treated with 0.68 ml of 10^{-2}M lactic acid during the bioassay. The two graphs show similar overall responses (mean number of larvae on treated paper from 1.5 to 5.0 hours), but early in the frass extract trials there were significantly fewer insects at rest on the control papers. The concentration of lactic acid on the frass treated paper (Figure 2) is equivalent to that on filter paper treated with a 0.68 ml aliquot containing lactic acid between 10^{-2}M and 10^{-3}M. The time required for stabilization in trials which indicated successful aggregation (Figures 2 and 3) was approximately 1.5 hr, at which point no further large increases in numbers on either treated or control test strips occurred. Variations occurring after stabilization generally resulted from insects falling from the filter paper into the arena or larvae which were initially inactive becoming active and responding to the filter papers at a later trial interval. In trials where repellency or an indifferent response was indicated stabilization occurred more gradually, but trends towards the final response can be seen at 1.5 hr.

Figure 4 illustrates the insect response to repellent 10^{-1}M lactic acid (wet). There was a significantly higher number of larvae on the control paper at 1.5 hours but the difference became more obvious at 2 hours. The response of the larvae to an indifferent chemical stimulus (10^{-3}M acetic acid) is illustrated in Figure 5. In this case stabilization is gradual with an increase in wandering insects occurring between 3.0 and 4.5 hours. This indicates that prolonged grouped stability was less likely in trials where no chemical message was perceived.

Tests indicated individual larvae were attracted to frass and lactic acid in the same manner as grouped larvae with counts taken at predetermined intervals. Table 4 gives the Chi-square values for a selected frass solution and 10^{-3}M lactic acid at 2 and 4 hour intervals.

Evaluation of first responding individuals (group tested) indicated that overall response of the larvae to treatment or control was not determined by the presence of other larvae (choice of first larvae reaching either test or control strips indicated no significant difference in preference), nor did first responding larvae remain on either treatment or control filter paper for the 300 second interval in a significantly different numbers. In fact, fewer than 60% of the first-responding treatment or control larvae remained for this amount of time. Individual larvae did not show any significant differences in their first choice but those selecting frass or lactic acid tended to remain longer on the paper; however, 31% of the observed individuals initially selecting test chemical stimuli left the papers only to return subsequently (data not shown, no significant difference).

Discussion

Both dried and wetted lactic acid test strips are the preferred substrates for the larvae of *T. molitor* at 10^{-2}M to 10^{-4}M concentrations. This range approximates the amount found in the fresh larval frass. Other chemicals present in the frass exert different behavioral effects when tested in isolation, but their efficacy is completely

masked by lactic acid when tested along with lactic acid at the biological concentration (Weaver et al. In Press, Section 4: Frass repellents- *Tenebrio*). The frass in entirety seems to be exclusively attractive.

The larval frass has a water content of 10.2 %. This indicates that the lactic acid in the frass is unlikely to be in solution, but more correctly, is substrate associated. This may in part account for the fact that the required interraction between pheromone and water (larvae only responding to papers wetted with aqueous acid solution) found in other studies (McFarlane et al. 1983, McFarlane and Alli 1986, McFarlane and Alli 1987) is not required for *Tenebrio*. The surrounding diet has a 9.8% water content, so environmental stimuli are unlikely to require water as a necessary component. Mealworms are also hygrotaxic in the stored product environment when a water source is available; ecologically they prefer damp surroundings. It is possible that they display two similar patterns of response to frass stimuli. In one case they will aggregate in response to frass in the appropriate concentration in a dry but species-marked environmental region. In the other case they may respond to moisture in a damp region, and may use the presence of lactic acid for the same purpose, to cluster. Group hygrotaxis is a form of aggregation in any case; the important distinction is that damp regions also containing frass could provide information on earlier infestation.

These components of the aggregation behavior bring to light an important issue. It is necessary to carefully consider all data provided by a particular bioassay. In the case of these trials it appears that these insects show classical attraction to lactic acid. However, the length of time required for response to occur and the relatively consistent variation of insects that do not respond to acetic acid indicates that these larvae respond to their substrate by contact sampling it in a relatively regular manner (Weaver and McFarlane 1989, Section 3: Aggregation behavior- *Tenebrio*). The response may be similar to that described by Burk and Bell (1973), for contact arrest of cockroaches. In this case we see no evidence of consistent arrest for the

majority of individuals, perhaps because the stimulus is uniformly distributed on the test substrate, rather than being localized. The *T. molitor* larvae wander over the surface of the papers in a random manner with the rate of activity decreasing through time (Weaver and McFarlane, 1989, Section 3: Aggregation behavior- *Tenebrio*). As this rate of activity decreases localized clusters of higher density form, particularly on DL-lactic acid treated substrates but also, with time, on control substrates (Weaver and McFarlane, 1989, Section 3: Aggregation behavior- *Tenebrio*). We suspect that yellow mealworm aggregations may involve mechanical interaction between individuals as well as other chemical messages. There can be little doubt that the overall response is acted out in concert with others behaving in the same manner. This complexity makes it difficult to view such chemicals as having a single effect. Their classification must depend, therefore, upon the relative effects upon both individuals and populations, not upon one or the other exclusively, as previously defined. The terms arrestant and aggregant might both be applied to DL-lactic acid for *Tenebrio* and both could be equally important to individuals and groups.

The response to lactic acid, a component of the frass only, may serve to indicate regions where infestations have been previously located. The long developmental time and conspicuous size of the larvae may make it advantageous for *T. molitor* to aggregate in these regions to avoid mechanical cleaning apparatus or cleaning procedures. This survival advantage may also be augmented by dispersion of the larvae when frass accumulates in amounts large enough to increase the lactic acid concentration into the repellent range. Such concentration increases could be spurred by further decomposition in the damp, dark regions *Tenebrio* inhabits. It is also of interest that acetic acid which is present in both frass and food induces no behavioral effects. This may be due to its environmental ubiquity precluding any directional advantage, but the lack of response to a volatile major component of both food and frass is unexpected, given the fact that both are favorable material for these larvae to inhabit.

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Table 1. Results of aggregation trials using frass extracts.

Chemical	Concentration	Mean A.I. (±S.E.).*	Significance**
Frass (dry)	50 mg/ml H_2O	0.72 ± 0.06^{a}	0.001
Frass (wet)	40 mg/ml H_2O	0.47 ± 0.07^{b}	0.001
Frass (dry)	30 mg/ml H_2O	0.29 ± 0.07^{bc}	0.001
Frass (wet)	20 mg/ml H_2O	0.26 ± 0.08^{bc}	0.001

*Means followed by the same letter are not statistically different at $P \leq 0.05$.

**Results of t-test for significant response to treated paper, i.e., a significant difference from zero (equal numbers on treatment and control). All indices are significant at $P \leq$ the value listed.

Based on 18 replicates of 20 larvae each. Raw data based on counts from time of approximate stabilization to trial end (1.5 - 5.0 hr).

Table 2. Results of *T. molitor* aggregation trials using lactic acid solutions.

Chemical	Concentration	Mean A.I.(±S.E.)*	Significance**
Lactic Acid (dry)	10^{-1}M	-0.39±0.06^{e}	0.001
Lactic Acid (dry)	10^{-2}M	0.35±0.07^{b}	0.001
Lactic Acid (dry)	10^{-3}M	0.60±0.06^{a}	0.001
Lactic Acid (dry)	10^{-4}M	0.26±0.05^{b}	0.001
Lactic Acid (dry)	10^{-5}M	-0.07±0.05^{d}	N.S.
Lactic Acid (dry)	10^{-6}M	0.04±0.05^{cd}	N.S.
Lactic Acid (wet)	10^{-1}M	-0.34±0.07^{e}	0.001
Lactic Acid (wet)	10^{-2}M	0.20±0.06^{bc}	0.005

*Means followed by the same letter are not statistically different at $P \leq 0.05$.

**Results of t-test for significant response to treated paper, i.e., a significant difference from zero (equal numbers on treatment and control). All indices are significant at $P \leq$ the value listed. N.S. Indicates responses to treatment and control papers are not statistically different.

Based on 18 replicates of 20 larvae each. Raw data based on counts from time of approximate stabilization to trial end (1.5 - 5.0 hr).

Table 3. Results of *T. molitor* aggregation trials using acetic acid solutions.

Chemical	Concentration	Mean A.I.(±S.E.)*	Significance**
Acetic Acid (wet)	10^{-1}M	0.05±0.06[a]	N.S.
Acetic Acid (dry)	10^{-2}M	0.07±0.05[a]	N.S.
Acetic Acid (wet)	10^{-3}M	0.05±0.05[a]	N.S.
Acetic Acid (dry)	10^{-4}M	0.02±0.05[a]	N.S.

*Means followed by the same letter are not statistically different at $P \leq 0.05$.

**Results of t-test for significant response to treated paper, i.e., a significant difference from zero (equal numbers on treatment and control). All indices are significant at $P \leq$ the value listed. N.S. Indicates responses to treatment and control papers are not statistically different.

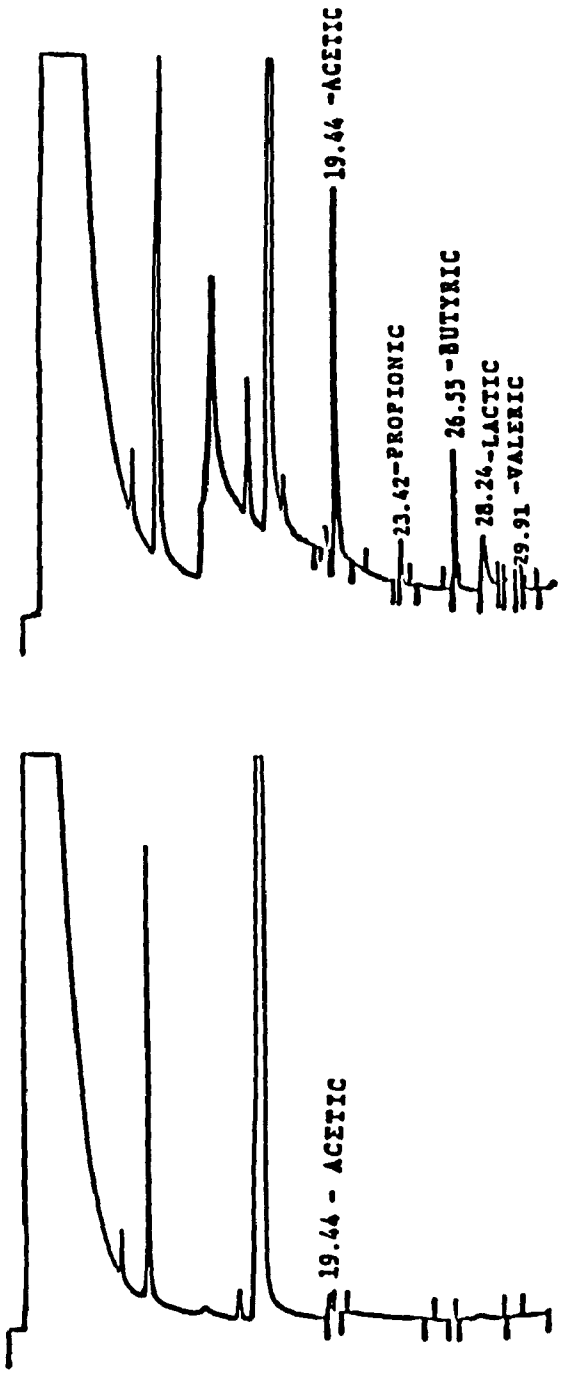
Based on 18 replicates of 20 larvae each. Raw data based on counts from time of approximate stabilization to trial end (1.5 - 5.0 hr).

Table 4. Results of trials using individual *T. molitor* larvae.

Chemical	Time	Treated[a]	Control
10^{-3}M Lactic Acid (Dry)	2 hr	30	13*
	4 hr	38	15**
Frass 30mg/ml (Wet)	2 hr	32	12**
	4 hr	41	14***

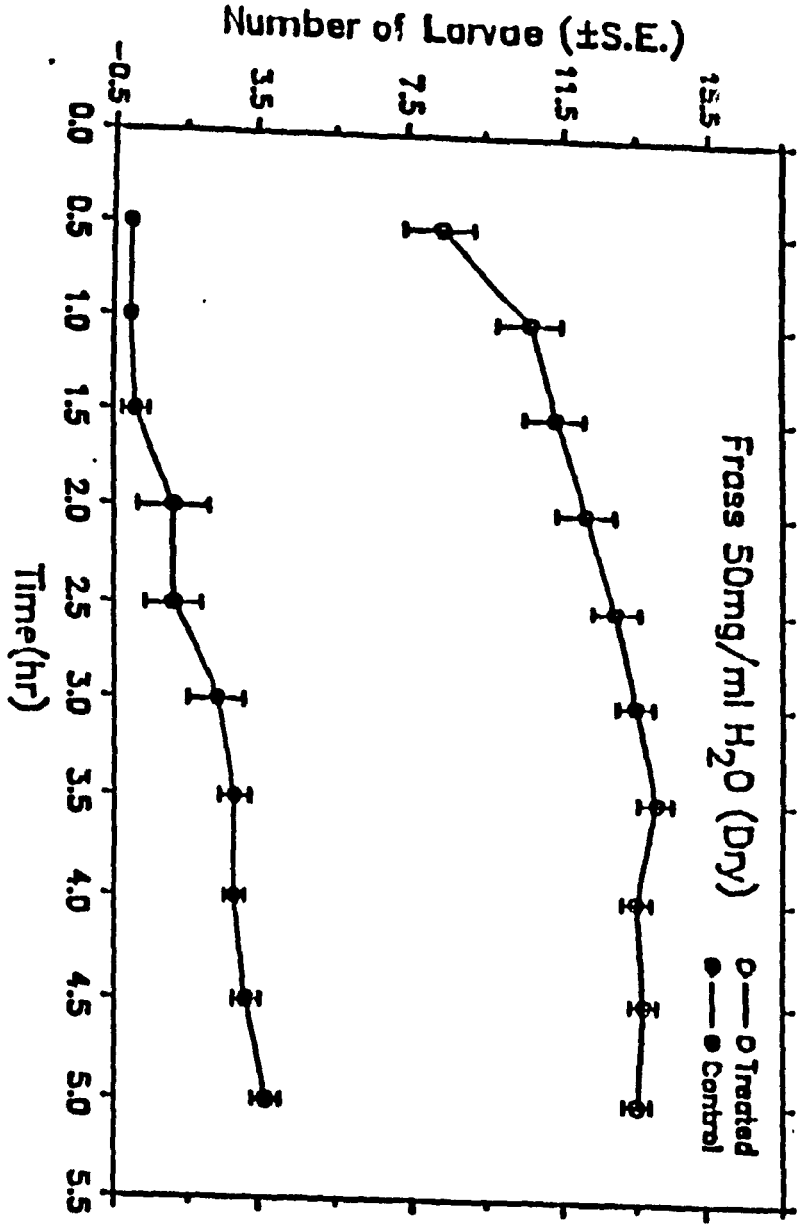
[a]Based on 5 trials of 12 replicates each.
*Treated significantly higher than control at $P \leq .025$.
**Treated significantly higher than control at $P \leq .005$.
***Treated significantly higher than control at $P \leq .001$.

Figure 1. Gas chromatograms of lactic acid and volatile fatty acid composition of *Tenebrio molitor* frass (upper) and diet (lower) samples. Common name and retention time of each acid is given over its representative peak.



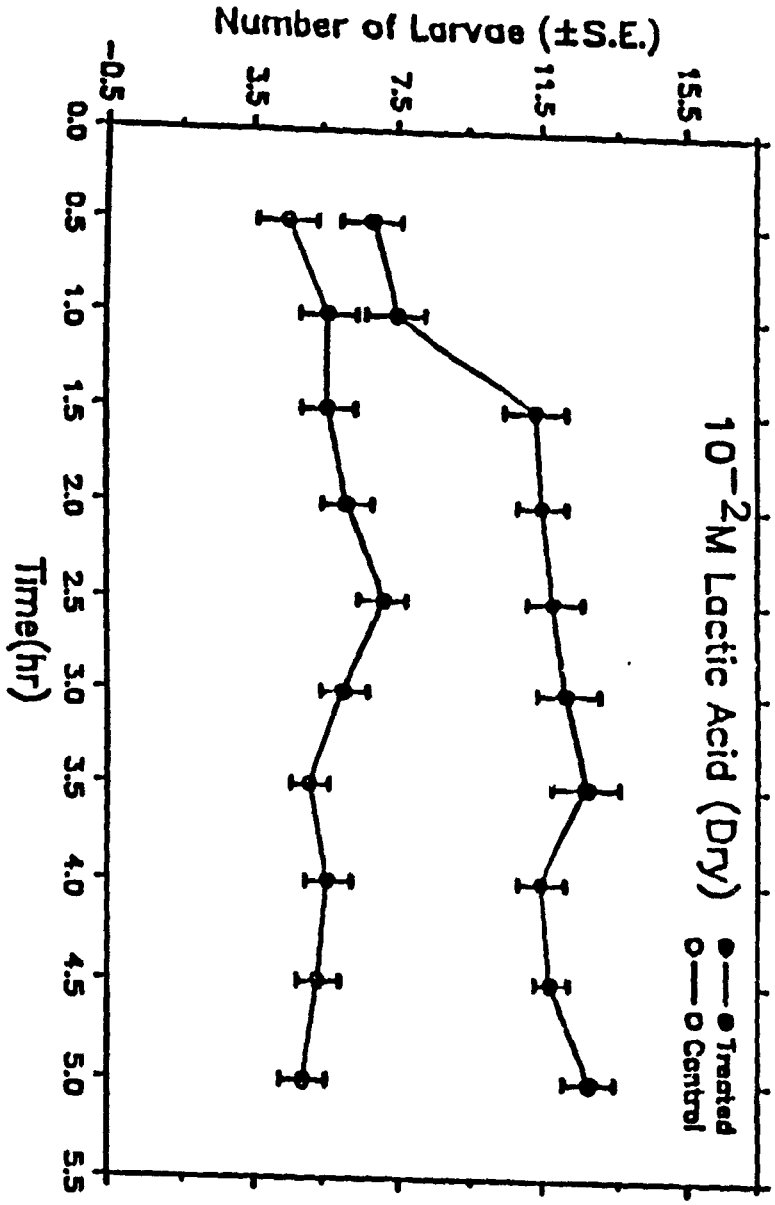
Frass-induced aggregation- *Tenebrio*

Figure 2. Mean number of larval mealworms (±S.E.) responding to papers treated with 50 mg frass/ml H_2O and dried. Based on 18 replicates of 20 larvae each.



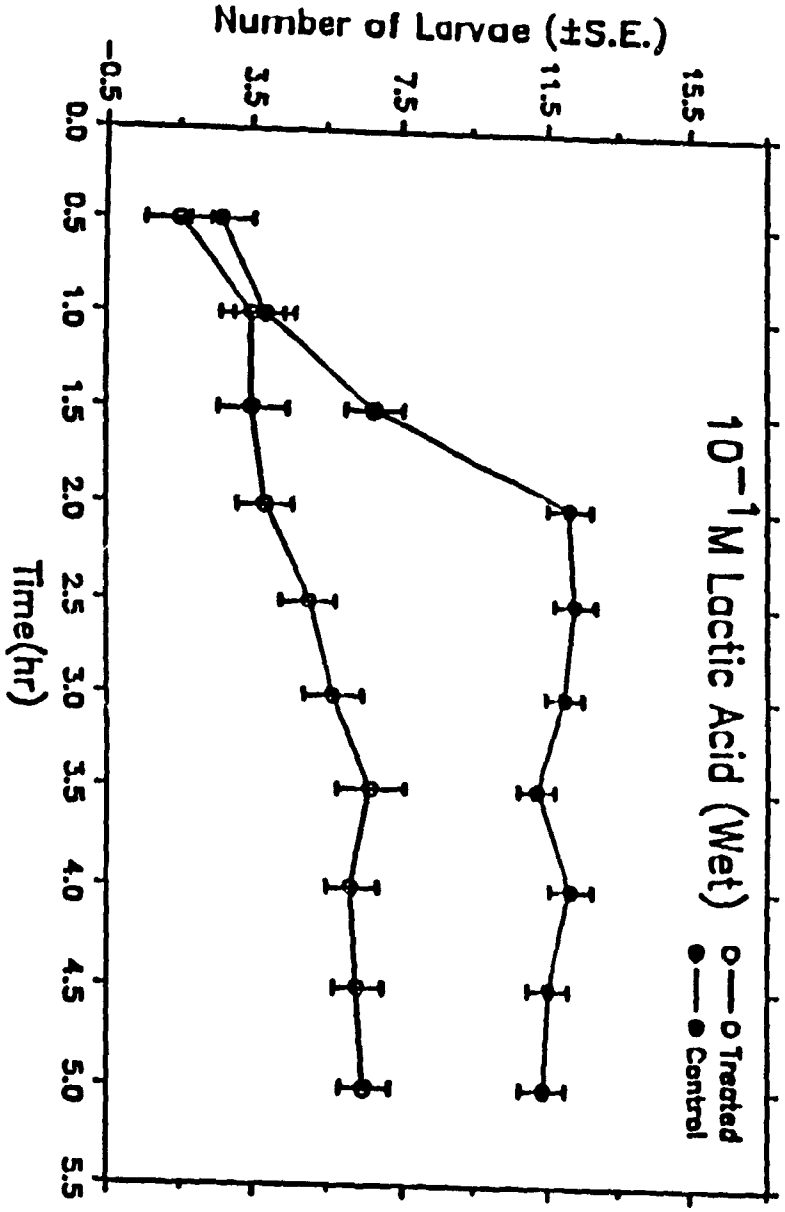
Frass-induced aggregation- *Tenebrio*

Figure 3. Mean number of larval mealworms (±S.E.) responding to papers treated with 10^{-2}M lactic acid and dried. Based on 18 replicates of 20 larvae each.



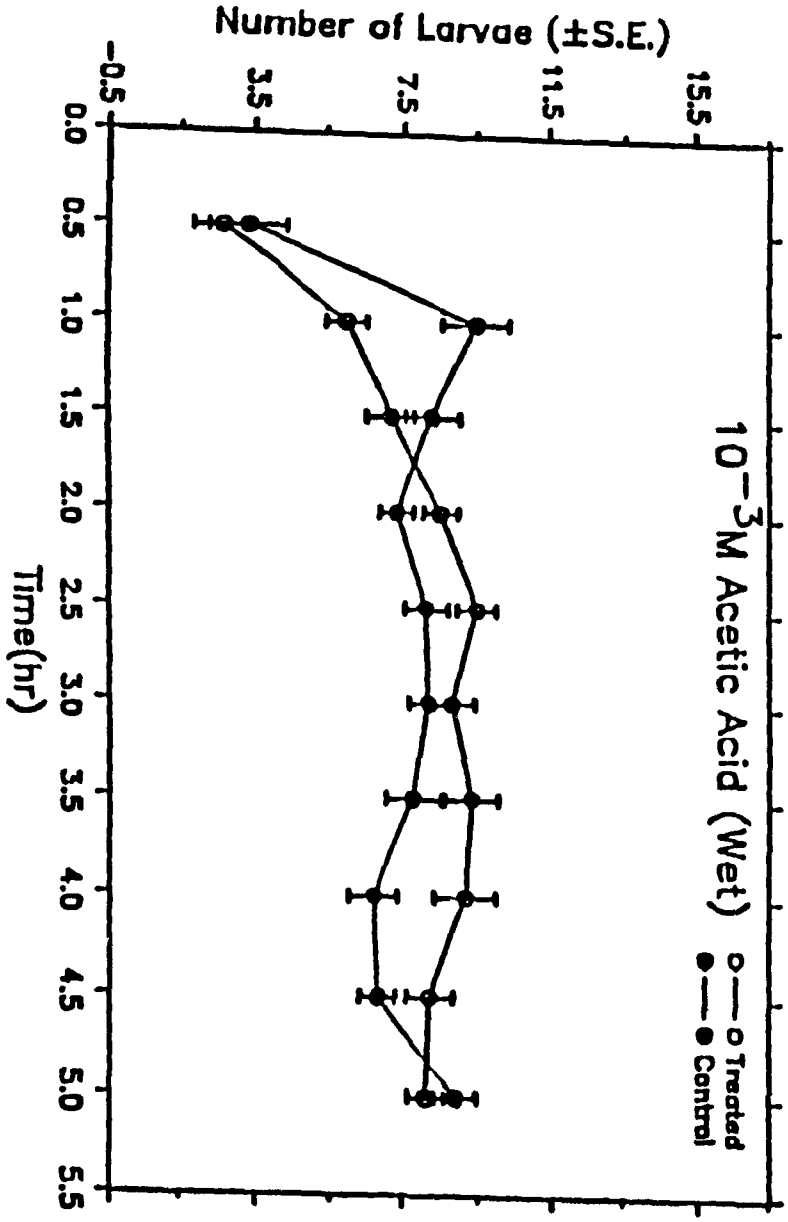
Frass-induced aggregation- *Tenebrio*

Figure 4. Mean number of larval mealworms (±S.E.) responding to papers wetted with 10^{-1}M lactic acid. Based on 18 replicates of 20 larvae each.



Frass-induced aggregation- *Tenebrio*

Figure 5. Mean number of larval mealworms (±S.E.) responding to papers wetted with 10^{-3}M acetic acid. Based on 18 replicates of 20 larvae each.



Frass-induced aggregation- *Tenebrio*

Connecting Statement II

Lactic acid in the frass of larval Tm was found to act as an arrestant that caused aggregate formation among grouped larvae. The arrest of the larvae suggested that stimuli were received by contact rather than by olfaction. This was evaluated in an olfactometer and by high magnification videotape recording. The effect of lactic acid upon grouped larvae was further examined in bioassays designed to quantify the interactions between larvae after contact had been established with a lactic acid stimulus (Section 3: Aggregation behavior- *Tenebrio*).

Section 3: Aggregation behavior- *Tenebrio*

Title

AGGREGATION IN YELLOW MEALWORMS, *Tenebrio molitor* L. (COLEOPTERA: TENEBRIONIDAE) LARVAE: II. OBSERVATIONS AND ANALYSES OF BEHAVIORAL PARAMETERS IN AGGREGATION.

Authors: Weaver, D.K. and J.E. McFarlane. 1989.
J. Chem. Ecol. 15: 1617-1627.

ABSTRACT Evaluation of the lactic acid attraction of individual and grouped larval *Tenebrio molitor* L. in an olfactometer indicated that olfaction is unlikely to be the chemoreceptive mode governing substrate choice or aggregation of these insects. High magnification videotaped sequences of mealworms on treated and control filter papers indicated that larvae sample the substrate by rapidly probing with mouthpart palpi in a manner similar to the leaf sampling of certain caterpillars. The reception of lactic acid stimuli may therefore involve contact chemoreceptors. The larvae frequently touch each other in a similar manner. Bioassays comparing the cumulative frequencies of distributions of mealworms on control and lactic acid-treated filter papers indicated significant differences with higher density clusters being found on the treated papers. Comparison of the control distribution with the expected distribution revealed an innate tendency to aggregate. The implications of these results are discussed with regard to the formation of mealworm clusters in the environment.

Introduction

Weaver et al. (1989, Section 2: Frass-induced aggregation- *Tenebrio*) have shown that DL-lactic acid is an excreted aggregation pheromone found in larval *Tenebrio* frass. It may also act as an epideictic pheromone (Wynn-Edwards 1962, Prokopy 1981) if one considers the dispersal ability of concentrations higher than those naturally occurring in the frass. McFarlane and Alli (1986) found lactic acid to be an excreted aggregant of *Blattella germanica*. L-lactic acid is an olfactory host attractant for *Aedes aegypti* (Acree et al. 1968, Davis and Sokolove 1976). Finger et al.(1965) found olfactory attraction was displayed between larvae of *Trogoderma granarium*, another stored products insect. The present study was undertaken to determine the chemoreceptive mode of yellow mealworms, with observations being conducted to determine whether olfaction or contact chemoreception was involved.

Experiments were also conducted to evaluate the density of distributions on lactic acid papers and on control papers using comparative bioassays. The possiblity of multi-chemical stimuli being involved in mealworm aggregations is discussed.

Materials and Methods

The larvae were reared as previously described (Weaver et al. 1989, Section 2: Frass-induced aggregation- *Tenebrio*).

Olfactometric analysis- A diagram of the olfactometer is shown in Figure 6. A Tygon T-type connector divided the purified airstream. Each branch was fitted with a 25 ml suction flask (H) to deliver test chemical stimuli (airflow rate 1.2 l per min). The treatment flask contained 10 ml of test solution; the control flask contained 10 ml of deionized, glass distilled water. In the case of dried test stimuli, 6 dried test strips (4 X 4 cm)were cut into 0.5 cm^2 pieces and placed in the test flask. Six dried distilled water test strips, prepared similarly, were placed in the control flask (flask positions were

randomized). Beyond this, in each branch a 30 ml plastic vial was mounted horizontally (I) with a 0.5 cm airstream hole on the the stimulus delivery side and a 1.5 cm hole on the opposing side for insertion of the bioassay tube. These vials functioned as traps for insects committed to a given airstream. The traps were connected to opposing sides of a common starting arena (K) by 15 cm of 1.5 cm diam. Tygon tubing (J) lined with fine mesh screening to facilitate larval movement. The starting arena vial was mounted vertically with two 1.5 cm diam. holes opposing each other at the base. The cap of the arena vial was fitted with an airstream outlet to avoid subjecting future experimental animals to test odors. Individual larvae or fourteen larval *T. molitor* were simultaneously introduced into the starting arena and their behavior was observed. Counts of mealworms in trap vials were taken at two and four hours. A solid CO_2 'smoke test' showed no evidence of airstream backmixing. Ambient air temperature varied between 23^{o} and 25^{o} C. The apparatus was disassembled after each usage and cleaned thoroughly using dilute acid, detergent and serial distilled water rinses.

