BIOACTIVITY AND CHARACTERIZATION OF SPRUCE BUDWORM LARVAL ORAL EXUDATE

by

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Bioactivity and characterization of spruce budworm larval oral exudate

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Abstract

The eastern and western spruce budworms, *Choristoneura fumiferana* (Clemens) and *C. occidentalis* Freeman, respectively, are major pests of coniferous forests in North America. In the laboratory, larvae readily produced an oral exudate in response to handling or encounters with conspecifics. Each larva produced approximately 2 μL of exudate at a time (defined as one larval equivalent), but production could be induced up to four times. Direct application of a single larval equivalent to the entrance of a silken feeding tunnel, or spray application of four larval equivalents to foliage bearing established larvae caused significant numbers of larvae to disperse from their feeding tunnels. A feeding bioassay was developed which demonstrated the repellency of larval oral exudate to conspecifics, and allowed further elucidation of bioactivity. The exudate was reciprocally active between the two *Choristoneura* species. Larvae were refractory to exudate for 24 h after producing exudate. Artificial diet-reared larvae were repelled by exudate from both diet- and foliage-reared larvae; foliage-reared larvae were repelled only by exudate from other foliage-reared larvae. The exudate was not repellent at <1 larval equivalent, suggesting that larvae which produce small amounts of exudate will not repel competitors effectively. Centrifugation did not separate the exudate into active and inactive fractions. Exudate from foliage-reared insects was persistent for 24 h, but not for 48 h. Exudate from diet-reared insects was persistent for >48 h, and could be frozen for at least a week with no reduction in bioactivity. Autoclaving the exudate destroyed the bioactivity. Artificial diet-reared larvae were repelled by exudate from larvae fed on agar and linseed oil (a minor component of the diet), but not by exudate from larvae fed on agar alone, or agar and other diet

components. However, linseed oil itself was not repellent, suggesting that the larvae metabolize one or more of the fatty acid glycerides in linseed oil into a bioactive derivative. Larval oral exudate apparently functions as an epideictic pheromone in spruce budworms. The precursor of the bioactive constituent may be a fatty acid component of foliage that is also found in linseed oil.

Dedication

For Ken and Anne McDougall, who made all the difference.

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1.0 GENERAL INTRODUCTION

1.1 LIFE HISTORY AND ECONOMIC IMPORTANCE OF SPRUCE BUDWORMS

The eastern and western spruce budworms, *Choristoneura fumiferana* (Clemens) and *C. occidentalis* Freeman, respectively, are two economically important coniferophagous members of the family Tortricidae. Both exhibit cyclical population dynamics, with periodic outbreaks during which population densities may greatly exceed those of associated species (Sanders, 1991). The factors which produce these cycles are not well understood, but host plant chemistry (Kimmins, 1971), weather (Wellington, 1950; Blais 1952), predators (Morris, 1963), parasites (Miller, 1963) and disease (Nielson, 1963) have all been implicated. *C. fumiferana* ranges over much of the boreal forest of North America. Its preferred hosts are balsam fir, *Abies balsamea* (L.) Miller, red spruce, *Picea rubens* Sargent, and white spruce, *Picea glauca* (Moench) Voss. *C. occidentalis* is found throughout the interior forests of Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, in western North America. Douglas-fir is its primary host, but it also feeds on a variety of other conifers which are sympatric with *P. menziesii* (Sanders, 1991).

Both species of spruce budworm have similar life cycles (Sanders, 1991). Eggs are laid in late summer, in masses on the underside of host foliage. They hatch about 8-12 days later, and the first instar larvae disperse by crawling along the branches or by dropping on silk threads. They do not feed at this stage. When they encounter a sheltered location, such as a bark crevice, they spin silken hibernacula, moult to the second instar, and enter diapause.

The second instar larvae emerge from the hibernacula in early spring, usually in synchrony with the host's bud-flush, and many disperse again on silk threads. When they find suitable locations, larvae mine into foliar or staminate flower buds, or into older needles if the buds are not yet beginning to flush. The later instars feed in the expanding foliar buds, usually webbing needles from one or more shoots together to form a silken feeding tunnel. If all young foliage is exhausted, the larvae are capable of utilizing older needles, but their size and adult fecundity are reduced (Mattson *et al.*, 1983). It is at this point in the life cycle that most of the damage is done to the host tree. Larvae which are disturbed, or which have consumed all the available foliage in an area, commonly drop from trees on silk threads. Those which land on suitable host trees in the understory are able to complete their development. Larvae which land or pupate on or near the ground are subject to heavy predation by other insects (Youngs and Campbell, 1984), spiders (Morris, 1963), birds (Torgersen and Campbell, 1982) and mammals (Morris, 1963). Larvae mature through six instars.

Pupation occurs in, or close to, the last feeding tunnel, and adults emerge in mid-summer. Female moths remain near the pupation site, and use a sex pheromone to attract males. The major components of this pheromone are (*E*)- and (*Z*)-11-tetradecenal, in both species (Sanders, 1991). Females usually deposit about half their eggs near the pupation site, then disperse. In the case of *C. fumiferana*, moths, including gravid females, may move > 20 km by taking advantage of local wind patterns (Greenbank, 1980). This long-range dispersal likely contributes significantly to outbreaks in previously unaffected areas. Much less is known about adult dispersal in *C. occidentalis*, but it probably exhibits similar behaviour (Sanders, 1991).

Trees which have sustained heavy defoliation for one or more years show significantly lower growth rates, both in height and girth, than protected trees (MacLean, 1985). Severe defoliation often results in the death of the top portion of the tree, where young foliage predominates, leading to stunting, forking, or bushing (Alfaro, 1986). In some cases, particularly those involving *C. fumiferana*, entire trees are killed (Shore and Alfaro, 1986). In 1990, *C. fumiferana* caused moderate to severe defoliation over 8.5 million ha in Canada (Moody, 1993). An additional 193,000 ha were defoliated by *C. occidentalis*. These losses make the spruce budworms two of the most economically important forest pests in North America. Chemical insecticides, including DDT, fenitrothion and aminocarb, were used widely in the past to control populations of eastern spruce budworms. Fenitrothion is still used in New Brunswick, but the only control method available to most forest health personnel is the spraying of high-density larval populations with *Bacillus thuringiensis* (Berliner), a bacterial pesticide (Cunningham and van Frankenhuyzen, 1991).

A large body of literature exists for both *C. fumiferana* and *C. occidentalis*, with over 650 papers published since 1984 (search of Agricola Database on CD-ROM, 1984-Sept. 1994, SilverPlatter 3.11). Despite this fact, and the probable importance of density-mediating behaviour in these insects, very little information is available on larval behaviour, particularly with respect to intraspecific interactions.

1.2 THE ROLE OF REPELLENTS IN THE BEHAVIOUR OF LARVAL LEPIDOPTERA

Like other animals, lepidopteran larvae compete intraspecifically for limited resources such as food and suitable habitat. When population densities increase, for instance during outbreaks of insects with cyclic population dynamics, the selective pressures due to intraspecific competition may become quite intense. Reductions in the quantity or quality of food available for each larva, and in the availability of suitable hiding places, or refugia, are the most obvious consequences of an overcrowded habitat. Above-optimal population densities may also result in physiological changes in the organism (Peters and Barbosa, 1977; Prokopy, 1981; Prokopy *et al.*, 1984), leading to altered developmental rates or reduced fecundity. These physiological changes probably occur as a direct result of the lowered availability of high-quality food. A reduction in the number of available refugia results in an increase in the vulnerability of the organism to predators and parasites, thus increasing mortality. Overcrowding may also increase the incidence of disease transmission (Jaques, 1962). These pressures can be expected to result in the development of adaptive mechanisms which reduce their impact on individual fitness. In particular, traits will be favoured which enable the individual to assess and respond to population density, or which provide effective defenses against conspecific competitors, thus regulating population density in the immediate area.

It is not surprising, therefore, that more and more examples of territoriality (Pianka, 1978) are being described for insects. While some larval lepidopterans demonstrate gregarious behaviour, or live in family groups (e.g. in the Lasiocampidae), most larvae are solitary. The mediation of

territorial interactions may take several forms in these insects. Physical combat has been documented for several lepidopteran species (Corbet, 1971; Peters and Barbosa, 1977; Berenbaum *et al.*, 1993), and is probably common in many others. Visually- (Rausher, 1979; Shapiro, 1981) and acoustically- (Doolan, 1981) mediated interactions are well known in other insects. Chemical mediation of population density has been well-documented in several species of bark beetles (Borden, 1993), and a variety of other insect taxa (Prokopy, 1981). However, such chemical mediation has received much less attention in the Lepidoptera than in other insects. Prokopy (1981) discusses the potential role of epideictic, or spacing, pheromones in the population biology of a few species of Lepidoptera. These semiochemicals serve to provide information about the density of potentially competing individuals. They may function as repellents, which cause insects to orient away from the source (Dethier *et al.*, 1960), feeding deterrents, which reduce or prevent feeding near their source (Dethier *et al.*, 1960), or oviposition deterrents (Prokopy, 1981).

The majority of known epideictic pheromones deter oviposition by conspecifics (Prokopy *et al.*, 1984). They are usually produced by ovipositing adults, and are used to mark an occupied resource. However, in a few instances the deterring chemicals are produced by larvae (Corbet, 1973). Deterrent kairomones may also be produced by the host plant in response to larval feeding (Schurr and Holdaway, 1970; Renwick and Radke, 1980; 1981).

Recently, researchers have begun to investigate the feasibility of exploiting these epideictic pheromones to aid in the regulation of pest insect populations below damaging thresholds (Borden, 1993). There are a number of constraints to artificial population regulation by this means, because

insects deprived of food or oviposition sites may eventually develop high response thresholds to the bioactive components, or may adapt to using different cues to obtain information about population density (Prokopy, 1981). However, there is high potential for judicious use of these semiochemicals.

Many insects produce various fluid secretions, particularly when they are disturbed or handled (Eisner and Meinwald, 1966). Researchers have documented several cases of insects, particularly the immature stages, which produce oral secretions (Davies and McCauley, 1970; Corbet, 1971; Eisner *et al.*, 1974; Loke and Ashley, 1984; Turlings *et al.*, 1990). In most cases, these oral exudates have been shown to serve as a defense against predators and parasitoids. The gregarious European pine sawfly, *Neodiprion sertifer* (Geoffroy), has a specialized diverticulum off the crop, where it sequesters resins from its host plant (Eisner and Meinwald, 1966). The sticky, terpene-rich fluid is then regurgitated if the larvae are threatened by predators or parasitoids. In some cases, oral exudates may also have kairomonal properties (Corbet, 1973; Loke and Ashley, 1984), acting as attractants for predators and parasitoids, or they may induce the host plant to produce compounds which have the same effect (Turlings *et al.*, 1990; Turlings *et al.*, 1991; Turlings and Tumlinson, 1992; Turlings *et al.*, 1993).

Oral exudate is important in mediating population density in the Mediterranean flour moth, *Ephestia kuehniella* Zeller. Corbet (1971) demonstrated that forced exposure to larval oral secretions increased larval development times and lowered pupal weights, thus probably reducing adult fecundity. In addition, the secretions acted as a kairomone for the ichneumonid parasitoid, *Venturia canescens* (Gravenhorst) (Corbet, 1971). Although this is the only documented case of an oral exudate functioning as

an epideictic pheromone, it seems likely that other insects might exhibit similar behaviour.

Larval spruce budworms were selected as candidates for further investigation of this behaviour, primarily because I observed that they readily produced an oral exudate when handled. Although they are found at very high population densities in outbreaks, they tend even then to be of solitary habit (L.E. Maclauchlan, pers.comm., B.C. Forest Service, Kamloops, B.C.). As well, they invest considerable time and energy in the production of a silken feeding tunnel. I hypothesized that a budworm larva would vigorously defend such an investment against potential conspecific competitors.

1.3 GENERAL OBJECTIVES

The objectives of my research were:

1. to determine the role of larval oral exudate in the intraspecific interactions of *C. fumiferana* and *C. occidentalis*,
2. to develop an effective laboratory bioassay technique to assess the repellency of oral exudate to larval spruce budworms,
3. to determine whether oral exudate functions as an intra- and/or interspecific repellent in the laboratory,
4. to elucidate what mechanisms, if any, exist to prevent an exudate-producing larva from repelling itself,
5. to determine the influence of rearing history and food source on exudate bioactivity,
6. to analyze and describe some of the physical properties of the exudate, and
7. to determine the chemical nature of the bioactive components of larval oral exudate.

2.0 INSECT SOURCES AND REARING METHODS

Laboratory-reared *C. fumiferana* and *C. occidentalis* were obtained as diapausing second instar larvae from the Forest Pest Management Institute, Canadian Forest Service, Sault Ste. Marie, Ontario. These source colonies have been maintained for many years at high population densities, on artificial diet, and under strictly controlled rearing conditions (Grisdale, 1970).

Wild *C. occidentalis* were collected in late May or early June from various field sites in British Columbia: near Kamloops in 1989 and 1992, near Kelowna in 1990 and 1991, and near Merritt in 1993 and 1994. Insects were collected as third instar larvae on the foliage of *P. menziesii*. Infested understory branches were clipped and stored in plastic garbage bags for transport. Eggs from wild *C. fumiferana* were also collected, by personnel from the Forest Insect and Disease Survey, Canadian Forest Service, in Sault Ste. Marie.

Larvae of both species were reared in the Insectary at Simon Fraser University, at approximately 24° C and 60% RH, with a 16:8 L:D photoregime. Most larvae were reared on an artificial, agar-based spruce budworm diet (Diet #9769, Bio-Serv Inc., Frenchtown, New Jersey). This diet was received as a dry mix, and prepared in small batches according to the package directions. While the diet was still molten, it was dispensed into 30 ml disposable dilution vials, using a kitchen baster. The vials were filled to a depth of about 1 cm. Once the diet had cooled, a fungicide composed of ethanol, sorbic acid, and methylparahydroxybenzoate (Chawla *et al.*, 1967) was applied to the exposed surface with a No. 2 artist's brush. When the fungicide had dried, two larvae were placed in each vial, and the vials were

capped with plastic lids. In spite of the application of surface fungicide, and the presence of formalin in the rearing medium, fungal and bacterial colonies were periodic problems necessitating the frequent transfer of larvae to clean vials of diet.

Other larvae were reared on foliage in cages. For *C. fumiferana* larvae, one-year-old seedlings of amabilis fir, *Abies amabilis* (Dougl.) Forbes, were obtained from the B.C. Forest Service's Campbell River Nursery, Campbell River, B.C. These seedlings were potted, and maintained in a greenhouse at Simon Fraser University during the winter, or outside in the summer. For *C. occidentalis* larvae, one- to three-year-old Douglas-fir seedlings were obtained from the B.C. Forest Service's Surrey Nursery, Surrey, B.C., and maintained as described above. Douglas-fir foliage was also collected from trees at several locations: near Simon Fraser University, at the Malcolm Knapp Research Forest in Maple Ridge, B.C., and at the various *C. occidentalis* field collection sites listed above. Cut branches from these trees were placed in jars of water and maintained in the laboratory. Potted seedlings or cut branches which bore actively growing foliage were individually infested with larvae. This method of rearing was difficult and time-consuming, and the resulting numbers of late instar larvae were highly variable and unpredictable.

Most of the larvae from field collections were reared to adulthood on artificial diet or foliage, and allowed to mate and oviposit. The resulting larvae were handled according to the procedures outlined by Grisdale (1984). They were provided with cheesecloth as a hibernation site, and kept under diapause-inducing temperatures of approximately 2° C for about six months. Diapause could then be terminated successfully by removing the larvae from refrigeration and supplying them with food.

3.0 ORAL EXUDATE AS A MEDIATOR OF BEHAVIOUR

3.1 INTRODUCTION

The production of oral exudates is widespread in larval Lepidoptera. These exudates are usually hypothesized to mediate interspecific interactions, acting for example as a defense against parasitoids or predators. The potential importance of these exudates in the mediation of intraspecific interactions has been shown by Corbet's (1971) work on *Ephestia kuehniella*. This epideictic role may be far more important than would be implied by the small body of literature available on the subject.

Certain characteristics would be expected of insects which might use larval oral exudate as an epideictic pheromone. They should be of solitary habit, although likely to be found at high population densities in nature. They would exploit resources which are limited in quantity or quality, or which are ephemeral. As well, they might be expected to construct and defend complex structures such as webs and feeding tunnels (Berenbaum *et al.*, 1993). Because such structures require a large expenditure of resources, larvae would be likely to defend them vigorously. Both *C. fumiferana* and *C. occidentalis* exhibit behaviour which suggests that their oral exudates could function as epideictic pheromones. In general, I have found only one larva in each developing bud, even though population numbers may be very high during outbreaks. Host tree foliage is only a suitable food resource for a short period of time before the needles toughen (Mattson *et al.*, 1983), and variations in tree phytochemistry may further reduce the availability of high-quality food (Kimmins, 1971). Larvae commonly web together needles from one or more developing shoots to form a silken feeding tunnel. Berenbaum *et al.* (1993) have shown that such silk structures can be a valuable resource which is defended vigorously by the resident larva. My own observations have shown that larvae of *C.*

fumiferana and *C. occidentalis* readily produce exudate when handled. Even under laboratory rearing conditions, larvae produced exudate during encounters with conspecifics.

Therefore, the objectives of this initial portion of my research were as follows:

1. to document the production of oral exudate by *C. fumiferana* and *C. occidentalis* larvae in response to handling and conspecific encounters,
2. to determine the frequency with which individual larvae could be induced to produce exudate in the laboratory, and the volume produced at each induction,
3. to determine whether the presence of conspecific oral exudate alters the behaviour of larvae on their natural foliage habitat, and
4. to assess the potential for increasing larval dispersal through the application of oral exudate to foliage.

3.2 MATERIALS AND METHODS

3.2.1 Observations on laboratory-reared larvae

During the rearing process, observations were made on 100 third to fifth instar laboratory-reared larvae of each species on artificial diet. Particular attention was paid to behaviour during handling, immediately following the introduction of two larvae to a new vial of rearing medium, and during encounters between wandering and established larvae. Handled larvae were touched on the head with the tip of a metal probe to simulate a conspecific encounter.

An additional 100 *C. fumiferana* larvae were touched repeatedly with a 5 μL micropipet, and any exudate was collected in the pipet. The frequency with which each of these larvae could be induced to produce exudate was recorded, as was the volume produced at each induction.

3.2.2 Larval behaviour on foliage

Two experiments were conducted. In the first experiment (Exp. 1), 40 fully-flushed shoots of Douglas-fir, approximately 10 cm long, were placed in water in 30 mL vials. Individual, field-collected, fourth and fifth instar *C. occidentalis* larvae were placed on each of these shoots, and allowed to establish for 24-48 h. Larvae were judged to be established when they had webbed several needles together to form a silken tunnel, and were feeding actively.

Each larva was randomly assigned to one of four treatment groups. Control larvae were undisturbed, other than having the shoot on which they were established relocated on the bench during randomization. Larval oral exudate was collected and pooled from other wild larvae, not used in the experiment, by touching the head region with a 5 μL micropipet. Two microlitres of the pooled exudate, approximately the amount produced by a single larval exudation (see Section 3.3.1), was applied inside the feeding tunnel and directly in front of the head of each larva in the second group. Larvae in the third group were treated similarly with 2 μL of distilled water. Larvae in the fourth group were touched with the tip of a probe until they produced a drop of oral exudate within the tunnel. Ten replicates of the four treatment groups were placed on a bench in a completely randomized design, consisting of four rows of 10 vials.

The behaviour of each larva was observed briefly and recorded at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, and 26 h after the treatments. Behaviours were grouped and rated on a scale of 1 to 10, with higher index values representing increased activity, and possibly an increased likelihood of an individual larva leaving its habitat (Table 1). The mean activity indices for the four treatments were compared at each observation time using the Kruskal-Wallis k-sample test (Steel and Torrie, 1980; Schlotzhauer and Littell, 1987). The numbers of larvae which had left the twigs (index value 10) and dispersed in each treatment group were

Table 1. Ranking of behaviours exhibited by *C. occidentalis* larvae over a 24 h period. Larvae placed on Douglas-fir shoots and allowed 24 to 48 h to establish prior to commencement of observations.

Index	Category	Behaviours observed
1	Inactive in tunnel	Body within tunnel, no movement
2	Active in tunnel	Body within tunnel, walking, turning, etc.
3	Maintenance	Silk spinning, combing, frass removal
4	Feeding in tunnel	Body within tunnel, feeding on needles projecting into tunnel or excised needle pulled into the tunnel from outside
5	Partly outside tunnel	Body partly exposed, no movement
6	Partly outside, feeding	Body partly exposed, feeding
7	Expanding tunnel	Spinning silk outside tunnel
8	Feeding outside	Body completely exposed, feeding
9	Active outside	Moving around on shoot
10	Leaving shoot	Spinning down, crawling off shoot

compared for each observation time using a $\chi 2$ goodness of fit test (Steel and Torrie, 1980). In all cases in this and subsequent chapters, $\alpha=0.05$.

In the second experiment (Exp. 2), the progeny of field-collected *C. occidentalis* were reared to the fourth or fifth instar on artificial diet. Larvae were then removed from the rearing vials and placed on small (approximately 5 cm long), flushed, cut shoots of Douglas-fir. The shoots were harvested from three-year-old trees forced to flush in a greenhouse, and were kept in 30 mL vials of water. Larvae were allowed 24 h to establish on the foliage. The same four treatments were applied to the larvae as in the first experiment, but the treatments were set out as 18 randomized complete blocks tested over two days. Air currents and disturbances were kept to a minimum over the course of the experiment. Larval behaviour was observed about 0.5 h after application of the treatments, and every hour thereafter for 8 h. Behaviours were ranked as in the first experiment, and the same statistical comparisons were used.

