ORIENTATION OF POLYPHAGOUS LEPIDOPTERA TO HOSTPLANT KAIROMONES

BY

ALLEN LAWRANCE

THESIS

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Master's Committee:

Professor May Berenbaum, Chair, Director of Research
Professor Lawrence Hanks
Professor Emeritus David Seigler

ABSTRACT

Although in some oligophagous lepidopterans oviposition kairomones from hostplants can also serve as larval feeding stimulants, the relationships in chemistry of attraction of larval and adult stages to hostplants have rarely been examined in polyphagous species. Volatile constituents are known to serve as attractants for ovipositing moths. These compounds differentially stimulate electrophysiological responses in antennae of male and female moths and can also serve as larval attractants. Similarly, ovipositing female adults and feeding larvae use contact cues for hostplant evaluation. In this study, I reviewed the literature to determine if any patterns of relationship between adult and larval host recognition cues in polyphagous Lepidoptera have been detected in previous studies (Chapter 1). I then conducted a series of behavioral assays with the navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), a highly polyphagous species, to determine if larvae of the navel orangeworm respond behaviorally to the same secondary compounds that elicit behavioral or electrophysiological responses in adult moths. In Petri dish arenas, navel orangeworm larvae showed a preference for 1-octen-3-ol and 2-phenylethanol, and aversion to ethyl benzoate out of a total of 9 tested compounds that are electrophysiologically active in male or female adults. The same behavioral assay was also used to investigate the role of volatiles emitted by a potentially mutualistic *Aspergillus* fungus in larval orientation behavior. Larvae were strongly attracted to methanolic extracts of almond meal spiked with fungal volatiles. In one case, larvae even preferred almond extract spiked with fungal volatiles over pure almond extract. This information may prove useful in refining current control systems for navel orangeworm; for example, my findings suggest that replacing lures when they become moldy may be counterproductive, and

that adding volatiles to pesticide formulations may be effective in attracting larvae to areas that have been treated, thereby increasing the likelihood of encountering the pesticide.

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I. COMPARISON OF CONTACT STIMULANTS FOR LARVAL AND ADULT LEPIDOPTERA

Introduction

The considerable mobility of adult Lepidoptera achieved through flight contrasts with the limited mobility of the wingless larval stages of members of this order. With the exception of those that exhibit ballooning behavior, which is characteristic of species primarily in the moth families Cossidae, Geometridae, Lymantriidae, Noctuidae, Psychidae, and Pyralidae (Zalucki et al. 2002), the majority of lepidopteran larvae rarely move great distances; for the most part, movements are restricted to within, rather than among, individual hostplants. Both life stages, however, depend to varying extents on contact chemoreception for assessing hostplant suitability—ovipositing females for host recognition and larvae for host evaluation. Neonate survival is thus highly dependent on the mother's ability to choose a suitable environment in which to lay her eggs and the neonate's ability to accept and feed on its hostplant (Renwick and Chew 1994). There are different sensilla used to assess leaf surfaces but ostensibly the same gustatory receptors are encoded in the genome at all life stages (Robertson 2003). Therefore, the question arises as to what extent the same cues are used across the life cycle.

At least in some species, a congruent sensory mechanism that exists in larval and adult stages allows a shared set of chemicals to serve as recognition cues eliciting either feeding or avoidance (Nishida 2005). While this sharing of chemical cues across life stages has been observed within some groups (particularly Papilionidae, the swallowtail butterflies), it has yet to be shown if this pattern, the shared kairomone hypothesis, applies across the order. One factor that might influence the nature of the chemically informative cues is the degree of dietary host breadth. Lepidopteran larval host ranges vary widely, encompassing monophagy to broad polyphagy, with most species able to utilize more than just one hostplant species but limited to

plants that are within the same family or that are chemically similar (oligophagy; Bernays and Chapman 1987). Because larval generalists are capable of feeding on a wide range of chemically disparate plants, general cues of nutritional suitability involving primary metabolites will likely be optimally informative. By contrast, larval specialists, particularly those that sequester plant chemicals for defense, may rely on specific secondary metabolites that are restricted in distribution; there should be strong selection on ovipositing females to respond to the same ecologically informative chemicals, as both life stages have a need to identify the same range of hostplant species. While host acceptance is controlled by a balance of both feeding stimulants and feeding deterrents (Chapman 2003), there is some support for this hypothesis; several reviews have concluded that oligophagous caterpillars are more responsive to deterrents than are polyphagous caterpillars (Bernays and Chapman 1987, 1994; Bernays et al. 2000; Jermy 1964; Zhang 2015). As well, glucosinolates sequestered by *Pieris brassicae*, serve as both feeding stimulants for larvae and oviposition stimulants for ovipositing adults (Loon et al. 1992).