Videotaping procedure- To determine how the larvae interact with each other and the test substrate, the aggregation bioassay was set up as previously described (Weaver et al. 1989, Section 2: Frass-induced aggregation- *Tenebrio*). Larvae were observed at 0.5 hr intervals and filmed through a Javelin JE 3010 color camera equipped with an EVA/Zoom lens (Javelin, Torrance, California). Taping was performed on a General Electric ICV 5025 videocassette recorder (Canadian General Electric, Montreal, Canada) at SP mode. Individual insects were observed for 1 min, if wandering, and 2 min, if arrested. Tapes were played back at 0.5 and 0.25 speed (real time) on a Hitachi CM-1481 monitor to quantify complex motions, particularly during wandering.

Bioassays for comparing population distributions- A 5 X 45.5 cm Whatman No. 1 filter paper strip was treated along its length with 3.5 ml of 5 X 10^{-3}M lactic acid and fixed into a cylindrical shape using a paper clip. This cylinder was introduced into a 19.2 cm diam. glass

specimen dish. The paper had been previously marked into 9 equal 5 X 5 cm grids using a carbon pencil. The cylinder was allowed to dry for 2 hours and then 30 mealworms were simultaneously introduced into the centre of the cylinder. Counts of the number of larvae per grid were taken at 2, 4, and 6 hours; 8 replicates were conducted, with arena positions randomized. Eight replicates of a similarly prepared distilled water control were conducted.

Statistical analysis- Analysis of the responses to the grouped olfactometer bioassay were based on a two-tailed test using Wilcoxon's Signed-Ranks Test for paired observations to determine if the number of insects responding to the chemical stimulus was significantly different from the number responding to the control. A Chi-square test was used to compare the responses of individual insects to lactic acid vs. the control (Sokal and Rohlf 1981). Treated versus control population distributions were compared using the Kolmogorov-Smirnov Two-Sample test, control versus expected Poisson distributions were analyzed using the Kolmogorov-Smirnov Goodness-of-Fit test (Sokal and Rohlf 1981). The T-test procedure was used for comparisons between mean numbers of insects/ grid on treated and control papers in the population distribution bioassay (SAS Institute 1988).

Results

The olfactometer bioassay indicates that larval *Tenebrio molitor* are unlikely to respond to airborne lactic acid molecules. Table 5 shows that yellow mealworms respond to both dried papers and solution-evolved stimuli (molecules in the air space above the surface of the liquid) in a manner not significantly different from the response to the control. This is true for both individual insects and groups of larvae. Table 6 illustrates the types of behavior displayed by *Tenebrio* larvae in the olfactometer bioassay. These data indicate that these larvae do show positive anemotaxis, with a total of 84.8% of the tested larvae

reaching the trap vials. It is unlikely, therefore, that any behavioral bias against olfactometric analysis was overlooked, particularly since the airflow rate is very low in relatively wide tubing.

Videotaping the larvae under high magnification revealed two predominant responses to the test chemical. Initially, mealworms wander over the filter paper surface with the following motion of the principal receptor organs: (i) antennae are rotated arbitrarily in all directions, making occasional contact with the papers, (ii) maxillary palpi in a synchronous co-ordinated tapping activity which generally contacts the paper 2 or 3 times per second, with occasional rates of up to 5 times per second and, (iii) labial palpi are moved in either synchronous or asynchronous fashion with contact occurring several times per second. Both maxillary and labial palpi contact the substrate for approximately 100 msec with a 100-500 msec lapse prior to the next contact. Occasionally, labial palpi may be moved in a similar fashion to the antennae with occasional paper contact rather than the above described "probing". These occasional contacts occur when the head capsule is turned for a directional change. The other major response is that of larval arrest in which the head capsule is curled forward so that antennae, maxillary and labial palpi are generally all in constant contact with the test substrate. Aggregating larvae may not remain totally arrested, but show occasional slow gregarious relocation. During wandering the larvae generally display bursts of "probing" activity rather than continuously doing so. Larvae frequently come into contact with each other. This contact also involves much "probing" with palpi and antennae, but is usually lacking the rapid synchronous maxillary tapping described above.

The results of the bioassay used to analyze distributions of *Tenebrio molitor* larvae are given in Figure 7. First, there were significant differences between control trials and those containing 5 X 10^{-3}M lactic acid test strips at each 2 hour interval. Secondly, these differences occurred at more than one larval density for each time interval. Differences become more pronounced with time. For example, at

2 hours 100% (cumulative frequency = 1.0) of the larvae on the control were at grid densities of 7 larvae per grid or less, whereas only 83.3 % of the larvae on the treatment papers were found at this density or less. At 6 hours 100% of the larvae on the control were located on grids containing 7 yellow mealworms or less, whereas only 71.8% of the larvae on the treated were found in grids containing ≤ 7 larvae. Finally, at both 4 and 6 hours there are treatment clusters up to 13 or 14 larvae per grid, whereas on the control at 4hr the highest density of larvae is 8 per grid; at 6 hr the highest density on the control paper is 6 larvae per grid.

There also is a tendency for a higher percentage of larvae to actually contact test substrates as opposed to control substrates, even when they are only presented one or the other. Two hundred and forty insects were used in total in all treatment and also in all control trials. In the control trials 174, 172, and 155 larval *Tenebrio* were found on the papers at 2, 4, and 6 hours respectively. In the lactic acid trials 198, 219, and 220 larvae were located on the papers at 2, 4, and 6 hours. The reduction in number of larvae at 6 hours in the control arenas is correlated with clusters that form at the base of the papers. These larvae (unstarved) defecate while on the papers. The fecal pellets accumlate to a substantial amount and it is in these collections of frass droppings that the mealworms group.

Figure 8 displays the differences in mean numbers of larvae on 5 x 10^{-3}M lactic acid grids and control grids for occupied grids (excluding those without insects) and for all grids (including those that were empty).

A comparison of the control distribution with the expected Poisson frequency is shown in Figure 9. The frequency of larvae at lower densities is less than expected for certain values at the recorded time intervals. The cumulative frequency reaches or exceeds the expected value only for grids containing a higher number of larvae (at the highest density of 6 larvae/ grid for 2 and 6 hours and at 8 larvae/ grid at 4 hr). This indicates that the larvae tend to group in higher densities than

theoretically expected. The cumulative frequency of larvae at lower densities also decreases with time on the control papers relative to the expected cumulative frequency. The larvae interact with each other in a similar manner, forming higher as opposed to lower density clusters, though with much lower density maximal clusters, than they do on treatment papers.

Discussion

Olfactometric analyses indicate yellow mealworms do not respond to airstreams containing lactic acid in concentrations that successfully aggregate the larvae in the aggregation arena. Thus, olfaction is unlikely to be the chemoreceptive mode of *Tenebrio* larvae. This is to be expected, since the dispersion of odor through the stored product mass would be uneven due to deflection away from unequal sized particles and collection in unevenly distributed air spaces. This would not provide a conventional concentration gradient for animal orientation. The lack of available water (Weaver et al. 1989, Section 2: Frass-induced aggregation- *Tenebrio*) also would not provide for much aqueous evaporation to aid in the volatilization of the relatively low-volatility lactic acid.

The "probing" behavior viewed through videotaping suggests that mouthpart palpi are utilized to test the substrate for chemical information. The rapid maxillary tapping is similar to that described for larval *Choristoneura fumiferana* by Albert and Parisella (1988). This may be a behavior characteristic of feral *Tenebrio molitor* larvae (not those associated with stored-products) that could be pre-adaptive for subtrate sampling in stored foodstuffs. This does not suggest that purely mechanical stimuli are also not involved in such behavior.

After these animals arrive on the stimulus substrate they tend to wander rapidly across it, occasionally sampling it by "probing" in the predescribed manner. This wandering brings the larvae into occasional contact. The individuals then tend to associate in a gregarious manner, with a conspicuous reduction in the rate of wandering which may lead to

group arrest. More often, however, individuals of these groups wander in a relatively random manner in an area in close association with the others; this wandering activity is much less rapid than that displayed after initial contact with the paper. This may involve an effect similar to that described by Burk and Bell (1973) for inhibition of cockroach locomotion by aggregation pheromone. Yellow mealworms may remain active because the bioassay does not provide localized regions of concentration. Frass accumulates to a very high density in *T. molitor* cultures so stimuli can be continuously contacted by moving larvae.

These data suggest that: (i) mealworms show an innate tendency to aggregate and (ii) since they prefer lactic acid substrates, they might also prefer to interact (possibly in a different manner) on such substrates. The analysis of cumulative frequency distributions (Figs. 7 and 9) indicate both are true. It must be emphasized that this assay of grouped interaction is addressing, by analogy , the toleration that conspecifics have for each other when they are in a large region conditioned by larval conspecifics, as opposed to one that had not. It is *not* demonstrating that high density clusters form around a localized pinpoint region that has been chemically marked. In that case such an observation would be expected because the chemical message would be force responding individuals to cluster on the conditioned spot. This grid assay (Fig. 7) show that secondary interaction results in enhanced clustering between individuals after they have responded to a chemical frass message. In addition, the movement of larvae into frass accumulated at the base of the control papers, but not from the treated papers is convincing. The larvae on the treated paper may not do so because the lactic acid on the papers already delivers a "frass message". Both treatment and control responding larvae encounter this accumulated frass during slow wandering; only the control larvae show any pronounced preference for it.

These data allow for the suggestion of a possible scheme for the formation of mealworm clusters in these bioassays: (i) initally, larvae wander through the arena in a random manner and select a substrate for

exploration, (ii) next, they remain upon this substrate or leave it after a period of rapid sampling, (iii) if they leave, they may or may not return, via future random wandering, depending on the nature of the information further sampling provides (Weaver et al. 1989). (iv) If groups of individuals select a substrate they will encounter each other and tend to form loosely cohesive groups. (v) The chemical nature of the substrate may affect the density per unit area that individuals will tolerate, particularly if DL-lactic acid, a component of larval conspecific frass, is present. A second alternative would be that the presence of lactic acid initiates "conspecific-seeking" behavior after positively reinforcing the quality of substrate for these animals.

However, clusters are also formed on control strips in a similar manner, but at lower densities per grid. This innate tendency may also involve chemical messages related to the close proximity or contact between individuals. This may be similar to the chemotactile/ mechanical aggregations among ovipositing locusts described by Norris (1970). This would mean that mealworm aggregation involves multi-chemical stimuli, with the unknown factor being a possible conspecific "recognition" or "interaction" factor (cf. Section 6: Cuticular lipids in aggregation.

It may be likely that the tendency for yellow mealworms to aggregate is related to an attraction to DL-lactic acid as an indicator of decomposing organic material suitable for these darkling beetles to feed upon in their feral state. The recent tendency of man to store harvested material may have provided abundant foodstuff for an existing behavior to lead to enhanced survival by inhabiting those cryptic locations where deteriorating foodstuffs and frass may abound. Atkins (1965) states it is beneficial for aggregation to be selected for in cases where a resource is located at low frequency or sparsely distributed.

Alternatively, a mechanism may have evolved during the last 5000-20,000 generations (*T. molitor* development time 3-12 months, 5000 years of post-harvest storage) whereby massive selection pressure occurred for attraction to a frass 'marker'. Those individuals present in

the main stored products mass contributed little to the gene pool if they were constantly removed along with the food material or by cleaning procedures, whereas those present in cryptic locations remained to breed in these areas where fecal material, exuviae and decomposing foodstuffs comprise nearly the entirety of the surrounding environment. These arguments might seem somewat group selectionist and therefore are subject to the usual criticisms (as in Haynes and Birch 1985), however there can be little doubt that random wandering through the long duration of development of *Tenebrio* larvae could greatly separate potential mating partners at the time of imaginal ecdysis, so there may well be individual advantages in the proximal associations caused by frass-induced arrest.

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Table 5. Results of the lactic acid olfactometer bioassay.

Chemical	Treated[a]	Control
10^{-1}M Lactic Acid (Dry)	6.64±0.50	5.79±0.75 N.S.
10^{-2}M Lactic Acid (Dry)	4.86±0.63	4.21±0.81 N.S.
10^{-3}M Lactic Acid (Dry)	5.14±0.69	4.86±0.38 N.S.
10^{-4}M Lactic Acid (Dry)	5.71±0.63	5.36±0.75 N.S.
10^{-1}M Lactic Acid (Wet)	5.50±1.13	6.14±0.44 N.S.
10^{-2}M Lactic Acid (Wet)	6.50±0.17	7.14±0.69 N.S.
10^{-3}M Lactic Acid (Wet)	0.42±0.06[b]	0.54±0.07 N.S.

[a]Mean number of larvae ± S.E., based on 8 replicates of 14 larvae each. Treatment not significantly different from control, Wilcoxon's Signed-Ranks Test for paired observations.

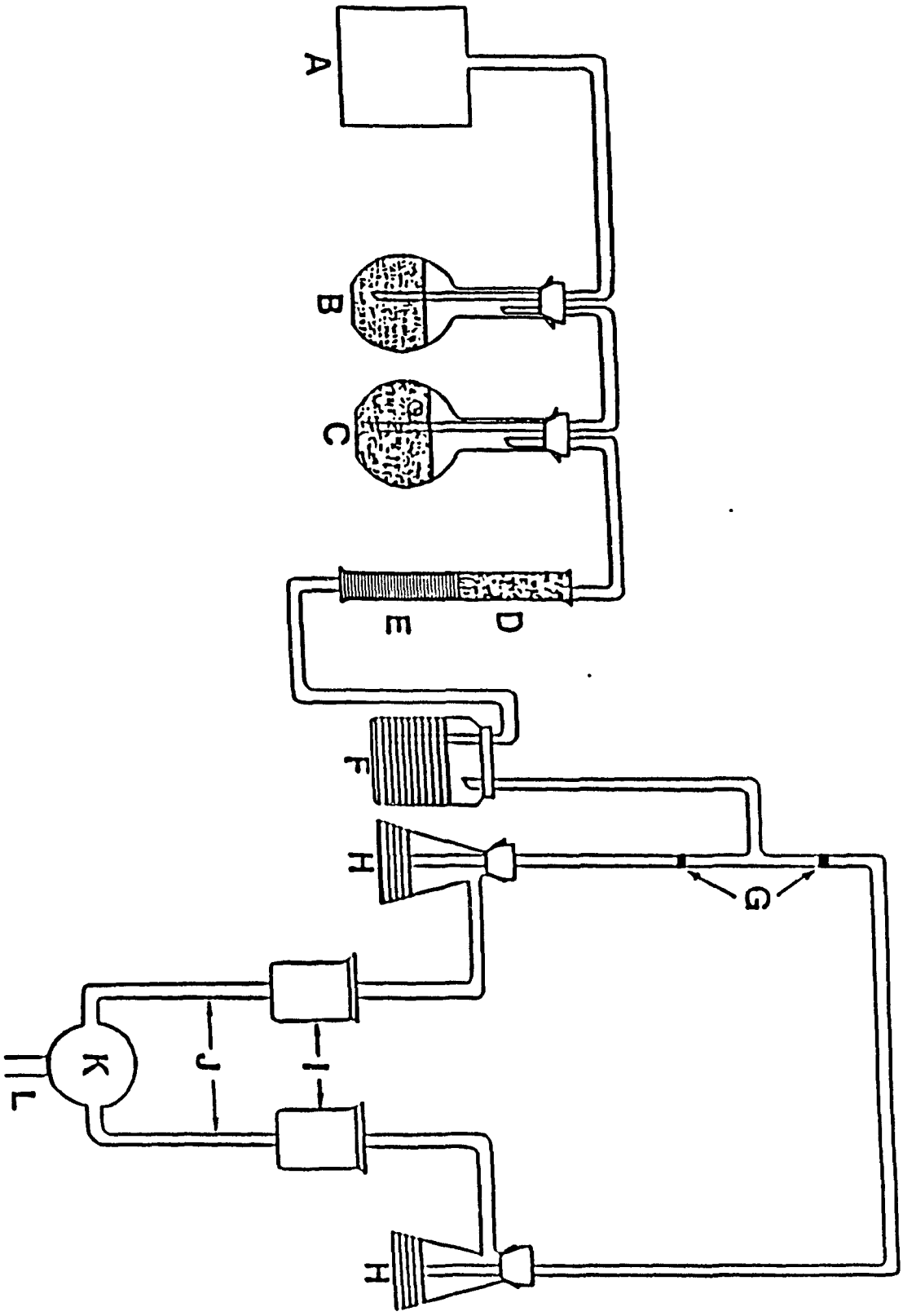
[b]Differences are not significant, based on 24 individual trials, Chi-square test.

Table 6. **Types of behavior of grouped *Tenebrio* larvae in an olfactometer.**

		%
a	Upwind crawling reaching trap in ≤2 hours.	63.4
b	Upwind crawling reaching trap in ≤4 hours.	9.8
c	Upwind crawling with ≥1 downwind shift; reaching trap in ≤4 hours.	11.6
d	Upwind crawling with ≥1 downwind shift; not reaching trap in ≤4 hours.	6.3
d	Quiescence, moulting or pupation.	8.9

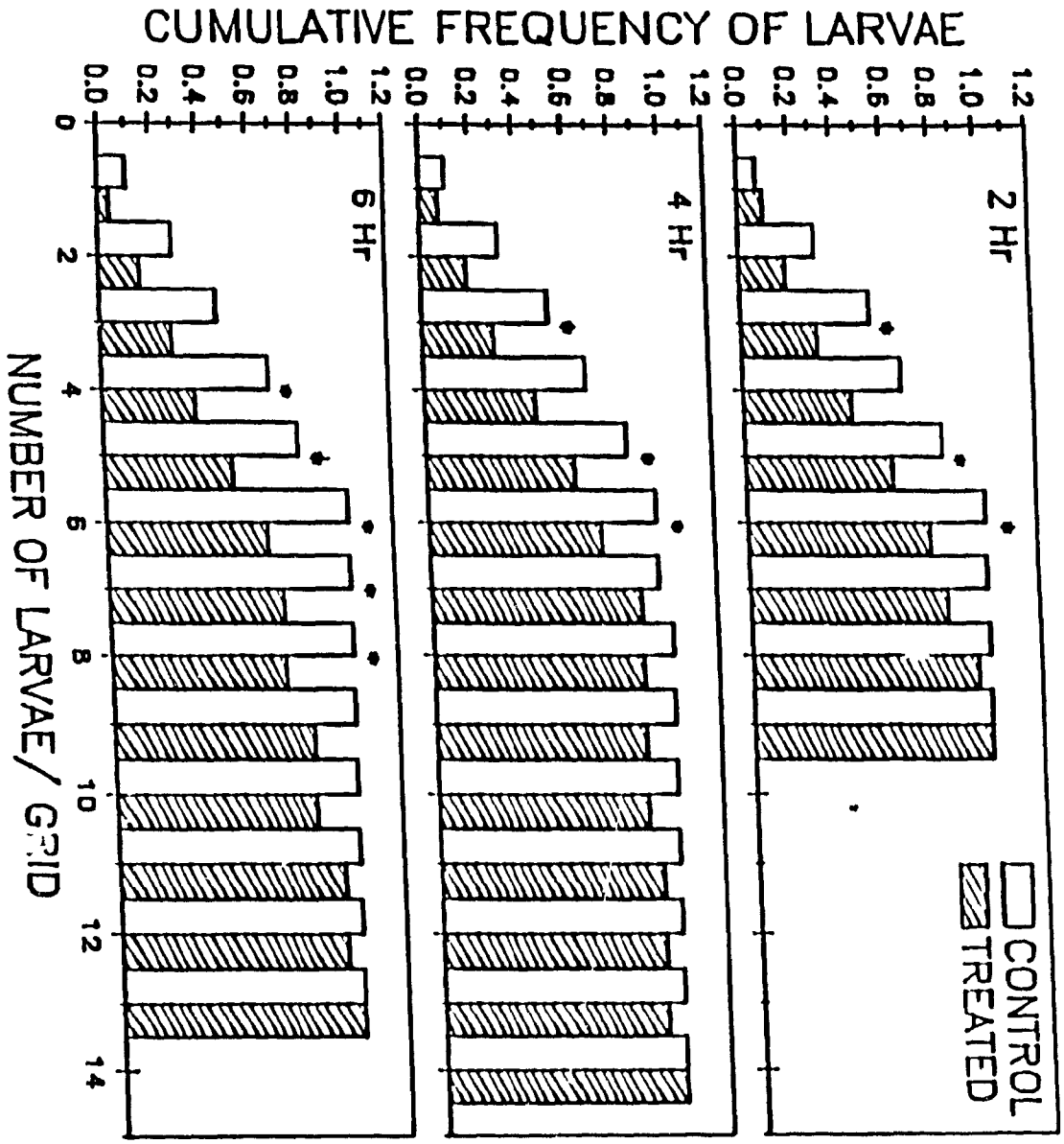
*N = 112 larvae.

Figure 6. Schematic diagram of olfactometer. A, vacuum pump coupled to rheostat, B, activated charcoal filter, C, drierite filter, D and E, gas purifier, F, gas washer, G, flowmeters, H, suction flasks (stimulus delivery), I, trap vials, J, Tygon crawl tubes, K, common starting arena, L, outlet to ventilation port. The vacuum pump pushes the airstream.



Aggregation Behavior- *Tenebrio*

Figure 7. Histogram comparing the cumulative frequency distributions of *Tenebrio molitor* larvae on control filter papers and those on filter papers treated with 5 X 10^{-3}M lactic acid at 2, 4, and 6 hours. Cumulative frequency at each abscissal point is the sum of all individuals at that density plus those at all preceding (lower) densities.
*Cumulative frequency of treatment significantly less than control at $P \leq .001$.
2 hours, n_1=174, N=240; n_2=198, N=240; $D_{.001}$=.20257.
4 hours, n_1=172, N=240; n_2=219, N=240; $D_{.001}$=.19862.
6 hours, n_1=155, N=240; n_2=220, N=240; $D_{.001}$=.20767.

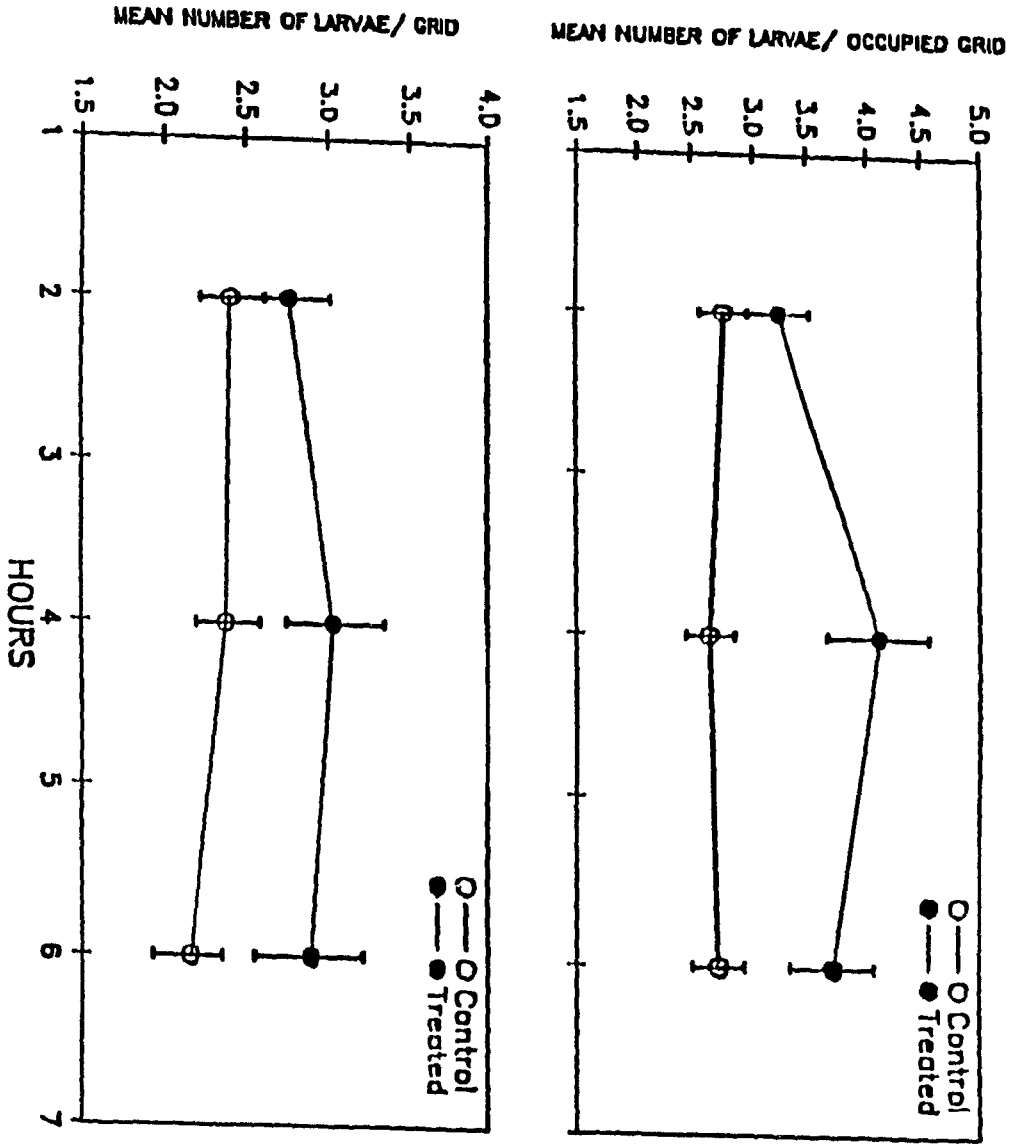


Aggregation Behavior- *Tenebrio*

Figure 8. Comparison of the mean number of larvae per occupied grid (top) and per all grids (bottom) on treatment or control papers at 2, 4, and 6 hours.

*For occupied grids treatment (5 X 10^{-3}M lactic acid) is significantly greater than control at $P \leq .001$ at 4 and 6 hours.

**For total grids treatment is significantly greater than control at $P \leq .01$ at 4 and 6 hour .



Aggregation Behavior- *Tenebrio*

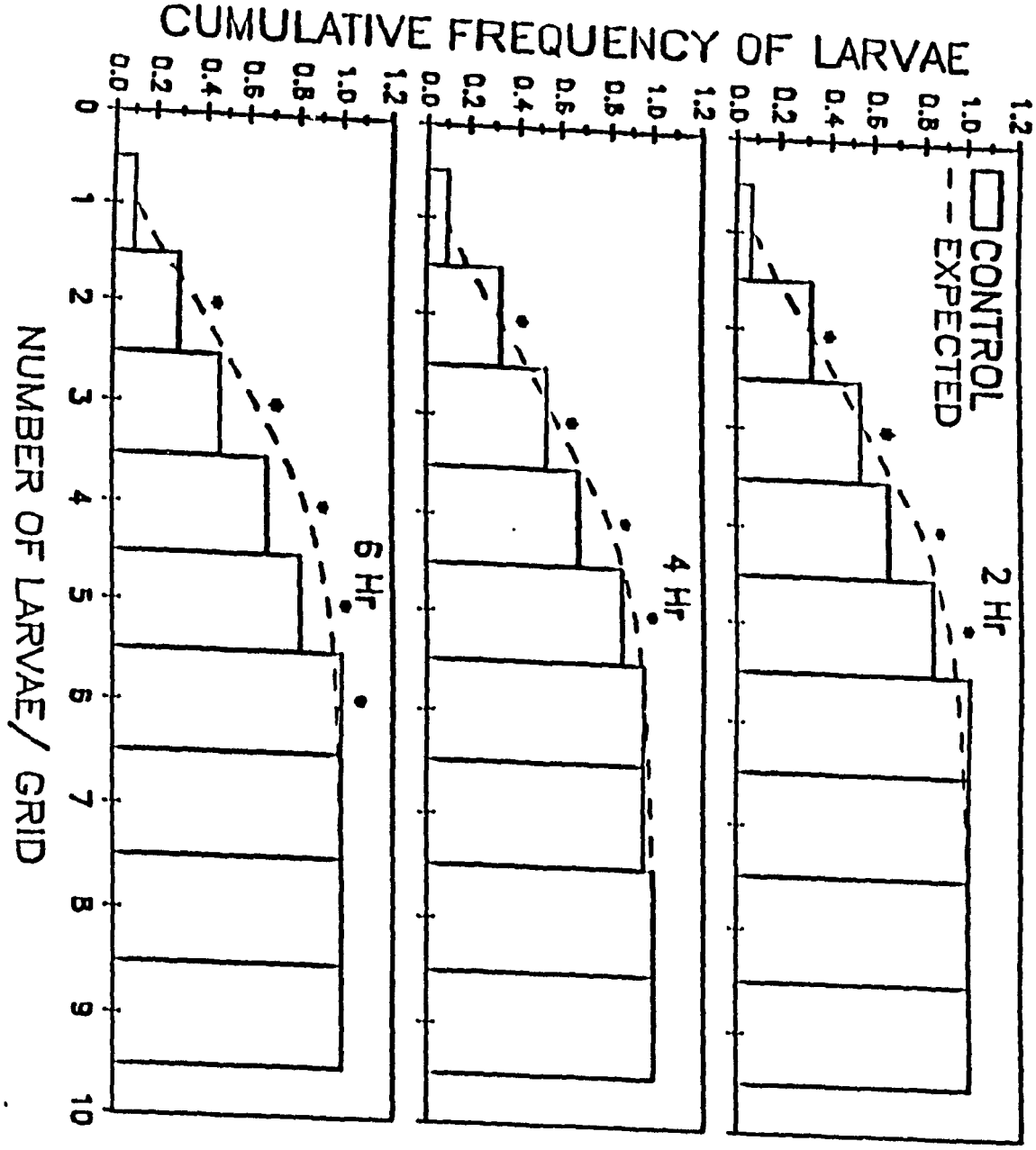
Figure 9. Comparison of a histogram of the cumulative frequency distributions of *T. molitor* larvae on control filter with a plot of the cumulative frequency of the expected Poisson distribution at 2, 4, and 6 hours. Cumulative frequency at each abscissal point is the sum of all individuals at that density plus those at all preceding (lower) densities.