3.2.3 Effect of spray applications of exudate

Two experiments were conducted using fully-flushed shoots of Douglas-fir, approximately 10 cm long, which were placed in water in 30 mL vials. Individual, field-collected, fourth and fifth instar *C. occidentalis* larvae were placed on these shoots, and allowed 24 h in which to establish.

In Exp. 3, larvae were randomly assigned to one of six treatment groups. Control larvae were disturbed as little as possible. Larval oral exudate was collected as described in Section 3.2.2, and 2 μL was applied inside the tunnel, directly in front of the head of each larva in the second group. Each of the remaining four groups was treated with distilled water, 0.5, 1.0 or 2.0 larval equivalents of oral exudate, applied to the outside of the larval tunnel using a small perfume atomizer. The total volume applied to each shoot was approximately 0.65 mL of exudate diluted in distilled water. The treated shoots were arranged in 21

randomized complete blocks. The numbers of larvae dispersing were recorded about 0.5 h after application of the treatments, and every hour thereafter for 8 h. In both Exp. 3 and 4, dispersing larvae were those which had left the shoot, or which were observed to be active on the shoot outside their tunnels. The numbers of dispersing larvae in each treatment group were compared for each observation time using a χ^2 test (Steel and Torrie, 1980).

Exp. 4 was conducted in the same manner as Exp. 3, except that the spray treatments consisted of distilled water and 4.0 larval equivalents. The treated shoots were arranged in 30 randomized complete blocks. The incidence of dispersal was observed and recorded at the same times, and the same statistical tests were used.

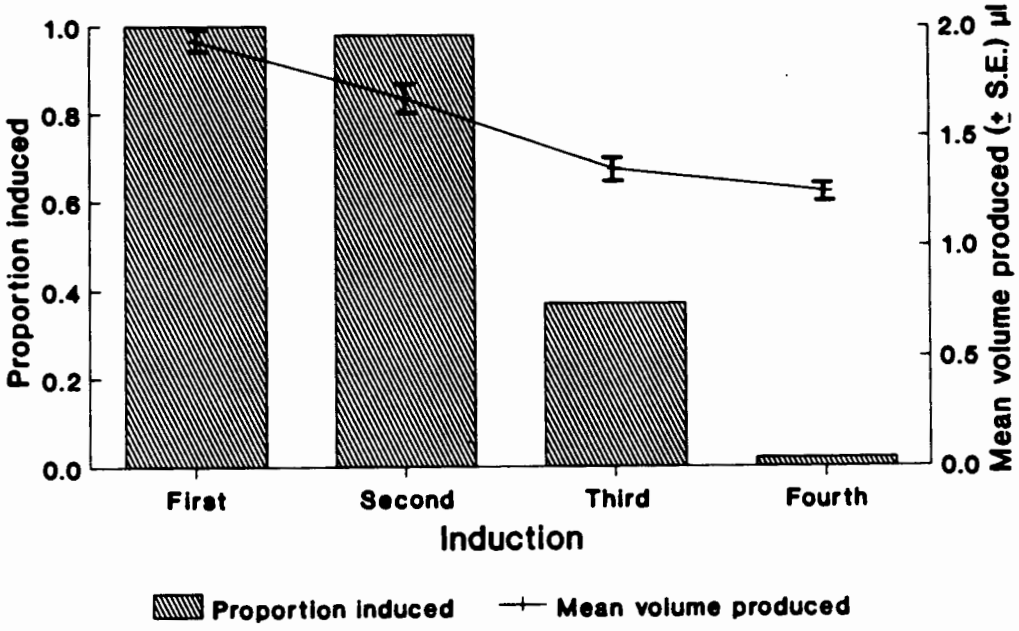
3.3 RESULTS

3.3.1. Observations on laboratory-reared larvae

Larvae of both species behaved similarly during the observation periods. When handled or touched on the head, all larvae reared up, opened the mandibles, bit at the probe, and attempted to wriggle backwards; 92% of *C. fumiferana* and 91% of *C. occidentalis* larvae produced oral exudate (n=100 for each species). Larvae which did not produce exudate were at the point of moulting to the next larval instar or pupae.

Each *C. fumiferana* larva was induced to produce exudate an average of 2.37 ± 0.06 ($\bar{x}$ ± S.E.) times, producing 1.92 ± 0.04 μL per induction (n=100). Almost all larvae were able to produce exudate twice within 2-3 min; about 40% produced a third drop and 2% a fourth drop (Fig. 1). The volume declined gradually with sequential production of exudate, but even the fourth drop was on average only 35% less than the first (Fig. 1).

Fig. 1. Proportion of *C. fumiferana* larvae induced to produce oral exudate one, two, three or four times, and mean volume of exudate produced at each induction, n=100.


Proportion induced
1.0
0.8
0.6
0.4
0.2
0.0
Mean volume produced (± S.E.) µl
2.0
1.5
1.0
0.5
0.0
First
Second
Third
Fourth
Induction
Proportion induced
Mean volume produced

Immediately following the introduction of two larvae to a fresh vial of diet, both insects were observed wandering around the vial. In the majority of the encounters observed for both species, one or both of the larvae reared up and opened the mandibles, and produced oral exudate (Table 2). There was no apparent correlation between size or sex and which larva reacted to the encounter first. The production of exudate by one larva always resulted in the second larva turning away, or wriggling backwards.

During rearing, an established larva would often abandon its initial feeding site and begin to wander. When this wandering resulted in an encounter with the remaining established larva, the established larva was no more likely to produce exudate first than was the wandering larva (Table 2). However, when the established larva backed away, it merely retreated further into its feeding tunnel, and thus did not appear to lose any territorial advantage.

3.3.2 Behaviours observed on foliage

A number of behaviours were exhibited by *C. occidentalis* larvae established on foliage during both experiments (Table 1). Most larvae spent the majority of the light hours inactive inside their tunnel. Larvae were commonly observed spinning silk within the tunnel. Some also spent time "combing" this silk with the claws of the prothoracic legs, presumably smoothing the inside walls of the tunnel. When a frass pellet failed to fall clear of the tunnel, the larva would turn around in the tunnel, pick up the pellet in its mandibles and remove it from the tunnel. These three activities of spinning, combing, and frass removal were grouped together as maintenance behaviours (Table 1). A few larvae were also seen spinning silk outside the tunnel, and expanding the tunnel area. Feeding was observed with the body inside, partly outside, and fully outside the tunnel. In several instances, larvae left the tunnel, chewed off a needle at the base, and used their mandibles to

Table 2. Numbers of established and wandering *C. fumiferana* and *C. occidentalis* larvae producing oral exudate during aggressive conspecific encounters under normal rearing conditions.

Status of larval establishment	Species	Number of encounters observed	Percent of encounters resulting in exudate production	
			Established larvae	Wandering larvae
Newly-introduced, both larvae wandering	*C. fumiferana*	42	—	86
	C. occidentalis	44	—	89
Both larvae established, one wandering after leaving feeding tunnel	*C. fumiferana*	14	57	43
	C. occidentalis	12	50	50

drag the food back into the tunnel. Occasionally larvae were inactive while partly outside the tunnel, but inactivity was never observed when the body was completely outside. The highest ranked activity was that of leaving the shoot, by either spinning down to the bench top on silk, or crawling or falling off the shoot. In Exp. 1, larvae were easily disturbed by air currents and activity in the room, and by the observer leaning over the insects in the front row to observe those in the back rows. All of these disturbances resulted in a brief cessation of activity for larvae in all treatment groups.

3.3.3 Analysis of activity

Fig. 2 shows the mean activity index for each treatment at each observation time for both experiments. In Exp. 1, larvae in the undisturbed and water-treated groups showed similar, low levels of activity throughout, while larvae in the exudate-treated group showed the highest level of activity. Larval activity was intermediate in the induced group. The differences between the treatment groups were most pronounced 3.5 and 6.5 h after the treatments. The insects were last observed about 26 h after the treatments were administered, but many had already eaten all the young foliage available. Activity had increased in all groups at this time, probably due to lack of food. As a result, this observation time was excluded from Exp. 2.

The mean activity indices for laboratory, diet-reared larvae did not follow the same trends in Exp. 2 (Fig. 2) as for field-collected larvae in Exp. 1. Significant differences between treatments were found only at 3.5 h after treatment, with the exudate-treated group showing the lowest level of activity.

In both experiments the frequency with which larvae left their feeding tunnels and dispersed was much higher after treatment with oral exudate from other larvae, or after they were induced to deposit oral exudate in their own tunnels

Fig. 2. Mean activity index, in two experiments, of *C. occidentalis* larvae established on foliage and exposed to no disturbance (control), 2 μL of distilled water, 2 μL of conspecific oral exudate, or 2 μL of individual's own exudate (induced). Exp. 1 used wild collected larvae, n=10. Differences between treatments were most pronounced at 3.5 and 6.5 h (Kruskal-Wallis test, P=0.06 and P=0.09, respectively). Exp. 2 used larvae reared on artificial diet, n=18. Significant differences between treatments were found at 3.5 h (Kruskal-Wallis test, P=0.02).

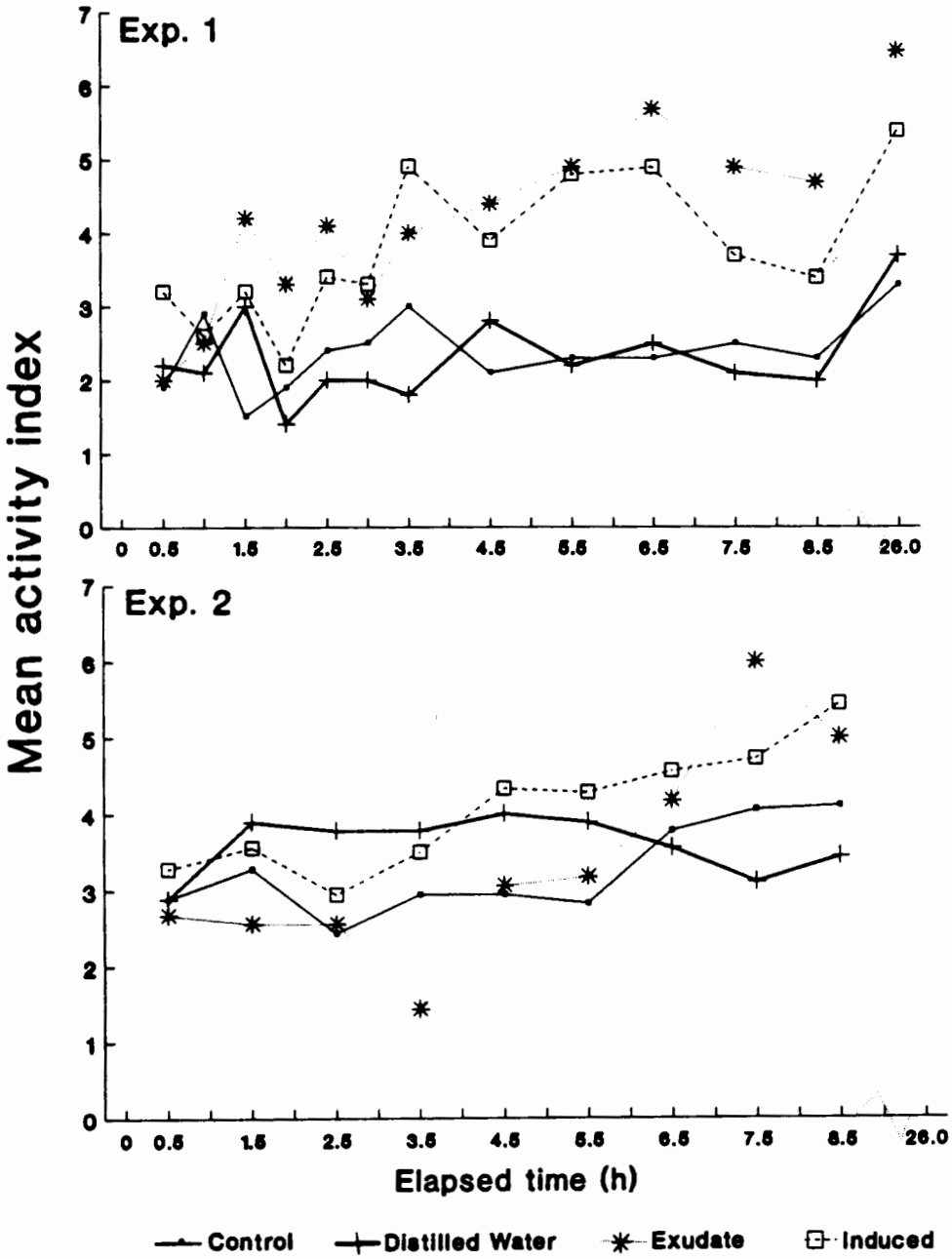
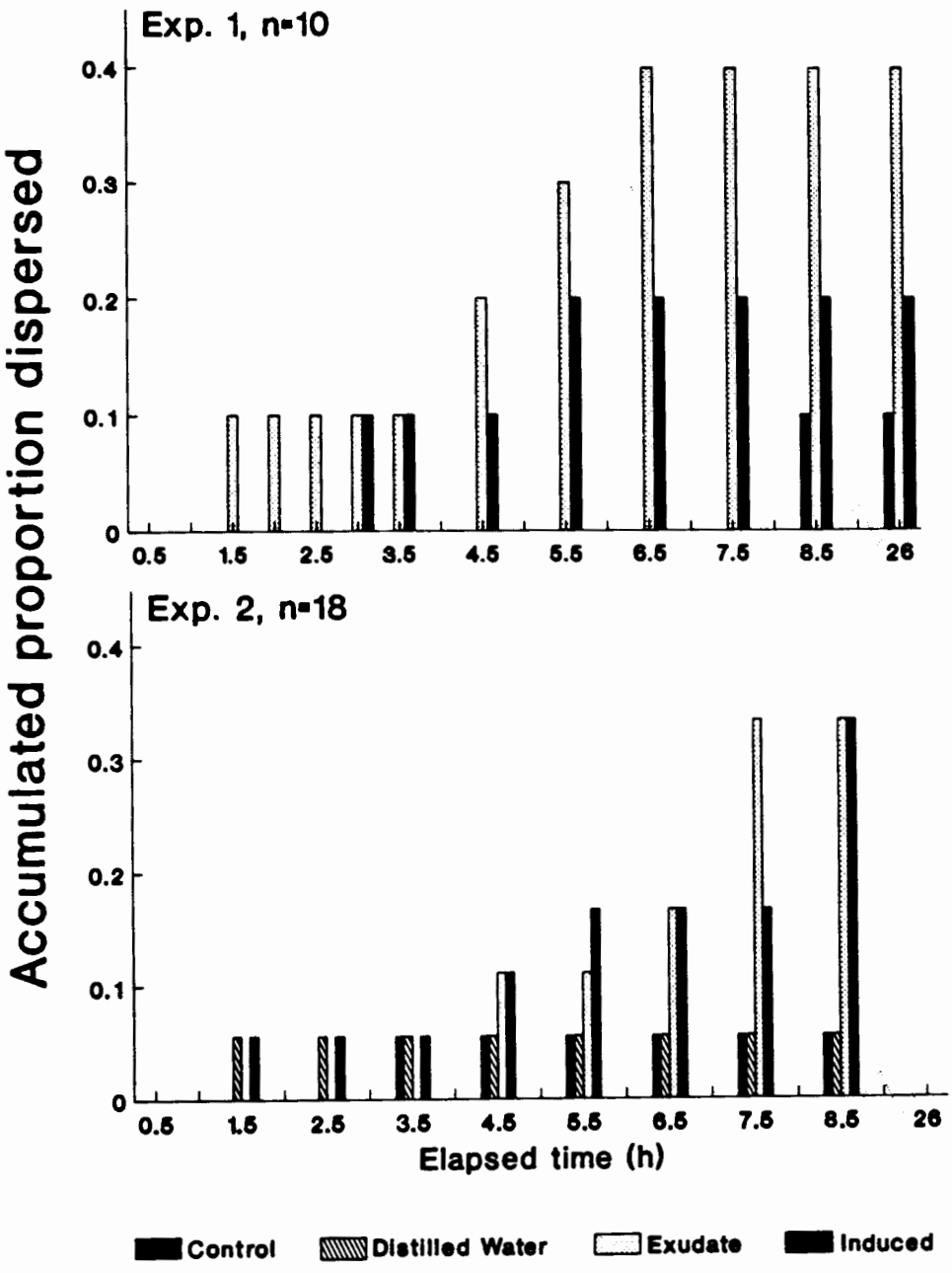

Exp. 1
Exp. 2
Mean activity index
Elapsed time (h)
0 0.5 1.5 2.5 3.5 4.5 5.5 6.5 7.5 8.5 26.0
Control
Distilled Water
Exudate
Induced

Fig. 3. Proportion of *C. occidentalis* larvae in two experiments dispersing from foliage after exposure to no disturbance (control), 2 μL of distilled water, 2 μL of conspecific oral exudate, or 2 μL of individual's own exudate (induced). Exp. 1 used wild collected larvae, n=10. Exp. 2 used larvae reared on artificial diet, n=18. Significant differences were found between treatments at 6.5 and 7.5 h after exposure in Exp. 1 ($\chi 2$ test, P=0.037 in both cases) and at 8.5 h in Exp. 2 (P=0.038).


Exp. 1, n=10
Exp. 2, n=18
Accumulated proportion dispersed
0.4
0.3
0.2
0.1
0
0.5
1.5
2.5
3.5
4.5
5.5
6.5
7.5
8.5
26
Elapsed time (h)
Control
Distilled Water
Exudate
Induced

(Fig. 3). The frequencies were significantly different between treatments at 6.5 and 7.5 h after treatment in Exp. 1 and after 8.5 h in Exp. 2.

3.3.4 Effect of spray applications of exudate

In Exp. 3, larval dispersal was highest after treatment with oral exudate from other larvae (Fig. 4). The frequencies were significantly different between treatments at 8 h after treatment. There was also a trend towards increasing dispersal in the group sprayed with 2 larval equivalents of oral exudate.

In Exp. 4, larval dispersal was increased in both the exudate-treated group and the group sprayed with 4 larval equivalents of exudate (Fig. 4). The frequencies were significantly different between treatments at 6 h after treatment.

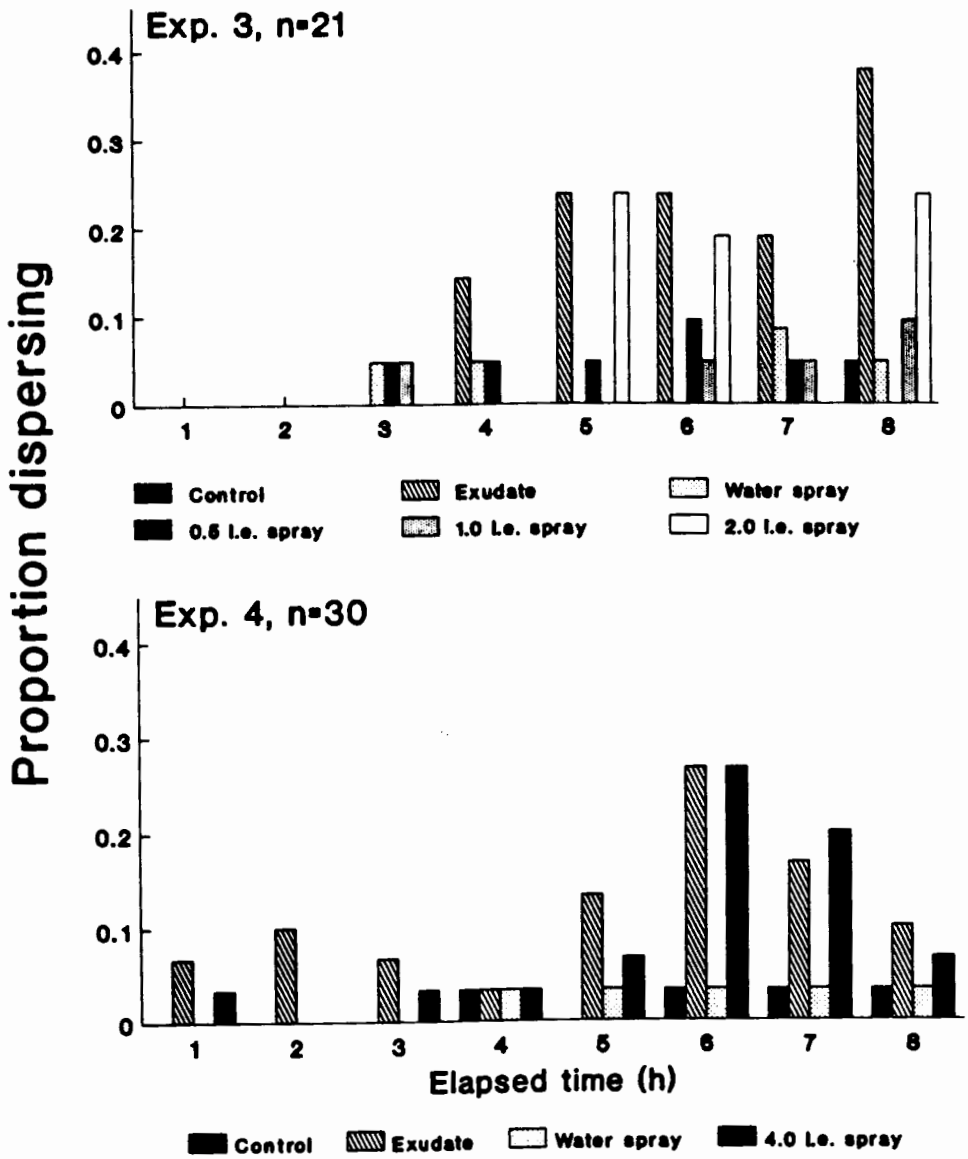
3.4 DISCUSSION

Almost all larvae of both species could be induced to produce exudate. Because the exudate is emitted orally, it is most probably either a product of the mandibular glands (Corbet, 1971; 1973) or a regurgitant (Eisner *et al.*, 1974; Turlings and Tumlinson, 1992). Because the gut is empty before moulting (Borror *et al.*, 1989), and because larvae on the point of moulting did not produce exudate, the oral exudate is most likely a regurgitant. The large volumes (up to 10 μL) sometimes produced by individual larvae also suggest that the exudate originates in the digestive tract.