To test my hypothesis—that, in Lepidoptera, both larval and adult stages of generalists are responsive behaviorally and/or electrophysiologically to nutritional cues whereas both larval and adult stages of specialists are responsive behaviorally and/or electrophysiological to specific secondary compounds—I conducted an analysis of the existing literature. I used the same data set to test the shared kairomone hypothesis, that larvae and adult lepidopterans are likely to react either behaviorally or electrophysiologically to at least some of the same hostplant kairomones.

Methods

I reviewed the literature in Web of Science, a citation index for literature from 1900 to 2016 using the search terms “contact chemoreception,” “Lepidoptera”, “larval”, “adult”, “oviposition stimulant”, “feeding stimulant”, and “hostplant kairomone”. In some cases, both

larval and adult kairomones were identified in the same study; in the majority of cases, however, different investigators conducted the studies on the different life stages. For the purposes of this study, species that are either monophagous or oligophagous were considered specialists and species that are highly polyphagous, those that make use of hostplants from multiple different families, were considered generalists.

After compiling the list, I conducted a Fisher's exact test to compare the frequency with which either of two kairomone categories (nutritional cues or secondary metabolites) was reported across both larval and adult stages in generalists and again in specialists. I also tested whether the frequency of species that were found to share at least one chemical signal between life stages was significantly greater than the expected frequency of no shared signals via Fisher's exact test.

Results

I found 28 papers that provided information on chemical cues used by conspecific larvae and adult lepidopterans. Fourteen species of Lepidoptera were included in the analyses; of these eight families were represented. Of the 14 species, eight of these were specialists and six were generalists (Table 1.1).

Of the generalists, 83.3% (5 of 6) rely only on nutritional cues and 16.7% (1 of 6) depend on secondary metabolites. In contrast, 75% of specialists (6 of 8) rely on secondary metabolites and only 25% (2 of 8) depend on nutritional cues alone to identify suitable hostplants. Significantly more specialists relied on secondary metabolites than generalists (Fisher's exact test $p=0.019$) and significantly more generalists relied on nutritional cues alone than specialists ($p=0.019$).

Almost half of the species were found to share at least one chemical signal between both adult and larval life stages (6 of 14). Comparing the frequency of 6 (n=14) species that share hostplant kairomones to the expected frequency of zero (n=14) species with shared kairomones given the null hypothesis that lepidopterans do not share hostplants produced a significant difference (p=0.016).

The specific cues varied among the specialists (Table 1.1). Of the eight specialists, three are moth species and five are butterfly species. For *Plutella xylostella* (Plutellidae), the diamondback moth, Loon et al. (2002), who conducted behavioral assays with fiberglass discs to measure feeding responses, found one glucosinolate was enough to induce feeding alone, but feeding was greatest when the glucosinolate was combined with a mixture of four flavonoids, all constituents of plants within Brassicaceae. Reed et al. (1989) conducted an oviposition preference assay and identified four glucosinolates that were effective at eliciting oviposition. For the ermine moth *Yponomeuta cagnagellus* (Yponomeutidae), Roessingh et al. (2000) tested whether dulcitol, a sugar alcohol present on the surface of its hostplant *Euonmyous europaeus,* was able to stimulate both larval feeding and adult oviposition. They found that it did stimulate larvae, but it was not sufficient in stimulating oviposition. Methanolic extracts from hostplants elicted oviposition, but the individual components have never been identified. For *Manduca sexta,* the tobacco hornworm, which is a specialist on *Nicotiana*, Nelson (1996) conducted behavioral assays on both adults and larvae to examine the role of *myo*-inositol in host identification and found the sugar initiated larval feeding but had no influence on oviposition.