*Cumulative frequency of the expected distribution (Poisson) of larvae significantly greater than the cumulative frequency of the larvae on control papers at the preceding larval density per grid; $P \leq .001$.

2 hours, n=174, $D_{.001}$=.14779.

4 hours, n=172, $D_{.001}$=.14865.

6 hours, n=155, $D_{.001}$=.15659.



Aggregation Behavior- *Tenebrio*

Connecting Statement III

Lactic acid in the frass of Tm was found to be an an excreted aggregant perceived by direct contact. Frass of Tm larvae contained volatile fatty acids which also have behavioral activity. The mode of chemoreception and quantification of the effective concentration of these chemicals was evaluated. The apparent conflict between the behavioral activities of these compounds was resolved by a bioassay utilizing a solution which mimics the quantities of these chemicals in the frass (Section 4: Frass repellents- *Tenebrio*).

Section 4: Frass Repellents- *Tenebrio*

Title

REPELLENCY OF VOLATILE FATTY ACIDS PRESENT IN THE FRASS OF LARVAL YELLOW MEALWORMS, *Tenebrio molitor* L. (COLEOPTERA: TENEBRIONIDAE), TO LARVAL CONSPECIFICS.

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ABSTRACT Frass of late instar larvae of *Tenebrio molitor* L. contained 0.0889g of butyric acid, 0.0279g of propionic acid, and 0.0175g of valeric acid per 100g. Grouped larvae were strongly repelled by butyric acid at the 10^{-1}M concentration. Lower concentrations of butyric acid were less repellent. Valeric acid was repellent at 10^{-1}M to 10^{-3}M concentrations, below which no tested concentration, including one identical to that occurring in prepared solutions of frass found to be attractive, displayed any effect. Isolated propionic acid was repellent at the concentration found in prepared solutions of frass, which were strongly attractive. 10^{-1}M, 10^{-3}M and 10^{-4}M propionic acid were also repellent. The implications of the repellency of these compounds to groups of mealworm larvae are discussed, with particular reference to the interaction between these frass components and others that have already been studied.

Introduction

Infestations of the yellow mealworm, *Tenebrio molitor* L., are generally found in damp, dark places where cereals may be decaying (Cotton 1963). These localized regions may contain large amounts of frass from several generations of *T. molitor*. Experiments have been conducted testing mealworm frass, and lactic and acetic acids, which are frass components, for behavioral effects (Weaver et al. 1989, Section 2: Frass-induced aggregation - *Tenebrio*; Weaver and McFarlane 1989, Section 3: Aggregation behavior- *Tenebrio*). The frass and lactic acid acted as aggregants and acetic acid had no behavioral influence (Weaver et al. 1989, Section 2: Frass-induced aggregation - *Tenebrio*). However, the chemical analyses of *Tenebrio* frass indicated the presence of volatile fatty acids in significant amounts, as were found in the frass of other omnivorous insects (McFarlane and Alli 1985). McFarlane (1984) found that frass volatile fatty acids were repellent to *Blattella germanica*. Lactic acid in the frass of *Blattella* was found to be aggregative (McFarlane and Alli 1986). These results indicated a variety of responses were possible with these chemical stimuli in the frass, with one component being dominant. In addition, propionic acid has been found to aggregate *Acheta domesticus* (McFarlane et al. 1983) and butyric acid has been identified as an aggregant for *Periplaneta americana* (McFarlane and Alli 1987). The present article deals with the behavioral responses caused by volatile fatty acids in the frass of larval *Tenebrio molitor*. In particular, experiments were conducted using serial dilutions of volatile fatty acids to determine activity at various concentrations. Experiments were also conducted using concentrations of acids identical to those occurring in behaviorally active frass samples. These frass samples, and lactic acid occuring in them, were found to be attractive (Weaver et al 1989, Section 2: Frass-induced aggregation - *Tenebrio*). Bioassay indicated the isolated volatile fatty acids were repellent. A mixture of volatile fatty acids and lactic acid was prepared to determine how the activity of these isolated components of frass was

manifested in the natural environment, where they occur concurrently. 10^{-1}M volatile fatty acids were also evaluated for olfactory responses in an active airflow olfactometer.

Materials and Methods

Yellow mealworms, *Tenebrio molitor* L., were raised on a diet of wheat bran, whole wheat flour, and brewer's yeast (50:45:5 w/w) at 25±1°C and 55±5% relative humidity and a photoperiodic regime of 14:10 light-dark. The density of all stages per filled 4.55l glass culture jar was not allowed to exceed 250 individuals. Collection of frass and diet and determination of volatile fatty acids in both were identical to those used in Weaver et al. (1989, Section 2: Frass-induced aggregation - *Tenebrio*). The bioassay arena was that used by Weaver et al. (1989, Section 2: Frass-induced aggregation - *Tenebrio*).

The test protocol involved a choice test using Whatman No.1 filter papers treated with 0.68 ml of test chemical in aqueous solution at the appropriate concentration versus a paper treated with 0.68 ml of water (distilled; de-ionized in both cases). The papers were allowed to dry 1.5 hours prior to testing. Dried papers were fixed in the arena opposing each other and twenty yellow mealworms (140-160 mg) were introduced simultaneously into the arena. The papers were examined at 0.5 hr intervals after the initiation of the trial and larvae on each paper were counted. Larvae were counted if they were actually on the paper or were touching it with their antennae, mouthparts, or legs. Trials were terminated after 5 hrs. Reagent grade acids (Anachemia Limited, Montreal, Canada) were used in preparation of all test solutions. Control trials indicated that these solvents showed no behavioral activity in comparison with HPLC grade acids (Fisher Scientific Company, Montreal, Canada).

An active airflow olfactometer (as in Weaver and McFarlane 1989) was used to evaluate the mode of chemoreception for these repellent chemicals. The test stimuli were derived from 2 shredded filter paper

strips treated with 0.68 ml of a 10^{-1}M aqueous volatile fatty acid solution and evaporated 0.5 hr prior to bioassay. Control stimuli were similarly prepared using filter paper strips treated with 0.68 ml distilled water.

Statistical comparisons between the various test stimuli were based on analysis of variance using Tukey's HSD test on mean aggregation indices (A.I.) calculated by subtracting the number of larvae on the control paper from that on the treated paper and dividing by the total number on the two papers. Index values may range from -1 (complete repellency) to +1 (all responding individuals on the treatment paper). A value of -0.2 corresponds to 50% more insects on the control paper and -0.33 corresponds to twice as many insects on the control as were on the treatment paper (Roth and Cohen 1973). Indices were based on ten observations per replicate, with twelve to twenty replicates being conducted per test chemical. Indices were analyzed by t-test for significant differences from a value of zero (SAS Institute 1988), which would represent identical numbers on treatment and control papers. Analysis of the responses to the grouped olfactometer bioassay were based on a one-tailed test using Wilcoxon's Signed-Ranks Test for paired observations to determine if the number of insects responding to the chemical stimulus was significantly less than the number responding to the control (Sokal and Rohlf 1981).

Results

Frass of late instar larvae of *Tenebrio molitor* L. contained 0.0889g of butyric acid, 0.0279g of propionic acid, and 0.0175g of valeric acid per 100g. These acids were not present in the diet .

The responses of late instar *T. molitor* to serial dilutions of those acids present in the frass and one not occurring in the frass but chemically similar (isobutyric acid) are shown in Table 7. 10^{-2}M and 10^{-3}M valeric acid were most strongly repellent. Two unusual effects were evident as well. The values of 10^{-2}M propionic acid and 10^{-2}M

isobutyric acid were both distinct from those for the concentrations surrounding them. 10^{-2}M propionic acid had a much higher (though statistically similar) A.I. value, and showed no significant repellency at all. Both 10^{-3}M and 10^{-1}M propionic acid are significantly repellent. Isobutyric acid was significantly repellent at 10^{-2}M and caused no effect at either 10^{-1}M or 10^{-3}M. This response to isobutyric acid indicates that these insects can respond to synthetic analogues of frass components.

The responses of grouped yellow mealworms to the amounts of isolated frass acids found in 20 mg frass/ml H_2O (13.6 mg frass/paper), as tested in Weaver et al. (1989), Section 2: Frass-induced aggregation - *Tenebrio* are shown in Table 8. Propionic acid was the only repellent chemical (when tested in isolation) found in this solution of frass, which had been found to exhibit overall attractancy (Weaver et al., 1989, Section 2: Frass-induced aggregation - *Tenebrio*).

An olfactometer bioassay (as described in Weaver and McFarlane, 1989, Section 3: Aggregation behavior- *Tenebrio*) using 10^{-1}M concentrations of the volatile fatty acids indicated they were significantly repellent to groups of large *Tenebrio* larvae. The results of the olfactometer trials are shown in Table 9.

A mimic solution composed of all acids (volatile fatty acids + lactic and acetic acids) isolated from the frass at the concentration in which they occurred in attractive 20 mg frass/ ml H_2O gave an A.I. value of 0.35 $\pm$.04. This showed significant attraction at the $P \leq 0.001$ level (based on 12 replicates).

Figure 10 shows the response of yellow mealworms to repellent 10^{-3}M propionic acid throughout the bioassay. Trial stabilization occurred rapidly and a large decrease in mealworms on the treatment and control papers occurred at 4.5 hr (consistent through 12 replicates).

Figure 11 shows the response of yellow mealworms to 10^{-3}M butyric acid, a non-effective concentration, throughout the duration of the bioassay. The response occurred much more slowly in this case than it did for a repellent chemical (approximately 2.5 hrs to have 75% of the insects responding versus 1.0 hr in Figure 1).

Discussion

The analyses of frass and diet samples indicate that these volatile fatty acids are produced within the larval mealworms, since they are not found in the diet, but are present in the frass. These chemicals may therefore be utilized as discriminants of areas that were previously inhabitated by conspecific larvae.

The bioassays conducted suggest that relatively high concentrations of these volatile acids are required to significantly repel yellow mealworms. In fact only propionic acid is active at the concentration that it actually occurs in behaviorally active frass solutions. The effect of the isolated acid is in apparent contradiction with that induced by aqueous extracts of frass containing this exact amount of it, since frass extracts and their lactic acid component have been previously demonstrated to be attractive/arrestant (Weaver et al 1989, Section 2: Frass-induced aggregation - *Tenebrio*). Therefore, a bioassay was conducted using the precise concentration of synthetic volatile fatty acids + lactic and acetic acids that occurred in active frass preparations, with the results indicating strong attraction to this mimic solution. These volatile fatty acids are present in frass samples in 1/3 to 1/10 of the amount(g) that lactic and acetic acids were observed in the frass (Weaver et al. 1989, Section 2: Frass-induced aggregation - *Tenebrio*). Therefore, it is not surprising that the overall response to a solution of volatile fatty acids + lactic and acetic acids designed to mimic an attractive frass stimulus (equivalent to 20 mg frass/ml H_2O) was aggregative.

However, there are factors other than instantaneous concentration involved here. Earlier experiments have established that lactic acid in the frass of *Tenebrio* does not have an airborne influence on behavior, but is instead likely to be perceived through contact chemoreceptors (Weaver and McFarlane 1989, Section 3: Aggregation behavior- *Tenebrio*). This result was probably due to the fact that lactic acid has very low volatility and probably does not volatilize from dilute aqueous solu-

tions or the surface of fecal pellets. These volatile fatty acids, however, did display repellent effects when tested on groups of larvae in an olfactometer at higher concentrations. It is evident that the larval mealworms must come into contact with the filter paper to aggregate in response to frass extracts or isolated lactic acid (Weaver and McFarlane 1989), Section 2: Frass-induced aggregation - *Tenebrio*, so it is likely that initial exploration is directed towards substrate evaluation, though this might be overcome by repellent airborne messages. It may also be likely that the concentration of airborne molecules from a treated filter paper or fecal pellet may not exceed the behavioral threshold until a larva is extremely close to it. The amount of volatile fatty acid on the paper (or arising from the fecal particles) and that at any given distance from source would decrease by volatilizing and subsequently dissipating with the passage of time. The attractive component is not subject to this reduction in quantity and can remain strongly attractive. Therefore frass in the environment can exert a pronounced behavioral influence through contact, without the prolonged manifestation of the repellency of these low concentration volatile components. This was similar to the effect noted by McFarlane (1984) when isolated volatile fatty acids in concentrations occurring in the frass were found to be repellent to larvae of the German cockroach, but frass conditioned papers and certain components of the frass were attractive. It is likely that the attractive chemical (lactic acid in both species) elicited a dominant behavioral response.

Therefore, the preference of the larvae for the control papers in these trials may involve an initial rejection of the substrate (through close range repellency) and then aggregation on the control paper, rather than only responding to a continuous repellent stimulus. Weaver and McFarlane (1989), Section 3: Aggregation behavior- *Tenebrio* have demonstrated an innate tendency for mealworms to aggregate on control papers in clusters of higher than expected densities, which means individuals may attract each other. Bioassays determining the orientation of animals that occur in groups naturally should be based on the

responses of groups primarily and subsequently on the the factors mediating this grouping. The existence of conspecifics in these bioassays in no way invalidates the significance of grouped larvae being repelled from a stimulus in these experiments, particularly if control vs. control trials offer the same possibility of behavioral influence through attraction to conspecifics and resultant distributions are equivalent.

Comparison of Figures 10 and 11 illustrates that trial stabilization required more time in those cases where the insects showed no orientation to a particular chemical. This was also observed in our earlier experiments with certain non-attractive concentrations of lactic acid (Weaver et al. 1989, Section 2: Frass-induced aggregation - *Tenebrio*). This may indicate that both repellent and attractive chemicals serve to orient *T. molitor* larvae more rapidly to suitable locations for cluster formation.

The preceding discussion centers on the nature of the interaction between the components in a given quantity of frass. This readily supports our hypothesis that frass acts as a chemical marker of safe refugia for these slow-developing insect larvae (Weaver et al 1989, Section 2: Frass-induced aggregation - *Tenebrio*). However, the rapid accumulation of fresh frass from a relatively high density of mature larvae may result in a considerable airborne concentration of volatile fatty acids. The repellency that would result could result in considerable ecological benefit, since large numbers of these relatively large slow-developing larvae might well have exhausted the available foodstuffs in that particular region. This would be the situation where larval dispersion might be the most beneficial. In addition, we have demonstrated that high concentrations of lactic acid are also repellent (Weaver et al 1989, Section 2: Frass-induced aggregation - *Tenebrio*), which might also occur under such conditions. Therefore, the repellent chemical message may be further enhanced by the probable epideictic (Prokopy 1981) nature of lactic acid. Regardless of the relative role

of the individual chemicals, very high concentrations of frass are likely to produce chemical mesages that differ from those of more moderate amounts.

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Table 7. Results of trials using serial dilutions of frass acids and isobutyric acid.

Acid	Concentration	Mean A.I.(±S.E.)*	Significance**
Butyric	10^{-1}M	-0.34±.03^{efg}	.001
Butyric	10^{-2}M	-0.22±.03de	.001
Butyric	10^{-3}M	-0.04±.03^{abcd}	N.S.
Butyric	10^{-4}M	-0.11±.04^{abcd}	.01
Butyric	10^{-5}M	-0.12±.05^{abcd}	.05
Propionic	10^{-1}M	-0.17±.05^{bcde}	.001
Propionic	10^{-2}M	-0.03±.05^{abcd}	N.S.
Propionic	10^{-3}M	-0.23±.03^{def}	.001
Propionic	10^{-4}M	-0.23±.04^{def}	.001
Valeric	10^{-1}M	-0.17±.04^{cde}	.005
Valeric	10^{-2}M	-0.49±.03^{g}	.001
Valeric	10^{-3}M	-0.43±.03^{fg}	.001
Valeric	10^{-4}M	0.03±.04^{a}	N.S.
Isobutyric	10^{-1}M	0.04±.03^{a}	N.S.
Isobutyric	10^{-2}M	-0.17±.04^{bcde}	.001
Isobutyric	10^{-3}M	0.06±.04^{a}	N.S.

*Means followed by the same letter are not statistically different at $P \leq 0.05$.

*Results of t-test for significant response to treated paper, i.e., a significant difference from zero (equal numbers on treatment and control). All indices are significant at $P \leq$ the value listed. N.S. Indicates responses to treatment and control papers are not statistically different.

Table 8. Results of trials using dilutions of frass acids equivalent to that found in an attractive concentration of whole frass extract (20 mg frass/ ml H_2O).

Acid	Amount(g)[1]	Mean A.I.(±S.E.)*	Significance**
Butyric	1.2×10^{-5}	$-0.05 \pm .04^a$	N.S.
Propionic	3.8×10^{-6}	$-0.22 \pm .04^b$	.001
Valeric	2.5×10^{-6}	$0.04 \pm .03^a$	N.S.

[1]Total amount of acid applied to filter paper in 0.68 ml of solution. Concentrations of solutions used were: butyric acid- 2.0×10^{-4}M, propionic acid- 7.5×10^{-5}M, valeric acid- 3.6×10^{-5}M.

*Means followed by the same letter are not statistically different at $P \leq 0.05$.

**Results of t-test for significant response to treated paper, i.e., a significant difference from zero (equal numbers on treatment and control). All indices are significant at $P \leq$ the value listed. N.S. Indicates responses to treatment and control papers are not statistically different.

Table 9. Results of olfactometer trials using volatile fatty acids.

Acid (10^{-1}M)	Treatment	Control	Ts Value[a]	Significance[b]
Butyric	11	41	0	.025
Valeric	7	49	0	.025
Propionic	4	50	0	.025

[a]Wilcoxon's Signed-Ranks Test for paired observations, all differences of like sign (the lowest value of $D = 0$).

[b]Treatment significantly less than control at $P \leq$ the given value. Calculations based on responding individuals for 6 replicates of 10 larvae each.

Figure 10. Mean number of mealworm larvae (±S.E.) responding to papers treated with 0.68 ml of 10^{-3}M propionic acid and dried 1.5 hr. Based on 12 replicates of 20 larvae each.

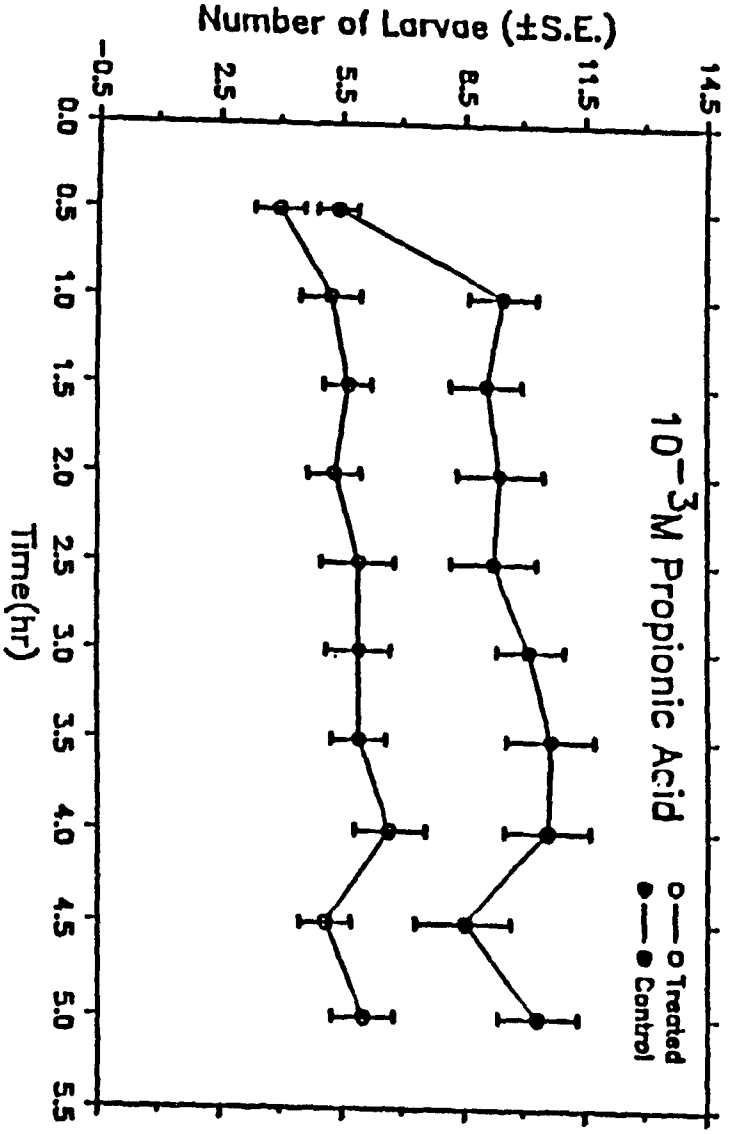
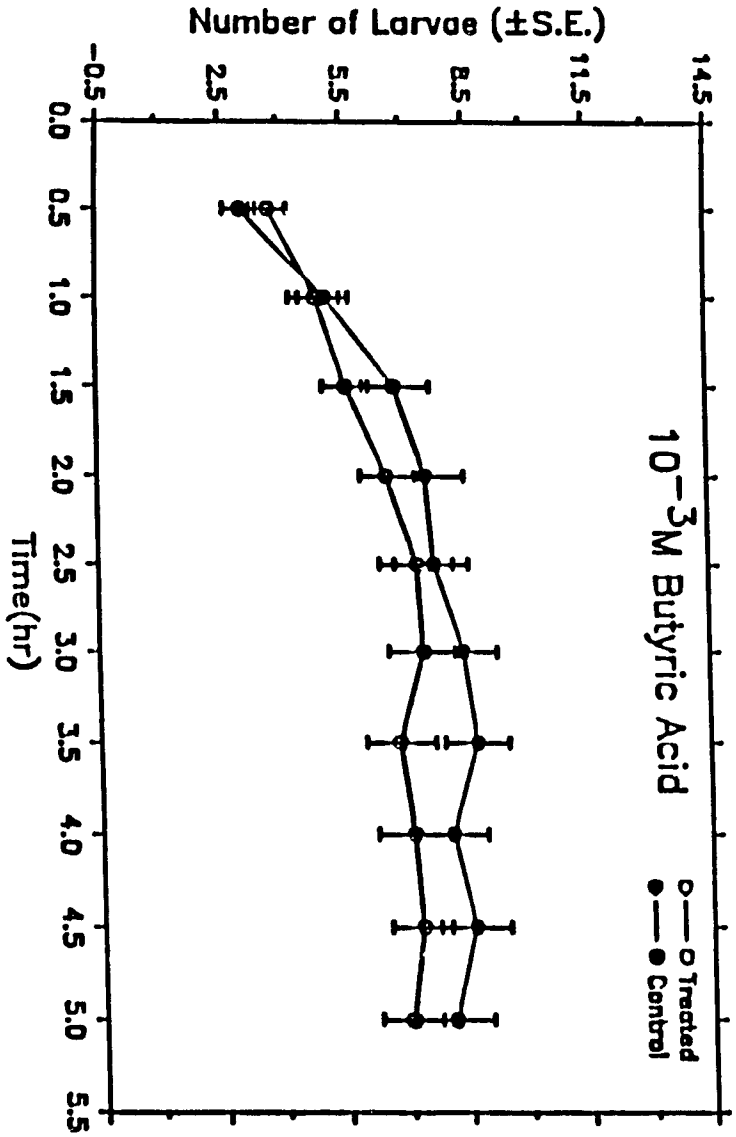

Number of Larvae (±S.E.)
-0.5
2.5
5.5
8.5
11.5
14.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
Time(hr)
10^{-3}M Propionic Acid
o—o Treated
●—● Control

Figure 11. Mean number of mealworm larvae (±S.E.) responding to papers treated with 0.68 ml of 10^{-3}M butyric acid and dried 1.5 hr. Based on 18 replicates of 20 larvae each.


Number of Larvae (±S.E.)
-0.5
2.5
5.5
8.5
11.5
14.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
Time(hr)
10^{-3}M Butyric Acid
o—— o Treated
●—— ● Control

Connecting Statement IV

There is considerable similarity between Ad and Tm in feral habitat choice and in synanthrophic habits (see Section 1: Literature review) as well. Ad prefer cryptic habitats in stored products and could also condition their surroundings with semiochemicals in the larval frass. The role of volatile fatty acids and lactic acid are evaluated for Ad, a smaller Tenebrionid that develops much more rapidly. The role of water in the semiochemical response is evaluated for these larvae, which display a preference for moist habitats (Section 5: Frass-induced aggregation- *Alphitobius*).

Section 5: Frass-Induced Aggregation- *Alphitobius*

Title

THE ROLE OF LIGHT, HUMIDITY AND LACTIC, ACETIC AND PROPIONIC ACIDS IN THE FRASS-INDUCED AGGREGATION OF LARVAL LESSER MEALWORMS.

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Submitted to Physiol. Entomol. August 30, 1989.

ABSTRACT Frass of late instar *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) was found to contain 0.378mg acetic acid, 0.828mg lactic acid, and trace propionic acid per g. Groups of larvae aggregated upon filter papers wetted with aqueous frass extracts. Clusters of larvae also formed on filter papers wetted with aqueous frass extracts and dried prior to bioassay. *Alphitobius* larvae aggregated on papers wetted with 0.68 ml of 10^{-3}M and 10^{-4}M lactic acid, but did not aggregate upon papers treated with the same solutions and dried. The amount of lactic acid on the papers treated with frass extracts is equivalent to the amount on papers treated with lactic acid in the 10^{-3}M to 10^{-4}M range. These insects also aggregated upon papers treated with 10^{-1}M propionic acid, either 'wetted' or 'dry'. Papers kept continually wetted with 10^{-2}M acetic acid were found to cause aggregation upon the control paper. Individual trials indicate that these concentrations of wetted lactic acid act as attractive stimuli while the same concentration of wetted and dried lactic acid acts as a dispersant. Evaluation of hygrotaxis indicates orientation towards a source of humidity, but no behavioral preference was displayed when larvae were allowed to contact the water source. Evidence is presented for the occurrence of negative phototactic responses by these larvae.

Introduction

The lesser mealworm, *Alphitobius diaperinus* (Panzer), is a cosmopolitan insect that infests commercial poultry houses (Pfeiffer and Axtell 1980). This species is a reservoir of avian pathogens, and is thus regarded as a pest (De las Casas et al. 1972, 1976). Late-instar larvae are also regarded as pests because of their tendency to leave the litter and tunnel into building insulation materials (Safrit and Axtell 1984, Vaughan et al. 1984), particularly when at high densities (Geden and Axtell 1987). It occasionally infests flour mills, grain elevators and feed mills in Canada (Sinha and Watters 1985). A visit to a poultry facility that had recently been treated for an infestation of lesser mealworms revealed that a high density of all developmental stages were overlooked during disinfestation procedures. These clusters of individuals were located in the poultry feed storage area and were concentrated in the detritus-containing spaces under forklift pallets (Weaver personal observation). The detritus consisted mainly of decomposing and fresh foodstuffs (predominately dust-size particles), insect frass, insect fragments and fragments of exuviae (Weaver personal observation). These conditions were virtually identical to those in which a large and long-standing infestation of *Tenebrio molitor* was observed in the feed storage area of a dairy facility (Weaver and McFarlane personal observations). Weaver et al. (1989), Section 2: Frass-induced aggregation - *Tenebrio* have demonstrated that yellow mealworm (*Tenebrio molitor*) larvae aggregate on filter papers treated with aqueous frass extracts and, also, on those treated with lactic acid, an isolated frass component. Weaver et al. (In Press), Section 4: Frass repellents- *Tenebrio*, also found that propionic acid, another frass component, repelled larval *Tenebrio*. The following article deals with the analysis of *Alphitobius* larval frass for volatile fatty acids and lactic acid and their possible role in the aggregation of these larvae. Analysis of the responses of the larvae to possible aggregation pheromones or dispersants may provide information useful for control.

This is particularly valid if one considers the possible role of accumulated 'frass markers' acting as indicators of safe, cryptic refugia for potential breeding populations (as hypothesized for *Tenebrio* in Weaver et al. 1989, Section 2: Frass-induced aggregation - *Tenebrio*) to re-infest poultry litter. This may also be relevant for those occasional 'secondary pest status' (in Sinha and Watters 1985) infestations of lesser mealworms in stored cereals and miscellaneous foodstuffs with associated fungi.

Materials and Methods

Lesser mealworms were maintained on a diet of wheat bran, whole wheat flour and brewer's yeast (50:45:5 w/w) at 25 ± 1^{o} C and $55\pm5\%$ relative humidity and a photoperiodic regime of 14:10 light-dark in 4.55 l glass culture jars.

The method of volatile fatty acid and lactic acid determination was similar to that of Weaver et al (1989), Section 2: Frass-induced aggregation-*Tenebrio*, however the conditons of the gas chromatographic analysis were: Varian gas chromatograph Model 3700 equipped with a flame-ionization detector; DB 17 fused silica (Chromatographic Specialities Ltd, Brockville, Ontario, Canada) capillary column (30 m X 0.25mm I.D.) nitrogen carrier gas (1 cm^3/ min; temperature programming: initial temperature =70^{o}C, initial hold = 8 min., program rate = 4^{o}C/min, final temperature = 140 ^{o}C, final hold = 10 min. The method of identity of the peaks and subsequent mass spectrometric confirmation are given in Weaver et al. 1989, Section 2: Frass-induced aggregation-*Tenebrio*

The bioassay method was similar to that used by Weaver et al. 1989, Section 2: Frass-induced aggregation- *Tenebrio*, except for using clothespins and hairpins to hold the papers. Ad larvae do not have sufficent mass to fell the papers. The test aqueous solution (0.68 ml) was applied to a filter paper and dried for 1 hr prior to testing. The control paper was wetted with 0.68 ml of distilled water and dried for 1 hr. In the case of the wetted papers, the strips of filter paper were

attached to 4.5 ml vials containing the test solution or distilled water by means of a cotton wick in the vial, thus ensuring that the papers did not dry. Thirty late-instar larvae (10-15 mg) were simultaneously introduced into the culture dish and were covered with an inverted cardboard box to maintain darkness. Larvae on the papers or touching them with mouthparts or legs were counted at 0.5 hr intervals. Trials were terminated after 5 hr and replicated six to twenty times.