On average, larvae produced slightly less than 2 μL of exudate when first induced. While they were capable of producing similar quantities on up to three more occasions, 2 μL would seem to be a reasonable estimate of one larval equivalent. This is approximately the quantity which would be encountered by most conspecific competitors.

These observations suggest that production of oral exudate is a mechanism for establishing and defending an individual's territory. Aggressive intraspecific

Fig. 4. Proportion of *C. occidentalis* larvae dispersing from foliage after exposure to no treatment (control), 2 μL of conspecific oral exudate, or aerosol applications of distilled water or 0.5, 1, 2 or 4 larval equivalents of exudate. For Exp. 3 and 4, n=21 and 30, respectively. Significant differences were found between treatments at 8 h after exposure in Exp. 3 (χ^2 test, $P<0.005$), and at 6 h in Exp. 4 ($P=0.005$).


Exp. 3, n=21
Proportion dispersing
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
Control
0.5 l.e. spray
Exudate
1.0 l.e. spray
Water spray
2.0 l.e. spray
Exp. 4, n=30
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
Elapsed time (h)
Control
Exudate
Water spray
4.0 l.e. spray

encounters involved the same behaviours provoked by handling and probing. These behaviours usually included rearing, opening the mandibles, biting, and finally exudate production, causing the retreat of one of the larvae. Such encounters occurred between two wandering larvae, or between a wandering and an established larva.

While it would be metabolically expensive to produce exudate, particularly if it is a regurgitant, Berenbaum *et al.* (1993) have shown that a silken structure, such as a feeding tunnel, can be a valuable resource which is worth defending. During the course of these experiments, several behaviours were observed which, to my knowledge, have not been reported previously (Table 1). Of particular interest is the frequency with which larvae were observed producing silk, expanding the tunnel, combing the tunnel walls and removing frass from the tunnel, supporting the hypothesis that the tunnel is a resource worth maintaining and defending from competitors. The habit of pulling an excised needle into the tunnel for feeding, rather than leaving the tunnel to feed, is further evidence of the tunnel's protective function and value.

The differences between wild and diet-reared larvae in level of activity provoked by exposure to exudate (Fig. 2), could be explained by several hypotheses. The switching of larvae from artificial diet to foliage may have affected larval behaviour, or possibly the quality of the regurgitant. The quality of the foliage resource may have been low in Exp. 2, because the buds were forced to flush prematurely under greenhouse conditions. Insects in Exp. 2 were exposed to minimal air currents and observer-induced disturbances. Possibly, the presence of exudate increases larval sensitivity to such disturbances, hence the greater response in Exp. 1. The 6-8 h delay in induction of dispersal provoked by exposure to the exudate (Fig. 3) may reside either in an alteration of the exudate over time, *e.g.* through oxidation of the bioactive components, or in a latent behavioural threshold being reached.

Although the results of Exp. 3 showed no significant effect of the sprayed exudate, a trend towards increasing dispersal was evident at the highest concentration (Fig. 4). Because much of the spray was lost during application, the larvae were probably exposed to much less than 2 larval equivalents of exudate, and possibly even less than 1 larval equivalent. This suggested that higher concentrations might be effective, and this was confirmed in the subsequent experiment. Larval dispersal was significantly increased by the concentrated spray administering four larval equivalents per treatment (Fig. 4).

The capacity for inducing larval dispersal in these experiments (Figs. 3, 4) suggests that it may be possible to induce larval dispersal through the application of oral exudate, or its active components, to trees. The development of the active constituents in the oral exudate of spruce budworms could lead to an effective pest management tool, either used alone to induce larval dispersal or as a bioirritant to increase exposure to low doses of a conventional insecticide. Before this work can be done, however, an effective laboratory bioassay must be developed, which can be used to assess the repellency of the oral exudate and its components.

4.0 THE REPELLENCY OF LARVAL ORAL EXUDATE

4.1 INTRODUCTION

The experiments described in Section 3.0 demonstrated that the presence of larval oral exudate affects the behaviour of conspecific larvae. However, they provided no understanding of the mechanism which altered behaviour. The most obvious hypothesis is that the exudate functioned as a repellent. To investigate the possible repellency further, an effective laboratory bioassay procedure was required. Therefore, the first objective of this portion of the study was to develop such a bioassay. Subsequent objectives were to determine the specificity of the exudate between the two *Choristoneura* species, and to determine whether an individual larva could "recognize" whether exudate came from itself or another larva. If the exudate is a conspecific repellent, a mechanism must exist to prevent an individual larva from repelling itself as it repels its competitors. It is possible that a larva can differentiate between its own exudate and the exudate of other larvae, but this does not seem plausible. More likely, the mechanism involves some form of sensory adaptation or habituation, which renders the exudate-producing larva unresponsive for a period of time. Proprioception of the partially empty gut may also be important if the exudate is a regurgitant, as seems probable. These hypotheses must be tested before definitive conclusions can be drawn about the role of larval oral exudate in territorial defense.

4.2 MATERIALS AND METHODS

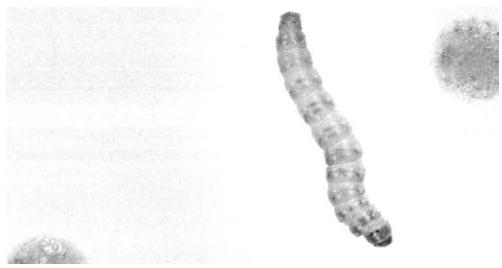
4.2.1 Bioassay development

To test the repellent effect of larval oral exudate, two drops of molten artificial diet (approximately 0.5 cm in diameter) were placed in the bottom of a 100 X 15 mm disposable petri dish, and allowed to solidify. In all bioassays, the petri dishes were inverted so that the diet feeding stations were on the "ceiling" of the petri dish chamber (Fig. 5).

In Exp. 1 and 2, the two feeding stations were placed 3 cm apart, edge to edge, in each dish. In Exp. 3 and 4, the two feeding stations were separated by only 1.0 cm. Each dish for Exp. 5 and 6 contained a single feeding station, placed at the centre of the chamber. Oral exudate was collected from diet-reared larval *C. fumiferana* (Exp. 1, 3 and 5) or *C. occidentalis* (Exp. 2, 4 and 6) by touching the head with a 5 μL micropipette. As much exudate as could be collected from two larvae was used to coat one of the two feeding stations in each dish in Exp. 1-4, and the single station in Exp. 5 and 6. Separate dishes, with one station treated with 5 μL of distilled water, served as controls.

A conspecific third to fifth instar diet-reared larva was then placed on moist filter paper on the floor of each chamber, and exposed to the feeding stations by placing the roof on the chamber. Test larvae which produced exudate while being transferred to the chamber were not used in the experiments. The larvae were left for 24 h under the above rearing conditions, and their final positions were then recorded. Any evidence of feeding or establishment on a particular station, such as feeding cavities, deposited silk, silk feeding tunnels or frass, was recorded. Larvae which did not establish on a feeding station were included in the sample, unless they had moulted or pupated over the 24 h test period. Larvae were, therefore,

Fig. 5. Bioassay chamber developed to test the bioactivity of larval oral exudate. Chamber consisted of a 100 X 15 mm disposable petri dish with two drops of artificial diet in the bottom of the inverted dish and a piece of moist filter paper in the lid. One of the artificial diet feeding stations was treated with exudate, and a single larva was confined in the chamber for 24 h.



categorized as not feeding, feeding on the untreated station, or feeding on the treated station.

Each experiment consisted of 20 experimental and 20 control replicates. The numbers of larvae in the three categories were compared between experimental and control dishes using Fisher's Exact Test for a 2 X 3 contingency table (Steel and Torrie, 1980; Schlotzhauer and Littell, 1987).

4.2.2 Interspecific repellency

In Exp. 7 and 8, third to fifth instar larvae of both species were tested in petri dish bioassays as described in Exp. 1. Pooled exudate from two larvae of one species was used to treat one of the stations in each dish. Larvae of the other species were then used as test insects. Control dishes were also set up, with one station treated with 5 μL of distilled water. There were 20 experimental and 20 control dishes for each species. Test larvae were left in the dishes for 24 h, and the results were assessed and analysed as in Exp. 1.

4.2.3 Self-recognition

Bioassay dishes for Exp. 9 were constructed as described in Exp. 1, except that the filter paper was omitted and the dishes were not inverted. Feeding stations were kept on the floor of the chamber to allow small larvae to reach them more easily. Both exudate donors and test insects were third to fifth instar, diet-reared *C. occidentalis* larvae. Dishes were divided among four treatments. In the control treatment, one station in each dish was treated with 2 μL of distilled water, and the test larva was not induced to produce exudate prior to the start of the experiment. In the first experimental treatment, one station was treated with 2 μL of conspecific exudate, and the test larva was not induced. Test larvae were induced to produce exudate in experimental treatments 2 and 3; one station in each dish received 2 μL

of exudate which was produced by either the test larva (treatment 2) or other, conspecific larvae (treatment 3). Test larvae were placed in all dishes immediately following treatment of the feeding stations. Each of the four treatments was replicated 40 times.

This experiment was repeated (Exp. 10) with dishes which had been treated and left on a bench for 24 h before test larvae were introduced. Test larvae were selected at the time of treatment and placed in individual vials of artificial diet for 24 h, then placed in the bioassay dishes. Each of the four treatments was replicated 40 times.

Because the exudate is most likely a regurgitant, the food that the larvae consume may have a significant impact on the repellency of the exudate and the responsiveness of the test larvae. Therefore, Exp. 9 and 10 were repeated using foliage-reared *C. occidentalis* larvae (Exp. 11 and 12).

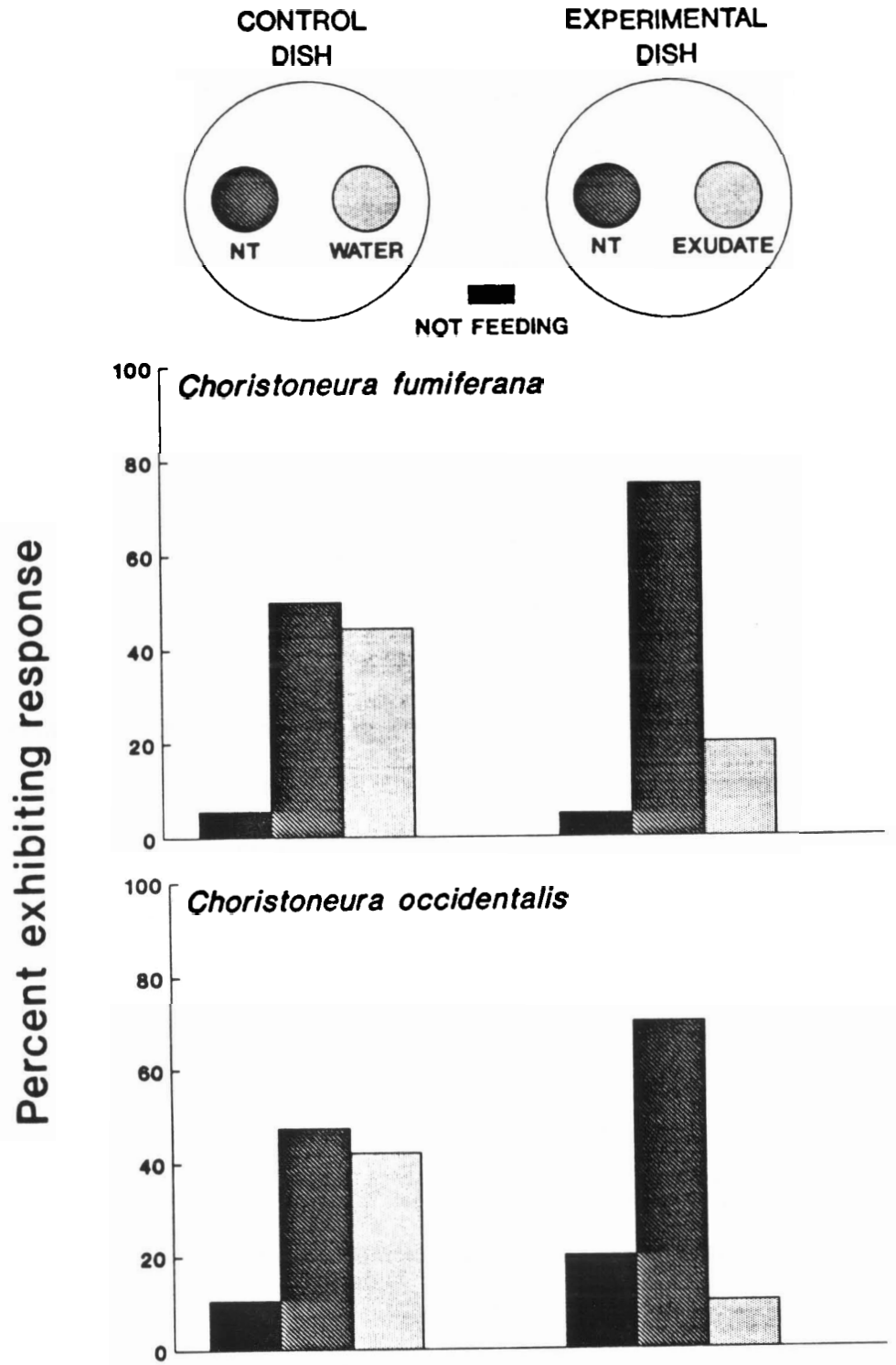
All test larvae from Exp. 9-12 were left in the bioassay dishes for 24 h. The results were then assessed and analysed as for Exp. 1.

4.3 RESULTS

4.3.1 Bioassay development

When the two feeding stations were separated by 3 cm (Exp. 1 and 2), the numbers of larvae in the three categories (not feeding, feeding on the untreated station, or feeding on the treated station) were significantly different between experimental and control dishes for both *C. fumiferana* and *C. occidentalis* (Fig. 6). In experimental dishes, most larvae fed on the untreated stations, while in the control dishes there were approximately equal numbers feeding on the water-treated stations and the untreated stations.

Fig. 6. Percentage of diet-reared *C. fumiferana* (Exp. 1) or *C. occidentalis* (Exp. 2) larvae not feeding, or feeding on one of two diet stations with centres separated by 3 cm. Circular schematic diagrams indicate placement of diet stations (not to scale) in petri dishes, with shading of treatment corresponding to the percent response bars below. Control dish (left) contains an untreated diet station (not treated = NT) paired with one treated with distilled water. Experimental dish (right) contains an untreated diet station (NT) paired with one treated with conspecific larval oral exudate. n=20. For both species, control and experimental distributions are significantly different, Fisher's Exact Test, P=0.034 and P=0.029, respectively.


CONTROL DISH
EXPERIMENTAL DISH
NT
WATER
NT
EXUDATE
NOT FEEDING
Percent exhibiting response
Choristoneura fumiferana
Choristoneura occidentalis
100
80
60
40
20
0

When the two feeding stations were separated by 1 cm, in Exp. 3 and 4, the numbers of larvae in the three categories were again significantly different between the experimental and control dishes for both species (Fig. 7). As in Exp. 1 and 2, very few larvae fed on the exudate-treated station. However, 50 and 75% of *C. fumiferana* and *C. occidentalis*, respectively, did not feed at all on either station in the experimental dishes. There was relatively equal feeding on untreated and water-treated stations in control dishes.

When only one feeding station, treated with conspecific oral exudate, was available (Exp. 5 and 6), 65 and 90% of *C. fumiferana* and *C. occidentalis*, respectively, did not feed at all (Fig. 8). Approximately 90% of all larvae of both species fed on the water-treated station in control dishes (Fig. 8).

4.3.2 Interspecific repellency

The results of Exp. 7 and 8 (Fig. 9) were very similar to those of Exp. 1 and 2 (Fig. 6), indicating reciprocal activity of larval oral exudate between the two *Choristoneura* species. The effect of *C. fumiferana* exudate on *C. occidentalis* larvae was particularly strong; no *C. occidentalis* larvae fed on diet stations treated with *C. fumiferana* exudate, and 45% did not feed at all.

4.3.3 Self-recognition

In Exp. 9 (Fig. 10), with no delay in exposure to treated diet stations, the numbers of larvae in the three categories were significantly different from the control dishes in only one case. When one station was treated with 2 μL of oral exudate from other larvae, and the test larva had not been induced to produce exudate, most test larvae fed on the untreated station. There was no significant difference between the treatment groups which used induced test larvae and the control group, whether the exudate was from the test larva (Fig. 10, experimental

Fig. 7. Percentage of diet-reared *C. fumiferana* (Exp. 3) or *C. occidentalis* (Exp. 4) larvae not feeding, or feeding on one of two diet stations with centres separated by 1 cm. Circular schematic diagrams indicate placement of diet stations (not to scale) in petri dishes, with shading of treatment corresponding to the percent response bars below. Control dish (left) contains an untreated diet station (NT) paired with one treated with distilled water. Experimental dish (right) contains an untreated diet station (NT) paired with one treated with conspecific larval oral exudate. n=20. For both species, control and experimental distributions are significantly different, Fisher's Exact Test, $P=0.026$ and $P<0.0001$, respectively.

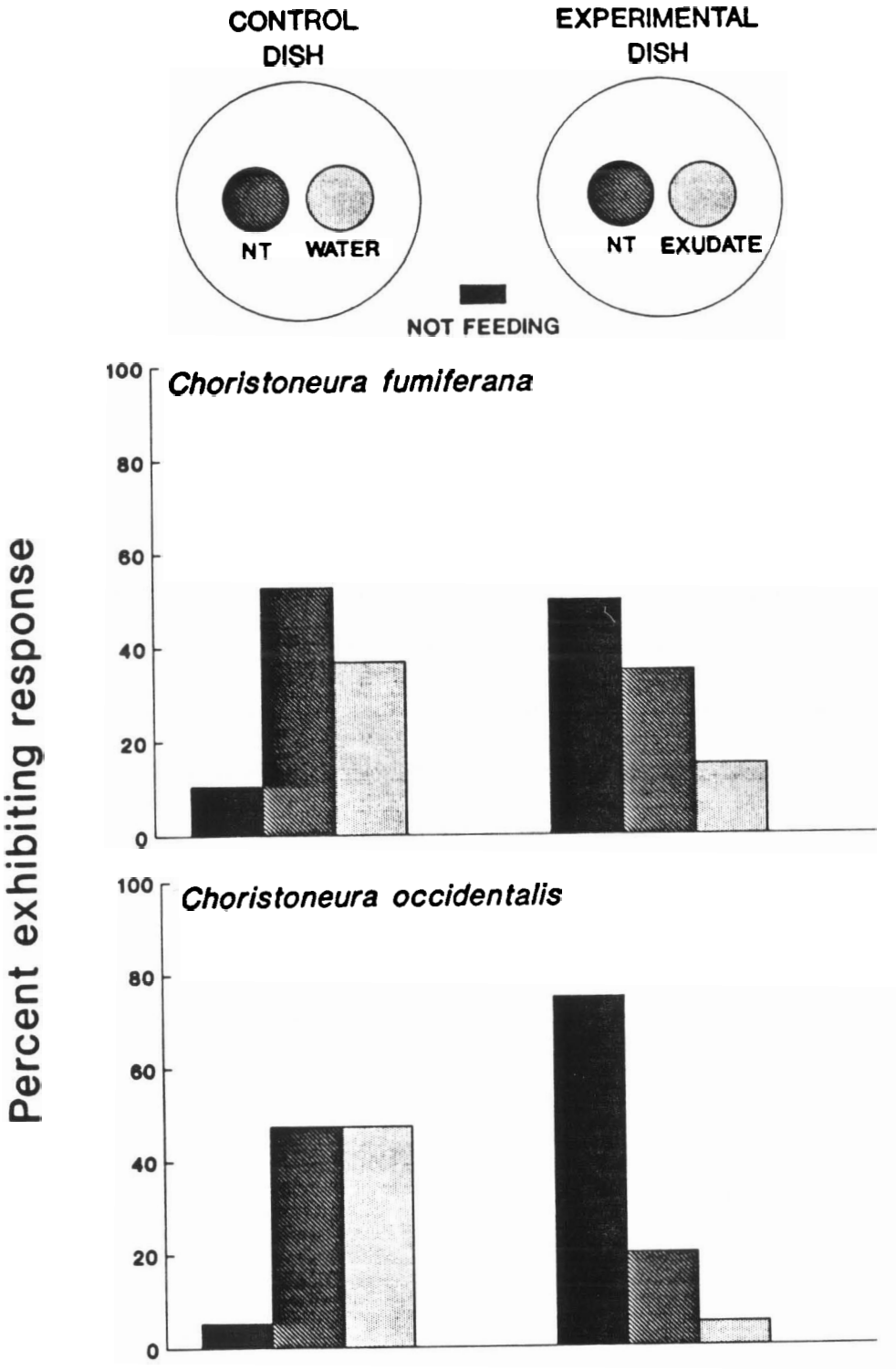

CONTROL DISH
EXPERIMENTAL DISH
NT
WATER
NT
EXUDATE
NOT FEEDING
Choristoneura fumiferana
Choristoneura occidentalis
Percent exhibiting response
100
80
60
40
20
0

Fig. 8. Percentage of diet-reared *C. fumiferana* (Exp. 5) or *C. occidentalis* (Exp. 6) larvae not feeding, or feeding on single diet stations. Circular schematic diagrams indicate placement of diet stations (not to scale) in petri dishes, with shading of treatment corresponding to the percent response bars below. Control dish (left) contains diet station treated with distilled water. Experimental dish (right) contains diet station treated with conspecific larval oral exudate. n=20. For both species, control and experimental distributions are significantly different, Fisher's Exact Test, $P<0.001$ and $P<0.0001$, respectively.