Of the five butterfly species, there were three species of Papilionidae, two species of Pieridae, and one species of Nymphalidae. The papilionids included representatives of two tribes (Graphiini and Papilionini). Zhang et al. (2015) found that a sugar, a chlorogenic acid, a

flavonoid glycoside, and a fatty acid, extracted from camphor tree (*Cinnamomum camphora*) leaves, all contributed to feeding stimulation of larvae of *Graphium sarpendon.* Li et al. (2004) tested electroantennogram and upwind orientation of adults to hostplant volatiles and found nonanal and decanal were attractive. In *Papilio xuthus*, Nishida (1987, 1990) and Oshugi et al. (1991) identified that a complex mixture of flavonoids, alkaloids, a sugar and a base stimulated oviposition in *Citrus* hostplants. Murata (2011) subsequently identified larval feeding stimulants and determined that a mixture of sugars, a betaine, a cyclic peptide, several lipids, and a polymethoxyflavone stimulated larval feeding via behavioral assays. Among pierids, Miles et al. (2004) identified a glucosinolate, gluconasturtiin from *Brassica oleracea*, stimulated larval feeding in *Pieris rapae*. Städler et al. (2008) found that any of nine different glucosinolates were able to induce oviposition, including the same glucosinolate that acts as a larval feeding simulant. Similarly, David and Gardiner (1966) found eight hostplant glucosinolates, isolated from *B. oleracea*, significantly induced feeding by larvae by *Pieris brassicae*. Loon et al. (1991) later confirmed that at least one larval feeding stimulant, sinigrin, induced oviposition. Finally, the nymphalid *Junonia coenia* responds to two iridoid glycosides, a class of chemicals common to its hostplants among four plant families: Plantaginaceae, Verbenaceae, Scrophulariaceae, and Acanthaceae in larval stages; one of these, catalpol, also stimulates oviposition in ovipositing butterflies (Bowers 1984; Pereyra and Bowers 1987).

Among the generalists (Table 1.1), three families are represented, all of which are moths. *Cydia pomonella* (Tortricidae), the codling moth, responds as a larva to the sesquiterpene farnesene, which is ubiquitous in plants, as a larva (Wearing and Hutchins 1972) and as an adult (Sutherland et al. 1997). As for the European cornborer, *Ostrinia nubilalis* (Crambidae), Binder and Robbins (1992) tested 28 hostplant compounds for oviposition stimulation and deterrence of

O. nubilalis and identified three sesquiterpenes that induced oviposition. Udayagiri and Mason (1997) tested extracts from *Zea mays* on oviposition behavior and found five n-alkanes that stimulate oviposition. Bartlett et al (1990) tested a variety of potential phagostimulants from artificial diets and found a number of sugars and amino acids stimulated feeding.

Oviposition and larval kairomones have been characterized for four generalist noctuids. A number of sesquiterpenes extracted from *Gossypium* spp. induced oviposition in *Helicoverpa armigera* but only when presented in combination and could not induce oviposition in isolation (Jallow et al 1999, Singh and Mullick 2002), whereas two sugars (Zhang 2013) and the flavonoid glucoside rutin (Blaney and Simmonds 1983) stimulated larval feeding. *Helicoverpa zea*, the corn earworm, is also stimulated to feed by rutin (Blaney and Simmonds 1983); in addition, alkanes, sesquiterpenes and fatty acids extracted from tomato (*Solanum* (*Lycopersicon*) *esculentum*) stimulated both feeding and oviposition in this species. Also stimulated to feed by rutin is *Heliothis virescens* (Blaney and Simmonds 1983); adults of this species, however, likely do not rely on chemical cues for hostplant identification, instead relying on physical traits (Ramaswamy et al. 2011). *Spodoptera littoralis*, the Egyptian cottonworm, also is induced to feed by rutin (Blaney and Simmonds 1983), although electrophysiologically adult antennae respond to two sugars, sucrose and glucose; caffeine, an alkaloid; and sodium chloride, a salt.

Discussion

It is important to recognize the limitations of this study. Of 28 articles, in only a small fraction (i.e. four) were studies examining both larval and adult kairomones conducted by the same investigator in the same study. However, 7 of 14 species did have at least one investigator examine kairomones for both life stages, although not in the same study. The small sample size of this study is also a reason for caution in interpreting the findings. Finally, in view of both the

limits of behavioral and chemical methodologies and the high likelihood of intraspecific variation in behavior, the list of identified kairomones (Table 1.1) does not contain every actual oviposition and feeding stimulant for each species.