Individual larvae were placed in 19.2 cm diam. glass specimen dishes with folded 14 X 3.8 cm filter papers to evaluate for attraction, arrest, repellency or dispersal (Dethier et al. 1960). These papers were wetted with 0.68 ml of solution and trials were conducted in a dimly backlit room and directly observed for 1 hr with the first choices of the larvae and number of contacts with treatment and control papers recorded.

Individual larvae were also tested for responses to water vapour and volatile stimuli from aqueous lactic acid in a two-choice pitfall olfactometer (Pierce et al. 1981). In these trials a cotton plug was wetted with 1 ml of 5 X 10^{-4}M aqueous lactic acid and the control was a cotton plug treated with 1 ml of distilled water. Water vapour responses were evaluated by comparing responses to a cotton plug treated with distilled water as above to one similarly treated and dried prior to bioassay. Dried cotton plugs that had been treated with 1 ml of 5 X 10^{-4}M lactic acid were also tested against dried plugs treated with 1 ml of distilled water. In all cases, the olfactometers were sealed with a lid to prevent insect escape and darkness was maintained by further covering the apparatus with a cardboard box. Trials were terminated after 2hr and the choices were recorded. Ambient temperature varied from 19-23^{0} C during all of the trials. Control vs. control trials showed no geomagnetic bias in either individual and group trials.

Sixty individual trials were conducted to assay possible phototaxis by placing individual larvae in 15 cm petri plates with 7 X 1.9 cm filter papers folded four times in a zigzag fashion (to form a 'w' connected to an 'm' by a common central side) placed in the middle

of the petri dish. Ten dishes were equally spaced around a circle with a 1 metre radius centered by a shrouded desk lamp aimed directly down at the center of the circle. The lamp shroud was 10 cm from the floor, preventing light from the 40 watt bulb directly illuminating the arenas. Larvae were introduced on the side of the filter paper proximal to the light and the light was turned on. Larvae were observed and their positions recorded after 15 minutes. No evidence of a temperature increase was seen at 1 metre from the light source during these trials.

Frass solutions were prepared by grinding the required amount of frozen frass in a mortar and pestle and adding it to the appropriate amount of de-ionized, distilled water. The resulting mixture was agitated in a vortex mixer for 2 min. and immediately subjected to Buchner filtration through a Whatman No. 1 filter disk. Aliquots were immediately applied to filter papers and dried 1 hr or the resulting solution was introduced into a 4.5 ml vial and attached to a filter paper by a cotton wick for immediate bioassay. Serial dilutions of acids were prepared using DL-lactic acid, obtained from the Fisher Scientific Company, Montreal and propionic and acetic acids from Anachemia, Montreal.

Statistical comparisons between the various test stimuli for grouped responses were based on analysis of variance using Tukey's HSD test on mean aggregation indices. Aggregation indices (A.I.) were calculated by subtracting the number of larvae on the control paper from that on the treated paper and dividing by the total number on the two papers. Index values may range from -1 (complete repellency) to +1 (all responding individuals on the treatment paper). A value of -0.2 corresponds to 50% more insects on the control paper and +0.33 corresponds to twice as many insects on the treatment as were on the control paper (Roth and Cohen 1973). Indices were based on ten observations per replicate with six to twenty replicates being conducted per test chemical. Indices were analyzed by t-test for significant differences from a value of zero (SAS Institute 1988), which would represent identical numbers on treatment and control papers. Chi-square

analysis was used to evaluate for differences in the treatment versus control choices in all trials using solitary larvae (Sokal and Rohlf 1981).

Results

Analysis of *Alphitobius* frass for lactic acid and volatile fatty acids indicated lactic acid at 0.828 mg/ g frass, acetic acid at 0.378mg/ g, and propionic acid at trace levels only. The insect diet was found to contain acetic acid at 0.261mg/ g. Lactic and propionic acids were not found to be present in the insect diet.

Extracts of frass were found to elicit aggregation of groups of late instar *Alphitobius* larvae at both tested dilutions, whether they were delivered as a continually wetted or wetted and subsequently dried stimulus (Table 10). The responses of grouped lesser mealworms to filter papers kept continually wetted through contact with a vial containing an aqueous solution of frass (22 mg/ml H_2O) are illustrated in Figure 12. It is evident that significant differences exist at 0.5 hr and that the differences become greater with the passage of time. Trials conducted to evaluate the response of thirty lesser mealworms to 20 mg *Tenebrio* frass/ml H_2O wetted and dried gave an A.I. value of $0.29 \pm .06$. Trials conducted to evaluate the response of twenty yellow mealworms (method of rearing and assay of Weaver et al. 1989, Section 2: Frass-induced aggregation- *Tenebrio*) to 50 mg *Alphitobius* frass gave an A.I. value of $0.23 \pm .05$. Both trials indicated species-specificity is not conferred by frass of these larvae. Eighteen replicates were conducted for each trial.

Trials conducted with 10^{-2}M lactic acid elicited aggregation on the control paper whether the filter paper stimulus was presented as continually wetted or was wetted and subsequently dried (Table 10). Wetted and subsequently dried lactic acid was found to cause aggregation on the control paper at 10^{-3}M and 10^{-4}M (with a much higher A.I. value), while the 10^{-5}M concentration was found to weakly elicit aggregation

(Table 10). For those trials in which the filter papers were kept continually wetted, the 10^{-3}M and 10^{-4}M lactic acid trials were found to elicit aggregation fairly strongly. Filter papers kept continually wetted with 10^{-5}M and 10^{-6}M lactic acid were also favored, with much lower A.I. values (Table 10). The responses of grouped larval lesser mealworms to filter papers kept continually wetted with 10^{-3}M lactic acid are shown in Figure 13. As in Figure 12 the differences are significant at 0.5 hr and become greater with the passage of time. The responses of grouped *A. diaperinus* larvae to filter papers wetted with 10^{-3}M lactic acid and dried prior to bioassay are given in Figure 14. These responses are distinct not only because the larvae aggregate on the control paper, but also in view of the fact that neither the treatment nor the control are preferred at 0.5 hr or 1.0 hr, but subsequently the differences become obvious.

Propionic acid was found to elicit aggregation at the 10^{-1}M concentration when presented either as continually wetted or wetted and immediately dried (Table 10). Other concentrations either continually wetted or wetted and subsequently dried were much less effective (Table 10). The responses of grouped *Alphitobius* larvae to papers wetted with 10^{-1}M propionic acid and immediately dried are given in Figure 15. A preference is again obvious at 0.5 hr with a subsequent strengthening of the response with the passage of time.

Acetic acid was found to cause aggregation of lesser mealworm larvae on papers kept continually wetted with the 10^{-2}M concentration (Table 10). No other tested concentrations of acetic acid showed any effect (Table 10). The responses of *Alphitobius* larvae to papers continually wetted with 10^{-2}M acetic acid are shown in Figure 16. Here we see no behavioral preference occurring until 2 hr. The responses of these larvae to a chemical inducing no behavioral effect (10^{-3}M acetic acid, wetted and subsequently dried) are given in Figure 17; no significant behavioral preference occured at any time, a situation analogous to control versus control trials (not shown).

Frass-induced aggregation- *Alphitobius*

Individual trials comparing the first choice preferences for filter papers kept continually wetted with 5 X 10^{-4}M lactic acid showed significant attraction (Table 11). This was further confirmed in trials using the same concentration of lactic acid in a pitfall olfactometer (Table 12). In both cases the control used was distilled water. Trials prepared using the same solutions, but with the stimulus allowed to dry showed no first choice behavioral effect in the arena bioassay (Table 11), or in the pitfall olfactometer (Table 12). Trials to evaluate first choice preferences for papers continually wetted with distilled water showed no attraction relative to a wetted and subsequently dried paper (Table 11). However, the same stimuli presented in a pitfall olfactometer resulted in a significant attraction to the distilled water vapor (Table 12). Trials using the arena bioassay to evaluate the first choice of individual larvae for wetted 10^{-2}M lactic acid indicated it was also more attractive than distilled water (Table 11), although aggregates were formed on the control paper in the group trial (Table 10).

Data illustrating the movements between the treated and control filter papers for 1 hr using 5 X 10^{-4}M lactic acid and distilled water, either as continually wetted or as a wetted and dried stimulus, are given in Table 13. The wetted treatment was contacted by 79.4% of the larvae, with 64.7 % contacting this paper only, and 41.2% were on the paper at the end of the trial. In these trials 26.5% of the mealworm larvae contacted the distilled water control, with 11.8% contacting the control only, and 8.8% were on the control at the end of the trial. In trials using a wetted and subsequently dried treatment, 70.5% of the larvae contacted the treatment paper, 29.4% contacted this paper only, and 14.7% were on this paper at the end of the trials. In these trials the control paper was contacted by 70.5% of the *Alphitobius* larvae, 29.4% contacted this paper only, and 38.2% were on the control paper at the end of the trial. This shows that with the wetted stimuli the percentage of larvae on the control paper relative to the treatment paper

decreases with time , whereas the percentage on the control paper increases relative to the treatment with the passage of time in the case of the wetted and subsequently dried stimuli.

Trials to evaluate the phototactic responses of larval *A. diaperinus* indicated that these larvae are negatively phototactic with 49 of 60 individual larvae moving to the less-lighted half of the petri dish within 15 minutes. The fact that the larvae usually came to rest in the faint shadows cast by the filter papers is convincing.

Discussion

The behavioral responses to 10^{-3}M and 10^{-4}M lactic acid are of particular interest because they represent the amount of lactic acid actually present on papers treated with frass solutions in these experiments. Conspecific frass is also attractive to *Alphitobius* larvae. Lactic acid is, in part, responsible for the frass-induced aggregation of these larvae as was shown by the response to the wetted filter papers. The repellency of wetted and subsequently dried filter papers treated with lactic acid contrasts with the attractiveness of similarly prepared frass-treated papers. This indicates that there must be a water-soluble chemical(s), other than lactic acid or the volatile fatty acids, present in the frass which exerts behavioral influence on these larvae, and dominates the lactic acid response, at least during extremely dry conditions.

The role of water in the aggregation response to lactic acid is similar to that observed by McFarlane and Alli (1986), and by McFarlane et al. (1983). In their experiments they found lactic acid to be either repellent or behaviorally ineffective to groups of larval German cockroaches when presented dried on papers, but attractive, at the same concentration, when presented wetted (McFarlane and Alli 1986). The same conditions were found to apply for propionic acid and larval *Acheta domesticus* (McFarlane et al. 1983). However, *Tenebrio* larvae responded equally to wetted or dried lactic acid stimuli at the concentrations

equivalent to that on frass-treated papers and these responses were likely to be initiated by actual contact (Weaver et al. 1989, Section 2: Frass-induced aggregation - *Tenebrio*).

The fact that lactic acid + water vapor was more attractive than water vapor alone (Tables 11 and 12) and that water vapor itself is attractive (Table 12) suggests that contact with water may be significantly responsible for the overall response to wetted lactic acid . However, in an arena where the larvae could actually contact water-wetted papers, there was no difference in contact preference for wetted as opposed to wetted and dried papers (Table 11). This indicates that insects favor an environment of higher as opposed to lower humidity and were not actually trying to either drink or remain on the wetted surface i.e., they fell into the pitfall olfactometer vials evolving water vapor. To summarize, therefore, it is obvious that a directed orientation is made towards aqueous 10^{-3}M to 10^{-4}M lactic acid and also that either subsequent arrest or repeated contacts are favored (Table 13) with the overall responses being very distinct from those caused by water alone.

In the case of wetted and subsequently dried papers treated with the same concentration, we see no evidence of behavioral preference in either first contacts or airstream preference (Tables 11 and 12). However, through time the insects come to prefer the control paper by leaving the treatment paper, possibly repeatedly (Table 13); however they do not remain on the control paper as readily as they do in the group trials (Figure 14). This is to be expected however, considering there is no chemical message to cause arrest of solitary larvae on this control paper. In the grouped situation, which is more representative of the natural condition, the presence of conspecifics reinforces the tendency for aggregate formation on a substrate. The lack of initial directed orientation and the lack of a significant olfactory response (Tables 11 and 12) indicated that this phenomenon was the result of dispersal, not repellency. This was also evident in the group trials where the initial responses indicated both papers were equally favored and the

overall response was manifested later (Figure 14). This demonstrated a different sort of behavior than that induced by stimuli that were more rapid in eliciting a response (Figures 12 and 13). The more rapid response suggested attraction, which was then confirmed by direct observation and olfaction tests (Tables 11 and 12).

The factors responsible for the role of water in the lactic acid response are complex. One might conclude that dried solutions are likely to cause localized crystal formations that could induce dispersion by exceeding receptor thresholds; however DL-lactic acid crystals melt at 16.8^{o}C and the lactic acid molecules are more likely to be in the liquid phase, adsorbed on the surface of the filter paper in a random manner after the evaporation of the water. Therefore one can assume that the presence of *some* water is integral to the relative arrest on the paper and also that water is required for this amount of lactic acid to volatilize enough to have an attractive effect, particularly in view of the fact that the absence of water causes dispersion, not repellency. The observations on hygrotaxis suggest that these larvae prefer a degree of humidity, so this dispersion from 'dried' lactic acid may be relevant only in situations that are biologically extreme, particularly since dried frass extracts retain their attractive nature (Table 10). This hypothesis is further augmented by the fact that the wetted 10^{-2}M lactic acid is initially attractive (first choices, direct observation) and the overall formation of aggregate on the control is the result of dispersion in both the wetted and the wetted and subsequently dried cases. In this case the dispersion is probably the result of concentration and occurs with or without the presence of water. This response also suggests that the lactic acid is of such significantly low volatility that the tenfold increase in concentration does not alter the airborne concentration above the attractive threshold, whereas the message received upon contact is probably in excess of the behavioral threshold. It is possible that these different behaviors may be caused by signals received by either single lactic acid receptors and water receptors or multiple receptor types.

The chemical analyses of frass and diet indicated that lactic acid and trace propionic acid are produced within the insect, as they were not present in the diet. Acetic acid is found in *Alphitobius* diet in two thirds of the amount it is present in the insect frass. Therefore, lactic acid and propionic acid are good candidates as chemical communicators in the frass because they are distinct from the surrounding environment. We have raised these insects on our *Tenebrio molitor* diet (Weaver et al. 1989, Section 2: Frass-induced aggregation - *Tenebrio*). It is of interest that another Tenebrionid could produce a significant amount of lactic acid in vivo, and that lactic acid is behaviorally active as well, though certainly under more restrictive conditions. These *Alphitobius* larvae produce lactic acid at 1/3 of the concentration in the frass that *Tenebrio* larvae do in their frass (Weaver et al. 1989, Section 2: Frass-induced aggregation - *Tenebrio*). We suspect that these chemicals occur as a result of microbial biosynthesis as has been found for other insects. For example, volatile fatty acids are the products of gut bacteria in *Acheta domesticus* (Ulrich et al. 1981) and *Periplaneta americana* (Bracke and Markovetz 1980), and certain of these volatile fatty acids are aggregants for the larvae of these species (McFarlane et al. 1983, McFarlane and Alli 1987).

The response to propionic acid at high concentrations is surprising, given the fact that it is not a major component of the *Alphitobius* frass. Du Toit (1987) cites that propionic acid is present at 10 parts per million in the air surrounding poultry houses so this may be important in the orientation of grouped *Alphitobius* larvae in poultry houses, particularly since they favor the chicken litter in the larval form. Similarly, the possible usage of propionic acid to preserve dried poultry manure for utilization as a feed additive (Cantoni and D' Aubert, 1978) may be influenced by this finding.

Wetted 10^{-2}M acetic acid is repellent to larval lesser mealworms. The amount of acetic acid on papers treated with this amount of acetic acid is in excess of the amount found on papers treated with the tested frass solutions. Lower concentrations do not produce this effect, so

this repellency is unlikely to be a part of the normal frass response of these insects. Weaver et al. (In Press), Section 4: Frass repellents-*Tenebrio* found that butyric and valeric acids were repellent to larval yellow mealworms at concentrations that were higher than that actually occurring on frass-treated filter papers, but not at those concentrations equivalent to the amount on frass-treated paper.

The observation that these lesser mealworm larvae are negatively phototaxic is not surprising since Geden and Axtell (1987) observed that climbing and tunneling behavior of the late-instars occurred primarily at night.

There is little evidence for species-specificity in the frass responses of these two species or in the most effective concentrations of lactic acid (Weaver et al. 1989, Section 2: Frass-induced aggregation - *Tenebrio*, and the present article). There can be little doubt that these compounds are "chemical substances that are released by an individual of a species and convey information to one or more receiving individuals of the same species" and are therefore pheromones (Katz and Shorey 1979, adapted from Karlson and Luscher 1959) and also are "substances produced by members of either or both sexes, that induce members of both sexes to aggregate" and thus fit the definition of aggregation pheromones (Borden, 1985). However, mention of sex is irrelevant in the case of immature insects, though it is the essence of the definition for adult insects, presumably to distinguish such compounds from sex pheromones. In the case of immature insects all conspecific-produced and directed behaviorally-active compounds are likely to to play a role in population orientation, but this definition of aggregation pheromone (Borden, 1985) may still distinguish these frass components and other compounds with similar behavioral activity from other compounds, for example, trail-marking pheromones of larval Lepidoptera (Fitzgerald and Peterson 1988) which elicit a different sort of pre-eusocial behavior in their environment. However, these frass compounds do exert behavioral influences that result in a coordinated orientation of members of the same species , but are noticeably distinct

from typical species-specific aggregation pheromones in that the close interaction of conspecifics after the initial behavioral response is elicited results in the aggregation (Weaver and McFarlane 1989, Section 3: Aggregation behavior- *Tenebrio*). This is evident because the same compound, i.e., lactic acid, elicits aggregation not only in related species such as *Tenebrio* (Weaver et al. 1989, Section 2: Frass-induced aggregation- *Tenebrio*) and *Alpnitobius*, but also in an unrelated species, *Blattela germanica* (McFarlane and Alli 1986) and at relatively similar concentrations in all cases. It would be inappropriate to conclude that any similarities in such pheromones be correlated to taxonomic relationship.

It is likely that frass and possibly lactic acid in the frass may act as chemical markers of safe refugia for populations of *Alphitobius diaperinus* larvae in the same manner as for *Tenebrio molitor* (Weaver et al. 1989, Section 2: Frass-induced aggregation- *Tenebrio*). The ecological benefit of remaining in such dark, cryptic earlier-infested regions may well be linked to the maintenance of potential breeding populations of these detritus feeding insects. These groups successfully avoid disinfestation procedures via the behavior induced by these pheromones in such locations. Such pheromone-marked refugia may act as subsequent point sources of re-infestation. It is of interest that the much smaller, rapidly-developing *Alphitobius* displays behavior that may favor persistence in an unstable environment in a similar manner as such a conspicuously large and slow-developing insect as *Tenebrio molitor*.

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Table 10. Results of trials using aqueous frass extracts and serial dilutions of isolated acids found in the frass.

Chemical	Concentration	Mean A.I.(±S.E.)*	Significance**
Frass (Wet)	22 mg/ml H_2O	$0.40\pm.02^{a}$	.001
Frass (Dry)	15 mg/ml H_2O	$0.31\pm.02^{abc}$	.001
Lactic Acid	10^{-2}M (Wet)	$-0.11\pm.03^{hij}$	.005
Lactic Acid	10^{-3}M (Wet)	$0.25\pm.02^{bcd}$	.001
Lactic Acid	10^{-4}M (Wet)	$0.21\pm.02^{cde}$	.001
Lactic Acid	10^{-5}M (Wet)	$0.10\pm.03^{ef}$	.001
Lactic Acid	10^{-6}M (Wet)	$0.11\pm.03^{def}$	.001
Lactic Acid	10^{-1}M (Dry)	$-0.28\pm.02^{k}$	.001
Lactic Acid	10^{-2}M (Dry)	$-0.23\pm.03^{jk}$	.001
Lactic Acid	10^{-3}M (Dry)	$-0.22\pm.03^{ijk}$	.001
Lactic Acid	10^{-4}M (Dry)	$-0.10\pm.03^{hij}$	.005
Lactic Acid	10^{-5}M (Dry)	$0.11\pm.03^{def}$	.001
Propionic Acid	10^{-1}M (Dry)	$0.25\pm.03^{bcd}$	.001
Propionic Acid	10^{-2}M (Dry)	$0.08\pm.03^{efg}$	.005
Propionic Acid	10^{-3}M (Dry)	$0.01\pm.03^{fgh}$	N.S.
Propionic Acid	10^{-4}M (Dry)	$0.00\pm.03^{fgh}$	N.S.
Propionic Acid	10^{-1}M (Wet)	$0.38\pm.03^{ab}$	.001
Propionic Acid	10^{-2}M (Wet)	$0.10\pm.03^{ef}$	.001
Propionic Acid	10^{-3}M (Wet)	$-0.09\pm.03^{hi}$	.005
Acetic Acid	10^{-1}M (Dry)	$-0.05\pm.03^{gh}$	N.S.
Acetic Acid	10^{-2}M (Wet)	$-0.18\pm.02^{ijk}$	.001
Acetic Acid	10^{-3}M (Dry)	$0.06\pm.04^{fg}$	N.S.

*Means followed by the same letter are not statistically different at $P\leq 0.05$.

**Results of t-test for significant response to treated paper, i.e., a significant difference from zero (equal numbers on treatment and control). All indices are significant at $P\leq$ the value listed.

N.S. Indicates responses to treatment and control papers are not statistically different.

Table 11. Results of trials evaluating the first choice contacts of *Alphitobius* larvae in a directly observed arena bioassay.

Chemical[1]	Concentration	Treatment[2]	Control[3]	Significance*
Lactic Acid	5×10^{-4}M (Wet)	25	6	.005
Lactic Acid	5×10^{-4}M (Dry)	18	16	N.S.
Lactic Acid	10^{-2}M (Wet)	23	9	.025
Water	55M	15	19	N.S.

*Results of chi-square test for significant first choice response to treated paper. N.S. Indicates responses to treatment and control papers are not statistically different. Data are from 34 replicates using solitary larvae.

[1]In each case an appropriate control was used, i.e., wet stimuli (aqueous solution) on papers were tested against distilled water on papers, dry stimuli (dried aqueous solution) on papers were tested against papers treated with distilled water and dried. Water was tested against papers treated with distilled water and dried. The continually wetted state was maintained by attaching a 4.5 ml vial containg the liquid to a filter paper by using a tightly-packed cotton wick. Aliquots of solutions to be dried were 0.68 ml.

[2,3]The number of solitary larvae choosing to make first contact with the filter paper.

Table 12. Results of trials evaluating the responses of solitary *Alphitobius* larvae in a pitfall olfactometer.

Chemical[1]	Concentration	Treatment[2]	Control[3]	Significance*
Lactic Acid	5 X 10^{-4}M (Wet)	38	12	.001
Lactic Acid	5 X 10^{-4}M (Dry)	22	26	N.S.
Water	55M	24	10	.05

*Results of chi-square test for significant response to treatment odor. N.S. Indicates responses to treatment and control papers are not statistically different. Data are from 60 replicates using solitary larvae for lactic acid, 40 replicates for water.

[1]In each case an appropriate control was used, i.e., wet stimuli (1 ml of aqueous solution on a cotton wick) were tested against 1 ml of distilled water on a cotton wick, dry stimuli were prepared using the same aliquots and allowing the cotton to dry prior to assay. Water was tested using 1 ml of distilled water on a cotton wick for the treatment, and a cotton wick similarly treated and dried was used for the control.

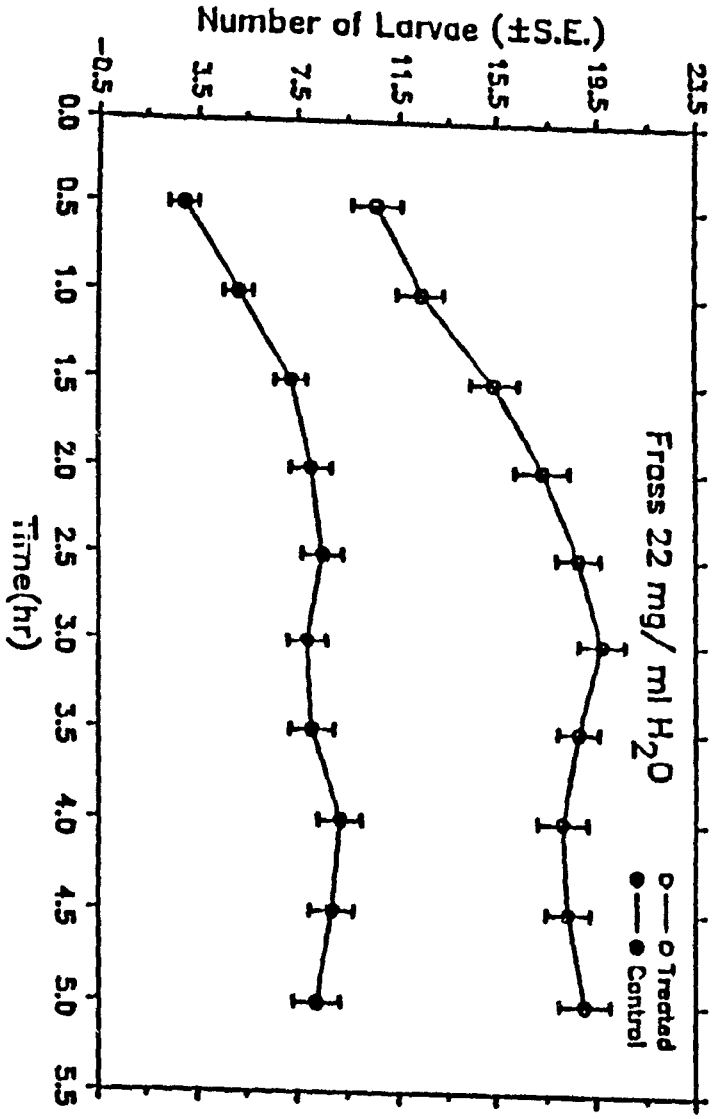
[2,3]The number of solitary larvae falling in the trap vials containing treatment or control stimuli.

Table 13. Results of trials evaluating the number of contacts with filter papers in a directly observed arena bioassay in 1 hour.

Alphitobius larvae making contact with:	Wet trials[1]	Dry trials
Neither paper	8.8	0
Treatment paper once only	26.5	23.5
Control paper once only	0	11.8
Treatment paper only $\geq$ 2 times	38.2	5.9
Control paper only $\geq$ 2 times	11.8	17.6
Both papers $\geq$ 1 time, first contact treatment	8.8	23.5
Both papers $\geq$ 1 time, first contact control	5.9	17.6
Treatment paper at trial end	41.2	14.7
Control paper at trial end	8.8	38.2

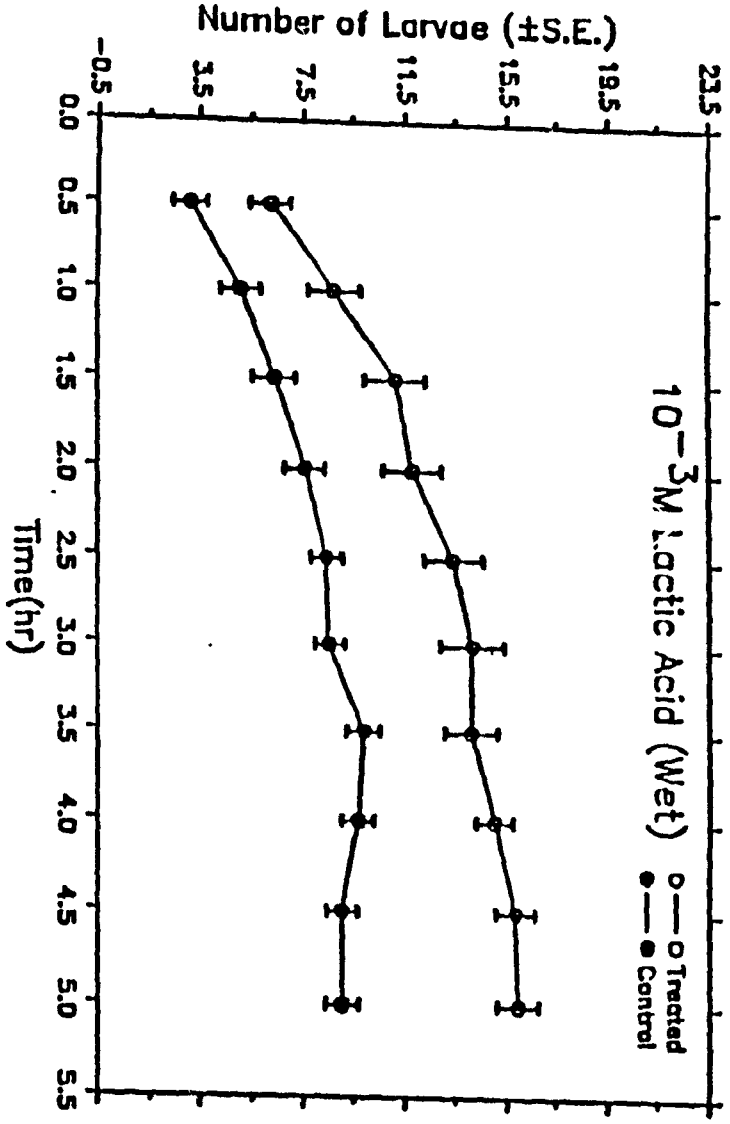
[1] Data are from 34 replicates using solitary larvae expressed as a percentage. Wet trials utilized a filter paper attached to a 4.5 ml vial containing 5 X 10^{-4}M lactic acid by means of a cotton wick for the treatment, and a similarly prepared apparatus containing distilled water for the control. Filter papers were treated with 0.68 ml of the same concentration of lactic acid and dried in the dry trials; the control was similarly prepared using distilled water.