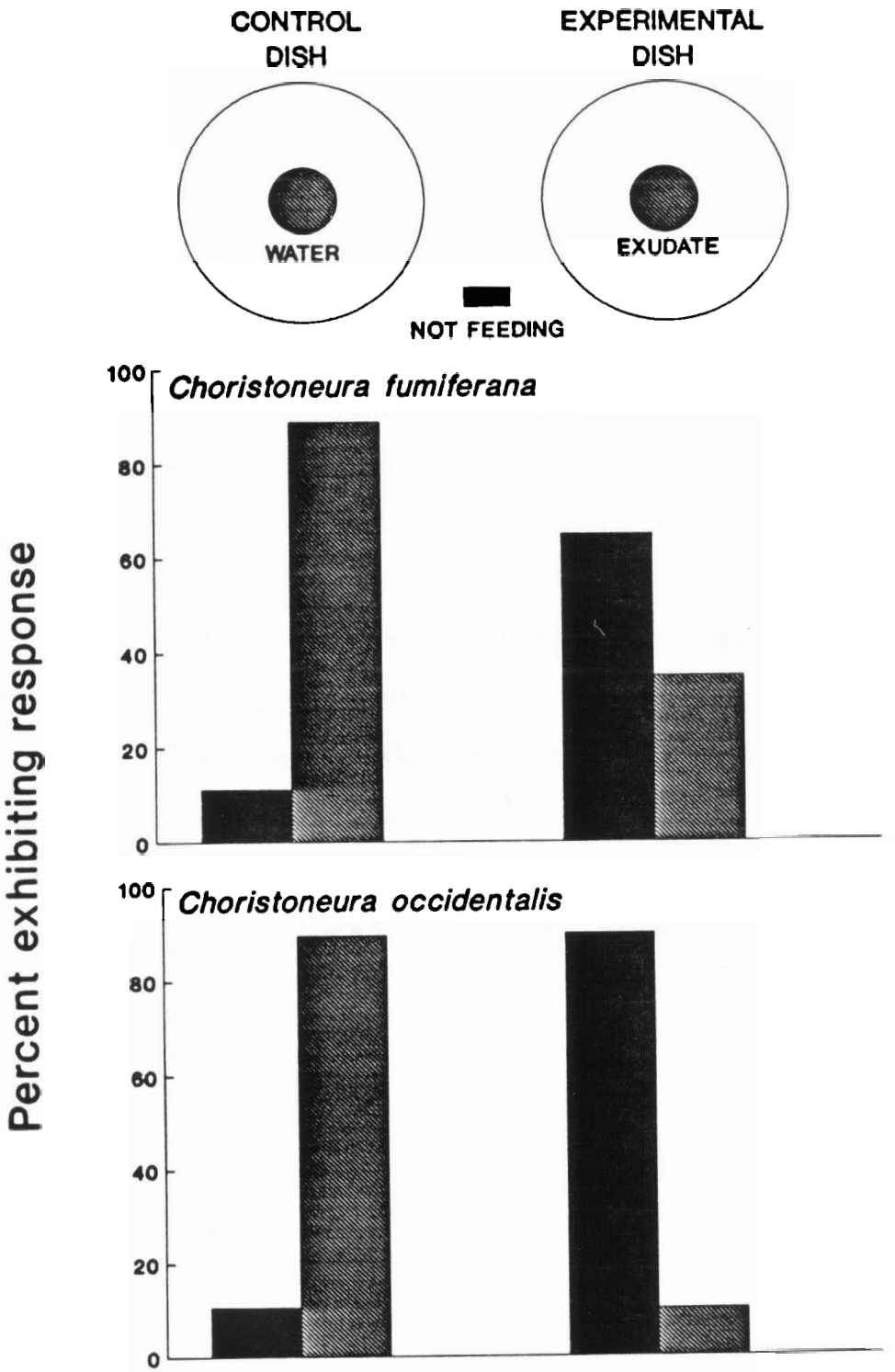

CONTROL DISH
EXPERIMENTAL DISH
WATER
EXUDATE
NOT FEEDING
Choristoneura fumiferana
Choristoneura occidentalis
Percent exhibiting response
100
80
60
40
20
0

Fig. 9. Percentage of diet-reared *C. fumiferana* (Exp. 7) or *C. occidentalis* (Exp. 8) larvae in interspecific bioassays not feeding, or feeding on one of two diet stations with centres separated by 3 cm. Circular schematic diagrams indicate placement of diet stations (not to scale) in petri dishes, with shading of treatment corresponding to the percent response bars below. Control dish (left) contains an untreated diet station (NT) paired with one treated with distilled water. Experimental dish (right) contains an untreated diet station (NT) paired with one treated with larval oral exudate from the other species. n=20. For both species, control and experimental distributions are significantly different, Fisher's Exact Test, $P=0.042$ and $P<0.003$, respectively.

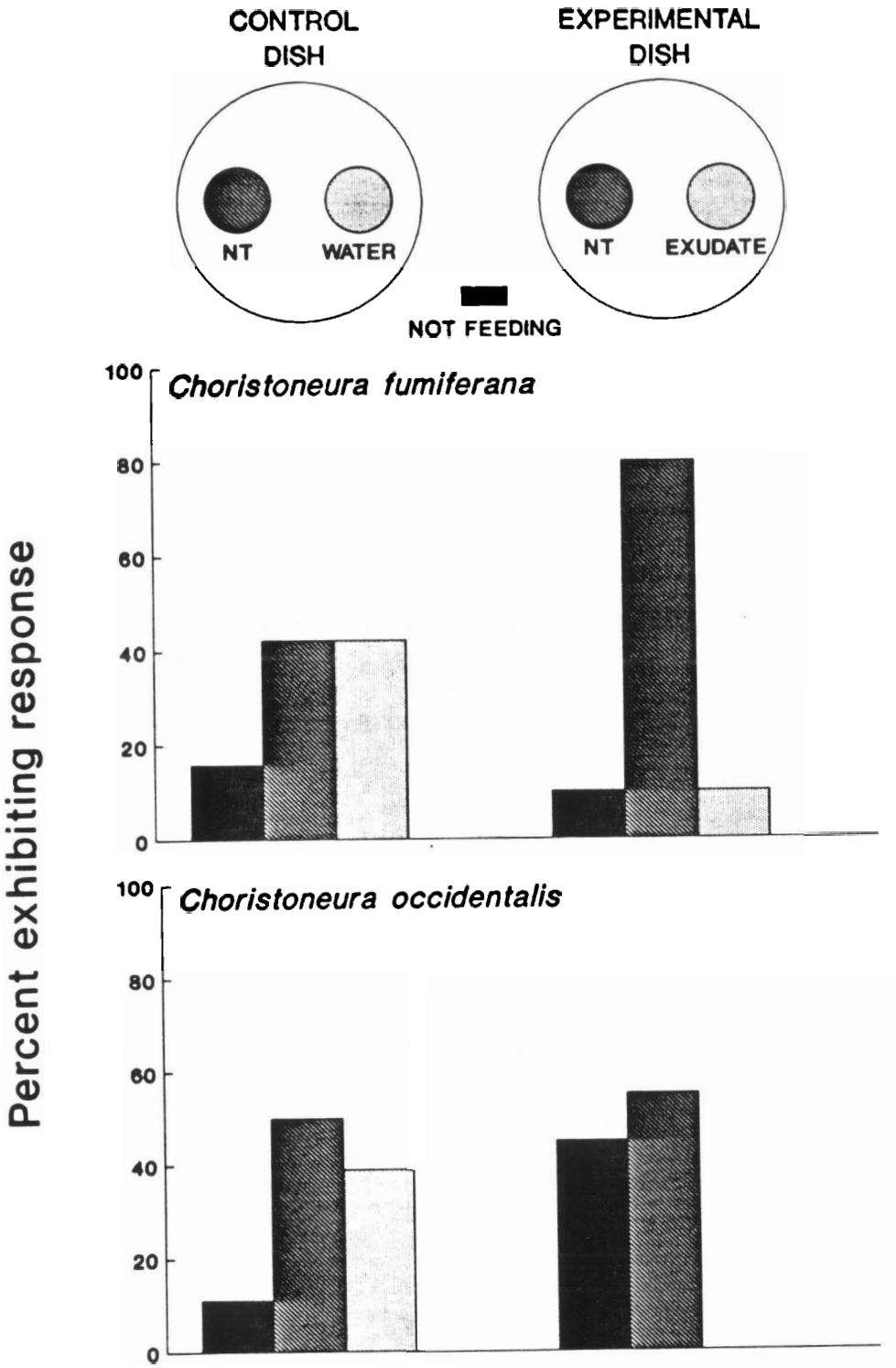
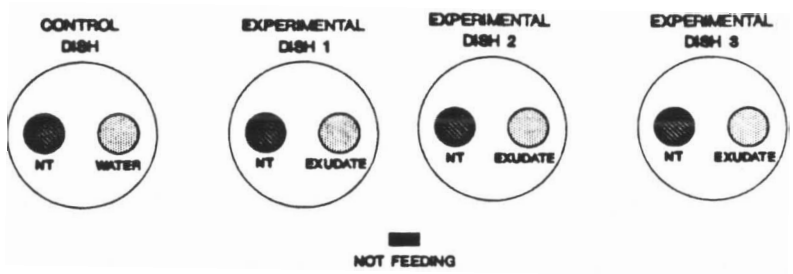
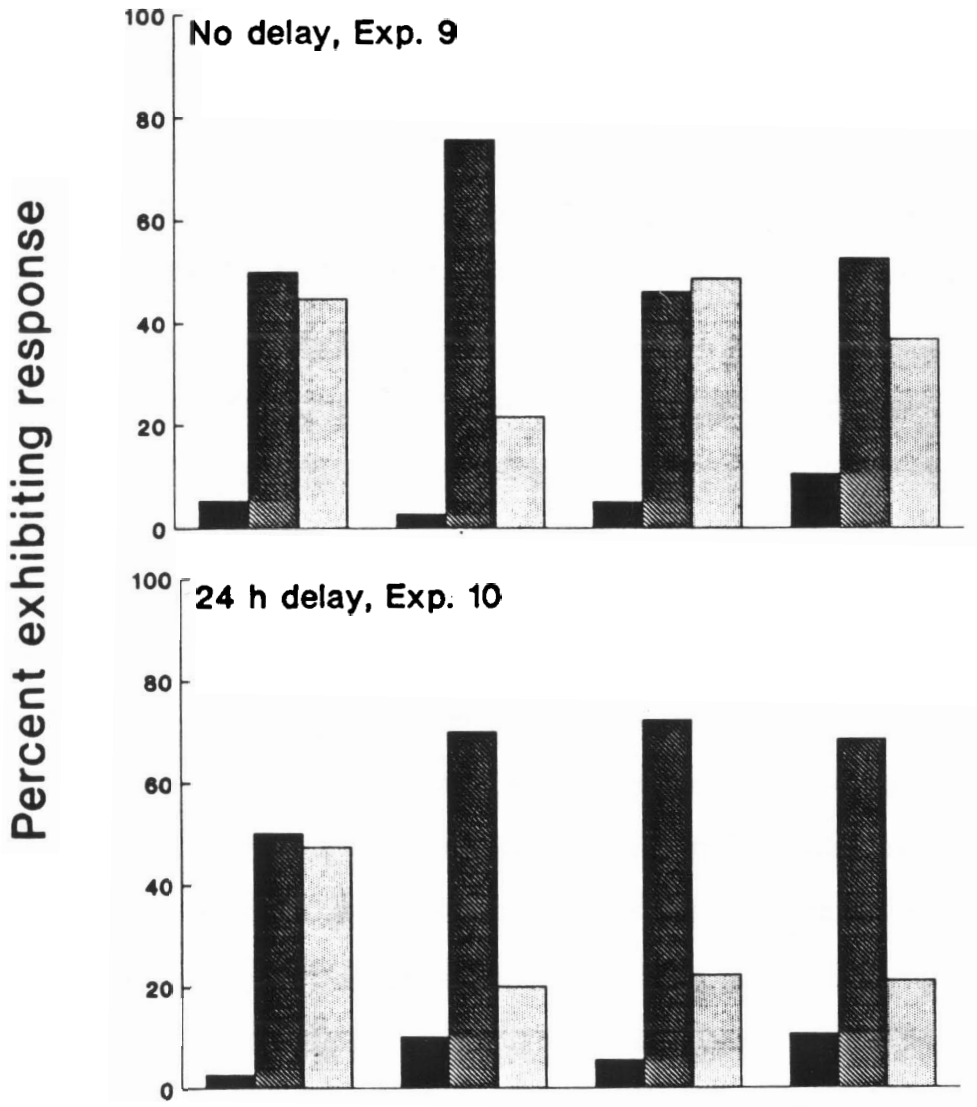

CONTROL DISH
EXPERIMENTAL DISH
NT
WATER
NT
EXUDATE
NOT FEEDING
Choristoneura fumiferana
Choristoneura occidentalis
Percent exhibiting response
100
80
60
40
20
0

Fig. 10. Percentage of diet-reared *C. occidentalis* larvae not feeding, or feeding on one of two diet stations with centres separated by 3 cm, either 0 or 24 h after treatment of stations and induction of exudate production. Circular schematic diagrams indicate placement of diet stations (not to scale) in petri dishes, with shading of treatment corresponding to the percent response bars below. Control dish (left) contains an untreated diet station (NT) paired with one treated with distilled water. Experimental dishes (right) each contain an untreated diet station (NT) paired with one treated with conspecific larval oral exudate. In dish 1, exudate was from another individual and the test larva was not induced to produce exudate. In dish 2, exudate was from the test larva (test larva induced). In dish 3, exudate was from another individual and the test larva was induced. n=40. With no delay in introduction of test larva (Exp. 9), only the distribution in experimental dish 1 is significantly different from the control (Fisher's Exact Test, P=0.047, 0.929 and 0.612, respectively). Following a 24 h delay (Exp. 10), all experimental distributions are significantly different from the control (P=0.024, P=0.048, and P=0.035, respectively).


CONTROL DISH
NT
WATER
EXPERIMENTAL DISH 1
NT
EXUDATE
EXPERIMENTAL DISH 2
NT
EXUDATE
EXPERIMENTAL DISH 3
NT
EXUDATE
NOT FEEDING


Percent exhibiting response
No delay, Exp. 9
24 h delay, Exp. 10
100
80
60
40
20
0

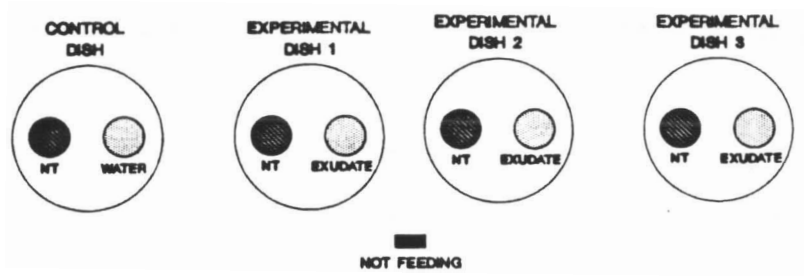
dish 2) or not (experimental dish 3). However, when larvae were placed in the dishes 24 h after the stations were treated (Exp. 10), all experimental treatments caused significant rejection of the treated feeding stations. Both induced and uninduced larvae fed on the untreated station most frequently, whether the exudate was from the test larva or not.

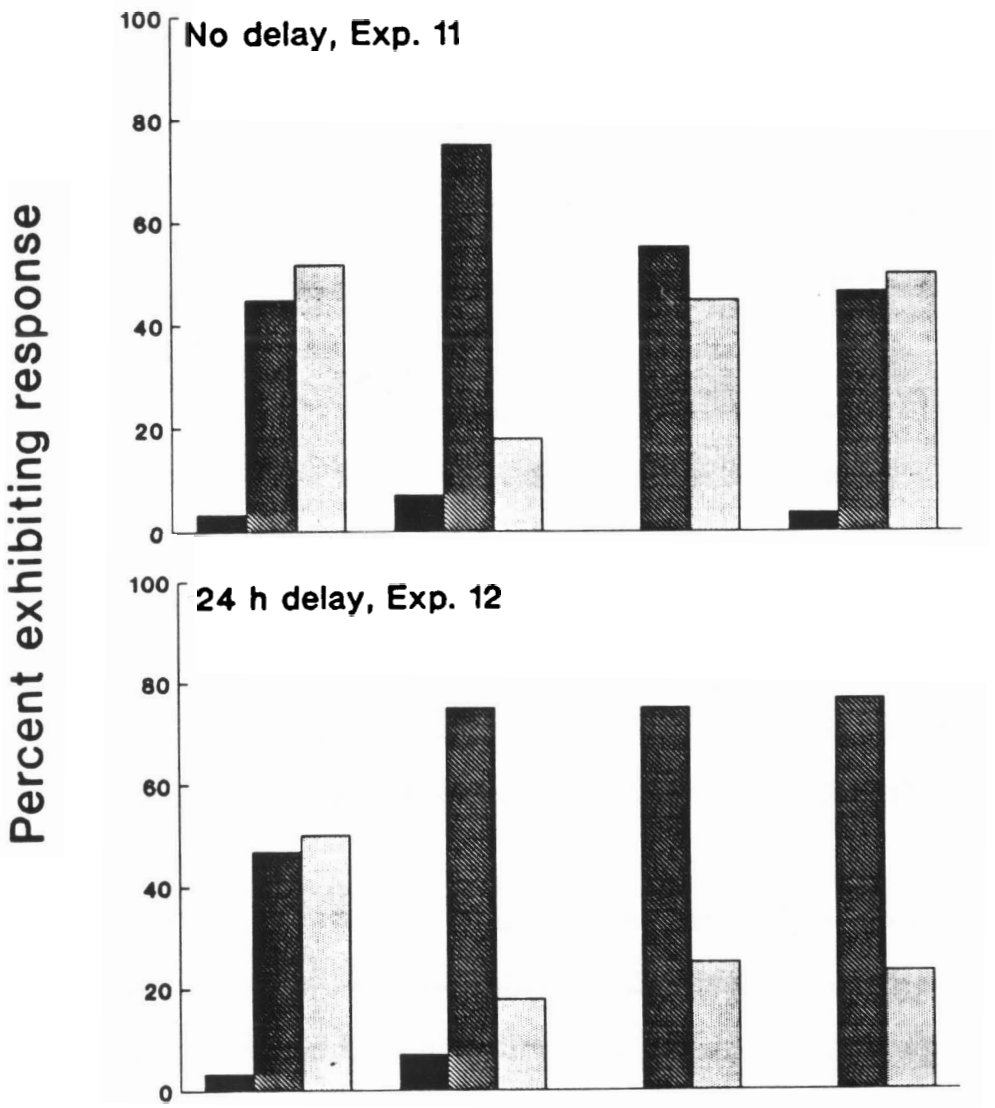
The results obtained when foliage-reared larvae were used in Exp. 11 and 12 (Fig. 11) were identical to those obtained for diet-reared larvae in Exp. 9 and 10 (Fig. 9).

4.4 DISCUSSION

The significant inhibition of feeding on stations treated with conspecific oral exudate when experimental and control stations were separated by 3 cm (Fig. 6), indicates that the oral exudate acts as a feeding deterrent. The inhibition of feeding on both treated and untreated stations when the stations were 1 cm apart (Fig. 7), suggests that the larval oral exudate also acts as a volatile repellent. Alternatively, larvae may be repelled by contacting the nearby treated station while attempting to establish on the untreated station. Partial repellency and feeding deterrency might also occur in *E. kuehniella*, causing the increased larval development times and lowered pupal weights observed by Corbet (1971). The power of the repellency and feeding deterrency of spruce budworm oral exudate is demonstrated by the high numbers of non-feeding larvae when presented with no choice other than a treated diet station (Fig. 8). However, these experiments used relatively large quantities of exudate, approximately 4-5 larval equivalents per treated station (Section 3.0), and the response may be correspondingly greater than might be expected under normal field conditions. As well, larvae were kept in a small chamber throughout the experiment, and were not given the opportunity to disperse away from the exudate.

Fig. 11. Percentage of foliage-reared *C. occidentalis* larvae not feeding, or feeding on one of two diet stations with centres separated by 3 cm, either 0 or 24 h after treatment of stations and induction of exudate production. Circular schematic diagrams indicate placement of diet stations (not to scale) in petri dishes, with shading of treatment corresponding to the percent response bars below. Control dish (left) contains an untreated diet station (NT) paired with one treated with distilled water. Experimental dishes (right) each contain an untreated diet station (NT) paired with one treated with conspecific larval oral exudate. In dish 1, exudate was from another individual and the test larva was not induced to produce exudate. In dish 2, exudate was from the test larva (test larva induced). In dish 3, exudate was from another individual and the test larva was induced. n=40. With no delay in introduction of test larva (Exp. 11), only the distribution in dish 1 is significantly different from the control (Fisher's Exact Test, P=0.032, 0.60 and 1.00, respectively). Following a 24 h delay (Exp. 12), all experimental distributions are significantly different from the control (P=0.025, P=0.043, and P=0.033, respectively).


CONTROL DISH
NT
WATER
EXPERIMENTAL DISH 1
NT
EXUDATE
EXPERIMENTAL DISH 2
NT
EXUDATE
EXPERIMENTAL DISH 3
NT
EXUDATE
NOT FEEDING


Percent exhibiting response
No delay, Exp. 11
24 h delay, Exp. 12
100
80
60
40
20
0

Dispersal has already been shown to be a common larval response to treatment with exudate (Figs. 2-4).