Despite this study's limitations, it does provide support for the shared kairomone hypothesis. Almost half of the species examined shared kairomones between life stages across five distinct families, the frequency of which is significantly higher than if there were no instances of shared kairomones. In addition, this literature analysis supports the hypothesis that host breadth has an effect on the shared chemical nature of kairomones. Even with a small number of studies conflicting with general hypothesis, the pattern holds true. Future studies may even strengthen support for this hypothesis as there are surely more stimulants to characterize for species included in this study. For example, in the study on *Manduca sexta* only one compound, *myo*-inositol, was tested, so it is unknown if this specialist responds to any other chemicals (Nelson 1996). For the purposes of this study, sugar alcohols such as *myo*-inositol, were not considered secondary metabolites as they serve an important function in insects as a secondary messenger, and in some cases, as with *Bombyx mori*, can be an essential nutrient (Chapman 2003). Changing the classification of this class of compounds to secondary metabolite or the identification of new feeding and stimulants for *Manduca sexta* would increase the support of the host breadth hypothesis.

A potential confounding factor in this study is that the use of defensive compounds as feeding and oviposition stimulants by lepidopterans may be a characteristic of butterflies, regardless of host breadth, as five out of eight specialists included were butterflies. Most butterflies are specialists to some degree as larvae. Even though *J. coenia* can feed on plants from a number of distinct families, it is considered a specialist in that it feeds only on plants

containing iridoid glycosides and sequesters these compounds (Bowers 1984). Characterizing the oviposition and feeding stimulants of more lepidopterans, especially those of key taxa, such as the generalist butterfly *Papilio glaucus*, a congener of many specialists with known larval and adult behavioral responses to kairomones, would address these questions.

Identifying specific gustatory receptors and examining expression patterns in sensory organs at all life stages is a high priority for understanding the chemical mediation of host acceptance across the life cycle of Lepidoptera. Tagging or producing knock downs of specific gustatory receptor (GR) genes paired with behavioral assays could definitively determine if the same GRs are expressed in both larval and adult life stages of lepidopterans during hostplant assessment. The ligands for very few GRs have been identified; their identification would help to reconstruct evolutionary histories of insects with GR gene trees, elucidate the ecological conditions surrounding adaptive radiations, and understand the genetic mechanisms underlying host shifts. Identifying the function of the full set of GRs in key taxa in a well-supported phylogeny can shed light on how host specificity evolves. Understanding the patterns of compounds detected by gustatory reception by lepidopterans can help give inform investigators on which compounds to focus their efforts on testing against unknown GRs.

This information can be useful in designing specific kairomone-based management tools. Using known feeding and oviposition stimulants in insecticide formulations could increase the amount of contact between control chemicals and the target pest by stimulating feeding by larvae on treated plant material or stimulating contact by ovipositing butterflies. Such a strategy could possibly be even more effective for species that share hostplant kairomones between life stages. Another possible strategy similar to mating disruption could be employed. Applying hostplant kairomones to non-hostplants in surrounding plots could induce the target pest in laying eggs or

attempting to feed on a plant not suitable for completing development. Outside of pest management, greater knowledge of kairomones could prove useful for facilitating rearing, whether for developing specific artificial diets for species that otherwise would not feed on a standard diet or increasing egg production for butterfly farms.

Table

Table 1.1. Summary of oviposition stimulants and larval feeding stimulants for lepidopterans that have had both examined.

Taxonomy	Host breadth	Life stage	Chemical class	Compound	Reference
Yponomeutidae					
Plutella xylostella	Specialist	Larva	Glucosinolate	sinigrin	Loon et al. 2002
			Flavonoid	kaempferol-3-*O*-β-D-sophoroside-7-*O*-β-D-glucoside aclyated at C-2' with caffeic acid or sinapic acid quercetin-3-*O*-β-D-sophoroside-7-*O*-β-D-glucoside acylated at C-2' with caffeic acid kaempferol-3-*O*-β-D-[β-D-glucopyranosyl(1→2) glucopyranoside]-7-*O*-β-D-[β-D-glucopyranosyl(1→4) glucopyranoside, acylated at C-2 with ferulic acid	
		Adult	Glucosinolate	allylglucosinolate, *p*-hydroxybenzylglucosinolate, 3-indolylmethylglucosinolate, 1-methoxy-3-indolylmethylglucosinolate	Reed et al. 1989
Yponomeuta cagnagellus	Specialist	Larva	Sugar	dulcitol	Roessingh et al. 2000
Tortricidae					
Cydia pomonella	Generalist	Larva	Sesquiterpene	α-farnesene	Wearing and Hutchins 1972
		Adult	Sesquiterpene	α-farnesene	Sutherland et al. 1977
Crambidae					
Ostrinia nubilalis	Generalist	Larva	Sugars	fructose, glucose, sucrose, maltose, raffinose	Bartelt et al. 1990
			Amino acids	arginine, alanine, leucine, aspartic acid, serine threonine, isoleucine, histidine, lysine, tryptophan, glycine	