Figure 12. Mean number of lesser mealworm larvae (±S.E.) responding to filter papers kept continually wetted with a solution of 22 mg frass/ ml H_2O. Based on 18 replicates of 30 larvae each.



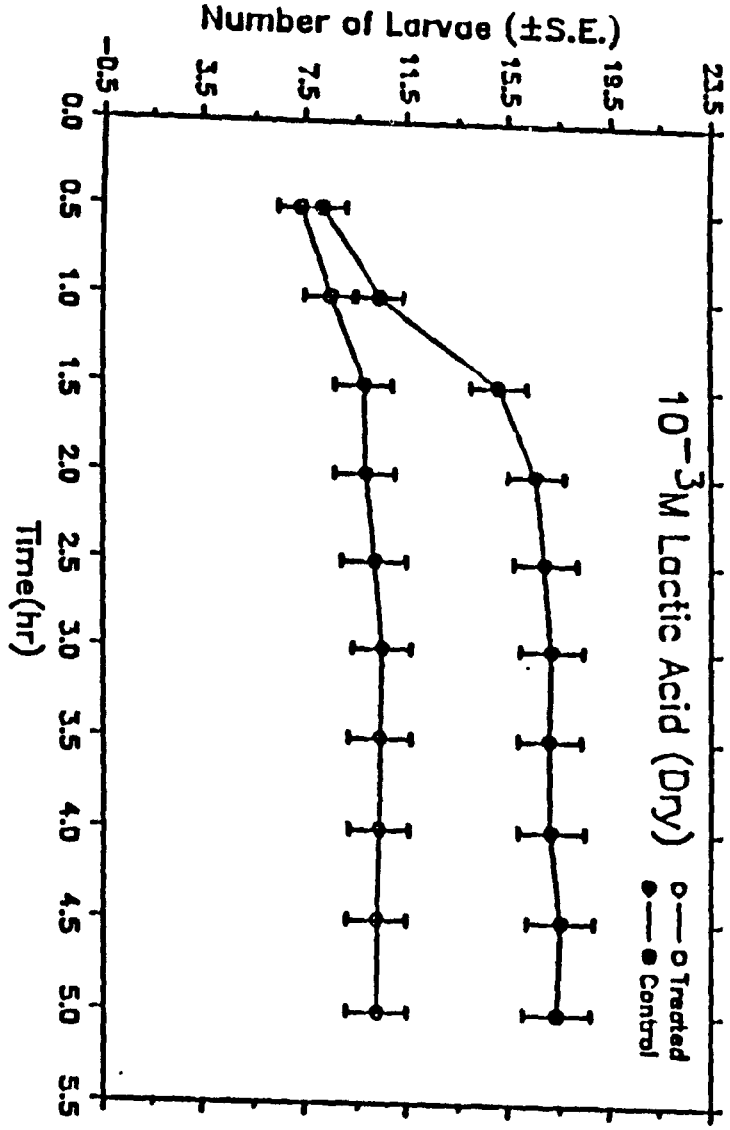
Frass-induced aggregation- *Alphitobius*

Figure 13. Mean number of lesser mealworm larvae (±S.E.) responding to filter papers kept continually wetted with a solution of aqueous 10^{-3}M lactic acid. Based on 18 replicates of 30 larvae each.



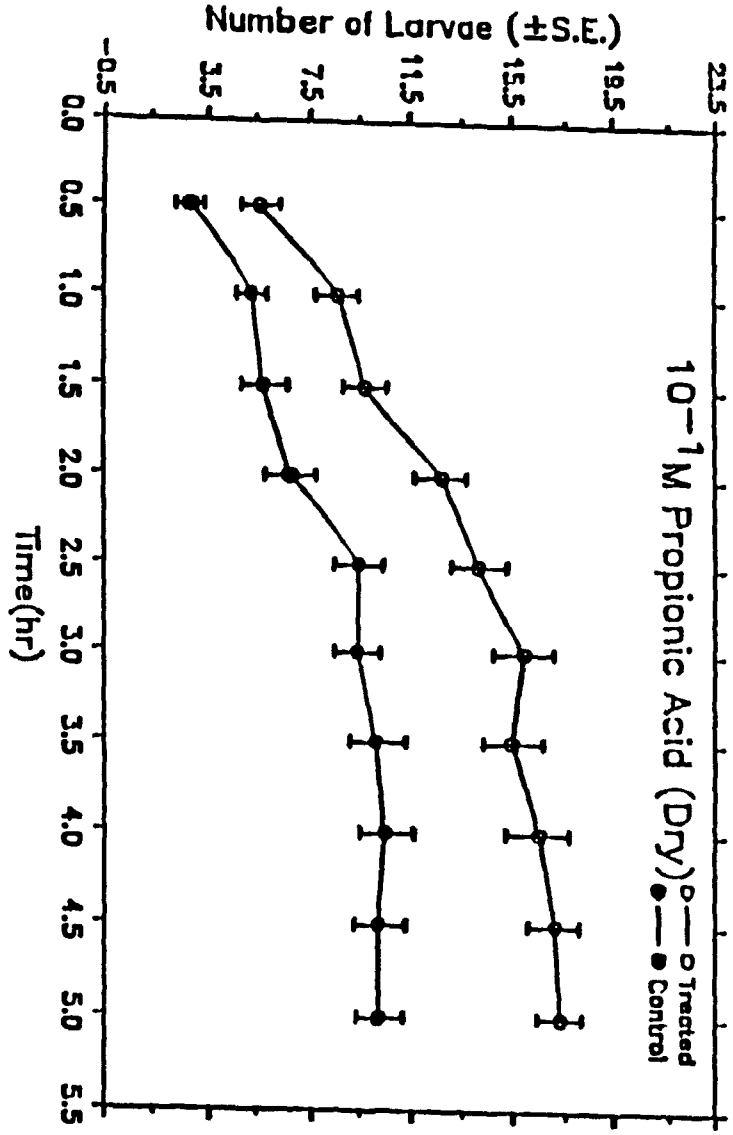
Frass-induced aggregation- *Alphitobius*

Figure 14. Mean number of lesser mealworm larvae (±S.E.) responding to filter papers wetted with 0.68 ml of a solution of aqueous 10^{-3}M lactic acid and dried prior to bioassay. Based on 20 replicates of 30 larvae each.



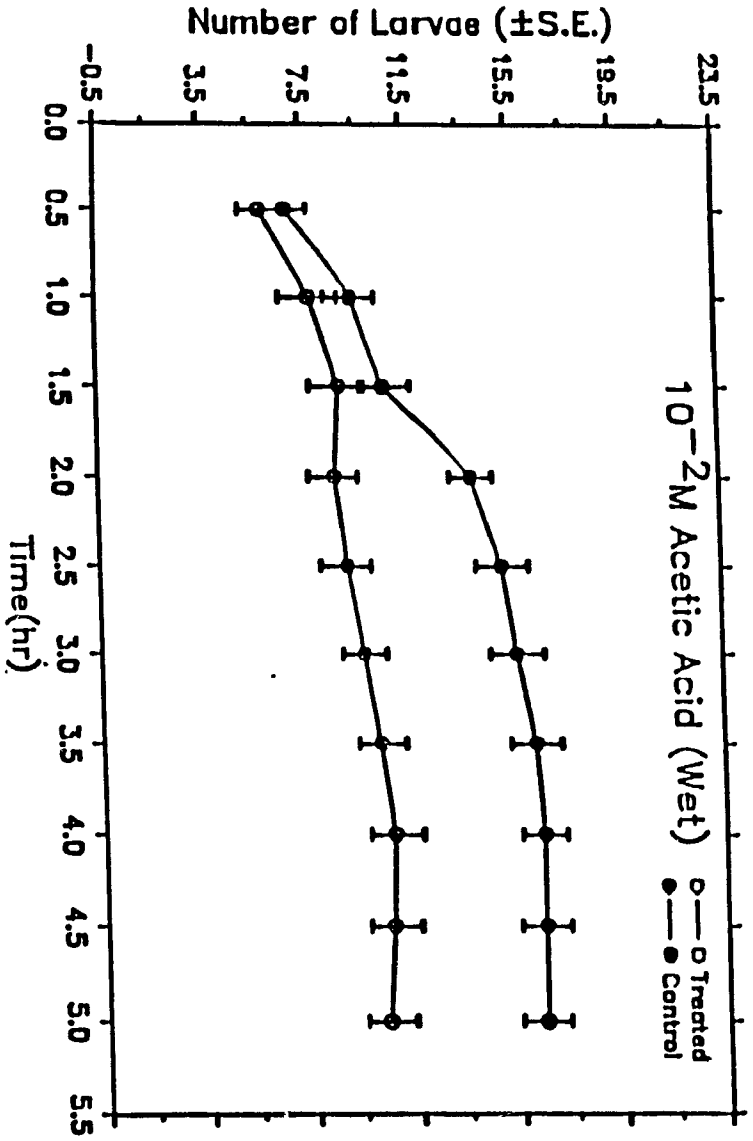
Frass-induced aggregation- *Alphitobius*

Figure 15. Mean number of lesser mealworm larvae (±S.E.) responding to filter papers wetted with 0.68 ml of a solution of aqueous 10^{-1}M propionic acid and dried prior to bioassay. Based on 18 replicates of 30 larvae each.



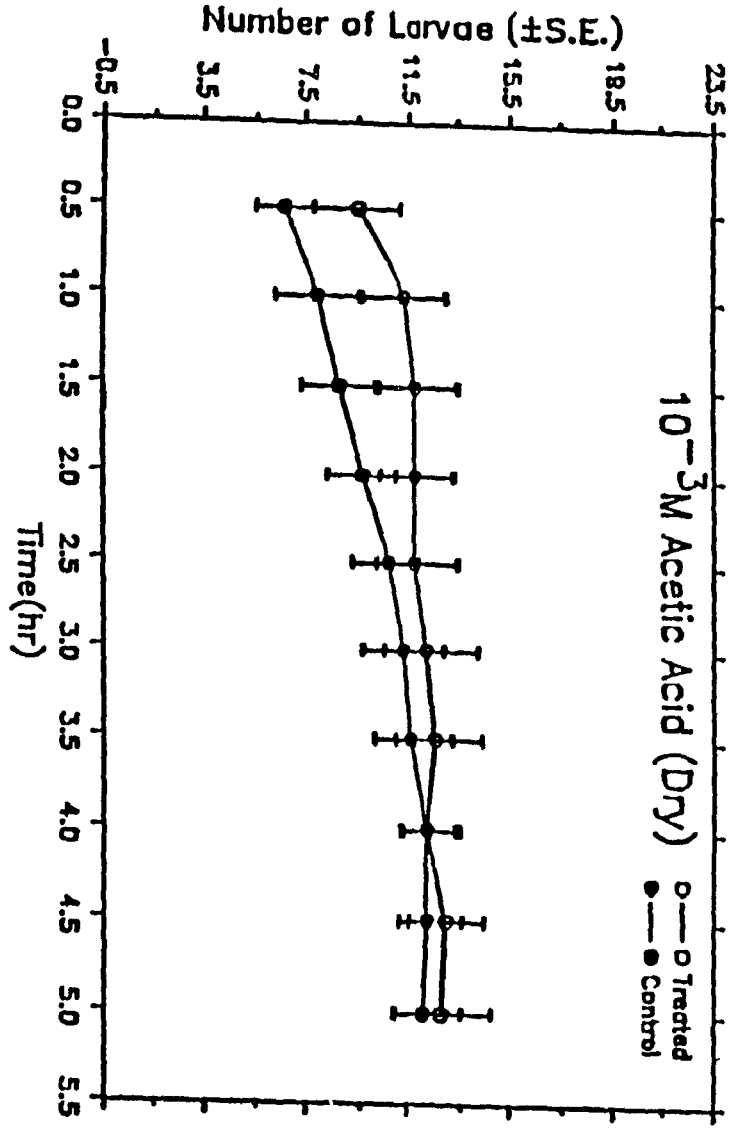
Frass-induced aggregation- *Alphitobius*

Figure 16. Mean number of lesser mealworm larvae (±S.E.) responding to filter papers kept continually wetted with a solution of aqueous 10^{-2}M acetic acid. Based on 18 replicates of 30 larvae each.



Frass-induced aggregation- *Alphitobius*

Figure 17. Mean number of lesser mealworm larvae (±S.E.) responding to filter papers wetted with 0.68 ml of a solution of aqueous 10^{-3}M acetic acid and dried prior to bioassay. Based on 6 replicates of 30 larvae each.



Frass-induced aggregation- *Alphitobius*

Connecting Statement V

The frass components evaluated and the frass itself have shown cross-specific activity for these larval mealworms, though they function as pheromones (Section 5: Frass-induced aggregation -*Alphitobius*). The higher density toleration of *Tenebrio* larvae upon lactic acid-treated substrates, plus the evidence that *Tenebrio* larvae respond to each other with time (Section 3: Aggregation behavior- *Tenebrio*), suggested that contact between individuals could facilitate clustering. This contact must provide a means of differentiation from that of random contact with objects, which would merely lead to thigmotaxis, or members of other species, because the formation of these clusters is the result of some form of conspecific communication, as was viewed in assay and videotaped observation (Section 3: Aggregation behavior- *Tenebrio*). This suggests that a pheromone may be involved. The role of cuticular compounds as recognition factors in mealworm aggregation is presented (Section 6: Cuticular lipids in aggregation).

Section 6: Cuticular Lipids In Aggregation

Title

CUTICULAR LIPID COMPONENTS AS CONSPECIFIC RECOGNITION FACTORS IN AGGREGATE FORMATION BY *Tenebrio molitor* L. LARVAE.

Authors: Weaver, D.K., A. Ali, J.E. McFarlane, and T.H. Chan.
In final preparation for J. Exp. Biol.

ABSTRACT Late-instar larvae of the yellow mealworm, *Tenebrio molitor* L., were attracted to cuticular lipid extracts of conspecific late-instar larvae presented on filter papers. Individual larvae and groups of larvae were attracted. Cuticular lipids were fractionated by flash column chromatography. Fractions were bioassayed for behavioral activity using individual larvae under direct observation for ninety minutes. A variety of behavioral parameters were collected by recording the number and duration of contacts with treated and control substrates. The results suggested that 8,9-pentacosanediol is the attractive component of the cuticular extracts. 8,9-pentacosanediol comprises approximately 60% of the larval epicuticle. Significant arrest preference after initial attraction occurred in the presence of 8,9-pentacosanediol, or when the fractions were recombined. Repeated visits to the filter paper play a role in arrest preference for 8-9 pentacosanediol treated substrates. Enhancement of the overall duration of substrate visitation occurred only with the recombined fractions, suggesting that perception of compounds other than the major one are required for a complete behavioral response, while only the major one serves as a close-range attractant. The role of these compounds as recognition factors in aggregate formation of grouped larvae is discussed, with emphasis upon contact between aggregating individuals. The role larval exuviae might play in environmental marking of suitable habitat is also discussed. Preliminary evidence for a cuticular lipid pheromone(s) in *Alphitobius* larvae is presented.

Introduction

Cuticular lipids are known to be the sex pheromones of certain Diptera (reviewed in Howard and Blomquist 1982, Blomquist and Dillwith 1985 and Lockey 1988). Grula et al. (1980) found that two species of co-existing congeneric Pierid butterflies are reproductively isolated by female responses to the presence or absence of cuticular esters in the males of each species. Cuticular hydrocarbons are components of the sex pheromone of the yellowheaded spruce sawfly *Pikonema alaskensis* (Bartelt et al. 1982). Unsaturated cuticular hydrocarbons serve as the sex pheromone of female *Aleochara curtula*, a staphylinid (Peschke and Metzler 1987). The azuki bean weevil, *Callosobruchus chinensis* L., was found to have a copulation release pheromone consisting of cuticular lipid components, that are distinct from the female sex pheromone (Tanaka et al. 1981). Copulation release activity is found in cuticular extracts from adult male and female *Tenebrio molitor* L., and this chemical is distinct from the female sex pheromone (Tanaka et al. 1986, 1988).

Cuticular lipids have been found to play a role in caste-and species-recognition in sympatric *Reticulitermes* species (Howard et al. 1982). Norris (1970) found that cuticular lipid components were in part responsible for aggregation in ovipositing female *Schistocerca gregaria*.

The cuticular lipid composition of adult *Alphitobius diaperinus* (Panz.) (Lockey 1979) and adult *Tenebrio molitor* L. (Lockey 1978) has been studied. Bursell and Clements (1967) studied the cuticular lipids of the larval exuviae of *Tenebrio molitor* L. They reported that >55% of the extracted lipid was 8,9-pentacosanediol.

Aggregation pheromones of insects are generally evaluated as being governed by factors that the insects release to signal the location of a favorable resource and also the presence of at least one conspecific by default (the individual that is releasing the pheromone). The success of this strategy is linked to the species-specificity of the pheromone or pheromone blend being conferr·d by chemical uniqueness, either in molecular structure or the blend ratio of the molecules released.

Weaver et al. (1989), Section 2: Frass-induced aggregation- *Tenebrio*, found that larvae of *Tenebrio molitor* aggregated upon substrates treated with aqueous extracts of conspecific larval frass, and that higher density clusters of individuals were formed upon such substrates than were formed upon control substrates (Weaver and McFarlane 1989, Section 3: Aggregation behavior- *Tenebrio*). With the passage of time, clusters also formed upon control substrates that were of a significantly higher density than expected and videotaped behavior indicated that close-range or contact chemical communication might be involved (Weaver and McFarlane, 1989, Section 3: Aggregation behavior- *Tenebrio*). The following article describes the role of cuticular lipids in larval yellow mealworm aggregation behavior. Preliminary evidence for the existence of a similar pheromone in *Alphitobius diaperinus* larvae is presented.

Materials and Methods

Yellow mealworms were reared as described previously (Weaver et al. 1989, Section 2: Frass-induced aggregation- *Tenebrio*). Lesser mealworms were reared as described previously (Section 5: Frass-induced aggregation- *Alphitobius*).

Late-instar larvae (140-160 mg- *Tenebrio*; 10-15 mg- *Alphitobius*) were removed from the culture and placed in glass specimen dishes for overnight starvation in the rearing incubator. This reduced the potential for frass contamination of lipid extracts considerably. After starvation, freshly moulted and injured larvae were removed and the number remaining were carefully counted. These indivduals were then subjected to a three-minute extraction in HPLC grade chloroform (Fisher Scientific Co., Montreal, Canada) on a 100 rpm shaker-table. The resulting solution was Buchner filtered through a Whatman No. 1 filter disc. The extraction was generally performed on @ 2000 larvae using 400 ml of chloroform in a 1000 ml Erlenmeyer flask. The extraction flask was rinsed three times with chloroform and the rinse solvent was poured over the cadavers in the Buchner funnel, total volume of rinse liquid

was @ 50 ml. The filtered extract was subjected to rotary evaporation under partial vaccuum and the remaining volume (5 ml) of solvent was evaporated under a stream of dry nitrogen. The mass of the total lipid extracted was then determined. For *Alphitobius* larvae the protocol was identical except for the amount of solvent used. These smaller insects (@ 2500) were initially extracted in 250 ml of chloroform.

The extracted *Tenebrio* lipid (100-150 mg) was then subjected to flash column chromatography using a 1.5 cm i.d. glass column containing 25 cm of packed silica Kieselgel 60 (230-400 mesh ASTM; BDH, Montreal, Canada). The column was packed in the eluting solvent (1:5 ethyl acetate:hexane). The sample was delivered to the column in 1.5 ml of 2:1 chloroform:methanol. The eluates were collected in 4 ml portions and separation was confirmed using thin-layer chromatography on pre-coated silica gel sheets (particle size, 2-25 X 10^{-6} m; thickness, 250 X 10^{-6} m, pore size- 60 Å; Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A.). The plates were developed in the eluting system and the lipid fractions were detected by reaction with a solution of 2.5:2.5:45:0.5 anisaldehyde:conc. H_2SO_4:ethanol:conc. acetic acid and subsequent exposure to a heated airstream. The extracted *Alphitobius* lipid was eluted on an identical system with the eluant being 1:9 ethyl acetate:hexane. The eluant systems were those selected after preliminary screening of a vaiety of systems.

After bioassay the behaviorally active fractions from both species were analyzed by thin-layer chromatography in higher and lower polarity solvent systems (1:3, 1:7 and 1:15 ethyl acetate:hexane). Only one *Tenebrio* fraction was behaviorally active; it appeared to consist of a large quantity of highly pure material. This suggested that it was 8,9-pentacosanediol, which Bursell and Clements (1967) found to compose greater than 55% of the larval exuviae. The present data suggested this compound represented more than 60% of the larval epicuticle, though the extraction was designed to extract pheromones in the surface lipid and may have left components of the inner cuticular lipid intact. The two

initial fractions of *Alphitobius* lipids that were behaviorally active were found to represent groups of closely related compounds and are currently being isolated and assayed further.

The nmr-spectra of the *Tenebrio* compound was obtained and was consistent with that of an aliphatic diol. Samples for ^{1}H nmr were run on a Varian XL-300 NMR Spectrometer with $CDCl_3$ as the solvent. Confirmation of the presence of hydroxyl groups was accomplished by the addition of trace D_2O to the sample under the same conditions. ^{13}C nmr spectra were obtained under the same conditions as the ^{1}H nmr. The mass spectra was obtained and was largely consistent with that described by Bursell and Clements (1967). Mass spectra were obtained on a Kratos MS 25 RFA mass spectrometer (Kratos, Manchester, England) using a direct insertion probe at 130°C. The conditions of mass spectroscopy were 70 eV at a source temperature of 200°C. The parent ion was confirmed using CI mass spectroscopy using NH_3 as the ionizing agent. Conditions of CI mass spectroscopy on a Hewlett-Packard 5980A mass spectrometer (Hewlett Packard Co., Avondale, Pennsylvania, U.S.A.) were 70 eV; samples were run using direct inlet with minumum temperatures of 65°C , 119°C, and 170°C.

Bioassays were conducted upon extracts and fractions using insect equivalent units. These represented the total yield of extract or fraction divided by the number of individuals they were obtained from; in all cases these are also reported as the amount of material (g) applied to the filter papers.

For bioassays using groups of either *Tenebrio* or *Alphitobius* larvae, twenty late-instar (140-160 mg) yellow mealworms or forty late-instar (10-15 mg) lesser mealworms were used. Bioassay arenas and filter papers were as described previously (Weaver et al. 1989, Section 2: Frass-induced aggregation- *Tenebrio*; Section 5: Frass-induced aggregation- *Alphitobius*). Extracted lipids were dissolved in the amount of chloroform required to deliver the desired insect equivalency upon the filter paper in 0.68 ml of solution. The solution was applied to the filter paper and the chloroform was allowed to evaporate for twenty minutes after which the papers were transferred to the arenas and the

larvae were introduced. Control papers were similarly prepared using 0.68 ml of chloroform. Arenas were kept in darkness and the number of larvae on the treatment or control paper were counted at 30 minute intervals.

For bioassays of fractions individual larvae of both species were viewed under direct observation in a dimly backlit room (Section 5: Frass-induced aggregation- *Alphitobius*). The biossays were conducted in 15 cm diam. glass petri dishes. *Tenebrio* bioassays used 10 X 3.8 cm Whatman No. 1 filter papers folded into a "W" with sides of equal length; for treatment papers the desired insect equivalency was delivered in 0.50 ml of chloroform and the chloroform evaporated for 20 minutes. Control papers were similarly prepared using 0.50 ml of chloroform. Filter papers were placed in the arenas with the open portions of the "W's" facing each other. The papers were placed 1 cm from the side of the dish. A late instar *Tenebrio* larva was introduced into the center of the arena and directly observed for ninety minutes. The data recorded were: the paper first contacted, the total number of contacts with either paper and the duration of each contact with either paper. *Alphitobius* bioassays were conducted under identical conditions with 1.9 X 7 cm filter papers folded into a "V" with arms of equal length and a vertex angle of 90^0. The papers were positioned as follows: < >, with the vertices 1 cm from the sides of the dish. The volume of chloroform used to deliver the appropriate insect equivalency was 0.17 ml and the larvae were observed for sixty minutes. The data collected were as for *Tenebrio*.

Fifty trials were attempted for each fraction and for control vs. control comparisons; at least forty-two insects responded in each case. A larva was used only once for behavioral assay. The position of treatment and control papers were randomized for all individual and group trials, however to compare accurately the various index values (described below) the random pattern used in the control vs control trials was followed in all fraction trials. The primary objective of randomization was to avoid potential directional bias caused by airstreams,

temperature gradients or geomagnetic orientation. This was achieved even if the random pattern was the same in all assays. Ten trials were conducted at once by direct scanning of all replicate jars. This leads to a consistent potential error in the recording of contact duration of @ 15 secs, but this occurs equally in all trials, so no bias results. In fact, this method is quite efficient for recording behavioral data from insects that move as slowly as these larvae do.

Aggregation indices were used to determine if the responses of grouped larvae to varying dilutions of lipid extracts were significantly different. Statistical comparisons between aggregation indices were based on analysis of variance using Tukey's HSD test on mean aggregation indices. Aggregation indices (A.I.) were calculated by subtracting the number of larvae on the control paper from that on the treated paper and dividing by the total number on the two papers. Index values may range from -1 (complete repellency) to +1 (all responding individuals on the treatment paper). A value of -0.2 corresponds to 50% more insects on the control paper and +0.33 corresponds to twice as many insects on the treatment as were on the control paper (Roth and Cohen 1973). Indices were based on ten observations per replicate with eighteen to thirty replicates being conducted per test chemical. Indices were analyzed by t-test for significant differences from a value of zero (SAS Institute 1988), which would represent identical numbers on treatment and control papers.

Chi-square tests were used to determine if the number of insects making first contact with lipid extracts or fractions was significantly different than the number making first contact with the control (Sokal and Rohlf 1981).

To determine if these compounds acted as olfactory arrestants post-contact, several new units were derived.

Indices of arrest (Ar.I.) were calculated to determine the amount of time each insect was in contact with the treatment (visiting) relative to the amount of time the insect was visiting the control. Ar.I. were calculated by subtracting the total duration of visits on the con-

trol paper from the total duration of visits on the treatment paper and dividing this by the total amount of time spent on both papers. Values could range from +1 (all visits were on the treatment) to -1 (all visits were on the control). This gives a numerical representation to each insect's relative duration of visits upon the two substrates, but equates indices regardless of their duration, i.e., an insect that spent only one minute on the control only (Ar.I.= -1) would give exactly the same value as an insect that spent fifty minutes on the control only (Ar.I.= -1). This unit accomodates the possibility of olfactory arrestment or contact arrestment in a defined spatial region (Kennedy 1978) by giving full weight to the individual preference for a substrate by relative duration of contact. Stated simplistically, an Ar.I. value determines if an insect prefers to remain on a particular paper

Contact indices (C.I.) were devised to aid in the determination of whether the total duration of visits upon the treatment relative to the total duration of visits upon the control could be a function of repeatedly re-establishing contact with a particular substrate during intermittent wandering. C.I. were calculated by subtracting the total number of contacts with the control paper from the total number of contacts with the treatment paper and dividing it by the total number of contacts with both papers. Values could range from +1 (contacts with the treatment only) to -1 (contacts with the control only). C.I. values equate relative values regardless of the size of the values in the numerator, thus an insect establishing contact with the treatment paper only once gives an index value of +1 as does an insect that establishes contact with the treatment repeatedly. A C.I. value treats each individual contact as an equivalent discrete value while ignoring the duration of visit as is used in determining an Ar.I. value. Stated simplistically, a C.I. value determines if an insect prefers to contact a particular paper, not just initially as is generally used in determining attraction (Dethier et al. 1960) but also repeatedly during a bioassay.

Finally, differences in displacement duration (D.D.) were calculated by subtracting the sum of the length of visits upon the control from the sum of the length of visits upon the treatment. This gives an absolute value for visit preference that accomodates the total length of time upon each paper. D.D. values can theoretically range from approaching 90 to approaching -90 for *Tenebrio* and from approaching 60 to approaching -60 for *Alphitobius* (some time is required for the larva to reach a paper in every trial). This value allows for determination of whether the relative spatial displacement (arrestment) of individuals contributes to a significantly longer visit. Stated simplistically, D.D. values indicate the overall length of time the insect prefers to remain upon a particular paper.

These values are all derived from the same raw data set (and for each fraction, from the same group of insects). Therefore, the different derived values can be accurately compared within a group and conclusions can be drawn about the contribution of duration of individual contacts and initial + subsequent attraction (repeated contacts) to the overall reponse to the fraction.