Choristoneura fumiferana and *C. occidentalis* larvae were reciprocally affected by exudate from larvae of the other species. Thus, the components in the exudate may be the same for both species. There would be no selection pressure for species-specific activity under field conditions, where larvae of the two species are found on different host trees and are allopatric (Harvey, 1985).

Immediately after being induced to produce oral exudate, larvae apparently entered a refractory phase, during which they did not respond to their own or other individuals' exudate (Figs. 9, 10). After 24 h, however, induced larvae had recovered their discriminating ability. The lack of response may be due to sensory habituation. Larvae may also be capable of removing exudate from themselves and their tunnels through grooming activity. Such mechanisms would enable larvae to use exudate to repel competitors from valuable resources without repelling themselves. Under field conditions, a refractory period of 24 h may be sufficient for most of the active components in the exudate to volatilize, degrade, or be washed away. However, in the laboratory, some larvae that had been induced to produce exudate in or at the mouths of their own feeding tunnels eventually abandoned the tunnels (Section 3.0). This dispersal may reflect a natural response in which the persistent occurrence of exudate (whether self- or otherwise-produced) could signal an overpopulated habitat.

Many avenues of investigation remain to be explored. These include determination of the role of natural host foliage in the production and potency of the exudate, the persistence and chemical nature of the exudate, and evaluation of the potential utility of the exudate or its components.

5.0 EFFECT OF COLONY SOURCE AND LARVAL DIET ON BIOACTIVITY OF ORAL EXUDATE

5.1 INTRODUCTION

In Section 3.0, differences were observed in the behavioural responses of larvae from different colonies to oral exudate. The differences were attributed in part to the rearing histories of the two colonies; the larvae used in Exp. 1 were reared on foliage throughout, while those used in Exp. 2 were reared on artificial diet and only transferred to foliage for the duration of the experiment. If the exudate is a regurgitant, it is likely that larval food could have a significant impact on the exudate's composition and bioactivity. Previous exposure to compounds in a food source might also alter the response thresholds of the larvae.

Larval oral exudate was found to be equally repellent to con- and heterospecific larvae in two-choice feeding bioassays (Section 4.0). However, in those experiments, insects were obtained from a laboratory colony which has been maintained at very high population densities for many generations. Since the oral exudate appears to act as a spacing mechanism, or epideictic pheromone (Prokopy, 1981), it is possible that selection during rearing might have diminished the ability of larvae to respond to the exudate.

The following experiments were undertaken to investigate the impact of colony source and rearing medium on the bioactivity of oral exudate in both *C. fumiferana* and *C. occidentalis*.

5.2 MATERIALS AND METHODS

5.2.1 Bioassay

Two drops of artificial diet, approximately 0.5 cm in diameter and separated by 3 cm, were used as feeding stations in the bottom of a 100 x 15 mm disposable plastic petri dish, as described in Section 4.0. A piece of moist filter paper was placed in the lid to help keep the diet moist and palatable, and the dish was inverted. Oral exudate was collected from two source larvae by touching the heads with a 5 μL micropipet, inducing them to produce exudate which was taken up in the pipet. The exudate was then used to coat one of the feeding stations in each dish. Separate dishes, with one station treated with 5 μL of distilled water, served as controls. A conspecific third to fifth instar test larva was then placed in the centre of the bottom of each petri dish chamber, and left undisturbed for 24 h under the above rearing conditions. At the end of that time, the stations were checked for any sigs of feeding or establishment, and the final positions of the larvae were recorded. Larvae which did not establish on a feeding station were included in the sample, unless they had moulted or pupated over the 24 h test period. Larvae were thus categorized as not feeding, feeding on the untreated station, or feeding on the treated station.

5.2.2 Experiments 1 and 2

Laboratory-reared and wild-collected *C. occidentalis* were tested for their responsiveness to each others' oral exudate. A total of approximately 5 μL of oral exudate was obtained from two larvae of the laboratory colony, and used to treat one of the feeding stations in each dish in Exp. 1. A wild larva

was then used as a test insect. This procedure was repeated 20 times, and 20 control dishes were also set up, using distilled water as the feeding station treatment and wild larvae as the test insects. In the reciprocal experiment (Exp. 2), the same volume of oral exudate was collected from two wild larvae and used to treat a feeding station in each dish. In this case, a laboratory-reared larva was used as a test insect. This procedure was repeated 20 times, and 20 control dishes were also set up, using distilled water as the feeding station treatment and laboratory-reared larvae as the test insects.

For each experiment, the numbers of larvae in the three categories were compared between experimental and control dishes using Fisher's Exact Test for a 2 X 3 contingency table (Steel and Torrie, 1980; Schlotzhauer and Littell, 1987).

5.2.3 Experiments 3-10

The rearing procedures resulted in eight colonies, four of each species: laboratory insects reared on artificial diet or foliage, and wild insects reared on artificial diet or foliage. In eight experiments (Exp. 3-10), test insects from each colony were presented with oral exudate from conspecific larvae of each of the four colonies, using the procedure described in Exp. 1 above. Separate dishes, with one station treated with 5 μL of distilled water, served as controls for each of the colonies. Larval responses were categorized as in Exp. 1. The numbers of larvae in the three categories in each of the groups of experimental dishes were compared with the numbers in the control dishes for the same species and colony within each experiment, using Fisher's Exact Test.

5.3 RESULTS

5.3.1 Experiments 1 and 2

When one station was treated with oral exudate from wild-collected *C. occidentalis* larvae, the numbers of laboratory-reared larvae in the three categories (not feeding, feeding on the untreated station, or feeding on the treated station) were significantly different between experimental and control dishes (Fig. 12). However, wild-collected larvae exhibited almost identical responses (Fig. 12) when presented with a choice between an untreated station and one treated with distilled water, or a choice between an untreated station and one treated with oral exudate from laboratory-reared larvae.

5.3.2 Experiments 3-10

Diet-reared *C. fumiferana* larvae from both the laboratory (Exp. 3) and wild-collected (Exp. 4) colonies responded similarly to the four oral exudate sources (Fig. 13). In all cases, the numbers of test larvae feeding on the treated station were significantly lower than in the corresponding control dishes. When larvae from either of these two groups were presented with arenas in which one feeding station was treated with oral exudate from diet-reared larvae, many larvae fed on the untreated station. However, for arenas in which one station was treated with oral exudate from foliage-reared larvae, there was instead a corresponding increase in the numbers of larvae not feeding, suggesting a more extreme response. Results were comparable for diet-reared *C. occidentalis* larvae from both the laboratory (Exp. 5) and wild-collected (Exp. 6) colonies (Fig. 14).

Fig. 12. Percentage of laboratory-reared (Exp. 1) or wild-collected (Exp. 2) *C. occidentalis* larvae feeding on a treated or untreated station or not feeding. Larvae provided with two separated stations, one untreated and one treated with distilled water (Control), or one untreated and one treated with oral exudate of two larvae from the other source colony (Exudate). Significant differences from the control distribution found only for laboratory-reared larvae exposed to exudate from wild-collected larvae (Fisher's Exact Test, $P<0.0001$ and $P=1.0$, respectively).

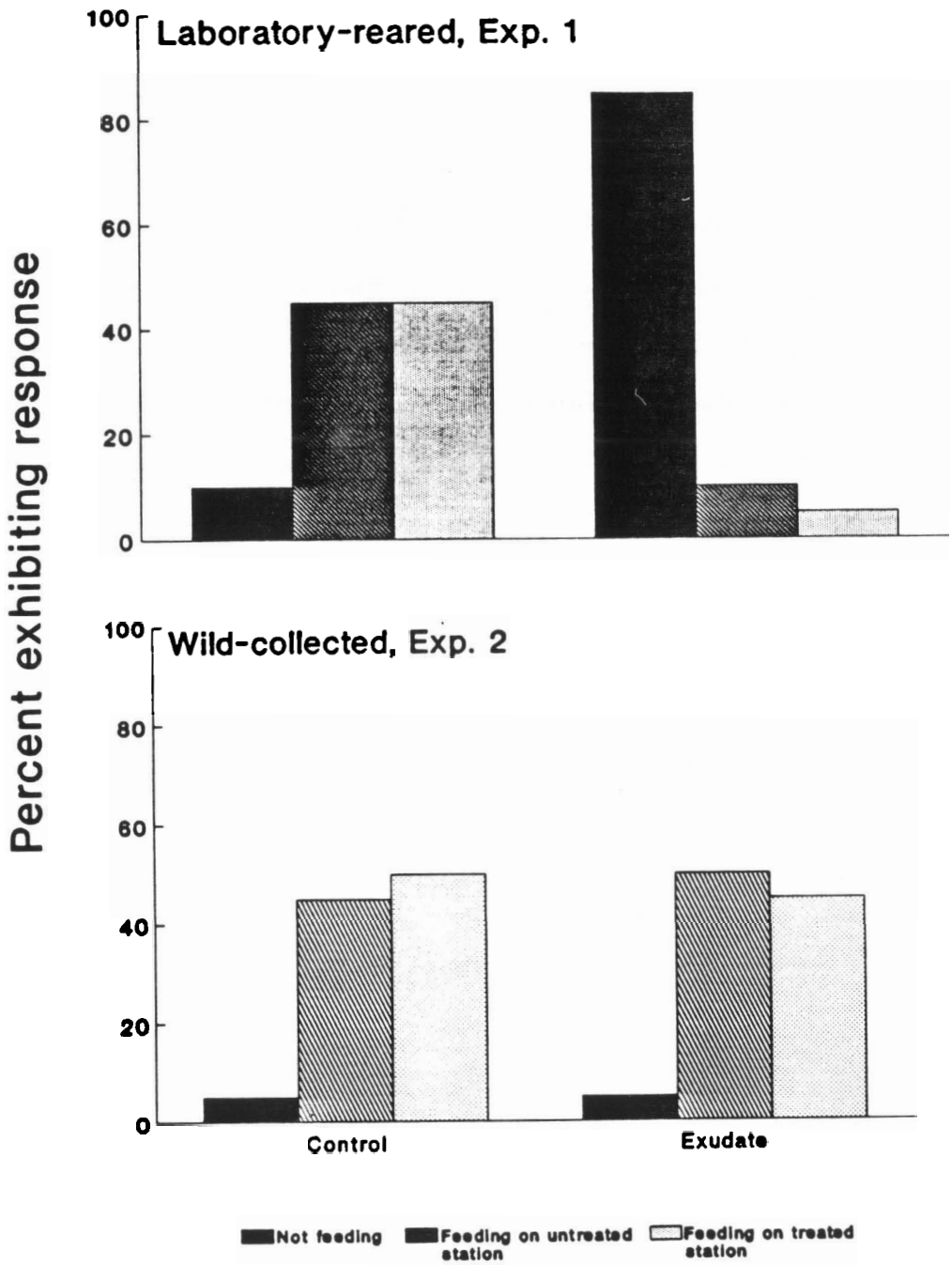

Percent exhibiting response
Laboratory-reared, Exp. 1
100
80
60
40
20
0
Wild-collected, Exp. 2
100
80
60
40
20
0
Control
Exudate
Not feeding
Feeding on untreated station
Feeding on treated station

Fig. 13. Percentage of artificial diet-fed *C. fumiferana* larvae from the laboratory population (Exp. 3), or the wild population (Exp. 4), feeding on a treated or untreated station or not feeding. Larvae provided with two separated stations, one untreated and one treated with distilled water (Control), or one untreated and one treated with oral exudate of two conspecific larvae from one of four source colonies. All distributions significantly different from their corresponding control distributions (Fisher's Exact Test, P=0.004, 0.003, 0.008 and 0.003, respectively, for laboratory-reared larvae; P=0.038, 0.020, 0.009 and 0.027, respectively, for wild-collected larvae).

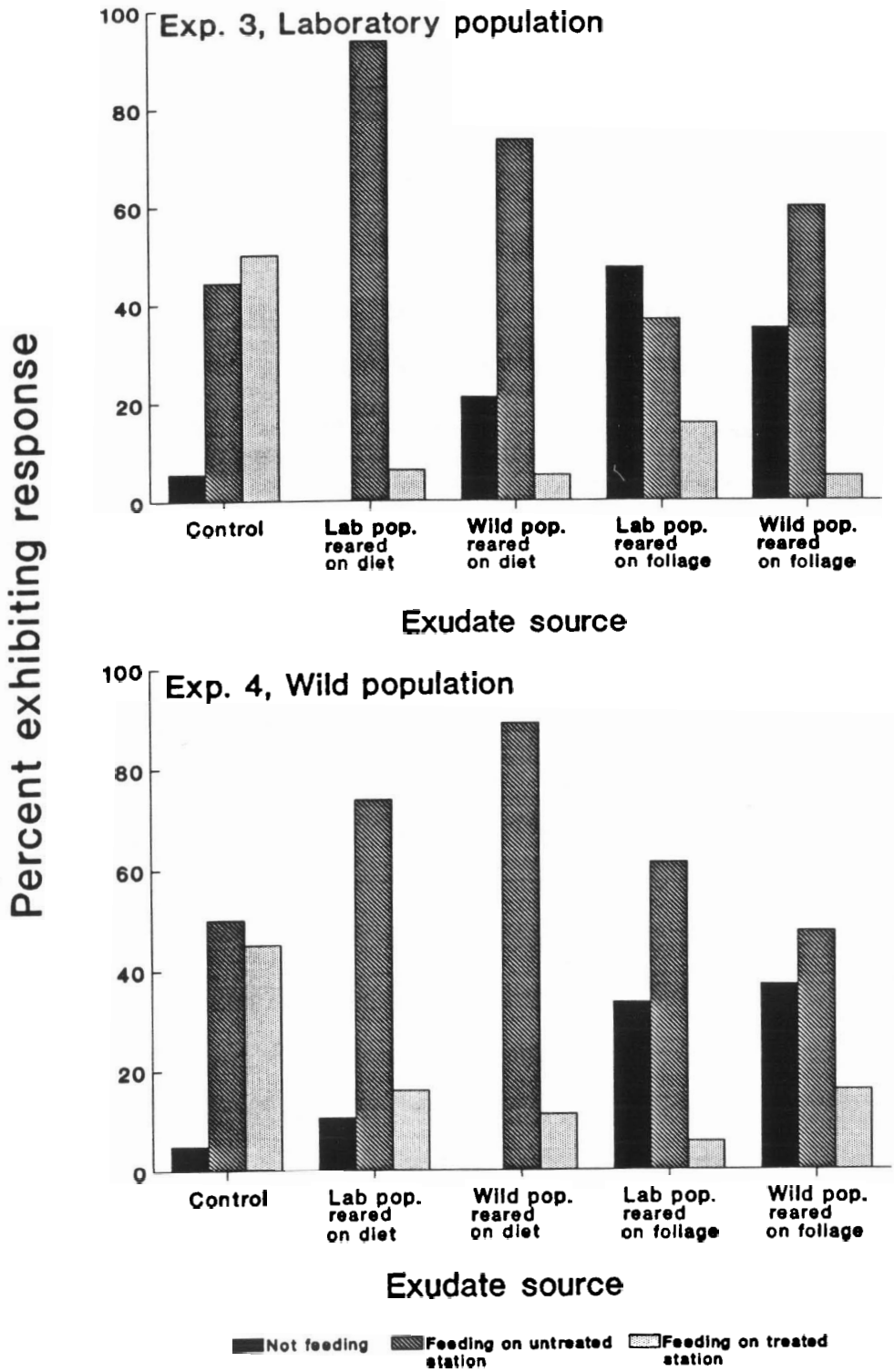
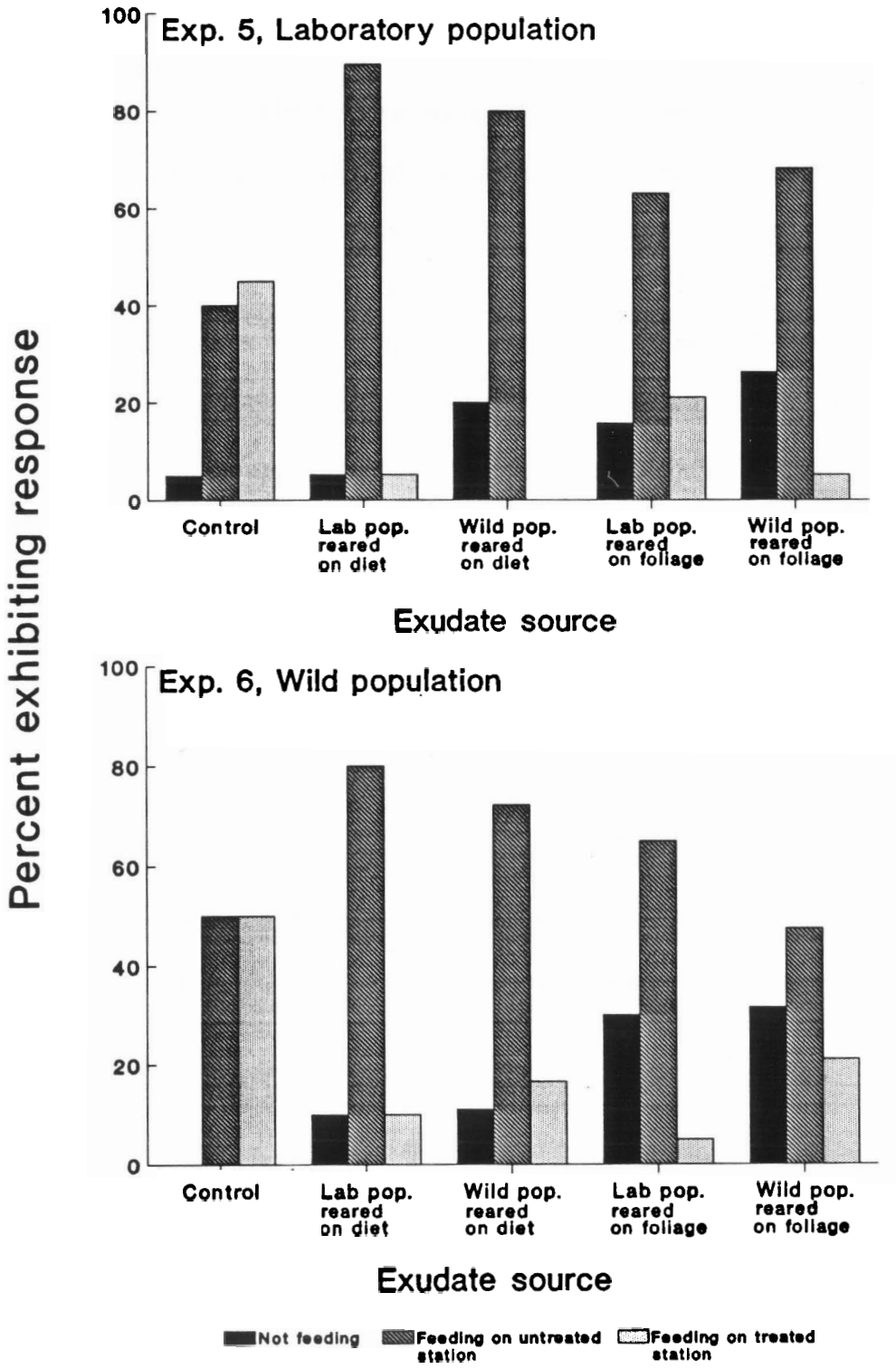

Exp. 3, Laboratory population
100
80
60
40
20
0
Control
Lab pop. reared on diet
Wild pop. reared on diet
Lab pop. reared on foliage
Wild pop. reared on foliage
Exudate source
Exp. 4, Wild population
100
80
60
40
20
0
Control
Lab pop. reared on diet
Wild pop. reared on diet
Lab pop. reared on foliage
Wild pop. reared on foliage
Exudate source
Percent exhibiting response
Not feeding
Feeding on untreated station
Feeding on treated station

Fig. 14. Percentage of artificial diet-fed *C. occidentalis* larvae from the laboratory population (Exp. 5), or the wild population (Exp. 6), feeding on a treated or untreated station or not feeding. Larvae provided with two separated stations, one untreated and one treated with distilled water (Control), or one untreated and one treated with oral exudate of two conspecific larvae from one of four source colonies. All distributions significantly different from their corresponding control distributions (Fisher's Exact Test, P=0.008, <0.001, 0.049 and 0.009, respectively, for laboratory-reared larvae; P=0.007, 0.035, <0.001, and 0.012, respectively, for wild-collected larvae).


Exp. 5, Laboratory population
100
80
60
40
20
0
Control
Lab pop. reared on diet
Wild pop. reared on diet
Lab pop. reared on foliage
Wild pop. reared on foliage
Exudate source
Exp. 6, Wild population
100
80
60
40
20
0
Control
Lab pop. reared on diet
Wild pop. reared on diet
Lab pop. reared on foliage
Wild pop. reared on foliage
Exudate source
Percent exhibiting response
Not feeding
Feeding on untreated station
Feeding on treated station

Foliage-reared *C. fumiferana* larvae from both the laboratory (Exp. 7) and wild-collected (Exp. 8) colonies responded similarly to the four oral exudate sources (Fig. 15). When larvae from either of these two groups were presented with arenas in which one feeding station was treated with oral exudate from diet-reared larvae, no significant difference was found from the control distribution. However, for arenas in which one station was treated with oral exudate from foliage-reared larvae, significantly fewer test larvae fed on the treated station than in the corresponding control dishes. This was accompanied by an increase in the numbers of larvae feeding on the untreated station. Results were comparable for foliage-reared *C. occidentalis* larvae from both the laboratory (Exp. 9) and wild-collected (Exp. 10) colonies (Fig. 16).

Because insects from both species and both colony sources responded identically to the various oral exudate sources, the results can be summarized on the basis of rearing medium alone (Fig. 17). Insects reared on artificial diet were repelled by oral exudate from both foliage-reared and diet-reared insects. However, insects reared on foliage were only repelled by oral exudate from other foliage-reared insects, and not by exudate from diet-reared insects. The most extreme response appears to be that of diet-reared insects to exudate from foliage-reared insects (Figs. 13, 14), because many did not feed on either station during the bioassay period.