Table 1.1 continued.

Taxonomy	Host breadth	Life stage	Chemical class	Compound	Reference
		Adult	Sesquiterpenes	α-farnesene, β-farnesene, α-humulene	Binder and Robins 1992
			Alkanes	hexacosane, heptacosane, octacosane, nonacosane, triatriacontane	Udayagiri and Mason 1997
Helicoverpa armigera	Generalist	Larva	Sugars	*myo*-inositol, sucrose	Zhang et al. 2013
			Flavonoid	rutin	Blaney and Simmonds 1983
		Adult	Sesquiterpenes	β-caryophyllene, β-bisabolol, α-humulene, myrcene, β-pinene	Jallow et al. 1999
				β-caryophyllene, α-humulene, α-bulnesene, α-guajene, α-muurolene, γ-muurolene	Singh and Mullick 2002
Helicoverpa zea	Generalist	Larva	Flavonoid	rutin	Blaney and Simmonds 1983
			Alkane	3-methylnonacosane, 2-methylnonacosane	Breeden et al. 1995
			Fatty acid	palmitic acid	
		Adult	Alkane	2-methyldotriacontane	
			Sesquiterpene	γ-elemene, (*E*,*E*)-farnesoic acid, β-bergamotenoic acid, curcumenoic acid	
			Alkane	3-methylhentriacontane, *n*-dotriacontane, 2-methyltriacontane, *n*-pentacosane	
			Fatty acid	7,10,13-hexadecatrienoic acid	
Heliothis virescens	Generalist	Larva	Flavonoid	rutin	Blaney and Simmonds 1983
Spodoptera littoralis	Generalist	Larva	Flavonoid	rutin	Blaney and Simmonds 1983
		Adult	Sugar	sucrose, glucose	Popescu et al. 2013
			Alkaloid	caffeine	
			Salt	sodium chloride	
Sphingidae					
Manduca sexta	Specialist	Larva	Sugar	*myo*-inositol	Nelson 1996
Papilionidae					
Graphium sarpendon	Specialist	Larva	Sugar	sucrose	Zhang et al. 2015

Table 1.1 continued.

Taxonomy	Host breadth	Life stage	Chemical class	Compound	Reference
			Chlorogenic acid	5-*O*-caffeoylquinic acid	
			Flavonoid glucoside	quercetin 3-*O*-β-glucopyranoside	
			Fatty acid	α-linolenic acid	
		Adult	Aldehyde	decanal, nonanal	Li et al. 2014
Papilio xuthus	Specialist	Larva	Sugar	D-glucose, D-fructose, D-sucrose	Murata et al. 2011
			Alkaloid	(-)-stachydrine	
			Sterol	1-linolenoylglycerol, 1-linoleoylglycerol, 1-octadecenoylglycerol, 1-stearoylglycerol, 1,2-dilinolenoyl-3-galactosyl-*sn*-glycerol	
			Polymethoxyflavone	isosinensetin	
			Cyclic peptide	citrusin I	
		Adult	Flavonoid	vicenin-2	Ohsugi et al. 1985
				hesperidin, rutin	Nishida et al. 1987, 1990
			Nitrogenous base	adenosine	
			Alkaloid	5-hydroxy-N_{ω}-methyltryptamine, bufotenin	
				(--)-synephrine	Ohsugi et al. 1991
			Sugar	(÷)-chiro-inositol	Ohsugi et al. 1991
			Alkaloid	(-)-stachydrine	
Pieridae					
Pieris rapae	Specialist	Larva	Glucosinolate	gluconasturtiin	Miles et al. 2004
		Adult		gluconasturtiin, glucocapparin, sinalbin, glucotropaeolin, sinigrin, glucoalyssinin, glucocheirolin, glucoerucin, glucoiberin	Stadler et al. 2008
Pieris brassicae	Specialist	Larva	Glucosinolate	glucoiberin, glucocheirolin, glucoerucin, sinigrin, glucoepparin, progoitrin, glucosinalbin, glucotropaeolin	David and Gardiner 1966

Table 1.1 continued.