The Ar.I., C.I. and D.D. values for the fractions are data that are not normally distributed. The index values actually follow the type of distribution known as the antimode (Sokal and Rohlf 1981) that occasionally occurs in behavioral tests (Martin and Bateson 1986). In these trials, this is likely to be a function of the insects preferring to be on a filter paper rather than the glass surface of the arena. However, 50 trials comparing control to control responses gave approximately equivalent distributions for the extreme values (Figures 24, 25, 26, and 27) and attractive fractions (by Chi-square) visibly altered this distribution greatly (Figure 25, upper left and upper right, for example). Therefore, Wilcoxon Rank Sums tests (Sokal and Rohlf 1981) were performed comparing the rank sums of the index values for each fraction to the rank sums for the control (Two-Sample test). Data analysis was conducted using the NPAR1WAY procedure of the SAS Institute (1988). This Wilcoxon Two-Sample procedure (with a continuity correc-

tion of .5) gave a value of Z, which is the sum of scores that is least different from that expected (under the null hypothesis that the rank sums of both samples are identical) divided by the standard deviation of the rank sums under the null hypothesis. Values of Z are approximately normal. The probability of a greater observed Z value (One-Tail Test) is extrapolated to give an approximate T-Test significance (SAS 1988).

Results

The quantities of the various fractions of a total cuticular lipid extract of *Tenebrio* larvae are given in Table 14. Fraction A was the only fraction to display significant biological activity and was present in a very high quantity. The quantities of the various fractions of a total cuticular lipid extract of *Alphitobius* larvae are given in Table 15. Fractions D and E showed significant biological activity. These fractions are multiple component mixtures and are present in much lower quantities than the biologically active compound present in *Tenebrio* larval cuticle.

The nmr spectra of *Tenebrio* fraction A is given in Figure 18. The large relative area at 1.3 ppm downfield is characteristic of an aliphatic chain. By comparing the peak averages for the triplet at 0.8 ppm (six terminal protons) to that for the hydroxyl singlet at 1.6 ppm downfield, it is evident that more than one virtually identical hydroxyl is present. Addition of D_2O during ^{1}H nmr confirmed the hydroxyl moities by their absence at ppm down field (Figure 19). ^{13}C nmr indicated that all carbons present were located in the aliphatic chain, no evidence of carbon containing functional groups or unsaturation was evident. The mass spectra of fraction A is given in Figure 20. The spectra is consistent with that described by Bursell and Clements (1967), though the parent ion peak at 384 and M-1 (383) are tiny, the ion peak at 365 is characterisic of M-1 and M-18 (loss of water). The parent ion was confirmed using chemical-ionization mass spectroscopy (Figure 21). The spectra gives a mass equivalent to the parent ion + 18 which commonly

occurs when NH_3 is the ionizing agent. In both mass spectra there are numerous tiny peaks indicating the existence of a homologous series of diols in trace quantities as described by Bursell and Clements (1967). The major fraction is therefore 8,9-pentacosanediol.

Trials comparing the reponses of grouped *Tenebrio* to selected equivalencies (20 - 0.1) of conspecific lipid extracts showed no significant differences due to differing concentrations. All trials indicated significant aggregation. The responses of grouped *Tenebrio* larvae to 0.1 insect equivalent of total lipid extract is shown in Figure 22. The response to a small quantity of material was quite strong. Trials comparing the reponses of grouped *Alphitobius* to selected equivalencies (20 - 1) of conspecific lipid extracts showed no significant differences due to differing concentrations. The responses of grouped *Alphitobius* larvae to 10 insect equivalents of conspecific larval extract is presented in Figure 23.

The first choice of individual *Tenebrio* larvae in attractancy trials is given in Table 16. Only the recombined fractions and fraction A were significantly attractive. The first choice of individual *Alphitobius* larvae indicated that both fractions D and E were attractive (Table 17).

All wandering larvae displayed a characteristic pattern of side to side turning of the head capsule while moving forward. The first choices indicated that both species did respond to these molecules as airborne stimuli, but directed motion towards the treated substrate was only observed within a distance of 1 cm from the filter paper. This was accomplished by a decrease in the rate of head capsule turning during forward exploration, and some individuals appeared to move faster during the directed behavior.

The *Tenebrio* larvae also wandered between the two filter papers to a considerable extent in most fraction trials, though in the case of attractive fractions this was much reduced. This is evident in Figure 24 which gives the number of visits/ paper per individual. The recombined fractions (upper left- page length oriented horizontally) and fraction A

(lower left) both show that more than half the responding larvae did not contact the control paper (0 visits/ paper), while very few did not contact the treatment paper. This contrasts sharply with the situation observed for the control (upper right, Figure 24) and for fraction E which induced no significant behavioral activity (lower right, figure 24). It is important to note (for interpretation of Ar.I. and C.I. values) that for the recombined fractions and for fraction A the number of insects not visiting the control paper is much greater than those visiting the treatment paper only once. Each insect that visited the treatment may also visit a control paper any number of times. This means that those larvae that visited only the treatment paper did so more than once in a significant number of such trials.

The Ar. I. values (Figure 25) for the recombined fractions (upper left) and the 8,9-pentacosanediol fraction (fraction A, lower left) reflect this; note the extreme distribution of the data and the peak at 0.8 - 1. This contrasts sharply with the equivalent peaks at the extremes for the control vs control trials (upper right, Figure 25) and the similar peaks for fraction D (lower right, Figure 25), which induced no behavioral activity. It is important to note that for the behaviorally active recombined fractions the number of individuals with Ar.I. values in the 0.8 - 1.0 range (upper left, Figure 25) is slightly greater than the number of insects not touching the control in the same trials (0 visits/paper; upper left, Figure 24). These additional higher Ar.I. values are for those insects that visited both papers at least once but spent a high proportion of the total visit time upon the treatment paper. Therefore, the overall data distribution in these trials is largely due to individuals that are spatially displaced and localized by only visiting the treatment paper, and, as was already presented, often visiting it repeatedly. The results of statistical analyses comparing the data for the Ar.I. responses to each fraction to that for the control vs control trials are given in Table 18.

The observation that those individuals that visited a paper more than once tended to do so more frequently upon the treatment papers in trials that induced significant behavioral activity, suggested that these repeated visits might play a role if what has been described as arrestment (Kennedy 1978) is induced by a reduction in velocity or an increase in the rate of turning or various combinations of these (Kennedy 1978). These were evaluated by simply determining if the relative number of contacts re-established by an individual i.e., recurring attraction to a stimulus, could facilitate arrest in a spatially defined region- upon a filter paper. Figure 26 shows the data distributions of the contact indices (C.I.) for behaviorally active recombined fractions (upper left) and the 8,9-pentacosanediol fraction (lower left). These are quite distinct from the distribution of C.I. values for the control vs. control trial (upper right) and for fraction C (lower right), which induced no significant behavioral activity. The distributions of the C.I. responses induced by behaviorally active fractions closely resemble the Ar.I. distributions indicating that the relative preference in duration of visits on an attractive substrate is correlated with a larvae visiting only it, and often doing so repeatedly. If the C.I. distributions of active fractions (Figure 26, upper and lower left) contained a larger number of intermediate values then the Ar.I. distributions of the attractive fractions (Figure 25, upper and lower left) might have been a function of the average duration of visits increasing. This was unlikely to occur since more than 65% of the insects visited only one paper in trials where behavioral activity was displayed (Figure 24, upper and lower left). The results of the statistical analyses of the C.I. responses elicited by all fractions compared to those induced in control vs. control trials is given in Table 19.

The comparison of the D.D. values induced by the recombined fractions (Figure 27, upper left) to those induced in control vs. control trials (Figure 27, upper right) shows a pronounced shift towards increasing duration for the distribution of the D.D. values of the recombined fractions. The distributions of D.D. values for fraction A (8,9-

pentacosanediol; lower left, Figure 27) and fraction B (lower right, Figure 27) showed no such shift. Only the D.D. values for trials using the recombined fractions were significantly different from those for control vs. control trials (Table 20), indicating that the 8,9-pentacosanediol plus at least one other fraction was required to significantly prolong arrest.

The *Alphitobius* larvae showed no obvious tendency to wander between the treatment and control papers and the incidence of repeated contacts with the paper of initial choice was much lower than for *Tenebrio*. Therefore, the relative and absolute spatial displacement are entirely linked to the initial choice even in the control vs control trial. This suggests that the arrest of these larvae may be governed entirely by tactile preference for the filter paper. The only comparison that can be made under such conditions are those evaluating the initial preferences to determine classical attraction (Dethier et al. 1960) using a Chi-square test (Table 17). The *Tenebrio* larvae wandered to a much greater extent during control vs. control trials (Figure 24, upper right compare 0 visits/paper with 1 visit/paper) so such tactile arrestment upon the filter paper contributes to a lesser degree.

Discussion

The data suggest that the attraction of *Alphitobius* and *Tenebrio* larvae occurs at close-range. In both species the attraction is due to specific compounds present in cuticular lipid fractions. The attractive fraction for the *Tenebrio* larvae is the one containing a large amount of 8,9-pentacosanediol, which is pure except for traces of a homologous series of higher and lower molecular weight diols, as was reported by Bursell and Clements (1967). It is surprising that such a high molecular weight compound could be significantly volatile, but cuticular lipids that act as sex pheromones have also been found to be perceived by close-range olfacation (Chaudhury et al. 1972, Muhammed et al. 1975) as well as by contact (Langley et al. 1975). The issue of perception

may be of relatively minor importance in the case of cuticular lipid pheromones because the attraction is close-range and contact is a subsequent occurrence of the behavior elicited, particularly during mating, but also when interaction occurs between aggregating individuals or members of a conspecific caste. However, the relative role of olfaction as opposed to contact will not be resolved until an electrophysiological study can be performed upon putative lipid contact chemoreceptors. Lipid olfactory receptors have already been investigated (Grula et al. 1980).

The responses of these two species suggest that discrimination of conspecifics can be accomplished by close-range perception or actual contact. This is an important consideration for the earlier observations made upon frass- and lactic acid-induced aggregation for these two species (Weaver et al 1989, Section 2: Frass-induced aggregation- *Tenebrio*; Section 5: Frass-induced aggregation- *Alphitobius*). The responses to frass and lactic acid confer little evidence of species-specificity, though both are pheromones. The observation that the *Tenebrio* larvae actively cluster together upon a preferred substrate (Weaver and McFarlane, 1989) indicates that aggregation in a preferred region for these larvae is the result of an attractive component plus further interaction among conspecifics as indicated by pheromones that are on their body surface. The trials with grouped *Tenebrio* larvae and *Alphitobius* frass (Section 5: Frass-induced aggregation- *Alphitobius*) thus resulted in a complete response because the attractive concentration of frass chemicals was present as were larval conspecifics, i.e., the *Tenebrio* larvae acted as though they were in a region conditioned with *Tenebrio* frass, because they are unable to discriminate that it was not conspecific frass. The presence of conspecifics as indicated by cuticular composition reinforced that it was conspecific frass when it was not. This may suggest that groups of wandering larvae might never actually determine if a region has been previously inhabited if they contact each other in favorable surroundings, but the presence of what appears to be conspecific-released material plus the conspecifics them-

selves is probably as efficient a mechanism as is required to indicate an ecologically favored habitat. A similar situation could occur with the presence of conspecific exuviae, which may be present in considerable numbers given the high number of larval moults by these two species. This would provide accurate information of earlier infestation by conspecifics without requiring their actual presence. The post-attraction clustering described for larvae of other species upon conspecific frass-conditioned papers (McFarlane and Alli 1986, 1987, McFarlane et al. 1983) could also be facilitated by specific responses to cuticular lipid pheromones.

The responses of the *Tenebrio* larvae during trials to determine spatial displacement and arrestment by attractive fractions indicate that the olfactory responses arrest the *Tenebrio* larvae in a region relatively proximal to the chemical source, through repeated attraction as described by Kennedy (1978). However, this orientation would appear to imply a "memory" component (Kennedy 1978) as well because the subsequent return to the treated filter paper (as opposed to ever reaching the control) occurs from a greater distance than the original directed orientation. External cues such as frass pellets or a slight cuticular lipid residue left as a trail as the mealworm drags its abdomen may facilitate this re-orientation, but both these cues and idiothetic (self-orienting) cues are required to display such "repeated visit" behavior.

The duration of total visits (D.D.) was significantly greater in the case of the recombined fractions only. This indicated that the response was greater when fractions that do not display significant behavioral activity by themselves are included in the bioassay. Thus the full response of *Tenebrio* is to the lipid blend or at least to more than one component. This is also evident when the bioassay data for fractions lacking significant behavioral activity are viewed (Figures 24-27). In several cases the data distribution is shifted towards the trend for the active fractions when compared with that of the control. It may be that these compounds could elicit behavioral activity, if

presented at higher concentrations. In any case, they enhance the response to the major lipid fraction containing 8,9-pentacosanediol. The two *Alphitobius* lipid fractions showing behavioral activity indicate that a blend is perceived by this species as well. In this case these compounds represent a much smaller perecentage of the total extract than did 8,9-pentacosanediol for *Tenebrio*.

Cuticular species-recognition factors have been found in social insects (see Blomquist and Dillwith 1985 for a review) and implied for aggregating adult insects (Norris 1970). The adults of *Tenebrio molitor* have a similar compound that serves as a species recognition factor as well as triggering copulatory behavior as has been reported (Tanaka et al. 1986, 1988). This species recognition function is valid because the active compounds are present in the cuticle of both sexes. There can be little benefit for the species if males attempt copulation with males. Females may also respond to these lipid extracts but these assays were not conducted (Tanaka et al 1986). There is preliminary evidence for caste-recognition factors in certain social insects as well (see Blomquist et al 1985). *Tenebrio* and *Alphitobius* are not social insects, but in their feral habitats in the nests of birds (Brendell 1975; Buck 1956, Britton 1940) or holes in rotting wood (Palm 1959) there may be co-existence of larval and adult forms (this certainly occurs in the synanthropic situations). These animals are nocturnally-active (Tyschenko and Sheyk Ba 1986; Geden and Axtell 1987) or occur in dark regions in stored products (Cotton and Wilbur 1984) so the responses of both adult and larval stages to stage-specific cuticular compounds could play a role in stage-recognition in interaction in darkness. Tschinkel and Willson (1971) reported that late-instar *Tenebrio* larvae delay pupation in response to increased contact, in particular, with other large larvae. It has been demonstrated that cuticular recognition facilitates clustering of *Tenebrio* larvae; it may also be that cannibalism-susceptible (Section 7: Effects of larval density) pre-molt larvae have

differing behavioral responses to conspecific larval cuticular components and use this recognition factor to facilitate dispersion and subsequently locate an isolated region to commence ecdysis safely.

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Table 14. Results of the fractionation procedure using flash column chromatography to separate larval *Tenebrio* lipid extracts.

Fraction	Quantity	Rf Value[1]	% Extract
A	.1039g	.11	64.7
B	.0366g	.35	22.8
C	.0039g	.16	2.4
D	.0007g	.20	.4
E	.0020g	.33, .25	1.2
X	.0133g	0	8.3

[1]The Rf value is based upon thin-layer chromatography performed using eluted fractions in the elution solvent system. The solvent system was 1:5 ethyl acetate:hexane.

Fraction E contains two partially resolved components. Fraction X is the methanol flush of all remaining material on the column after fractionation.

The percent recovery from the fractionation procedure was 91.2%; % extract values given represent the percentage of recovered material. 1547 larvae were extracted.

Table 15. Results of the fractionation procedure using flash column chromatography to separate larval *Alphitobius* lipid extracts.

Fraction	Quantity	Rf Value[1]	% Extract
A	.0488g	.41	55.6
B	.0028g	.37	3.2
C	.0033	.20	3.7
D	.0042	.14	4.8
E	.0045	.06	5.1
X	.0242	0	27.5

[1]The Rf value is based upon thin-layer chromatography performed using eluted fractions in the elution solvent system. The solvent system was 1:9 ethyl acetate:hexane.

Fraction X is the methanol flush of all remaining material on the column after fractionation.

The percent recovery from the fractionation procedure was 87.4%; % extract values given represent the percentage of recovered material. 2362 larvae were extracted.

Table 16. Results of attractancy trials evaluating the responses of solitary *Tenebrio* larvae to fractionated lipids from conspecific larvae by direct observation.

Fraction[1]	Amount in g	Treatment[2]	Control[3]	Significance*
Recombined	1.04×10^{-4}	38	11	.001 (13.80)
A	6.71×10^{-5}	34	14	.01 (7.52)
B	2.36×10^{-5}	27	17	N.S. (1.84)
C	2.52×10^{-6}	32	17	N.S. (4.00)
D	4.52×10^{-7}	21	24	N.S. (0.36)
E	1.29×10^{-6}	27	17	N.S. (1.84)
X	8.60×10^{-6}	24	18	N.S. (0.60)
Control	--	27	22	N.S. (0.33)

*Results of chi-square test for significant response to fraction odor. Significance is for probability that treatment is greater than control at P> the value given. Chi-square value is given in parentheses. N.S. Indicates responses to treatment and control papers are not statistically different at $P \geq .05$. Data are from 50 replicates using solitary larvae.

In all cases the amount in g equals 5 insect equivalents.

[1]In each case an appropriate control was used, i.e., control filter papers were treated with the equivalent amount of chloroform used to deliver the insect equivalency. Chloroform was evaporated for twenty minutes prior to bioassay.

[2,3]The number of solitary larvae establishing initial contact with the treatment or with the control filter paper.

Table 17. Results of attractancy trials evaluating the responses of solitary *Alphitobius* larvae to fractionated lipids from conspecific larvae by direct observation.

Fraction[1]	Amount in g	Treatment[2]	Control[3]	Significance*
Recombined	3.72×10^{-5}	35	11	.001 (11.5)
A	2.07×10^{-5}	30	19	N.S. (2.04)
B	1.19×10^{-6}	28	20	N.S. (1.02)
C	1.40×10^{-6}	28	22	N.S. (0.5)
D	1.78×10^{-6}	36	14	.005 (8.82)
E	1.91×10^{-6}	33	17	.05 (4.5)
X	1.02×10^{-5}	28	22	N.S. (0.5)
Control	--	26	23	N.S. (0.08)

*Results of chi-square test for significant response to fraction odor. Significance is for probability that treatment is greater than control at P> the value given. Chi-square value is given in parentheses. N.S. Indicates responses to treatment and control papers are not statistically different at $P \geq .05$. Data are from 50 replicates using solitary larvae.

N.S. Indicates responses to treatment and control papers are not statistically different. Data are from 50 replicates using solitary larvae. In all cases the amount in g equals 5 insect equivalents.

[1]In each case an appropriate control was used, i.e., control filter papers were treated with the equivalent amount of chloroform used to deliver the insect equivalency. Chloroform was evaporated for twenty minutes prior to bioassay.

[2,3]The number of solitary larvae establishing initial contact with the treatment or with the control filter paper.

Table 18. Results of the comparison of index of arrest (Ar.I.) values for *Tenebrio* larvae in trials using fractions of conspecific larval lipids (5 insect equivalents).

Fraction	Z[1]	Prob>Z[2]	Significance[3]
Recombined	3.67	0.0002	0.0004
A	2.19	0.0286	0.0310
B	1.05	0.2934	0.2962
C	1.53	0.1261	0.1294
D	-0.54	0.5886	0.5889
E	1.48	0.1368	0.1403
X	0.85	0.3949	0.3972

[1]The value of the sum of scores that is least different from that expected (under the null hypothesis that the two samples come from poulations that do not differ in location) divided by its standard deviation under the null hypothesis. These values are the results of tests comparing the index of arrest for individual fractions for those of control vs. control trials.

[2]The probability of a higher value of Z. Values of Z follow an approximately normal distribution.

[3]Significance is T-test approximation.

Table 19. Results of the comparison of contact index (C.I.) values for *Tenebrio* larvae in trials using fractions of conspecific larval lipids (5 insect equivalents).

Fraction	Z[1]	Prob>Z[2]	Significance[3]
Recombined	3.84	0.0001	0.0002
A	2.16	0.0310	0.0335
B	0.75	0.4518	0.2962
C	1.21	0.2262	0.2291
D	-0.67	0.5006	0.5023
E	0.98	0.3285	0.3310
X	0.91	0.3629	0.3653

[1]The value of the sum of scores that is least different from that expected (under the null hypothesis that the two samples come from populations that do not differ in location) divided by its standard deviation under the null hypothesis. These values are the results of tests comparing the index of arrest for individual fractions for those of control vs. control trials.

[2]The probability of a higher value of Z. Values of Z follow an approximately normal distribution.

[3]Significance is T-test approximation.

Table 20. Results of the comparison of absolute differences (D.) in preferences of *Tenebrio* larvae for the treatment papers in trials using fractions of conspecific larval lipids (5 insect equivalents).

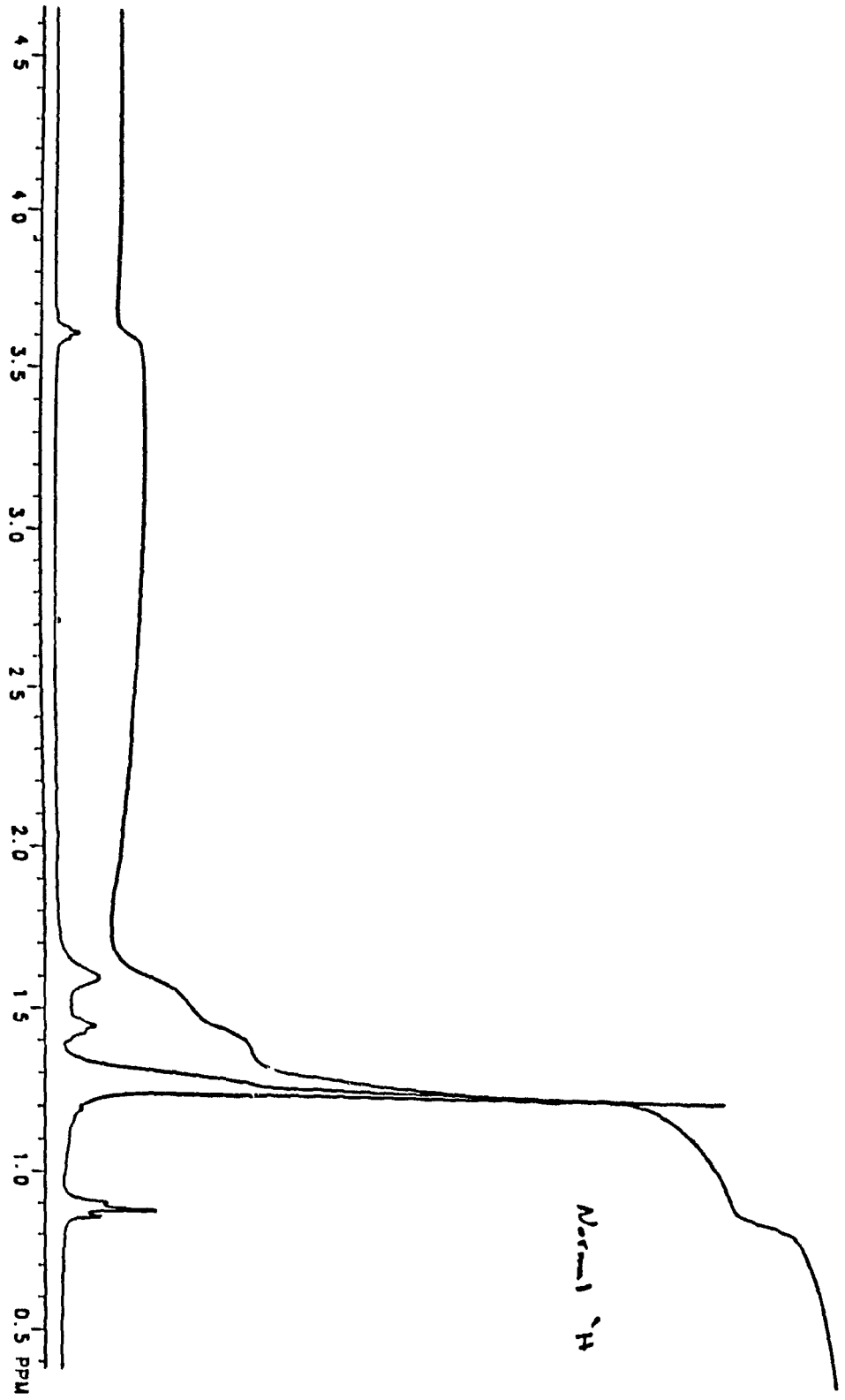
Fraction	Z[1]	Prob>Z[2]	Significance[3]
Recombined	2.10	0.0361	0.0387
A	1.22	0.2241	0.2270
B	0.88	0.3783	0.3806
C	1.06	0.2897	0.2924
D	-0.76	0.4491	0.4510
E	1.49	0.1368	0.1403
X	0.81	0.4191	0.4212

[1]The value of the sum of scores that is least different from that expected (under the null hypothesis that the two samples come from populations that do not differ in location) divided by its standard deviation under the null hypothesis. These values are the results of tests comparing the index of arrest for individual fractions for those of control vs. control trials.

[2]The probability of a higher value of Z. Values of Z follow an approximately normal distribution.

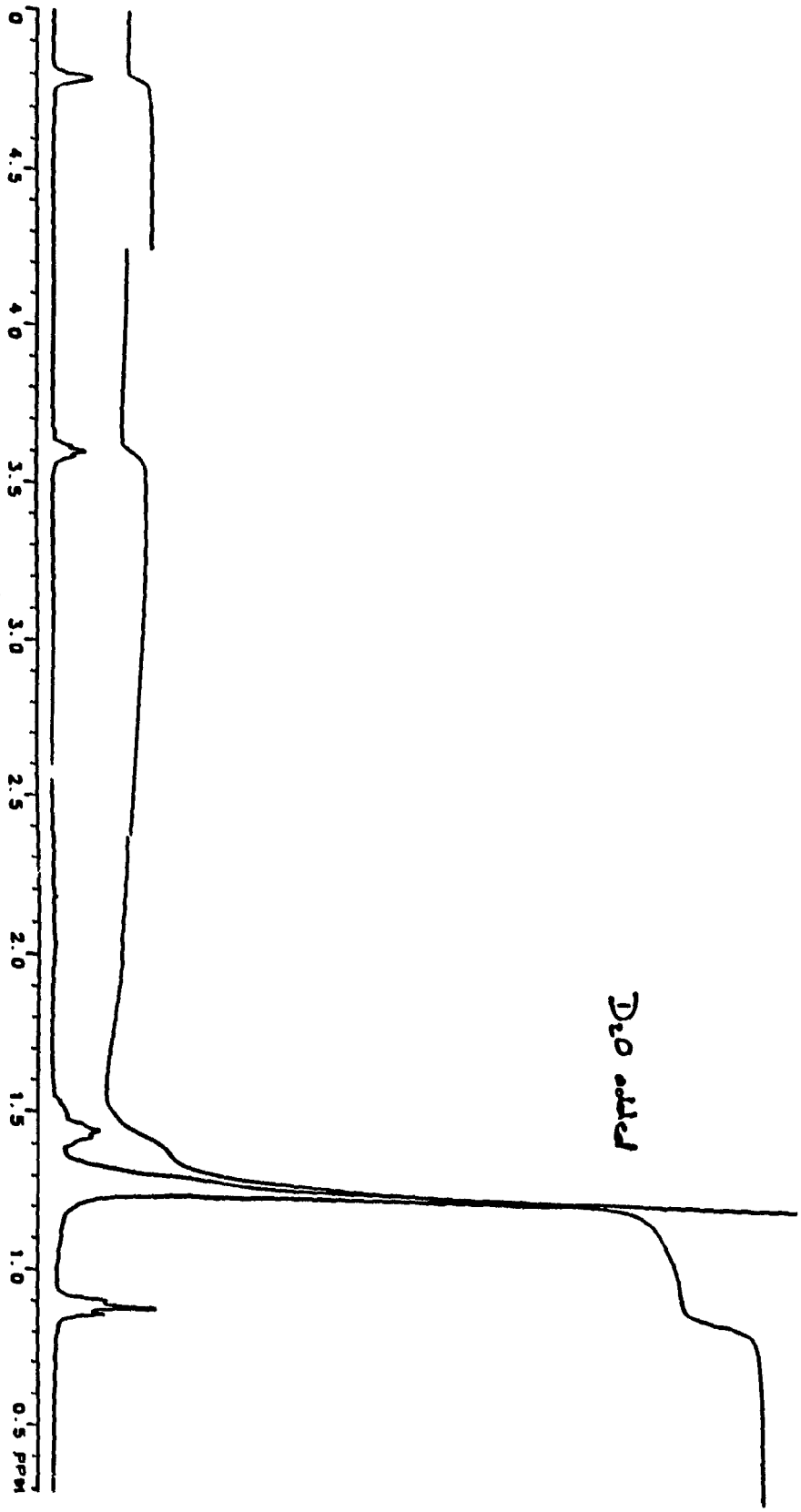
[3]Significance is T-test approximation.

Figure 18. ^{1}H nuclear mass resonance spectra of fraction A, *Tenebrio* larval lipid. Note the characteristic hydroxyl singlet at 1.6 p.p.m. downfield. Comparison to the relative area for the triplet for the terminal protons at 0.8 p.p.m. downfield indicates that two closely related hydroxyl groups are likely. The spectra is consistent with that of 8,9-pentacosanediol.