5.4 DISCUSSION

The results from Exp. 1 and 2 (Fig. 12) appear to indicate that laboratory-reared larvae respond to oral exudate from wild-collected larvae, but that wild larvae do not respond to oral exudate from laboratory-reared

Fig. 15. Percentage of foliage-fed *C. fumiferana* larvae from the laboratory population (Exp. 7), or the wild population (Exp. 8), feeding on a treated or untreated station or not feeding. Larvae provided with two separated stations, one untreated and one treated with distilled water (Control), or one untreated and one treated with oral exudate of two conspecific larvae from one of four source colonies. Significant differences from corresponding control distributions found only for those larvae exposed to exudate from foliage-reared insects (Fisher's Exact Test, P=1.00, 1.00, 0.018 and 0.030, respectively, for laboratory-reared larvae; P=0.724, 0.629, 0.016 and 0.007, respectively, for wild-collected larvae).

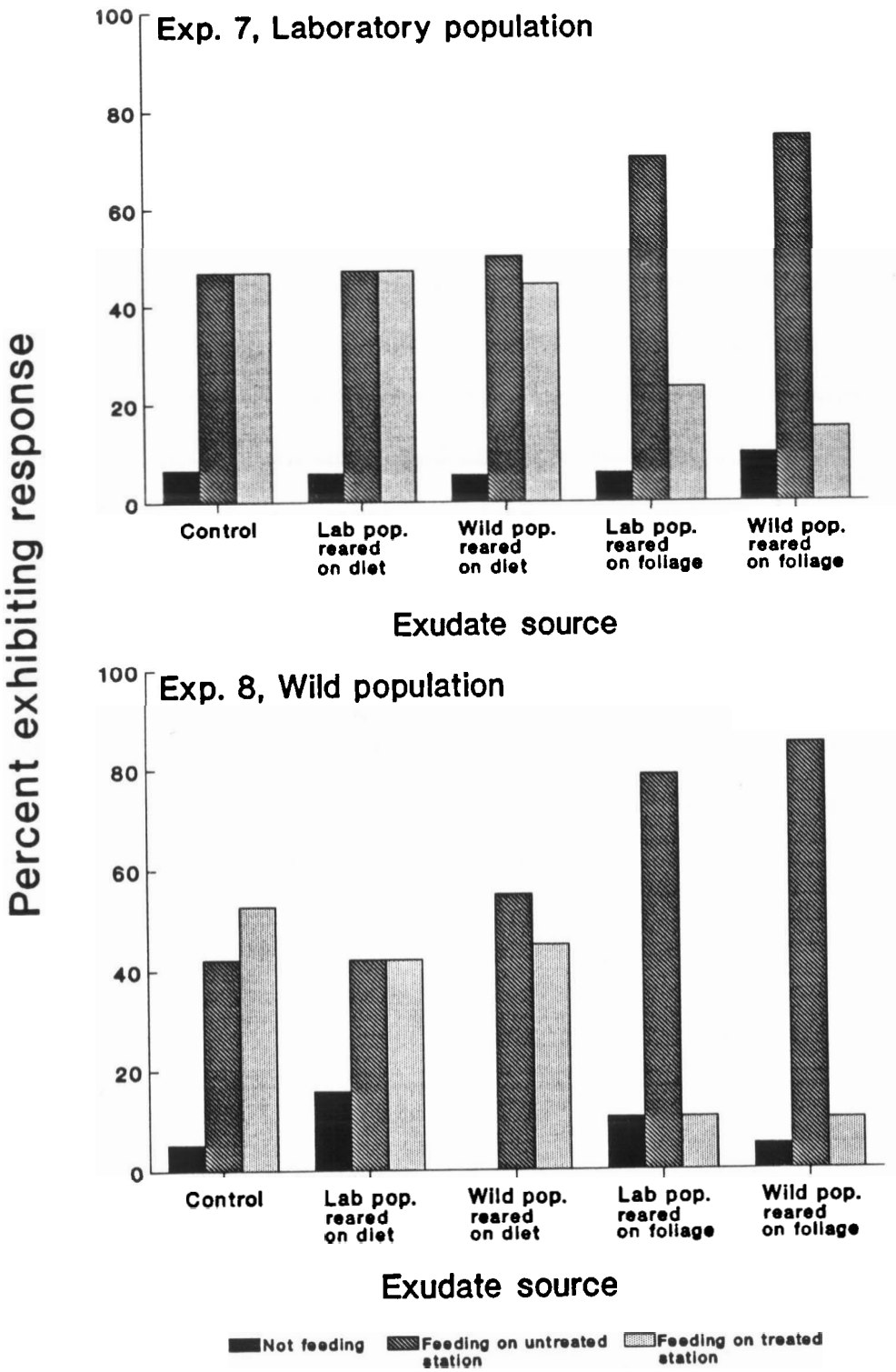

Exp. 7, Laboratory population
100
80
60
40
20
0
Control
Lab pop. reared on diet
Wild pop. reared on diet
Lab pop. reared on foliage
Wild pop. reared on foliage
Exudate source
Exp. 8, Wild population
100
80
60
40
20
0
Control
Lab pop. reared on diet
Wild pop. reared on diet
Lab pop. reared on foliage
Wild pop. reared on foliage
Exudate source
Percent exhibiting response
Not feeding
Feeding on untreated station
Feeding on treated station

Fig. 16. Percentage of foliage-fed *C. occidentalis* larvae from the laboratory population (Exp. 9), or the wild population (Exp. 10), feeding on a treated or untreated station or not feeding. Larvae provided with two separated stations, one untreated and one treated with distilled water (Control), or one untreated and one treated with oral exudate of two conspecific larvae from one of four source colonies. Significant differences from corresponding control distributions found only for those larvae exposed to exudate from foliage-reared insects (Fisher's Exact Test, P=1.00, 1.00, 0.027 and 0.007, respectively, for laboratory-reared larvae; P=0.328, 1.00, 0.010 and 0.002, respectively, for wild-collected larvae).

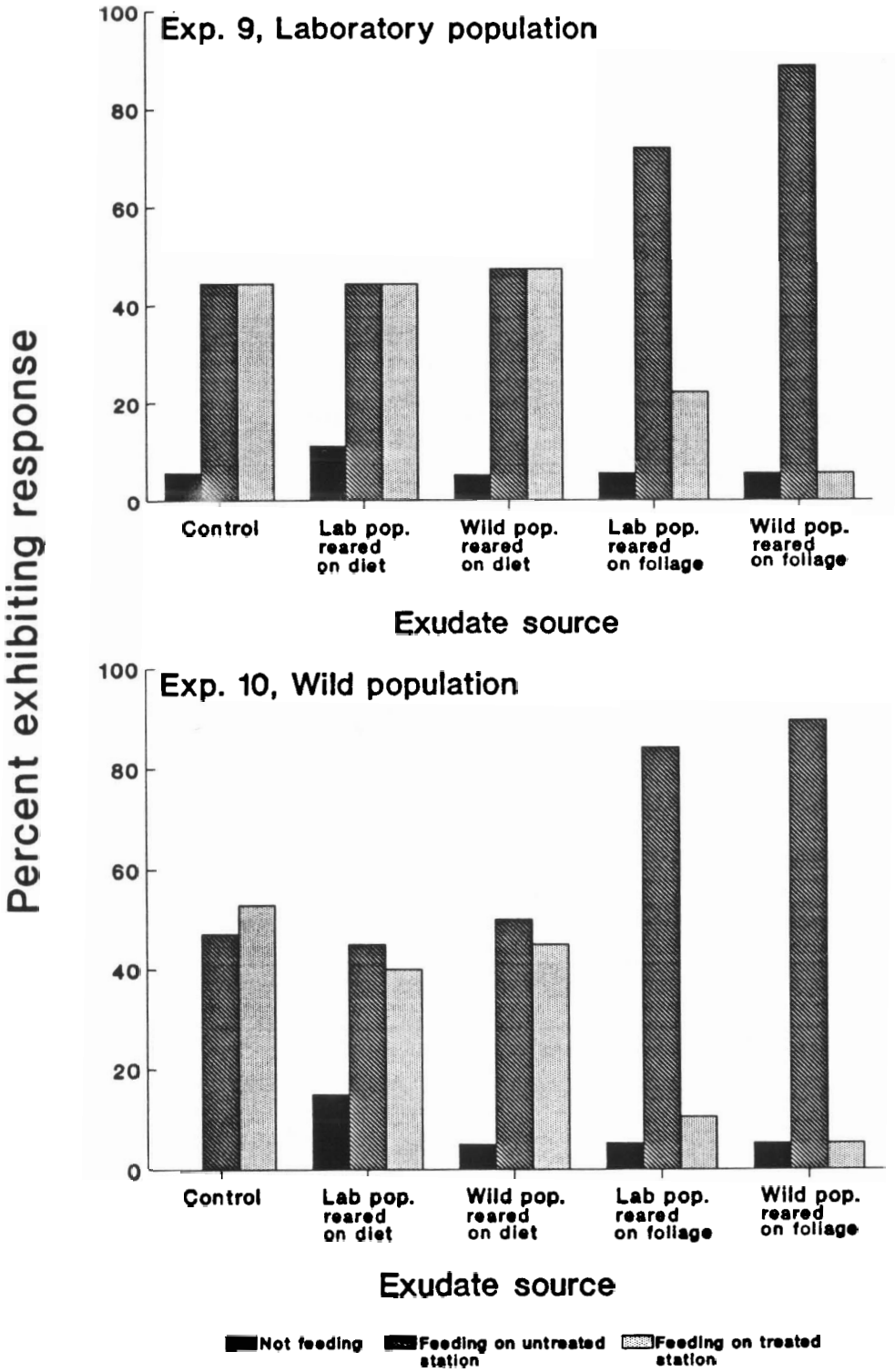

Exp. 9, Laboratory population
100
80
60
40
20
0
Control
Lab pop. reared on diet
Wild pop. reared on diet
Lab pop. reared on foliage
Wild pop. reared on foliage
Exudate source
Exp. 10, Wild population
100
80
60
40
20
0
Control
Lab pop. reared on diet
Wild pop. reared on diet
Lab pop. reared on foliage
Wild pop. reared on foliage
Exudate source
Percent exhibiting response
Not feeding
Feeding on untreated station
Feeding on treated station

Fig. 17. Summary of the sixteen combinations of test insect and oral exudate source used to determine the relative importance of rearing medium and colony source on the repellency of larval oral exudate. The same combinations were tested for both *C. fumiferana* and *C. occidentalis*, with the same results. Asterisks indicate a significant feeding deterrent effect of the source exudate (Fisher's Exact Test, $P<0.05$); NS indicates no significant effect.

SOURCE / TEST		DIET		FOLIAGE	
		LAB	WILD	LAB	WILD
DIET	LAB	*	*	*	*
	WILD	*	*	*	*
FOLIAGE	LAB	NS	NS	*	*
	WILD	NS	NS	*	*

larvae. However, Exp. 3-10 (Figs. 13-16) demonstrate that this is a direct result of differences in larval diet between the two colony sources.

The response in Exp. 7-10 by larvae reared on foliage only to oral exudate from foliage-reared insects, and not to oral exudate from diet-reared insects (Figs. 15, 16) indicates that there are differences in the composition of larval oral exudate from the two sources, or in the concentrations of biologically active constituents. Larval response may also depend, in part, on previous exposure to, and experience with, the repellent components.

The response of diet-reared larvae in Exp. 3-6 to oral exudate from foliage-reared larvae (Figs. 13, 14) appears to be more intense than the response of foliage-reared larvae in Exp. 7-10 to the same oral exudate (Figs. 14, 15). The latter consists of a preference for the untreated feeding station, while the former consists largely of a refusal to feed at all over the 24 h test period. This result suggests that the oral exudate from foliage-reared larvae contains more of the repellent components than the exudate from diet-reared larvae, or that it contains additional components, possibly derived from foliar constituents in the diet. It also suggests that the repellent from foliage-reared larvae is slightly volatile, lessening a larva's response to any food in the immediate vicinity of the exudate.

My results do not support the hypothesis that selection for diminished responses to the oral exudate occurred through many generations of crowded larval rearing on artificial diet. Rather, the ability to produce and respond to oral exudate is heritable and retained, but may be overridden by the need to feed when in crowded conditions in a rearing program. Under less crowded conditions, exposure to the exudate causes a significant portion of a population to disperse away from an occupied feeding habitat (Figs. 3, 4). A diet-derived repellent that acted on heterospecifics in the same genus

(Section 4.0), and possibly on insects in other genera, would not only provide a spacing mechanism for conspecific insects in high-density populations in nature, but would also mediate competition between unrelated species feeding on the same host, even when population densities were low.

6.0 QUALITATIVE ANALYSIS OF ORAL EXUDATE

6.1 INTRODUCTION

The preceding sections have provided insights into the role of oral exudate in the behavioural ecology of spruce budworms, and some of the factors which might affect its bioactivity. It may ultimately prove possible to exploit this activity to aid in the management of spruce budworms. However, after biologists have developed bioassay techniques and characterized the behaviour mediated by a pheromone, the course of research emphasis passes to chemists, who then isolate, identify and if necessary synthesize the active components (Brand *et al.*, 1979). This chemical research will be necessary to obtain sufficient quantities to conduct large-scale trials of the utility of these components in inducing larval dispersal. To facilitate achievement of this goal, the following experiments were conducted to determine some properties of the exudate.

It is possible that there may be more efficient techniques for acquiring large amounts of oral exudate than collecting from individual larvae, depending on the actual source of the exudate. To this point, most of the evidence suggests that the exudate is a regurgitant. However, salivary gland secretions or components of the silk might also be critical in repellency, and previous experiments have not excluded this possibility. The lower threshold of repellency must also be determined, preferably in terms of larval equivalents as defined in Section 3.0. Chemical analysis could be expedited by determining whether the active components are carried in the exudate in a suspension or a solution, because this knowledge will help to restrict the search to appropriate compounds. It is also important to learn more about the behaviour of the active components of the exudate in the laboratory, under a variety of conditions. For example, knowledge is

needed about the thermostability of the repellent, its persistence under bioassay conditions, and the length of time it may be stored without losing activity.

6.2 SOURCE OF EXUDATE

6.2.1 Materials and Methods

Bioassay dishes were constructed as described in Section 4.0, with both filter paper and dish inversion omitted. All insects used in this experiment were third to fifth instar diet-reared *C. occidentalis* larvae from the laboratory colony. Larvae were frozen in liquid nitrogen for approximately 1 min, then dissected while still frozen. The frozen contents of the foregut were separated from the surrounding gut tissues, removed intact, and placed in a glass vial. Only foregut contents were extracted, because they would be the first to be exuded when a larva regurgitated. A drop of distilled water was then applied to the preparation, and both of the labial, or silk, glands were removed and placed in a second glass vial. Oral exudate was collected from live larvae, using a 5 μL micropipet, and placed in a third glass vial. All three vials, each with the pooled material from 20 larvae, were stored at approximately -4°C for 3-4 days. This procedure was repeated several times, to obtain enough material to replicate the experiment sufficiently.

After the vials were removed from storage, 4 μL of distilled water per larval equivalent was added to each vial, and the contents were thoroughly mixed with a glass rod. This dilution or suspension in water was necessary to reduce the viscosity sufficiently to allow the preparations to be dispensed using a micropipet.

One of six treatments was applied to one feeding station in each dish, as follows: 2 μL of distilled water, 2 μL of freshly collected conspecific exudate, 2 μL of stored exudate, 2 μL of the foregut preparation, 2 μL of the labial gland preparation, or 2 μL of a preparation containing equal volumes of the labial gland and foregut preparations. Due to the dilution, therefore, the stimulus consisted of

0.5 larval equivalents, as defined in Section 3.0, in each treatment. Forty dishes (replicates) of each of these treatments were used. An uninduced test larva was placed in each dish, and left undisturbed for 24 h. The results were then assessed and analyzed as described in Section 4.0.

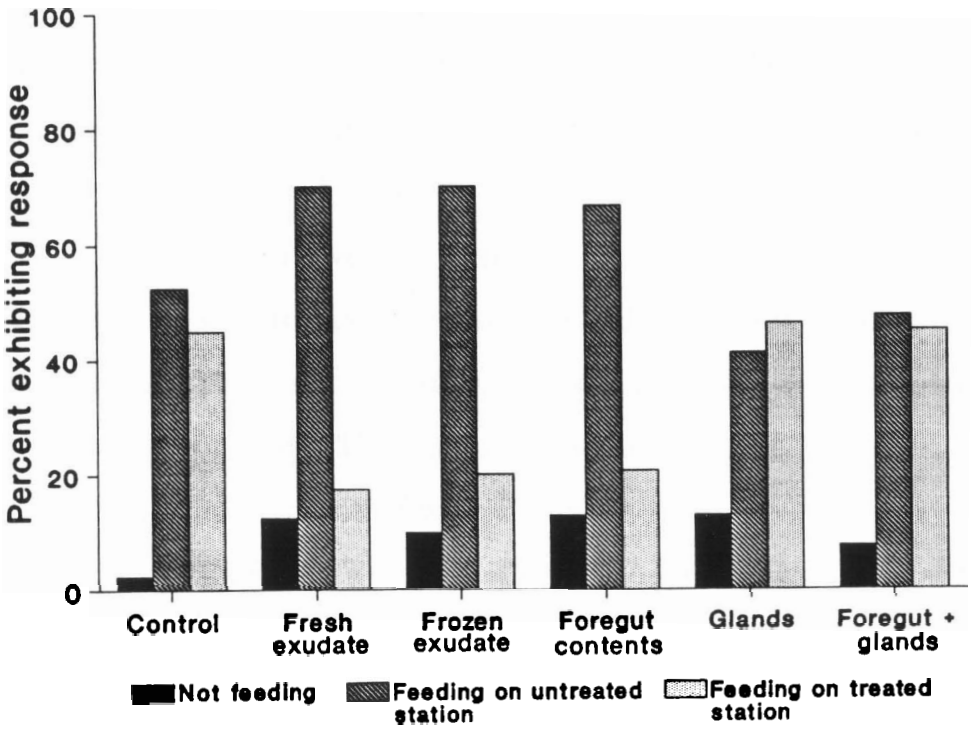
6.2.2 Results and Discussion

Significantly more larvae established on the untreated station when the other station was treated with fresh exudate, frozen exudate, or foregut contents alone, than in the control dishes (Fig. 18). The results from dishes treated with the gland preparation alone or foregut contents plus gland preparations were not significantly different from those in the control dishes (Fisher's Exact Test, $P>0.05$).

The lack of bioactivity in the 50:50 mixture of foregut and gland preparations was probably because the active foregut components in the mixed preparation, at 0.25 larval equivalents, were simply diluted below the lower threshold of bioactivity. These results also indicate that storing the exudate for 4 days at -4°C does not reduce the bioactivity.

This experiment demonstrated that larval oral exudate is a regurgitant, with no repellent components being obtained from sources other than the gut in diet-reared larvae. While it is possible to extract the gut contents directly, using the technique outlined here, it is probably most efficient to collect the exudate using a micropipet. Such collections are non-destructive. Larvae are able to produce exudate again within 24 h of being induced to produce it the first time (pers. obs.), and an individual larva would, therefore, be able to produce far more exudate if kept alive than if killed and dissected.

Fig. 18. Percentage of artificial diet-fed *C. occidentalis* larvae not feeding, or feeding on one of two diet stations with centres separated by 3 cm. Larvae provided with two stations, one untreated and one treated with 2 μL of distilled water (Control), or one untreated and one treated with 2 μL of fresh exudate, stored exudate, foregut preparation, labial gland preparation, or a combined foregut plus gland preparation. n=40. Significant differences from the control found in dishes treated with fresh exudate, stored exudate, and the foregut preparation (Fisher's Exact Test, P=0.012, 0.038, 0.034, 0.193 and 0.712, respectively).