Taxonomy	Host breadth	Life stage	Chemical class	Compound	Reference
		Adult	Glucosinolate	sinigrin, glucobrassicin	Loon et al. 1992
Nymphalidae					
Junonia coenia	Specialist	Larva	Iridoid glycoside	aucubin, catalpol	Bowers 1984
		Adult		catalpol	Pereyra and Bowers 1987

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II. LARVAL NAVEL ORANGEWORM, *AMYELOIS TRANSITELLA*, ORIENTATION TO HOSTPLANT KAIROMONES

Introduction

Although the vast majority of lepidopterans are oligophagous, many of the most important economic pests are polyphagous species. How adults and larvae of species that utilize a broad range of phytochemically diverse hostplants evaluate hostplant suitability has not been well-characterized. One such polyphagous species is *Amyelois transitella* (Lepidoptera: Pyralidae), the navel orangeworm and its facultative fungal associate *Aspergillus flavus*. The New World native navel orangeworm is highly polyphagous, utilizing multiple, chemically diverse plant families throughout its range from the southern United States through Central America into South America. Along with native hosts in Fabaceae and a few genera in other families (Sapindaceae, Rubiaceae, Asparagaceae), hosts include a diversity of exotic crop plants, including orange, grapefruit (Rutaceae), almonds, peach, apple, pear (Rosaceae), figs (Moraceae), dates (Arecaceae), pomegranates (Lythraceae), pistachios (Anacardiaceae), and English walnut (Juglandaceae; Heinrich 1956; Niu et al. 2011). The predominant larval feeding mode among species in the genus *Amyelois* and related phycitine pyralid genera is internal feeding in fallen fruits, primarily of legumes and secondarily in fruits of other families (Heinrich 1956).

Infestation of fruit by navel orangeworm is almost invariably associated with *Aspergillus* infection (Palumbo 2014). Evidence that this association is mutualistic includes discoveries that: 1) the moth is a vector for *A. flavus* spores (Palumbo et al. 2014); 2) development in caterpillars is delayed without the fungus (Siegel et al. 2010; Bush 2015); 3) larval development is faster in the presence of a toxic phytochemical (xanthotoxin) when the fungus which degrades xanthotoxin is also present in the diet (Bush 2015); 4) fungal growth is enhanced on Navel

orangeworm frass (Bush 2015); 5) ovipositing moths are electrophysiologically responsive to conophthorin, an *Aspergillus* volatile (Beck et al. 2014).

In this study, I examined whether volatile phytochemicals that are electrophysiologically active in adult stages of navel orangeworm affect behavior of the larvae. To aid in the design of a practical field trapping lure for navel orangeworm management, Beck et al. (2012) examined almond volatiles for their attractiveness to navel orangeworm moths (Table 2.1). A blend of the almond volatiles1-octen-3-ol, ethyl benzoate, methyl salicylate, acetophenone, and conophthorin attracted both male and female moths under field conditions. Follow-up electroantennogram screening studies of 21 compounds emitted by almonds at hull split demonstrated electrophysiological activity primarily in females of a majority of these compounds. Several of the compounds that were electrophysiologically active are associated with mechanically damaged plants (1-octen-3-ol) or plants contaminated by fungi (conophthorin), suggesting a role for fungal mutualists of navel orangeworm in adult host orientation behavior.

In view of the behavioral and physiological evidence of activity of almond-associated volatiles in adults, I conducted bioassays with newly hatched first instar navel orangeworm caterpillars to determine if larvae of this polyphagous species display similar chemo-orientation behavior to this array of chemicals.