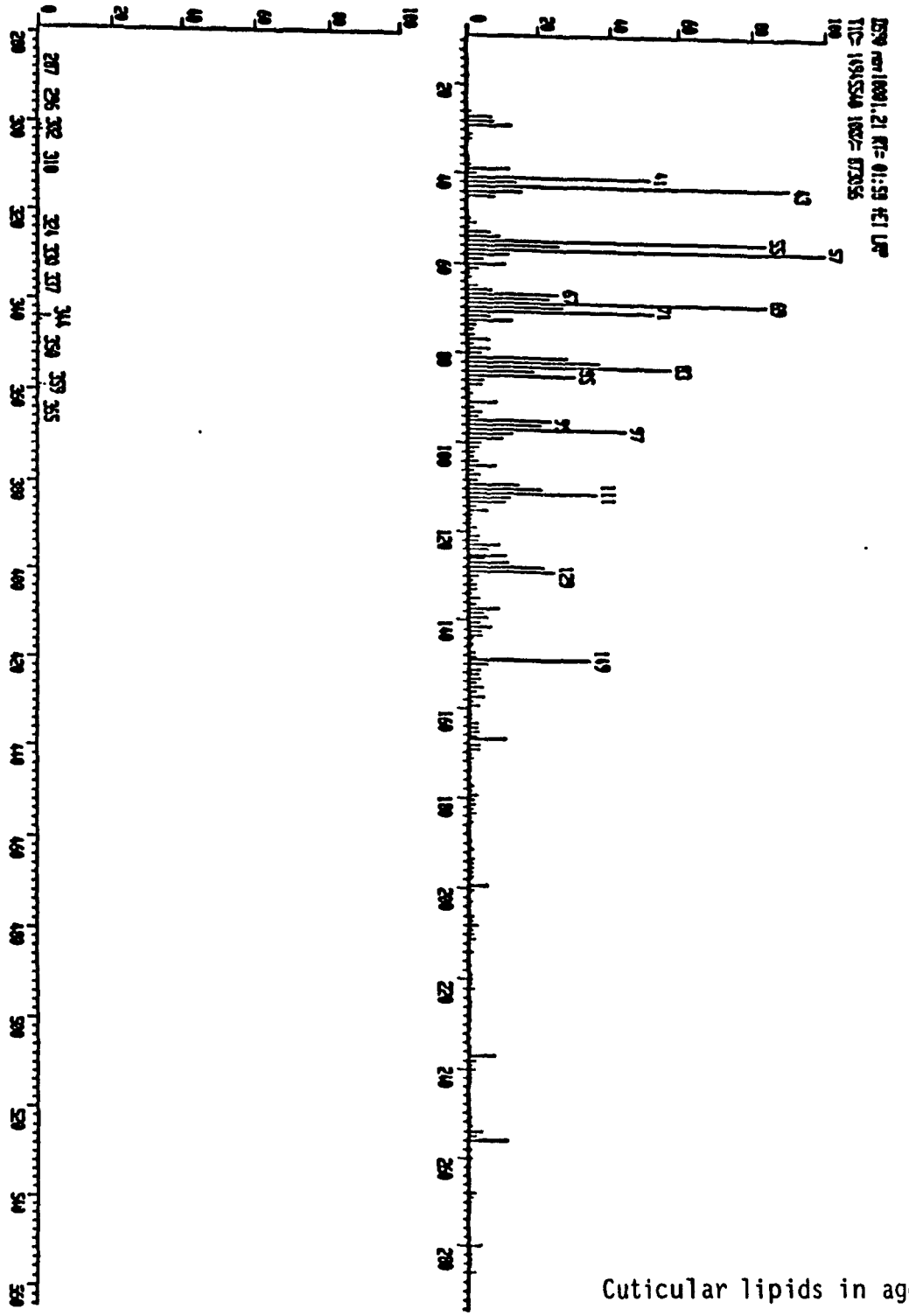
Cuticular lipids in aggregation

Figure 19. ^{1}H nuclear mass resonance spectra (D_2O added) of fraction A, *Tenebrio* larval lipid. Note that the singlet for the suspected hydroxyl groups at 1.6 p.p.m. has vanished; thus the functional groups giving rise to the singlet are likely to be hydroxyl groups. The spectra is again consistent with that of 8,9-pentacosanediol.



Cuticular lipids in aggregation

Figure 20. The electron impact mass spectra of fraction A. The characteristic splitting pattern of 8,9-pentacosanediol is evidenced by the M-18 ion at 365 which gives the loss of water (from the M-1 ion) characteristic of hydoxyl groups. A peak for the parent ion is only evident as a trace. The spectra as a whole is consistent with that of 8,9-pentacosanediol.

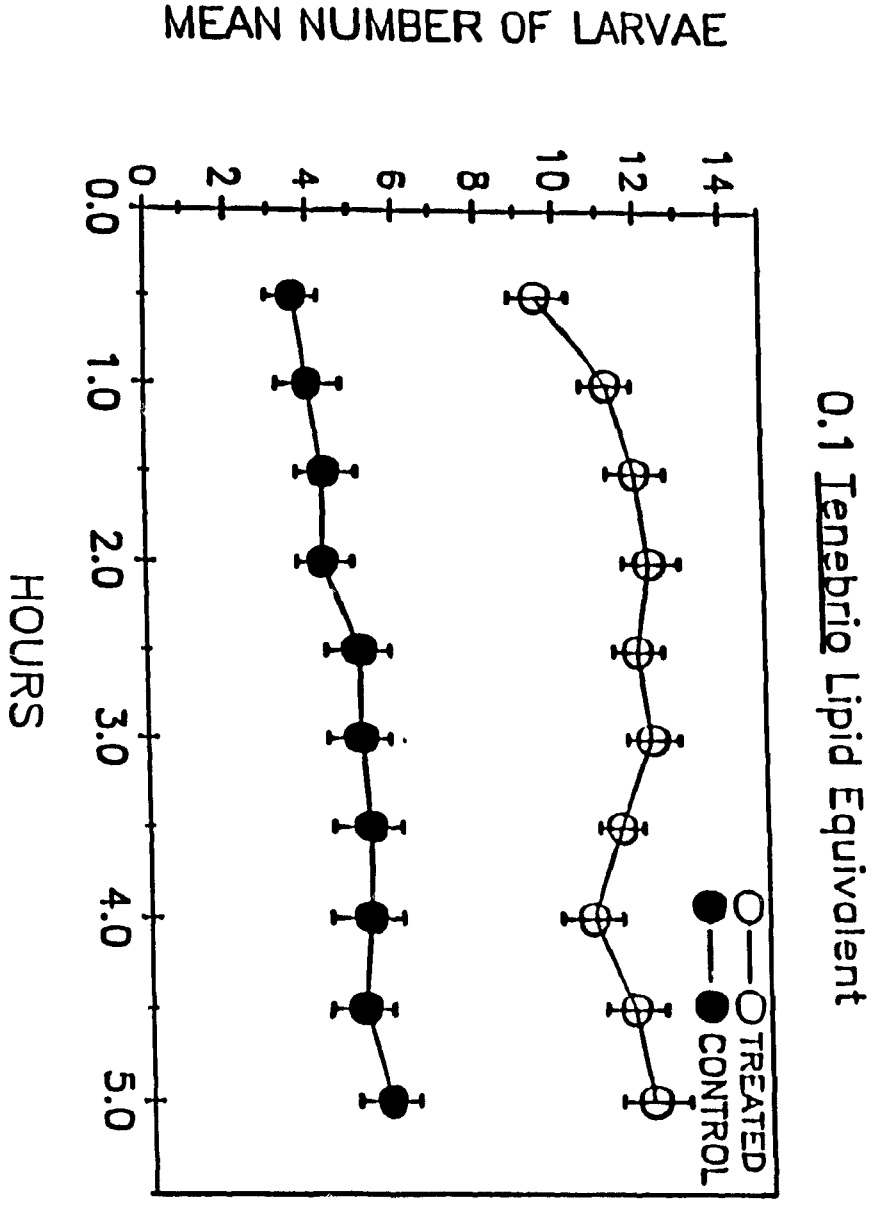


Cuticular lipids in aggregation

Figure 21. The chemical ionization mass spectra of fraction A. The ionizing agent was NH_3. The parent ion is not evident, instead we see the M+18 ion that often occurs when this gas provides the ionizing environment. The spectra is again consistent with that of 8,9-pentacosanediol.

FRN:SCAN#: 6269 29 TIME (MIN): 1.43 PEAKS:19 463415
BASE PK: 402 BASE PK x TI: 68.97 TOT. IONS: 3597
% rel abund
100
50
190
240
290
340
% rel abund
100
50
340
390
440
490
amu (m/e)

Figure 22. Mean number of yellow mealworm larvae (±S.E.) responding to filter papers treated with 0.1 lipid equivalents (13.9×10^{-6}g) of conspecific larval lipid extract. The aggregation index (A.I.) for this trial was 0.47±.06. This indicates significant aggregation at the P>0.001 level. Based upon 18 replicates of 20 larvae.



Cuticular lipids in aggregation

Figure 23. Mean number of lesser mealworm larvae (±S.E.) responding to filter papers treated with 10 lipid equivalents (68.9×10^{-6}g) of conspecific larval lipid extract. The aggregation index (A.I.) for this trial was 0.31±.05. This indicates significant aggregation at the P>0.001 level. Based on 30 replicates of forty larvae each.

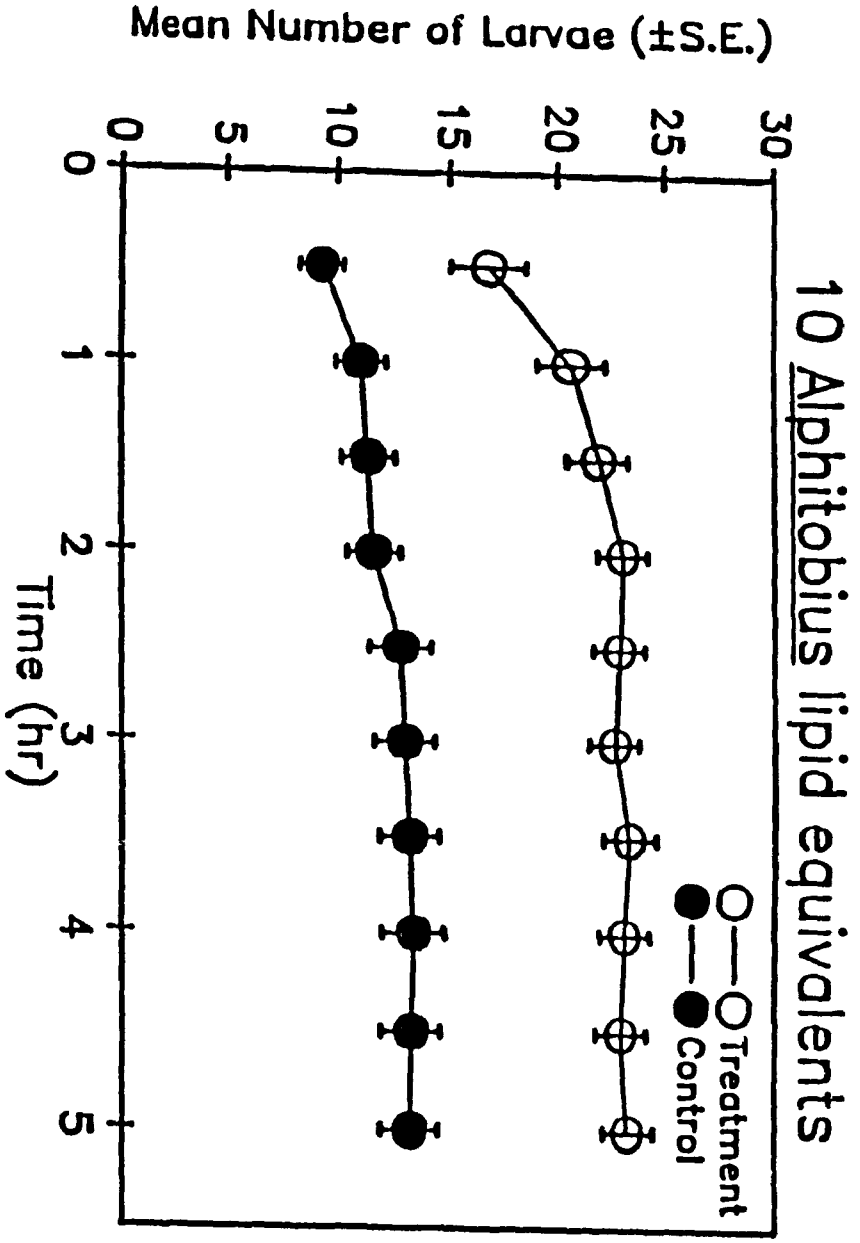
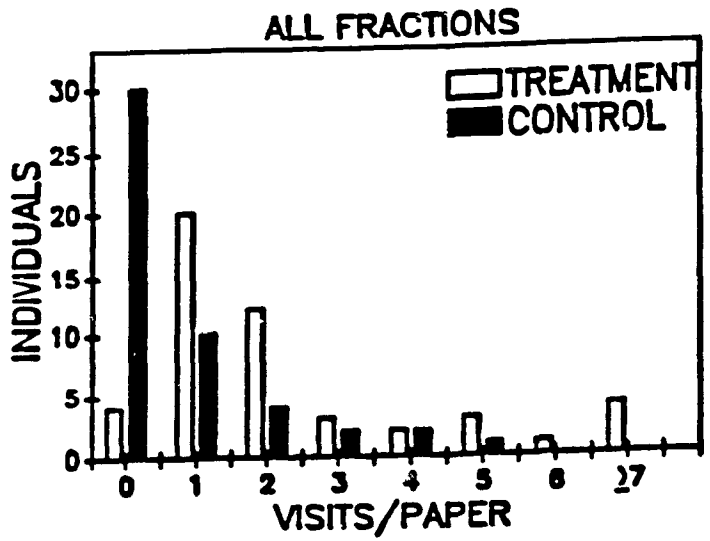
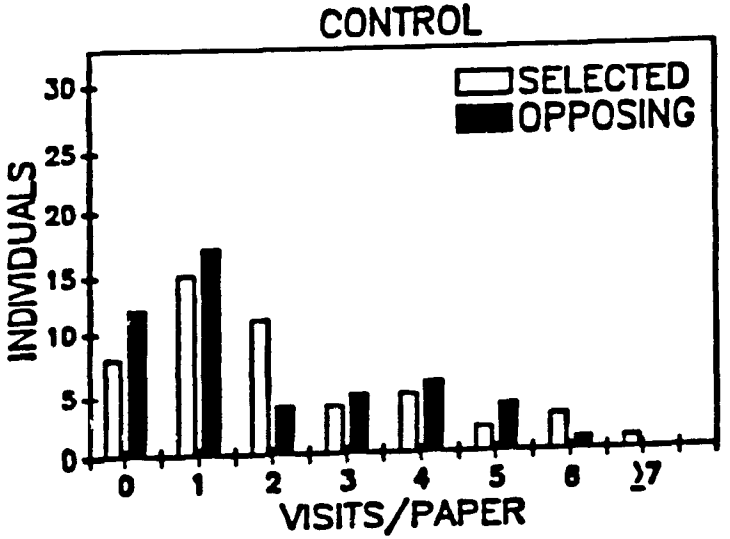
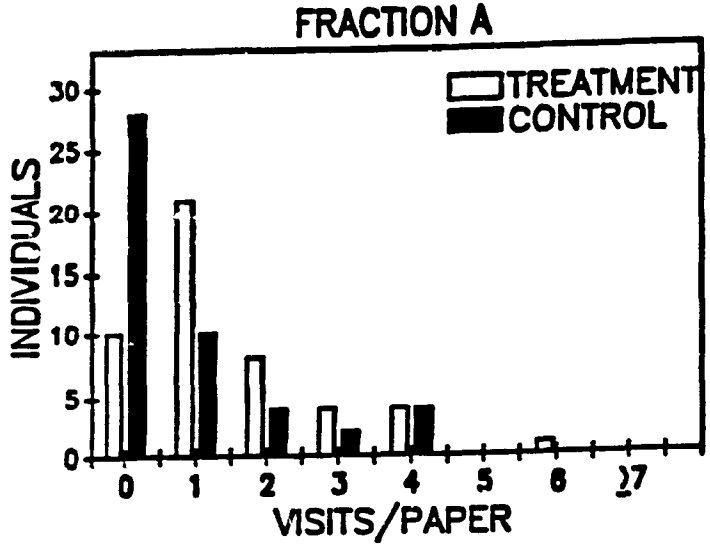

Mean Number of Larvae (±S.E.)
0
5
10
15
20
25
30
0
1
2
3
4
5
Time (hr)
10 Alphitobius lipid equivalents
Treatment
Control

Figure 24. Plot showing the number of visits to both treatment and control papers in trials using selected *Tenebrio* lipid fractions. The figures are: upper left- recombined fractions; lower left- fraction A containing 8,9-pentacosanediol; upper right- control vs. control trials; lower right- fraction E. The data distributions of the fractions on the left side of the Figure are those for the behaviorally significant fractions. Fractions are presented as 5 insect equivalents.


ALL FRACTIONS
TREATMENT
CONTROL
INDIVIDUALS
VISITS/PAPER
0 1 2 3 4 5 6 ≥7
0 5 10 15 20 25 30


CONTROL
SELECTED
OPPOSING
INDIVIDUALS
VISITS/PAPER
0 1 2 3 4 5 6 ≥7
0 5 10 15 20 25 30


FRACTION A
TREATMENT
CONTROL
INDIVIDUALS
VISITS/PAPER
0 1 2 3 4 5 6 ≥7
0 5 10 15 20 25 30

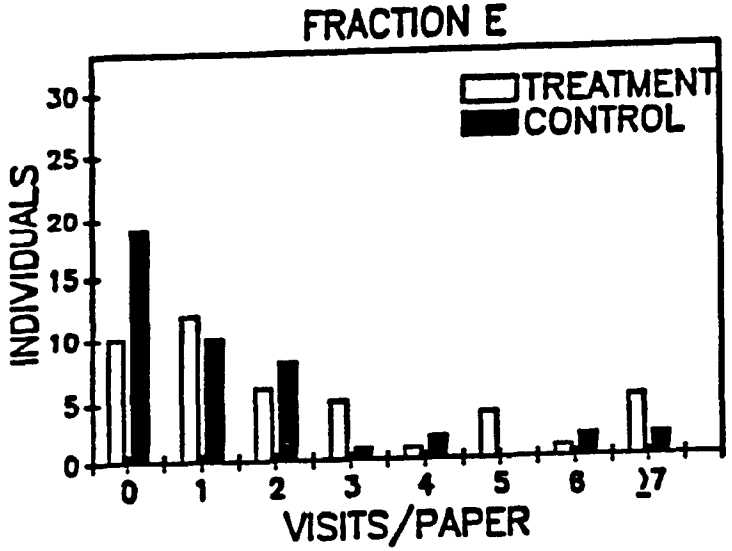
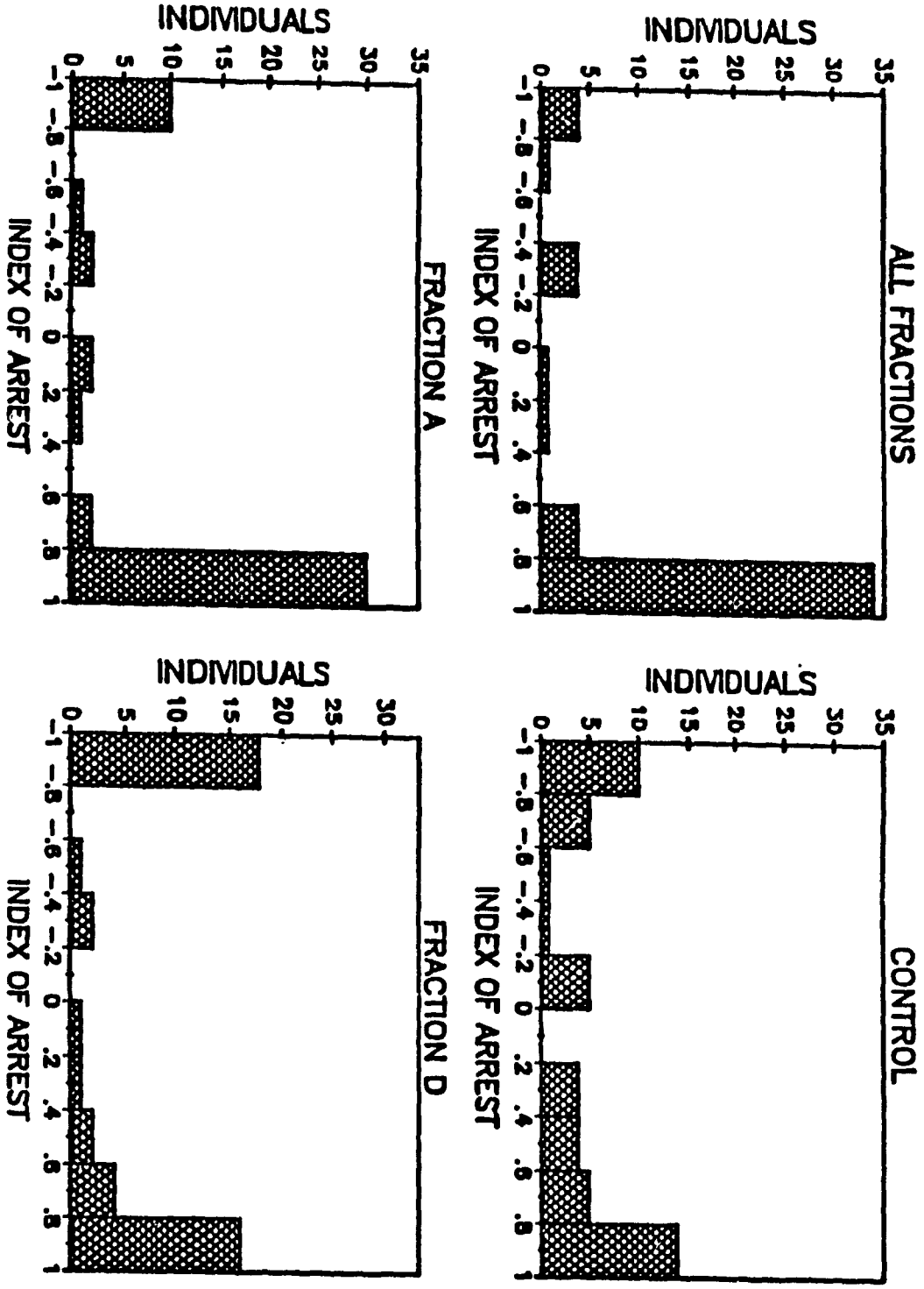
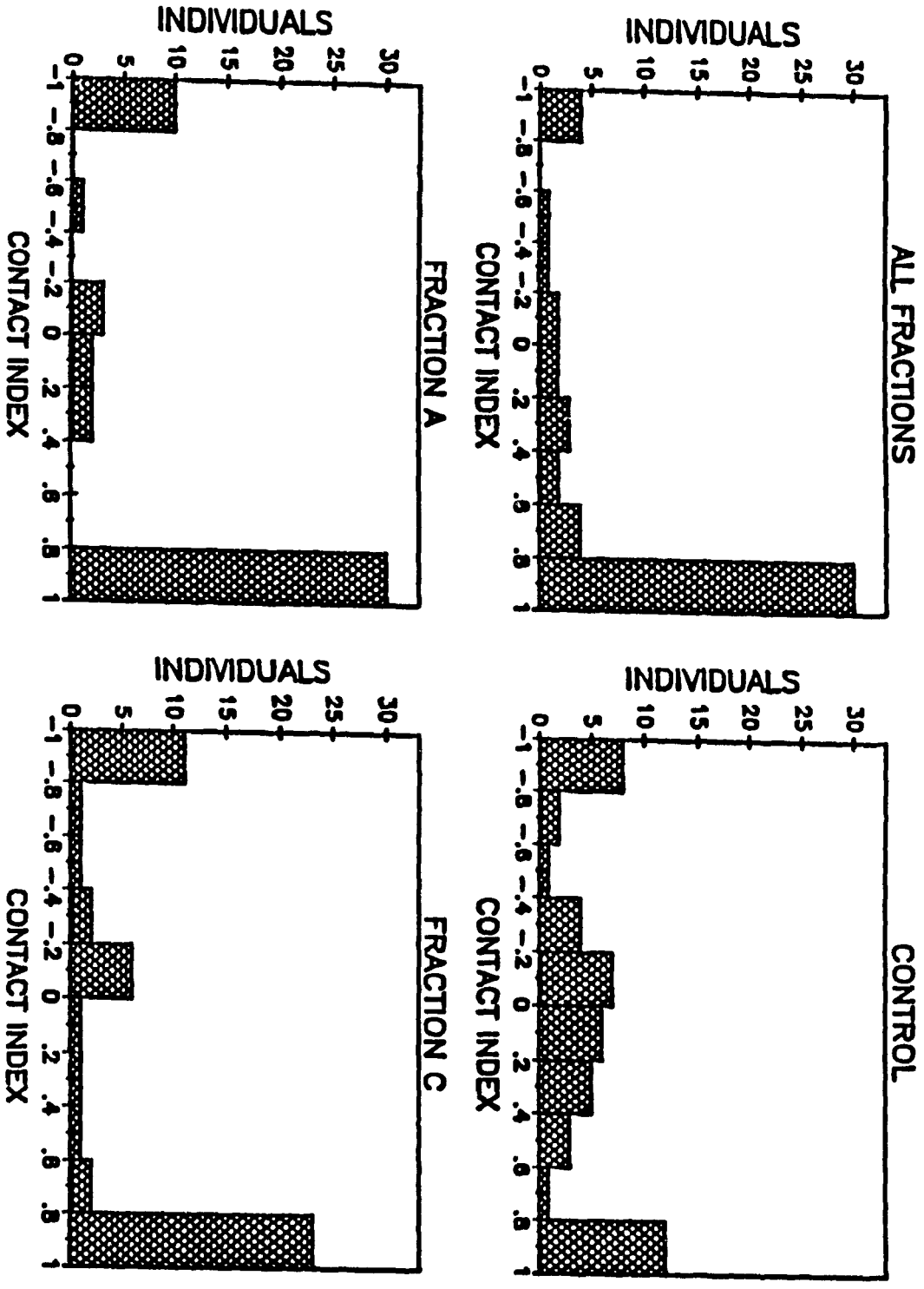

FRACTION E
TREATMENT
CONTROL
INDIVIDUALS
VISITS/PAPER
0 1 2 3 4 5 6 ≥7
0 5 10 15 20 25 30

Figure 25. Plot showing the relative degree of arrest (Ar.I.) to the treatment papers in trials using selected *Tenebrio* lipid fractions. The figures are: upper left- recombined fractions; lower left- fraction A containing 8,9-pentacosanediol; upper right- control vs. control trials; lower right- fraction D. The data distributions of the fractions on the left side of the Figure are those for the behaviorally significant fractions. Fractions are presented as 5 insect equivalents.



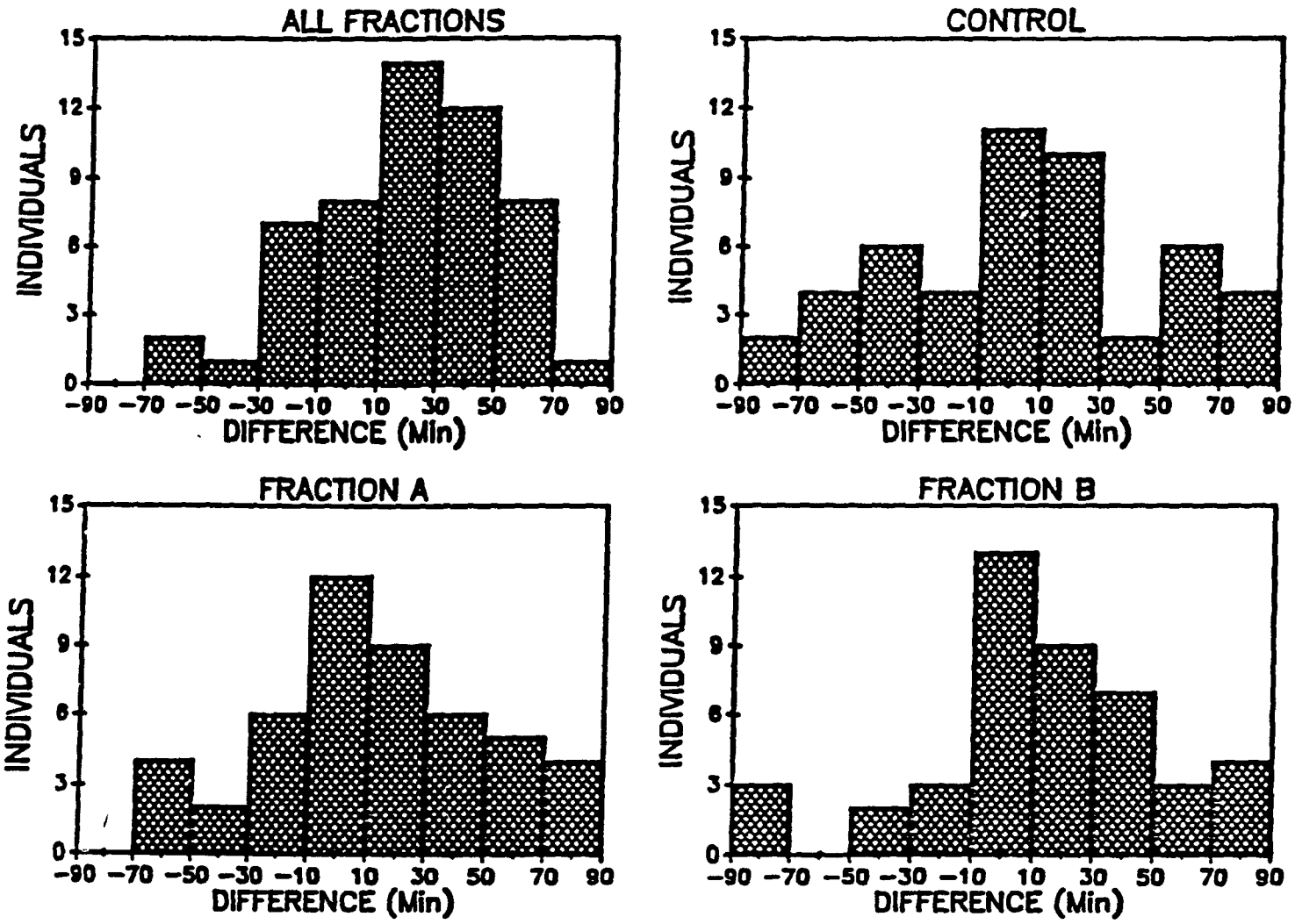
Cuticular lipids in aggregation

Figure 26. Plot showing the relative number of visits (Contact Index-C.I.) to the treatment papers in trials using selected *Tenebrio* lipid fractions. The figures are: upper left- recombined fractions; lower left- fraction A containing 8,9-pentacosanediol; upper right- control vs. control trials; lower right- fraction C. The data distributions of the fractions on the left side of the Figure are those for the behaviorally significant fractions. Fractions are presented as 5 insect equivalents.