Percent exhibiting response
100
80
60
40
20
0
Control
Fresh exudate
Frozen exudate
Foregut contents
Glands
Foregut + glands
Not feeding
Feeding on untreated station
Feeding on treated station

6.3 DOSE-RESPONSE

6.3.1 Materials and Methods

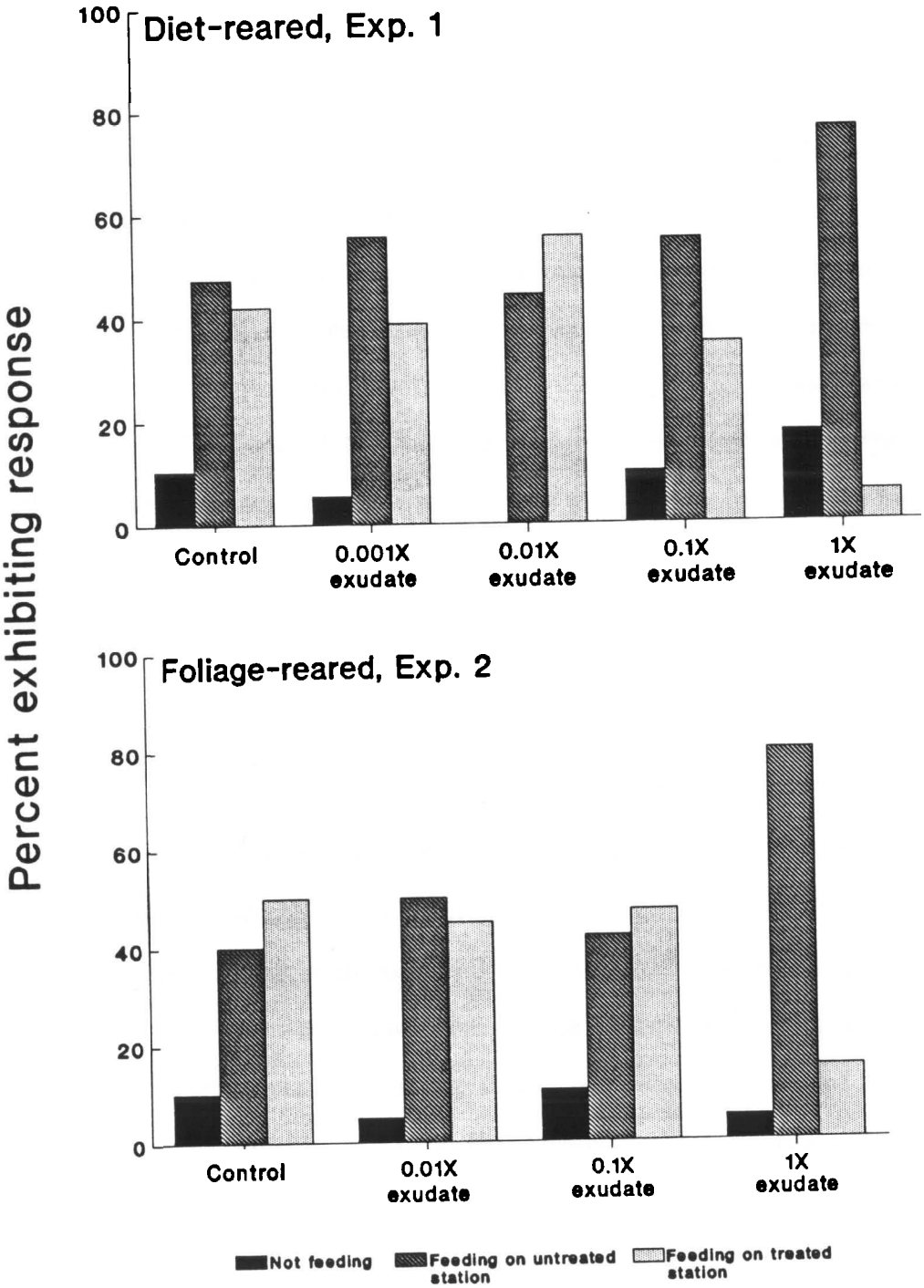
This experiment employed a modified bioassay designed to preclude any interaction between organic solvents and the diet stations, if such solvents were found to be needed during chemical isolation procedures. Circular glass cover slips (18 mm diam.) were attached to the dishes using chloroform, such that diet stations on the cover slips had their centres 3 cm apart. Exudate treatments were applied to the glass in a ring around the outer edge of the cover slip. A drop of molten artificial diet was then applied to the center of each cover slip, within, but not contacting the exudate treatment ring. No filter paper was used, and the dishes were not inverted because large larvae were capable of pulling the cover slips off the dishes, or reaching up to the food without ever contacting the treatment ring.

Exp. 1 used third to fifth instar diet-reared *C. fumiferana*, from the laboratory colony. Exudate was collected from larvae using a 5 μL micropipet, and pooled in a glass vial kept on ice. The exudate was then diluted 0-, 1-, 10-, 100- and 1000-fold by serial dilution in distilled water. One station in each bioassay dish was treated with 2 μL of distilled water or 2 μL of one of the exudate dilutions. Twenty dishes were prepared for each of the five treatments. An uninduced test larva was placed in each dish, and left undisturbed for 24 h. Exp. 2 used third to fifth instar wild-collected *C. occidentalis*, reared on foliage. Exudate was collected, and the treatments set up and replicated as in Exp. 1. The results of both experiments were assessed and analyzed as in Section 4.0.

6.3.2 Results and Discussion

When the exudate was diluted by any tested amount, the numbers of larvae in the three response categories were not significantly different between experimental and control dishes in either experiment (Fig. 19). Only larvae in

Fig. 19. Percentage of artificial diet-fed *C. fumiferana* or foliage-fed *C. occidentalis* larvae not feeding, or feeding on one of two diet stations with centres separated by 3 cm. Larvae provided with two stations, one untreated and one treated with 2 μL of distilled water (Control), or one untreated and one treated with 2 μL of a 1-, 10-, 100-, or 1000-fold dilution of conspecific exudate. n=40. Significant differences from the control found only in dishes treated with whole exudate (Fisher's Exact Test, P=0.043, 1.00, 0.513, 0.897 and 0.043, respectively, for diet-reared larvae; P=0.805, 1.00 and 0.032, respectively, for foliage-reared larvae).


Diet-reared, Exp. 1
Foliage-reared, Exp. 2
Percent exhibiting response
100
80
60
40
20
0
Control
0.001X exudate
0.01X exudate
0.1X exudate
1X exudate
Not feeding
Feeding on untreated station
Feeding on treated station

dishes treated with undiluted exudate showed responses which were significantly different from those of larvae in the control dishes, with most larvae establishing on the untreated feeding station.

These results show clearly that application of one larval equivalent, as defined in Section 3.0, is necessary to elicit a response from test larvae in the laboratory bioassay. Because this biologically realistic dose is necessary for bioassays, it is probable that large amounts of starting material will be needed for chemical fractionation, bioassay and analysis (Brand *et al.*, 1979), if the active constituents in the exudate are to be identified.

My results also have implications in the behavioural ecology of spruce budworm larvae. An individual is repelled by approximately the amount of exudate that it would sense if it encountered another larva, but not by any lesser amount. Therefore, a larva that is diseased, has not fed well, has grown slowly, or for other reasons produces less than one larval equivalent of exudate, is unlikely to be effective at repelling conspecifics that could outcompete it in nature.

6.4 CENTRIFUGATION

6.4.1 Materials and Methods

Bioassay dishes were constructed as described in Section 6.2.1. Exp. 1 used third to fifth instar diet-reared *C. occidentalis* larvae, from the laboratory colony. Exp. 2 used third to fifth instar wild-collected *C. occidentalis*, reared on foliage. For both experiments, exudate was collected from larvae using a 5 μL micropipet, and pooled in three Eppendorf tubes kept on ice. Two tubes were centrifuged at 13,000 rpm for 5 min in a Micro-Centaur benchtop Eppendorf centrifuge. The supernatant was drawn off one tube with an Eppendorf Pipetman automatic pipet and retained, and distilled water was added to the pellet fraction to bring it back to the pre-centrifugation volume. In the second tube, the pellet was resuspended in the

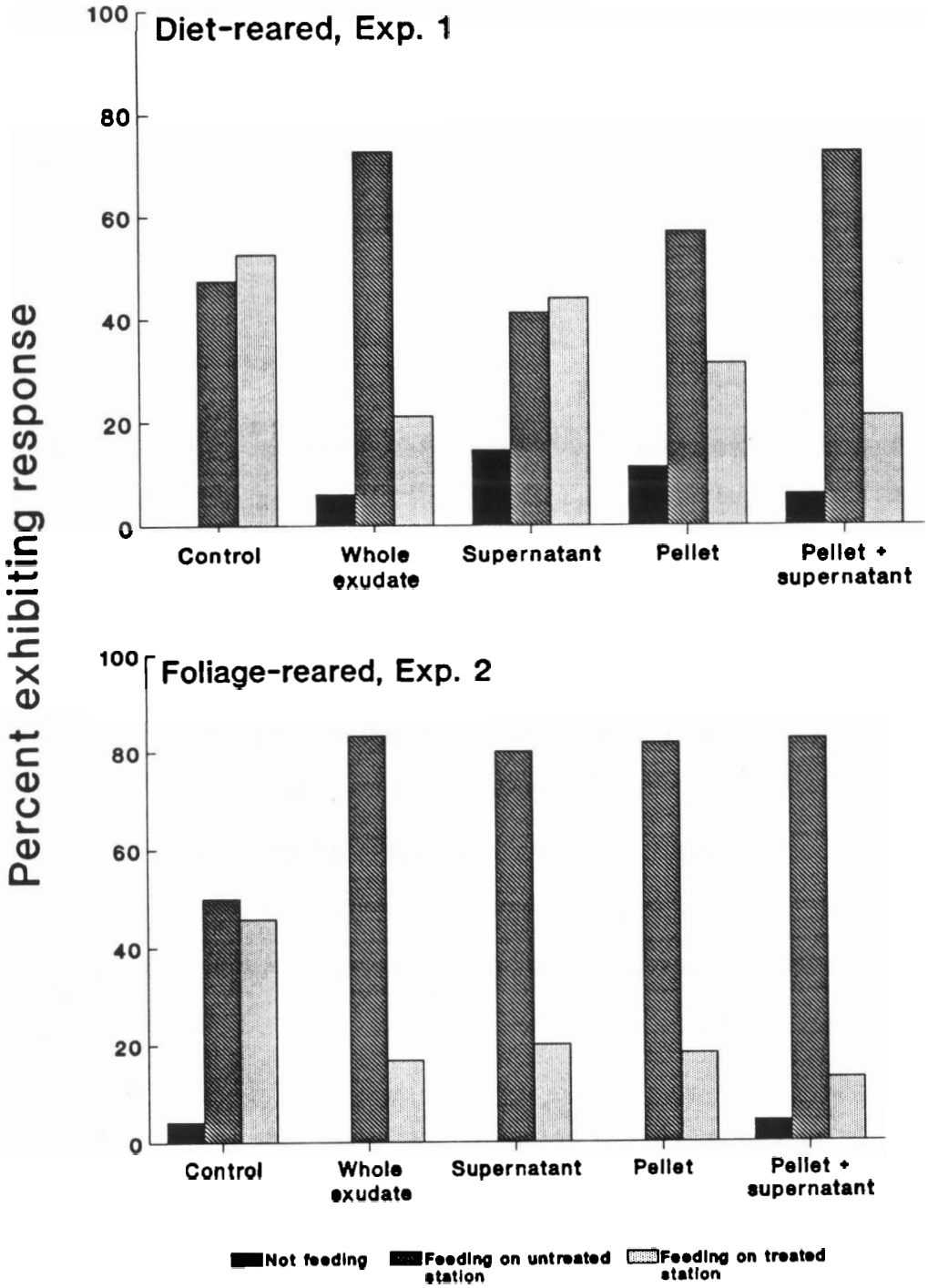
supernatant with an automatic pipet. The third tube was left untreated. One station in each bioassay dish was treated with 2 μL of either distilled water, whole exudate, supernatant, pellet suspension, or centrifuged, reconstituted exudate. Forty dishes (replicates) were prepared for each of the 5 treatments. An uninduced test larva was placed in each dish, and left undisturbed for 24 h. The results were then assessed and analyzed as described in Section 4.0.

6.4.2 Results and Discussion

In Exp. 1, the numbers of larvae in the three response categories were significantly different between control dishes and dishes treated with either whole or reconstituted exudate (Fig. 20), with fewer larvae establishing on the treated than untreated feeding stations. There was no difference between control dishes and dishes treated with supernatant. Some reduction in the numbers of larvae feeding on the treated station was observed in dishes treated with the pellet resuspension, but this was not significant (Fisher's Exact Test, $0.05<P<0.10$). However, in Exp. 2, the numbers of larvae in the three response categories were significantly different from the control dishes in all treatments (Fig. 20).

The results from these experiments were not conclusive. Centrifugation did not destroy the bioactivity of exudate from either diet- or foliage-reared larvae, since reconstituted exudate was as deterrent as whole exudate. Exudate from diet-reared insects appeared to have some of the repellent components carried as a suspension. However, the particulate matter alone was not as repellent as whole exudate. Exudate from foliage-reared insects appeared to have the repellent components equally distributed between the pellet and the supernatant, with both fractions being as repellent as whole exudate. It is possible that the centrifugation used was not sufficient to separate the materials in the exudate, and that better results might have been achieved by using higher centrifugation speeds or centrifuging the samples for a longer period of time. However, since the exudate

Fig. 20. Percentage of artificial diet (Exp. 1) or foliage-fed (Exp. 2) *C. occidentalis* larvae not feeding, or feeding on one of two diet stations with centres separated by 3 cm. Larvae provided with two stations, one untreated and one treated with 2 μL of distilled water (Control), or one untreated and one treated with 2 μL of either whole exudate, supernatant, pellet suspension, or reconstituted exudate. n=40. Significant differences from the control found for both whole and reconstituted exudate in Exp. 1 for artificial diet-reared larvae, and in all dishes for foliage-fed larvae in Exp. 2 (Fisher's Exact Test, P=0.043, 0.280, 0.183 and 0.043, respectively, for diet-reared larvae; P=0.011, 0.017, 0.026 and 0.030, respectively, for foliage-reared larvae).


Diet-reared, Exp. 1
Foliage-reared, Exp. 2
Percent exhibiting response
100
80
60
40
20
0
Control
Whole exudate
Supernatant
Pellet
Pellet + supernatant
Not feeding
Feeding on untreated station
Feeding on treated station

apparently consists of a partially digested substrate, it is also possible that a portion of the repellent component is still associated with the particulate matter suspended in the exudate, while the remainder is free in solution. The results from Section 4.0 suggested that diet-reared larvae may produce lower concentrations of repellent than foliage-reared insects. Dividing the repellent between two fractions could, therefore, reduce the concentration below the response threshold in the case of diet-reared insects. However, enough material could still be retained in both fractions of the exudate from foliage-reared insects to elicit a response.

6.5 PERSISTENCE

6.5.1 Materials and Methods

Bioassay dishes were constructed as described in Section 6.2.1. Exp. 1 used third to fifth instar diet-reared *C. fumiferana* larvae, from the laboratory colony. Exp. 2 used third to fifth instar wild-collected *C. occidentalis* larvae, reared on foliage. On three consecutive days, exudate was collected from larvae using a 5 μL micropipet, and pooled in a glass vial kept on ice. One feeding station in each dish was treated with 2 μL of either distilled water or exudate. Exudate was added to the exudate-treated dishes 0, 24 or 48 h prior to the introduction of test larvae. The water-treated dishes were used immediately (0 h delay). All dishes had larvae added on the same day. Twenty dishes (replicates) were prepared for each of the four treatments in each experiment. An uninduced test larva was placed in each dish, and left undisturbed for 24 h. The results were then assessed and analyzed as described in Section 4.0.

In Exp. 3, bioassay dishes were constructed in the same manner, and third to fifth instar laboratory, diet-reared *C. fumiferana* larvae were used. Exudate was collected from larvae using a 5 μL micropipet, and pooled in glass vials kept on ice. One vial was used immediately. The other vials were stored at approximately -4°C

for one week or one month. One feeding station in each dish was treated with 2 μL of either distilled water, fresh exudate, week-old exudate, or month-old exudate. Twenty dishes were prepared for each of the four treatments. An uninduced test larva was placed in each dish, and left undisturbed for 24 h. The results were then assessed and analyzed as described in Section 4.0.

6.5.2 Results and Discussion

In Exp. 1, the numbers of larvae in the three response categories were significantly different (Fisher's Exact Test, $P<0.05$) from the control dishes in all treatments (Fig. 21). More larvae fed on the untreated station in exudate-treated dishes for all delay times. The bioactivity of exudate from diet-reared larvae persisted for at least 48 h under laboratory conditions.

In Exp. 2, the numbers of larvae in the three response categories were significantly different from the control dishes in all treatments except the 48 h delay (Fig. 21), indicating persistence for >24 h, but <48 h, under laboratory conditions.

The numbers of larvae in the three response categories in Exp. 3 were significantly different from the control dishes in all treatments except the 1 month storage (Fig. 22). Exudate from diet-reared insects can, therefore, be stored at -4°C for at least a week, but less than one month.

These experiments indicate that exudate from both diet- and foliage-reared larvae deteriorates over time, although the breakdown appears to be faster in the latter case. While this deterioration may be mediated by microbial activity, it seems likely that the repellent components will also be subject to the action of enzymes contained in the regurgitant.

Fig. 21. Percentage of artificial diet-fed *C. fumiferana* (Exp. 1) or foliage-fed *C. occidentalis* (Exp. 2) larvae not feeding, or feeding on one of two diet stations with centres separated by 3 cm. Larvae provided with two stations, one untreated and one treated with 2 μL of distilled water (Control), or one untreated and one treated with 2 μL of exudate and left on a benchtop for 0, 24, or 48 h. n=20. Significant differences from the control found in all dishes (Fisher's Exact Test, P=0.019, 0.036 and 0.019, respectively) for diet-reared larvae, and in dishes left for 0 or 24 h (P=0.010, 0.033 and 0.284, respectively) for foliage-fed larvae.

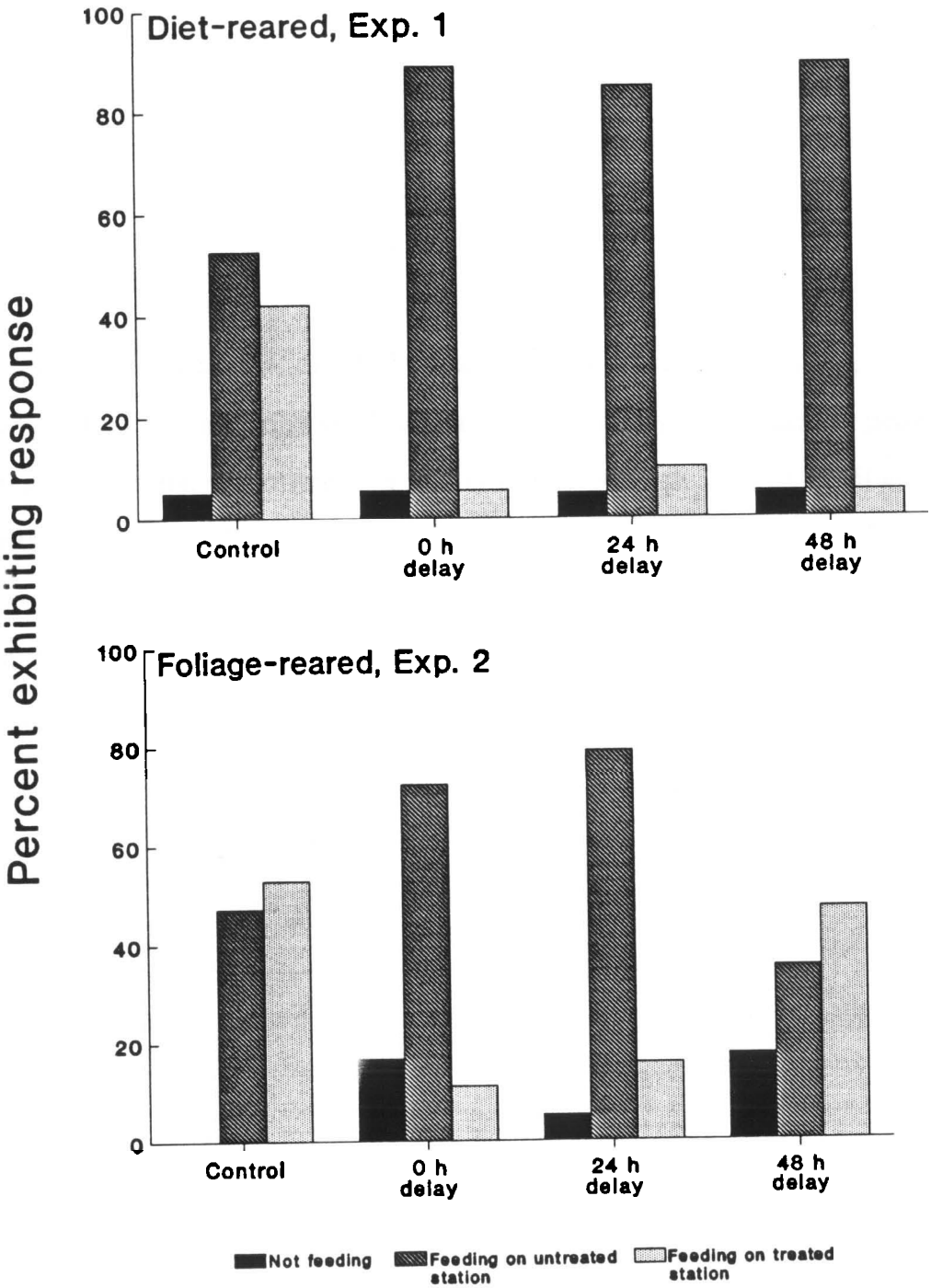
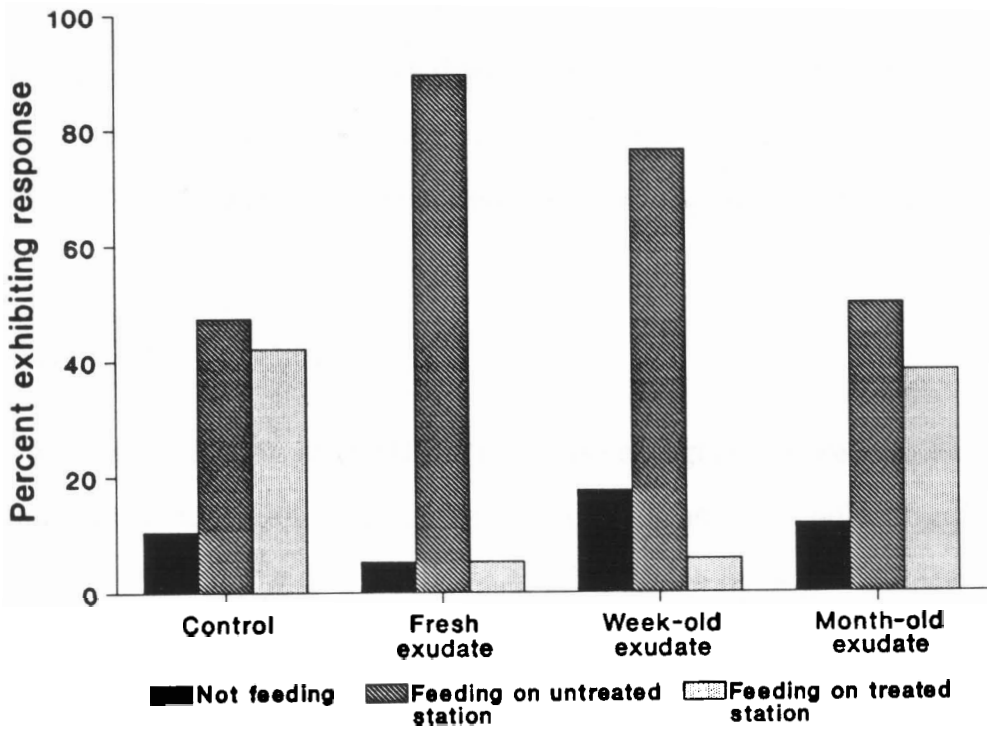

Percent exhibiting response
Diet-reared, Exp. 1
100
80
60
40
20
0
Control
0 h delay
24 h delay
48 h delay
Foliage-reared, Exp. 2
100
80
60
40
20
0
Control
0 h delay
24 h delay
48 h delay
Not feeding
Feeding on untreated station
Feeding on treated station

Fig. 22. Percentage of artificial diet-fed *C. fumiferana* larvae not feeding, or feeding on one of two diet stations with centres separated by 3 cm. Larvae provided with two stations, one untreated and one treated with 2 μL of distilled water (Control), or one untreated and one treated with 2 μL of either fresh exudate, or exudate stored at -4°C for 1 week or 1 month. n=20. Significant differences from the control found in dishes treated with fresh exudate and exudate stored for 1 week (Fisher's Exact Test, P=0.011, 0.043 and 1.00, respectively).