Materials and Methods

Navel orangeworm colony: Navel orangeworm used in this study were shipped as eggs on moistened paper towels in Ziploc© bags from a laboratory colony maintained by the USDA-ARS Parlier laboratory, where they are reared on a modified wheat bran diet at a constant temperature of 28 ± 4^{o}C with a 16:8 (L:D) hours photoperiod (Finney and Brinkman 1967; Demkovich et al. 2015). The eggs were stored under similar conditions in the University of

Illinois at Urbana-Champaign Department of Entomology insectary. First instar larvae were chosen for bioassay within 24 hours of hatching.

Aspergillus flavus culture: To test attractiveness of *A. flavus*, I used a culture of atoxigenic *A. flavus* (AF36) maintained at the University of Illinois at Urbana-Champaign by Daniel Bush; this culture originated from infected wheat seeds sent to the University of Illinois at Urbana-Champaign by Themis Michailides (University of California, Davis). The *A. flavus* culture was grown on potato dextrose agar (PDA) at room temperature (~24^{o}C). For bioassays, an experimental medium was prepared by mixing almond meal with PDA; this medium was composed of 400 mL of water, 15.6 g of PDA (Sigma-Aldrich, St. Louis, MO), 21.88 g of Bob's Red Mill (Milwaukee, OR) almond meal, and 0.057 g of streptomycin (Sigma-Aldrich) for ~10 (8.5 cm diameter) plates. An agar plug 5 mm in diameter from a sporulating culture one to two weeks old was used to inoculate the almond PDA plates. To create a fungus-free control treatment, media were treated with1 mL of 10% formaldehyde (diluted from 36.5-38.0% stock) (Macron, Center Valley, PA).

Chemical preparations: The chemicals used in the bioassay were purchased as follows: methyl salicylate, ethyl benzoate, methanol, 2-phenylethanol, and 1-octen-3-ol from Sigma-Aldrich (St. Louis MO, USA); β-caryophyllene from TCI (Tokyo Chemical Industry, Portland OR, USA); and farnesene from Sigma-Aldrich Fine Chemicals (St. Louis MO, USA). Conophthorin, provided by Dr. Matthew Ginzel from Purdue University, was collected from lures manufactured by Contech Inc. (Victoria BC, Canada). These compounds were each diluted in methanol to achieve solutions with concentrations of 1% and 0.1%.

I created a methanolic extract of *A. flavus* 100 mg of *A. flavus* scrapings from the top of the colony to 10 mL of methanol. I also created an almond meal extract by adding 1g of almond

meal to 10 mL of methanol. The mixtures were vortexed for 30 seconds and then set aside for approximately one hour to allow solids to settle. I then decanted the supernatant and used this solution for the bioassay. I also prepared a combination of both extracts by combining the two solutions in a 1:1 ratio.

Behavioral bioassay: I tested larval orientation in a two-choice still air assay with methanolic dilutions pipetted onto 6±0.5 mm filter paper discs, created with a hole punch from grade 1 Whatman® qualitative filter paper. Immediately prior to each trial, 2 µg of a methanolic dilution or extract, enough to saturate the filter paper disc, was pipetted on one paper disc and methanol on the other. Bioassays were carried out in arenas fabricated from plastic Petri dishes (100 mm diameter, 15 mm tall; Figure 2.1). I divided each dish in half with a line drawn down the center on the outside of the dish with a permanent marker and placed a filter paper disc on each side of the dish, farthest away from the center line. The methanol was allowed to evaporate before a single first instar larva was placed in the center of the Petri dish using a soft paintbrush. The paintbrush was washed with ethanol and allowed to air dry before each trial.

The location of the larva, either control or treatment side of the arena, was recorded at one-minute intervals for five minutes. Taking observations at one-minute intervals allowed the neonates ample time to travel the diameter of the arena. Observations were recorded for five minutes; beyond this time period a certain proportion of larvae escaped from the arena in pre-trials. To minimize any positional effects, the location of the treatment was alternated between left and right sides of the arena between trials. In one set of bioassays, almond meal extract was compared against a 1:1 solution of *A. flavus* and almond meal extract. Another set compared almond meal extracts spiked with conophthorin and 1-octen-3-ol (0.01 µl / 1 µl almond meal

extract) to both methanol and almond meal extract controls. A total of 40 trials were conducted for each compound bioassayed.

Statistical analysis: To test if the summed number of larvae on each side of the arena at each minute interval significantly differed, a logistic regression model was applied to the data using the generalized linear model function in R version 2.15.2 (Hothorn and Everitt 2014) with time points as explanatory variables. A separate regression was run for each two-way orientation assay.