Cuticular lipids in aggregation

Figure 27. Plot showing the absolute differences (D.D. values) in individual preference for the treatment papers in trials using selected *Tenebrio* lipid fractions. The figures are: upper left- recombined fractions; lower left- fraction A containing 8,9-pentacosanediol; upper right- control vs. control trials; lower right- fraction B. The data distributions of the fractions on the upper left side of the Figure is that for the only behaviorally significant fraction. Fractions are presented as 5 insect equivalents.


ALL FRACTIONS
CONTROL
FRACTION A
FRACTION B
INDIVIDUALS
DIFFERENCE (Min)
0
3
6
9
12
15
-90 -70 -50 -30 -10 10 30 50 70 90

Connecting Statement VI

The evaluation of chemicals that influence population distribution and orientation is generally governed by bioassay procedures that focus on the short term effects upon insect location. The long term effects of increased density may enhance or inhibit a variety of physiological parameters, which may be important in defining the overall benefit that pheromones have upon a population. The effect of initial larval density upon growth and development of Tm is investigated under optimal conditions to provide basic information on the role of grouping in Tm development (Section 7: Effects of larval density- *Tenebrio*).

Section 7: Effects Of Larval Density- *Tenebrio*

Title

THE EFFECT OF LARVAL DENSITY ON GROWTH AND DEVELOPMENT OF *Tenebrio molitor* L.

Authors: Weaver, D.K., and J.E. McFarlane
Submitted to J. Insect Physiol. on October 17, 1989.

ABSTRACT Day-old larvae of *Tenebrio molitor* were reared at densities of 1, 2, 5, 10, and 20 per 455 ml rearing jar. After one month, larvae reared at the density of 20 were significantly larger than those at the density of 2, which were smallest. Numerically higher average mass was correlated with increasing density after one month of development. Isolated female pupae and isolated adult females were significantly larger than those at densities of 10 and 20 individuals per jar. At these later stages numerically higher mass of females was correlated with decreasing density. No significant difference was found between average mass of males at pupation or adult emergence with varying densities, nor was there any difference in time to pupation or time to adult emergence stage at any density for either sex. Female pupae were significantly larger than male pupae at densities of 1 and 2 individuals per jar; adult females were signifcantly larger than adult males when reared in isolation. Percent survival after one month was statistically similar at all densities, while at pupation and adult emergence isolated individuals had statistically greater percent survival than those at high densities. At each density percent survival at one month was statistically higher than that found at pupation and adulthood; no difference was observed for percent survival between these later developmental stages. Observations indicated that cannibalism and incomplete larval-pupal and pupal-adult transformations occurred more frequently at higher densities.

Introduction

The effects of temperature and photoperiod on larval development and pupation have been investigated for *Tenebrio molitor* L. (Tyshchenko and Sheyk Ba 1986). In addition, the effect of duration of exposure to unfavourable hygrothermic condition has been studied for later developmental stages (Punzo and Mutchmor 1978). Tschinkel and Willson (1971) found that mechanical contact between individuals (and to a lesser extent with chains resembling larvae in size) was responsible for the delay in the onset of pupation for late-instar larvae placed together at high densities at this stage. This would allow for the location of an isolated region, via larval wandering prior to the quiescent (and cannabalism-susceptible) pre-pupal stage and pupal stage. This region, perhaps indicated by an absence of frass, would be suitable for the commencement of metamorphosis (Tschinkel and Willson 1971).

Recently, late-instar *Tenebrio molitor* larvae have been found to arrest on filter papers treated with aqueous frass extracts and on those treated with DL-lactic acid, which results in aggregate formation for grouped larvae (Weaver et al. 1989, Section 2: Frass-induced aggregation- *Tenebrio*). Weaver and McFarlane (1989), Section 3: Aggregation behavior- *Tenebrio*, found that higher density clusters formed on filter papers treated with lactic acid than on water treated filter papers, indicating that frass components may play a role in density tolerance. This is of particular interest when one considers that other species that are attracted to frass components, for example, *Acheta domesticus* (McFarlane et al. 1983) gain developmental enhancement (group effects) through the presence of other conspecifics (McFarlane 1962).

Weaver et al. (1989), Section 2: Frass-induced aggregation- *Tenebrio*, suggested that frass may function as a chemical indicator of safe refugia for such conspicuous insects by preventing groups of larvae from wandering away from such locations. This would likely result in slower development through competition alone, ignoring completely the effect of feeding upon conspecific-conditioned and utilized foodstuffs.

The following experiment addresses what effect the presence of conspecifics has on early and late development of *Tenebrio* and also allows for some interpretation of the effects of frass-induced grouping and of the frass itself on larval development since the amount of frass increases with increasing density, and with increasing larval size through time. This approach is used for simplicity; trials allowing choice of rather than confinement at high density or using pre-conditioned material would be preferred, but such potentially heterogenous experimental designs should be conducted only after the results of the simplest case scenario are known.

Materials and Methods

The stock culture originated from an infestation at the Macdonald College dairy facility. New genetic material was added by purchasing yellow mealworms from Wards Biological Supply, Mississagua, Ontario, Canada and from various local pet stores. The stock culture was maintained on a diet of wheat bran, whole wheat flour, and brewers yeast (50:45:5 w/w) at 30±1°C and 55±5% relative humidity. The photoperiodic regime was 14:10 light-dark. Two water vials (9.3 X 2.5 cm o.d.) filled with distilled water and plugged with absorbent cotton were provided biweekly as a water source. The diet was introduced into the 4.55l rearing jars with the flour and brewers yeast mixed in a bottom layer and the bran in the upper layer. This prevented the water being absorbed by the flour if the vial was shifted down into the diet by insect burrowing. Diet decomposition was avoided in this manner. The density of mobile stages per filled rearing jar was not allowed to exceed 250 individuals. Jars were covered with fine mesh screening to prevent the escape of the mobile stages.

Adult females (newly-emerged) and males were removed from the stock culture and placed in 455 ml glass ointment jars containing enriched white flour and a water vial as described above. Eggs were removed daily by sieving and placed in petri dishes. The experiment was

commenced when day old larvae (@ .00003 g) were transferred into 455ml ointment jars containing whole wheat flour and wheat bran (70:30 w/w; 400 ml) in two layers as above with a single water vial changed biweekly. Larvae were introduced using a soft bristle brush and only those individuals whose cuticle had attained the characteristic tanned color of larger individuals were used. Jars were covered to prevent escape of the mobile stages. Replicates for the entire experiment were prepared using larvae that had hatched the same day. Incubator conditions were as above, for the stock culture. The position of the ointment jars were randomized in the incubator.

All diet materials were supplied by Ogilvy Flour Mills, Montreal, Canada after initial attempts using supermarket and bulk-food store purchased materials resulted in high larval mortality. This excluded the possibility of wholesaler treatment of storage facilities with insecticides that, present in trace amounts only, might kill the susceptible newly hatched larvae.

The larvae were removed at one month and weighed. After 50 days jars were searched every two days for pupae and adults which were sexed (method of Bhattacharya et al. 1970) and weighed. Competitive interactions such as feeding on freshly moulted larvae, quiescent larvae or pupae were cursorily examined at this time. Abnormalities in metamorphic transformations were also noted at this time. The pupae were generally found on the top of the bran layer and thus were quickly returned to their original location. In several cases where the pupae were missed and only the adult weight was recorded pupal values were included as missing data in the statisical model. Adults and cadavers were removed from the jars.

The experiment was replicated twice to completion. Another partial replicate was the initial experiment in which times to pupation were liberally assumed to be sixty days, thus pupae and adults were present in some replicates at first search and these jars containing adults were removed due to the uncertainty of the age and therefore, mass, of these indivduals. The data for one month old larvae and any

jars containing only larvae and/or pupae at sixty days for this repetition were included in the statistical model. All repetitions of the experiment were not statistically heterogenous and were thus pooled.

Statistical analysis of differences between the sexes in developmental time or mass at specific densities were based on T tests. Differences between mass or development time at varying densities for either males or females were analyzed using the Tukey-Kramer multiple comparison procedure for unequal sample sizes, differences between masses of month-old larvae were similarly analyzed. Comparisons between percent survival data per replicate jar were conducted on arcsine-transformed values using the Tukey-Kramer procedure with measures of variability being reported as back-transformed asymmetrical confidence limits rather than standard error as elsewhere. The experiments were conducted using unequal numbers of replicates and comparisons are based upon harmonic means; although such procedures are generally robust, this should be considered when marginal differences exist in comparisons. All statistical analyses were performed using the general linear models procedure of the Statistical Analysis System (SAS Institute 1988)

Results

Varying the density that one day old larvae were reared to adulthood had an effect on a variety of physiological parameters.

After one month larvae at the density of 20 were significantly larger than those at the density of two individuals per jar (Table 21). Larvae reared at all other densities were intermediate between these two values and were statistically similar to both extreme values. However, numerically higher mass was associated with higher density with the values for densities of 2 or 1 larvae per jar being exchanged in the decreasing order of values (Table 21).

At the time of pupation varying density had exerted an effect on the females with those reared in isolation being significantly larger than those reared at densities of 10 or 20 individuals per jar (Table

22). All other values were intermediate between these and were not significantly different from either the smaller or larger values. wever, at this time numerically higher mass was correlated with decreasing density, an opposite observational trend from that seen at one month (Table 22). At the densities of 1 and 2 individuals per jar female pupae were larger than male pupae (Table 22). No significant differences were observed between male pupae at differing initial larval densities (Table 22).

At adult emergence increasing density again resulted in females at densities of 20 and 10 larvae per jar being significantly smaller than those reared in isolation (Table 23). Females from intermediate initial larval densities had mean masses that did not differ from those for solitary or high density-reared individuals. Again, as an observation, numerically higher mass is correlated with decreasing density (Table 23).

Times to pupation and adult emergence for all densities and both sexes are given in Table 24. Mean developmental time is statistically similar at all densities within sexes and within densities for both sexes. Direct comparison of pupal to adult values for developmental times are impossible because of mortality as pupae (especially for individuals slow to pupate), occasional synchrony of pupation and adult emergence for some individuals at group densities, and occasional missing values for pupae overloooked during searches. Therefore, mean values can actually appear higher for pupae than for adults (Table 24) although for any individual this is clearly impossible.

Percent survival data per replicate jar at one month, at pupation and at adult emergence are given in Table 25. After one month of development no significant differences were found with varying density (Table 25). The percent survival at all densities was very high at this stage. At pupation and at adult emergence individuals reared in isolation had significantly higher mean percent survival than thosed reared

at densities of 10 and 20 larvae per jar. Higher percent survival was observed to be correlated with decreasing density at these developmental stages as well (Table 25).

Analysis of the mean percent survival per replicate jar at the three developmental stages showed that larvae had significantly higher percent survival than pupae or adults within each density (Table 26). This indicates that with the passsage of time there is a statistically significant decrease in percent survival at optimal as well as less than optimal densities. Though it is impossible for percent survival to increase with the passage of time, there is no reason to assume that percent survival would be significantly less with continuing development under optimal conditions. No significant differences were observed between percent survival at pupation and adult emergence within any density.

During searches at the density of 20 individuals per jar cannibalism was directly observed by large larvae upon other large larvae (probably quiescent pre-puae in most cases) 11 times and upon pupae 5 times. The damage to large larvae resulted in holes in the outer integument that varied from slightly less than 1 mm to several mm in diameter. These were generally located on the dorsal surface several body segments behind the head capsule. Six more larvae with such wounds were observed at this density. The haemolymph generally leaked from these wounds and the larvae frequently died (5 out of 6), presumably from subsequent septicaemia. Microbial growth was observed during later searches on such wounds. Often after such wounding, a larva would die and form a fully sclerotized cadaver without further injury. However it was also observed that the entire internal tissues and and > 50% of the integument were consumed in large larvae that showed no evidence of post-mortem sclerotization. Four such cadavers were found in jars containing 20 individuals.

In addition 3 individuals were found at this density that had incomplete larval-pupal tranformations. These were slower developing individuals and they all died. This highest density also had 8 females

and 4 males that retained pupal characters after adult emergence. These were weighed as adults if they survived the process, which six of the females and all of the males did.

At the density of 10 individuals per jar 7 acts of cannibalism were observed upon larvae by larvae and 2 upon pupae by larvae. One 'wounded' individual was found and subsequently died. Two adult females were observed to have retained pupal characters and both survived.

At the density of 5 individuals per jar cannibalism was observed twice by larvae upon other larvae.

At lower densities no cannibalism was observed nor was there any evidence of incomplete transformation between developmental stages.

The above data were the result of observations during the search for adults and pupae every second day and represent incidence values for these search times only, and are not intended to assess the overall contribution of these processes in group dynamics. However, search efficiencies are roughly equivalent for all jars so qualitative comparisons can be made.

Discussion

The enhanced growth of month old larvae at higher densities is interesting. Obviously, individuals in this situation are potential competitors and potential competition may result in increased diet consumption and increased weight gain. A similar situation is found in *Acheta domesticus* where isolated two week old larvae are significantly smaller than those raised in contact with conspecifics (McFarlane 1978). However, such a response is difficult to explain in terms of an appropriate mechanism, particularly for one day to one month old *Tenebrio*. The larvae are initially tiny and are still quite small at 30 days, particularly in 400 ml of particulate diet material, so how do they sense the presence of conspecifics? How does this trigger enhanced feeding? Frass may play a role, but all jars have frass accumulations, so it would require higher quantities of frass and possibly non-self frass.

Direct contacts may also play a role, but again this would first require the ability to discern contact with conspecifics and subsequently determine the number of conspecific individuals by some means. It is also very difficult to determine if there is significant interaction between small larvae at all.

Late instar mealworms are coprophagous, possibly as a result of their general feeding behavior, if not selectively (Weaver personal observation). This suggests that early instars might benefit from more rapid diet conditioning with excreta. This might increase bacterial colonization of the gut which could enhance utilization of ingested material.

The enhanced early growth of high density individuals is soon overcome by what can be assumed to be competitive disadvantage for larger individuals. This disadvantage is likely due to increased (and due to experimental design, forced) contact between individuals, rather than competition for the food resource itself. The food supply is considerably in excess of the requirements of 20 larvae. At all densities volume per individual decreases with increasing consumption and increased size, so interaction between individuals increases rapidly. This not only leads to aggressive encounters between individuals and subsequent cannibalism, but may also lead to increased activity wandering to less stressful surroundings. Such relatively isolated locations may be favored for feeding, but are probably more important for larval moults. *Tenebrio molitor* larvae generally have a variable, high number of larval moults (9-20) and develop slowly in grain stores (281-629 days) (Cotton 1927). Even under optimal conditions hatch to imaginal ecdysis is at least 79 days and there were 11-14 larval instars (Urs and Hopkins 1973). It is during the non-mobile period prior to a moult, ecdysis, and immediately post-ecdysis that these insects are most susceptible to acts of aggression/cannibalism in the larval stage as well as the pre-pupal stage (as stated in Tschinkel and Willson 1971). This was evident in group rearing where there was a significant absence of

cadavers of large and intermediate larvae, though some small and large cadavers were found at all densities. At high densities the cadavers represented only a small portion of the 'missing' larvae.

Therefore, it is likely that the decreased mass of the higher density reared individuals may be in part due to increased non-feeding time prior to pupation. It is generally assumed that either slower-growing individuals feed upon faster-developing individuals and subsequently increase developmental times, or the inverse, faster developing individuals consume the slower, decreasing the developmental time (Stinner et al. 1977). However, the results suggest that density affects the mass of females, not the duration of the larval stage. Size may also be influenced in a similar manner. The data at one month suggests that density enhances early larval growth and also that adult females are larger in isolation than solitary males. Males and females develop at the same rate at all densities. This suggests that females grow faster (become larger in the same amount of time). The increased growth rate requires that these individuals moult more frequently, and are therefore more susceptible to cannibalism. After these larger and more frequently moulting females are removed cannibalism may occur approximately equally upon both sexes, due to roughly equivalent size and moult frequency, until competitive pressure is reduced. The data set is not large enough to see if the sex ratio would be altered. There can be little doubt that even if it were, this would be most beneficial because reproductive capacity would be decreased in a crowded situation. The removal of the females with the highest potential fecundity (largest) is beneficial in the same way.

At high densities, the reproductive potential of these large insects may also be influenced in another way. Schmialek (1961) found that farnesol and farnesal, which display juvenile hormone activity, are found in *Tenebrio* frass. Tschinkel and Willson (1971) suggest that that these compounds may delay pupation under crowded conditions. The increased incidence of earlier stage characters being retained by the later stages that was observed is probably an effect of these compounds,

particularly since this was observed for the slowest developing individuals at the highest densities only. Wigglesworth (1958) describes the assay for juvenile hormone activity on *Tenebrio* pupae with one of the characteristic results being severely crumpled elytra. The entire abdomen was pupal in all the abnormal specimens that were observed at high density and the elytra were severely crumpled. Abrasion or puncture of recently moulted pupae was required to achieve this effect in hormone assay, but the larvae in our experiments may have consumed this material and certainly may have had prolonged topical exposure when tunneling as well as numerous more intense exposures during moults. These were the slower growing individuals, who would subsequently not be able to reproduce.

The data also showed that there were a significant number of slower growing larvae at higher density, though this did not influence the mean developmental time greatly. Tyschenko and Sheyk Ba (1986) report the presence of two races in mealworm larvae reared under the same conditions, those that pupate without delay and those that delay, but without a clearly expressed gap between the categories that would define a diapause. It may be that individuals that tend to grow slower will be more susceptible to exogenous material with JH activity. This would certainly occur with increasing density of individuals allowing the frass concentration to increase. The frass concentration increases relative to foodstuff with the passage of time, via consumption and defecation, even for isolated individuals, so what may be a tendency can have an increasingly pronounced effect with time for all individuals.

The *Tenebrio* in these experiments grew from hatch to adult in as little as 57 days. Efforts were made to incorporate new genetic material into our culture and these insects probably do not represent a particular strain, though the developmental time is considerably less than the value of 79 days which has been previously reported for a specialized strain (Urs and Hopkins 1973). The availabity of free water is the only possible explanation for the rapid development (Urs and Hopkins 1973), which seems plausible since mass hygrotaxis to a renewed water

supply is generally seen, as well as frequent individual consumption of water by all stages of larvae, as was seen by Mellanby and French (1958) and Murray (1968), as well. The diet in the experiments presented here was offered in stratified layers to facilitate this, but larval foraging patterns indicate that both layers are equally accessible. Pupation generally occurs at the top of the bran layer as well, thus reducing moulting difficulty and making location easier.

Finally, our results suggest that the tendency of late-instar yellow mealworms to aggregate in frass contaminated regions (Weaver et al. 1989, Section 2: Frass-induced aggregation- *Tenebrio*) may induce competition and have little direct physiological benefit to the aggregating individuals. There may be population survival benefits in ensuring that a potential breeding population remains in a safe location, but aggregation in a short term bioassay may be sufficiently distinct from group-rearing in a restricted space for any further interpretation.

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Table 21. Mass of larvae reared at varying densities at one month.

Density	Mass in g[a] (±S.E.)	
1	0.0378±.0033[ab]	(73)[b]
2	0.0362±.0029[b]	(76)
5	0.0415±.0023[ab]	(97)
10	0.0442±.0021[ab]	(118)
20	0.0457±.0019[a]	(145)

[a]Mean values followed by the same letter are not statistically different at $P \leq 0.05$, Tukey-Kramer procedure for unequal sample sizes.

[b]Values in parentheses represent the number of individuals weighed.

Table 22. Mass of pupae obtained from rearing larvae at varying densities.

	Females	Males
Density	Mass in g[a] (±S.E.)	Mass in g[b] (±S.E.)
1*	0.2132±.0068[a] (27)[c]	0.1888±.0061[a] (20)
2*	0.1982±.0065[ab] (21)	0.1730±.0056[a] (23)
5	0.1981±.0069[ab] (27)	0.1831±.0058[a] (30)
10	0.1838±.0060[b] (31)	0.1896±.0056[a] (33)
20	0.1778±.0056[b] (42)	0.1770±.0062[a] (34)

[a]For females, mean values followed by the same letter are not statistically different at $P \leq 0.05$, Tukey-Kramer procedure for unequal sample sizes.

[b]For males, mean values followed by the same letter are not statistically different at $P \leq 0.05$, Tukey-Kramer procedure for unequal sample sizes.

[c]Values in parentheses represent the number of individuals weighed.

*At the densities indicated, females are significantly larger than males at $P \leq 0.05$, T-test procedure.

Table 23. Mass of adults obtained from rearing larvae at varying densities.

Density	Females Mass in g[a] (±S.E.)		Males Mass in g[b] (±S.E.)	
1*	0.1817±.0064	(30)[c]	0.1607±.0050	(27)
2	0.1701±.0055	(28)	0.1559±.0063	(26)
5	0.1700±.0045	(34)	0.1533±.0052	(33)
10	0.1582±.0056	(33)	0.1569±.0051	(33)
20	0.1517±.0065	(28)	0.1504±.0054	(25)

[a]For females, mean values followed by the same letter are not statistically different at $P \leq 0.05$, Tukey-Kramer procedure for unequal sample sizes.

[b]For males, mean values followed by the same letter are not statistically different at $P \leq 0.05$, Tukey-Kramer procedure for unequal sample sizes.

[c]Values in parentheses represent the number of individuals weighed.

*At the density indicated, females are significantly larger than males at $P \leq 0.05$, T-test procedure.

Table 24. Time to pupation and adult emergence of individuals reared at varying densities.*

	Females		Males	
Density	Pupae	Adult	Pupae	Adult
1	83±7 (27)	86±7 (30)	81±9 (20)	80±7 (27)
2	70±4 (21)	71±3 (21)	77±7 (23)	79±6 (26)
5	80±9 (27)	76±6 (34)	71±5 (30)	74±5 (33)
10	73±4 (31)	76±4 (33)	76±6 (33)	80±6 (33)
20	75±3 (42)	81±5 (28)	83±6 (34)	93±7 (26)

*No significant differences for mean values between sexes at any density or for between densities for either sex. Values are reported as mean number of days ± standard error. Values in parentheses indicate the number of individuals.

Table 25. Percent survival of individuals per replicate jar reared at varying densities at one month, pupation and adult emergence.

At one month

Density	% Survival[a]	# of Jars[d]
1	98.6[a] (97.2, 99.6)	80
2	99.4[a] (97.7, 100)	40
5	98.0[a] (94.2, 99.8)	21
10	96.1[a] (89.7, 99.5)	13
20	95.1[a] (85.9, 99.6)	8

At pupation

Density	% Survival[b]	# of Jars[d]
1	93.9[a] (86.2, 98.6)	69
2	89.9[ab] (76.2, 98.1)	34
5	82.5[abc] (60.5, 96.7)	19
10	59.6[bc] (29.1, 86.5)	12
20	47.5[c] (13.5, 82.7)	8

At adult emergence

Density	% Survival[c]	# of Jars[d]
1	93.9[a] (86.1, 98.6)	69
2	89.9[ab] (76.1, 98.2)	34
5	77.0[ab] (53.4, 93.9)	19
10	56.3[bc] (25.8, 84.3)	12
20	32.4[c] (4.7, 70.0)	8

[a,b,c]At each developmental stage, mean values for percent survival followed by the same letter are not statistically different at $P \leq 0.05$, Tukey-Kramer procedure for unequal sample sizes. The values in parentheses are back-transformed 95% confidence limits.

[d]Number of replicate jars used in statistical model.

Table 26. A comparison of percent survival per replicate jar for all developmental levels at each density.

Density	Stage*	% Survival	# of Jars[a]
1	1 month	98.6 (96.7, 99.7)	80
1	pupae	93.9 (90.1, 96.7)	69
1	adult	93.9 (90.1, 96.7)	69
2	1 month	99.4 (96.4, 99.9)	40
2	pupae	89.9 (81.5, 96.0)	34
2	adult	89.9 (81.5, 96.0)	34
5	1 month	98.0 (93.7, 99.9)	21
5	pupae	82.5 (72.8, 90.4)	19
5	adult	77.0 (66.5, 86.0)	19
10	1 month	96.1 (89.0, 99.6)	13
10	pupae	59.6 (45.2, 73.2)	12
10	adult	56.3 (41.8, 70.2)	12
20	1 month	95.1 (86.7, 99.5)	8
20	pupae	47.5 (32.8, 62.4)	8
20	adult	32.4 (19.3, 47.0)	8

*For each density % survival is significantly greater at 1 month than at pupation or adult emergence at $P \leq 0.05$, Tukey-Kramer procedure for unequal sample sizes. The values in parentheses are back-transformed 95% confidence limits. No significant difference occurs between % survival values at pupation and at adult emergence.

[a]Number of replicate jars used in statistical model.

General Conclusions

The specific conclusions that are relevant to each group of experiments have been discussed in the appropriate sections and the more important findings of the entire study have been listed in the "Claim To Originality". However, some general comments regarding the overall findings and their implications to the chemical ecology of larval mealworms are appropriate at this point.

The role of excreted fermentation products in the orientation of mealworm larvae. It has been clearly demonstrated that larval mealworms of both species respond to the frass of conspecific larvae. The demonstration that this chemical conditioning of the environment can be accomplished by larvae is exciting, perhaps only because the chemical communication between insect larvae has not been seriously undertaken previously for this order. This may be due to the fascinating complexity of pheromone production among adult stored products Coleoptera. However, the environmental marking by larval Tenebrionidae that elicits behavioral responses in conspecific larvae clearly plays a role in the overall population orientation. It has been proposed that such marking serves to indicate safe refugia for reservoirs of potential breeding individuals. This marking is a relatively efficient way to quickly label unutilized surroundings. There should be some caution taken in interpreting these results. These fermentation products are probably fairly common in the environments that these insect larvae inhabit in both their feral and synanthropic modes of existence. The feral existence of these species in birds' nests or rotting wood closely parallels their preferences for poultry litter or decomposing harvested material, particularly since all of these surroundings are probable sources of semiochemical fermentation products. This would imply that the behavioral responses to these compounds may be due to the presence of these compounds in the preferred natural habitat of these larvae and the response to conspecific frass containing these pheromones is derived from the be-

haviors induced by these exogenous natural semiochemicals. The larvae have no way of distinguishing what the sources of these compounds are nor does it matter since, in either the surrounding exogenous material (from environmental decay or contamination by vertebrate feces) or the excreted pheromone case, ecologically favorable surroundings are indicated. The fact that these larvae can evolve these behaviorally active chemicals could easily be due to consumption of and subsequent internal establishment of bacteria that produce fermentation products under gut conditions; repeated coprophagy could ensure the same outcome from generation to generation. Certainly, under the unstable conditions of stored foodstuffs, selection could enhance the tendency of particular populations to respond to these compounds by the simple removal of those individuals that do not remain arrested in the safe region defined by such pheromones.

The relative role of water and olfaction in these pheromone-induced orientations. Both of these species favor damp locations ecologically. The fact that dry filter papers plus lactic acid act as a dispersant for *Alphitobius* larvae may be a behavioral response to a situation that these individuals rarely encounter. It is of interest that these larvae respond to airborne lactic acid stimuli, while *Tenebrio* larvae, which show a consistent response to lactic acid in the presence or absence of water, do so only through contact. Therefore the volatility of lactic acid is likely to be enhanced by the presence of water. It is also of interest that the response of the *Tenebrio* larvae, which are well known for their ability to endure dry conditions, would allow for a response to the pheromone under both favorable and less favorable conditions. In general, low volatility compounds are involved in the aggregation responses of these larvae, suggesting that close-range interaction governs the pheromone-induced aggregation of these species.

The role of cuticular lipids in the aggregation of these species. The demonstration that cuticular lipids play a role in the stage-specific recognition between these groups of larval conspecifics

provides a mechanism for the determination of whether what appears to be a favorable location is currently inhabited by conspecifics, or with the presence of exuviae, if it has been. This is a chemical basis that confers elements of species specificity to the more general orientation caused by frass-evolved pheromones. It may seem unusual that aggregation is occurring despite potential increased competition, however the relative proximity of potential mates, even if they are still developmental stages away from breeding may be a net benefit to individuals and subsequently to the species. The recognition of conspecific larvae that occurs may also facilate orientation away from potential aggressors during vulnerable periods, such as pre-molt quiescence and ecdysis.

The need for future research. There is much room for future work with respect to the relationships between the chemical ecology, behavior and physiology of these mealworm larvae. *Tenebrio* and *Alphitobius* larvae were both primarily evaluated as late-instar larvae. More thorough research is needed to determine when these aggregation responses commence in the larval development. Do day old larvae recognize the presence of day old conspecifics? It would also be of considerable benefit to determine if these volatile fatty acids and lactic acid are produced by specific bacterial species in the digestive tract of these insect larvae. Similarly, is possible gut colonization by bacteria facilitated by the occurrence of coprophagy early in larval development? Finally, a great deal more research should be conducted to determine the effects of grouping as determined by semiochemical influences in conditioned as well as unutilized resources, particularly by varying the density of larvae in foodstuffs that have been exploited to various degrees. This should be done on a larger scale to view the spatial dispacement of individuals in addition to determining the various physiological parameters during development.