Percent exhibiting response
100
80
60
40
20
0
Control
Fresh exudate
Week-old exudate
Month-old exudate
Not feeding
Feeding on untreated station
Feeding on treated station

6.6 AUTOCLAVING

6.6.1 Materials and Methods

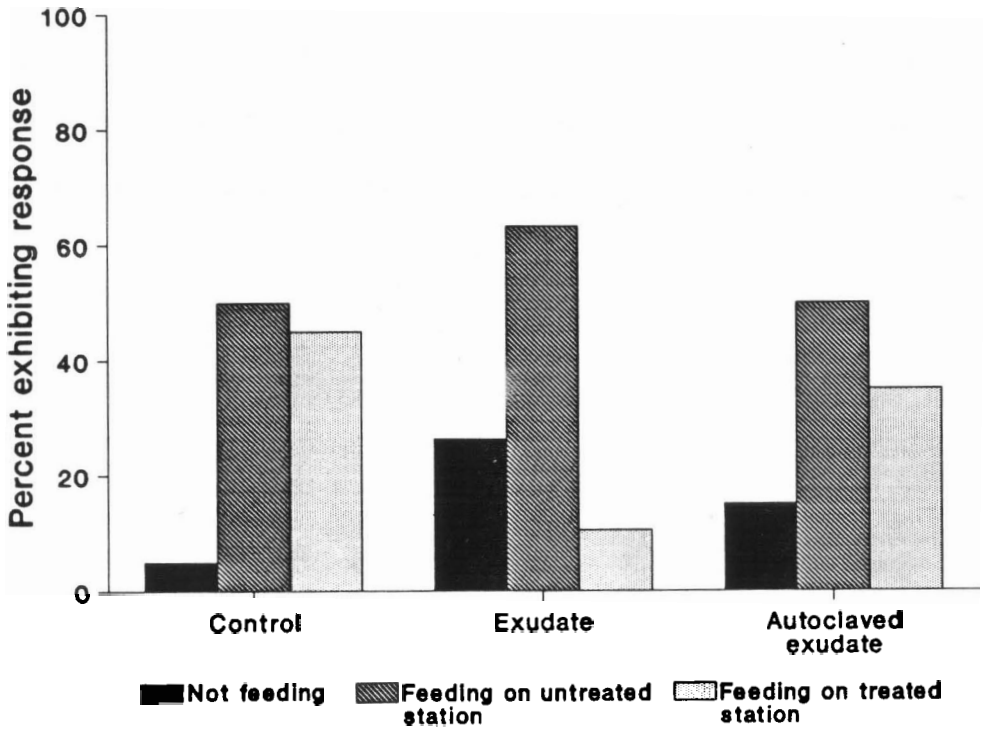
Bioassay dishes were constructed as described in Section 4.0, with both filter paper and dish inversion omitted. All insects used in this experiment were third to fifth instar diet-reared *C. occidentalis* larvae from the laboratory colony. Oral exudate was collected from larvae using a 5 μL micropipet, and pooled. Half the exudate was autoclaved for 15 min at a pressure of 15 kPa, 122°C. One feeding station in each dish was treated with 2 μL of distilled water, 2 μL of fresh exudate, or 2 μL of autoclaved exudate. Twenty dishes (replicates) were prepared for each of the three treatments. An uninduced test larva was placed in each dish, and left undisturbed for 24 h. The results were then assessed and analyzed as described in Section 4.0.

6.6.2 Results and Discussion

The numbers of larvae in the three response categories were significantly different from the control dishes in the fresh exudate treatment only (Fig. 23). The results from dishes treated with autoclaved exudate were not significantly different from the control dishes.

Autoclaving appears to destroy the bioactivity of the exudate. Because the conventional use of an autoclave is sterilization by protein denaturation, these results may suggest that the repellency is caused by a protein. However, a number of other materials may be significantly altered by high temperatures. Few conclusions can be drawn about the nature of the repellent materials, but it is clear that their nature can be altered by certain laboratory procedures, and care will be needed to isolate and identify them.

Fig. 23. Percentage of artificial diet-fed *C. occidentalis* larvae not feeding, or feeding on one of two diet stations with centres separated by 3 cm. Larvae provided with two stations, one untreated and one treated with 2 μL of distilled water (Control), or one untreated and one treated with 2 μL of either fresh exudate or autoclaved exudate. n=20. Significant differences from the control found only in dishes treated with fresh exudate (Fisher's Exact Test, P=0.035 and 0.604, respectively).


Percent exhibiting response
100
80
60
40
20
0
Control
Exudate
Autoclaved exudate
Not feeding
Feeding on untreated station
Feeding on treated station

6.7 SUMMARY

The experiments in this section provide considerable insight into the nature of the bioactive constituent(s) in the larval oral exudate of these two *Choristoneura* spp. Its origin is in the digestive tract. It persists for up to 48 h at room temperature, and survives storage when frozen for at least one week. However, it is thermally unstable under pressure at 122°C. When centrifuged, the bioactive component(s) does not partition completely, suggesting that some fraction remains associated with the particulate matter in the exudate, while some is in solution. Lastly, below a biologically realistic concentration of one larval equivalent, bioactivity is lost. Because diet can influence bioactivity (Section 5.0), and because the components of the artificial diet are known, modification of this diet could potentially provide further insight into the nature of the bioactive component(s).

7.0 EFFECT OF DIETARY COMPONENTS ON EXUDATE REPELLENCY

7.1 INTRODUCTION

When undertaking an isolation and identification program (Brand *et al.*, 1979), it is helpful for a chemist to know the class of compound which is most likely to be biologically active. While the results of Section 6.0 provided inferences about some properties of the bioactive components of larval oral exudate, they did not provide strong evidence as to the chemical nature of the repellent material. However, given that the exudate is a regurgitant (Section 6.0), and that its repellency is strongly influenced by larval rearing medium (Section 5.0), it seems probable that repellency is associated in some way with one or more of the dietary components. The following experiments were designed to test this hypothesis for diet-reared larvae.

7.2 MATERIALS AND METHODS

A list of the ingredients in the artificial diet was obtained from H.J. Siegfried, Bio-Serv, Inc., Frenchtown, NJ. The proportions of the ingredients used were based on other artificial diets for spruce budworm larvae (McMorran, 1965; Grisdale and Wilson, 1991). Initially, the three most complex ingredients, casein, linseed oil and wheat germ, were chosen for testing.

For Exp. 1, four agar-based media were prepared using the same techniques employed to make the artificial diet for the main colony. In the first, nothing was added to the agar preparation. The others contained casein, linseed oil, or wheat germ in the same proportions as in the complete artificial diet (3.5%, 0.5% and 3.0%, respectively). These media were poured into the bottoms of rearing containers

and allowed to set. Nothing else was added to them, and they were not treated with fungicide. Third to fifth instar artificial diet-reared *C. occidentalis* larvae from the laboratory colony were placed on the media, and allowed to feed for at least two days, after which exudate was collected using a 5 μL micropipet from larvae reared on each of the media, as well as from larvae reared on normal artificial diet.

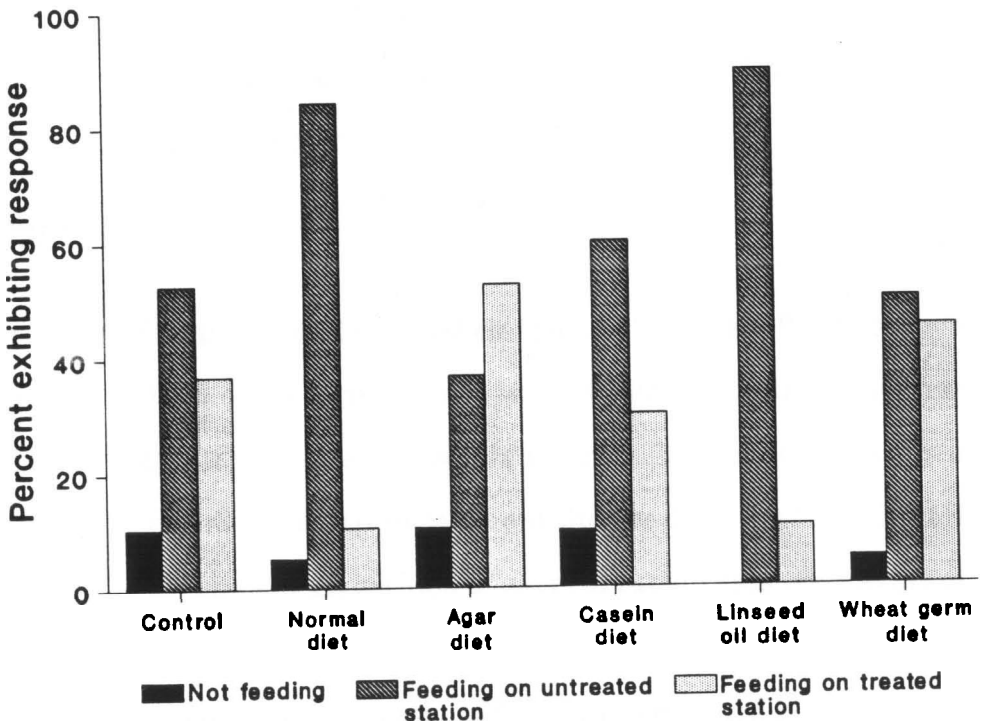
Bioassay dishes were constructed as described in Section 4.0, with both filter paper and dish inversion omitted. One feeding station in each dish was treated with 2 μL of either distilled water, exudate from normal artificial diet-reared larvae, or exudate from larvae on one of the four agar media. An uninduced, artificial diet-reared test larva was then placed in each dish, and left undisturbed for 24 h. The results were assessed and analyzed as described in Section 4.0.

Based on the results from Exp. 1, raw linseed oil was tested for repellency. Bioassay dishes were constructed as described in Section 4.0, with both filter paper and dish inversion omitted. One feeding station in each dish was treated with 2 μL of either distilled water, a 0.5% or 1.0% shaken suspension of raw linseed oil in distilled water, or 100% raw linseed oil. An uninduced, artificial diet-reared test larva was then placed in each dish, and left undisturbed for 24 h. The results were assessed and analyzed as described in Section 4.0.

7.3 RESULTS

In Exp. 1, significantly more larvae established on the untreated station in dishes treated with exudate from normal artificial diet-reared larvae, or exudate from larvae fed on a diet of agar and linseed oil (Fig. 24). Exudate from insects fed on agar alone, agar with casein or agar with wheat germ did not produce distributions of feeding activity significantly different from those in the control dishes.

Fig. 24. Percentage of artificial diet-fed *C. occidentalis* larvae not feeding, or feeding on one of two diet stations with centres separated by 3 cm. Larvae provided with two stations, one untreated and one treated with 2 μL of distilled water (Control), or one untreated and one treated with 2 μL of exudate from larvae reared on normal artificial diet, or 2 μL of exudate from larvae fed on agar alone, agar + casein, agar + linseed oil or agar + wheat germ. n=20. Significant differences from the control found in dishes treated with exudate from larvae reared on normal artificial diet and exudate from larvae fed on agar + linseed oil (Fisher's Exact Test, P=0.025, 0.667, 0.895, 0.036 and 0.802, respectively).


Percent exhibiting response
100
80
60
40
20
0
Control
Normal diet
Agar diet
Casein diet
Linseed oil diet
Wheat germ diet
Not feeding
Feeding on untreated station
Feeding on treated station

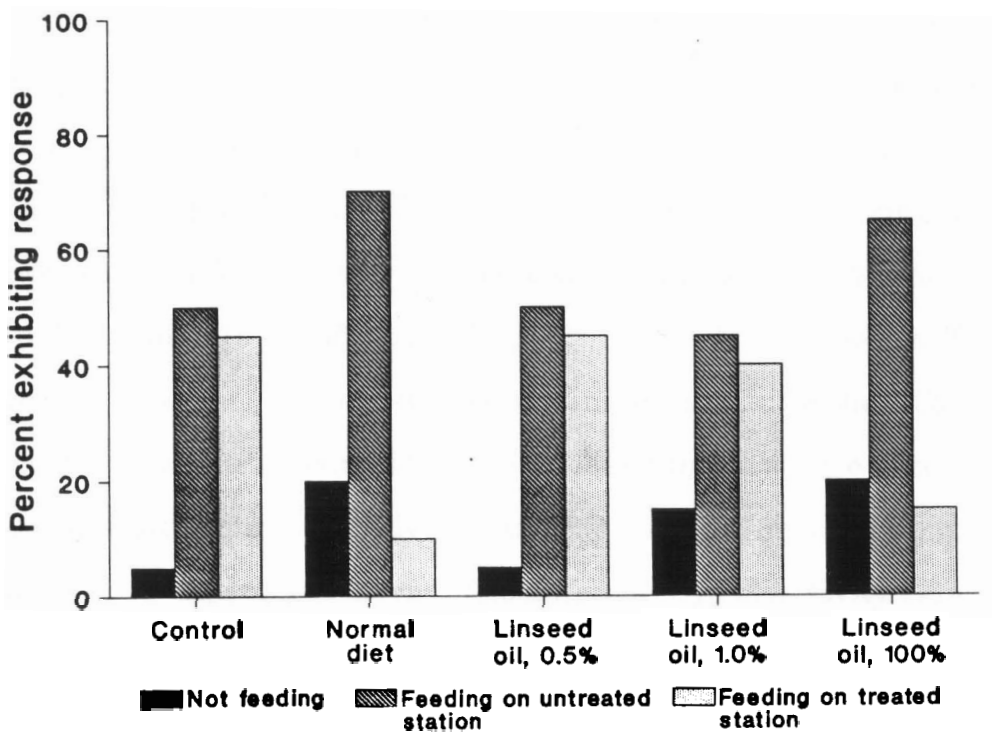
Neither a 0.5% nor a 1.0% suspension of linseed oil in Exp. 2 produced distributions of feeding activity which were significantly different from those in the control dishes (Fig. 25). Results from dishes in which one station was treated with exudate from normal artificial diet-reared insects were significantly different from those in the control dishes. Two microlitres of 100% raw linseed oil also appeared to be repellent (Fig. 25). However, when these dishes were evaluated, it was noted that large quantities of silk were present around almost every treated station. I noticed several larvae attempting to approach the treated stations, and turning back when they encountered the slippery, oiled surface. These observations suggest that the larvae were not repelled by the chemical nature of the linseed oil, but were deterred by its physical characteristics.

7.4 DISCUSSION

The results of Exp. 1 and 2 showed clearly that the repellent component of oral exudate in artificial diet-reared larvae is associated with the raw linseed oil contained in the diet, but that the oil itself is not repellent at the concentrations found in the diet. Linseed oil is expressed from the seeds of the flax plant, *Linum usitatissimum* L. It consists of glycerides of several fatty acids (Budavari *et al.*, 1989), including linolenic (55%), linoleic (17%), oleic (13%), palmitic (9%), stearic (5%) and myristic (1%) acids (Gunstone, 1958). Linolenic acid is an essential nutrient for lepidopterans, as without it they do not complete pupal development properly (Dadd, 1985). Moreover, myristic (tetradecanoic) acid is the precursor to the major component of the *C. fumiferana* sex pheromone, (*E*)-11-tetradecenyl acetate (Wolf and Roelofs, 1987).

Following ingestion of fatty acid glycerides, such as those contained in linseed oil, lipases in the insect gut hydrolyze them to free fatty acids and glycerol (Applebaum, 1985). ß-oxidation is used to remove 2-carbon units from the free fatty

Fig. 25. Percentage of artificial diet-fed *C. occidentalis* larvae not feeding, or feeding on one of two diet stations with centres separated by 3 cm. Larvae provided with two stations, one untreated and one treated with 2 μL of distilled water (Control), or one untreated and one treated with 2 μL of exudate from larvae reared on normal artificial diet, or 2 μL of either 100% raw linseed oil, or 0.5% or 1.0% raw linseed oil suspended in distilled water. n=20. Significant differences from the control found only in dishes treated with exudate from larvae reared on normal artificial diet (Fisher's Exact Test, P=0.031, 1.00, 0.805, and 0.115, respectively).


Percent exhibiting response
100
80
60
40
20
0
Control
Normal diet
Linseed oil, 0.5%
Linseed oil, 1.0%
Linseed oil, 100%
Not feeding
Feeding on untreated station
Feeding on treated station

acids, and these are then converted to acetyl-S-CoA (Downer, 1985). Because recently-ingested foregut contents are as repellent as the regurgitant (Fig. 18), larvae are probably responding to a newly-released free fatty acid or a relatively simple derivative thereof.

The results of these experiments provide strong evidence for the involvement of a fatty acid or its derivative in the bioactivity of exudate from artificial diet-reared larvae. The nature of the repellent components of exudate from foliage-reared insects is still not known. However, the predominant fatty acids in current-year needles of *Picea abies* are linolenic, linoleic and palmitic acids (Senser, 1982), present mainly in the form of galactolipids. The concentrations of these acids change seasonally, with increasing levels of unsaturated fatty acids functioning as a mechanism of frost resistance for the tree (Senser, 1982). In the spring, when spruce budworm larvae feed actively, linolenic acid comprises 52.1%, linoleic acid 12.7% and palmitic acid 15.0% of the total lipids in current-year needles. These concentrations are similar to those reported for linseed oil (Gunstone, 1958), except that higher concentrations of palmitic acid were found in the spruce needles. Therefore, similar acids may be involved in the repellency of exudate from both artificial diet- and foliage-reared larvae. The differences in bioactivity between exudate from diet- and foliage-reared larvae (Fig. 17), could be explained simply by differences in the relative concentrations of fatty acids in the diet.

8.0 CONCLUSIONS

The importance of epideictic pheromones in the behaviour of larval eastern and western spruce budworms has been shown clearly by my work. Specifically, this work has met the original objectives in the following manner.

1. Both *C. fumiferana* and *C. occidentalis* larvae were shown to produce oral exudate in response to encounters with conspecifics. When larvae were induced to produce the exudate, they produced slightly less than 2 μL, and could be induced up to four times within a few minutes. Application of fresh exudate within the feeding tunnel increased larval dispersal after a 6 to 8 h delay.
2. An effective two-choice feeding bioassay was developed to assess the repellency of oral exudate to larval spruce budworms.
3. Oral exudate was shown to function as an intraspecific repellent in the laboratory. It also functioned interspecifically, suggesting that even though eastern and western spruce budworms are allopatric, other insects which are competing with a larva for the same food source may also be repelled.
4. A larva which produces exudate did not normally repel itself from its habitat, primarily because it entered a refractory phase during which it was unable to respond to its own or other individuals' exudate. This mechanism might allow larvae to use exudate to repel competitors from valuable resources, such as the feeding tunnel, without forcing them to abandon the resource themselves.

5. While rearing history was not found to have any impact on the bioactivity of the exudate, larval food source had a marked effect. Larvae reared on artificial diet were repelled by exudate from both artificial diet- and foliage-reared larvae. However, foliage-reared larvae were only repelled by exudate from other foliage-reared larvae. These results indicate that there are differences in the composition of exudate from the two sources, or in the concentrations of the biologically active constituents. It may also suggest that previous experience of larvae with the exudate may be a factor in repellency.
6. Larval oral exudate was shown to be a regurgitant rather than a labial gland secretion. One larval equivalent was necessary to elicit a response from test larvae in the laboratory bioassay. The biologically active components were not damaged by centrifugation, although neither artificial diet- nor foliage-reared larval exudate could be separated into active and inactive fractions by this means. Exudate from foliage-reared larvae was persistent for >24 h, but <48 h. Exudate from artificial diet-reared larvae was persistent for >48 h, and could be stored at -4°C for >1 week but <1 month without losing activity. Autoclaving destroyed the bioactivity of exudate from artificial diet-reared larvae.
7. The biologically active portion of artificial diet-reared larval oral exudate was shown to be linked to the presence of linseed oil in the diet. However, linseed oil itself was not found to be repellent. These results suggest that the repellent component is a fatty acid, or a derivative. Finally, although this hypothesis has not been tested for exudate from foliage-reared larvae, it seems probable that the biological activity in nature stems from a similar compound.

The experiments described above have improved understanding of larval behaviour under a variety of conditions, and have demonstrated that it may be possible to manipulate that behaviour using the part of insects' own communication system. Further research in this area could lead to the development of new and more effective management techniques than those that are presently available.

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