Results

Of the nine individual compounds assayed, three elicited a significant response—that is, they were found more often than random on the side with the test compound (attractant/arrestant) or on the side with the control (repellent). Of these responses, 1-octen-3-ol and 2-phenylethanol were attractive or arrestant to larvae and one compound, ethyl benzoate, repelled larvae (Table 1). Individual time points for assays showing a significant effect include: 1-octen-3-ol at 1, 4, and 5 minutes ($p < 0.05$); 2-phenylethanol at 5 minutes ($p < 0.04$); ethyl benzoate at 2 ($p < 0.01$), 3, and 4 minutes ($p < 0.05$); almond meal extract + conophthorin at 4 and 5 minutes ($p < 0.001$) when tested against methanol, at 5 minutes ($p < 0.05$) when tested against unaltered almond meal; and almond meal extract + 1-octen-3-ol at 4 and 5 minutes ($p < 0.05$) when tested against methanol.

Larvae were not significantly attracted to the fungus-associated compound conophthorin (Table 2.2, Figure 2.3) but this chemical did interact synergistically with almond meal in that the combined solution was significantly attractive or arrestant to larvae when compared against both methanolic and almond extracts (Table 2.3, Figure 2.2). Larvae were also attracted to 1-octen-3-ol (Table 2.2, Figure 2.2), but there was no synergistic interaction observed between 1-octen-3-ol

and the methanolic almond extract. Larvae significantly oriented toward the almond extract solution spiked with 1-octen-3-ol when compared to methanol, but there was no significant preference when compared against pure almond extract (Table 2.2, Figure 2.2, Figure 2.3). Farnesene, β-caryophyllene, α-humulene, and methyl salicylate also failed to produce a significant orientation response (Table 2.2, Figure 2.3). Larvae did not display a significant response in any trials testing the lower concentration of 0.001µL compound per 1 µL methanol (Table 2.2, Figure 2.3).

Discussion

In contrast with some previous studies of hostplant evaluation by larvae of oligophagous species, I found the larvae of navel orangeworm oriented to hostplant volatiles that are attractive and electrophysiologically active in adults. Whether this behavior represents attractant activity of arrestant activity is difficult to determine, in this still-air assay. An attraction to both 2-phenylethanol and 1-octen-3-ol on the part of larvae is consistent with the general feeding behavior of this species, individuals of which, although highly polyphagous, tends to feed preferentially on fungus-infected or fallen fruits of tree species. Thus, orientation behavior on the part of the larva to signals indicative of hostplant tissue damage, such as hull split, or fungus infection should act to increase the likelihood that larvae can establish themselves in host fruit. Hull split is a period of particular vulnerability of nut crops because entry into the nut or fruit is greatly facilitated. It follows that larvae would benefit from the ability to use volatile chemical cues to locate these hull splits. The attractive compound 2-phenylethanol is associated with mechanically damaged hostplants and additionally the attractive compound 1-octen-3-ol is associated with decomposing hostplants (Beck et al. 2012). Not surprisingly, only the higher concentrations of both solutions (1%) were attractive. Most of the compounds tested comprise

between 1-5% of the volatiles collected from almonds during hull split (Beck 2014). The lower concentration of 0.01% was likely too low to represent realistic volatile levels in the field.

In terms of compounds that repelled larvae, the one identified in this study has been shown to be fungicidal; repellency might reflect the mutualistic association between *Aspergillus* fungi and navel orangeworm. Ethyl benzoate inhibits both *Aspergillus* growth and aflatoxin release (Chipley and Uriah 1980). The navel orangeworm is extraordinary in its ability to tolerate diets contaminated with high levels of aflatoxin compared to other lepidopterans (Niu et al. 2009). This avoidance of ethyl benzoate suggests that larvae orient to volatiles that indicate hostplants that are suitable both for larval and fungal growth. Similar behavior has been documented in other fungus-insect mutualisms; workers of the leafcutter ant *Atta cephalotes*, for example, avoid terpenes that are toxic to their mutualistic fungus (Hubbell et al. 1983; Howard et al. 1988, 1989).

Even though they can vector *Aspergillus* fungi, navel orangeworm neonates may benefit by finding a host already colonized by the fungus, as they develop more slowly when it is absent (Siegel et al. 2010; Bush 2015; personal observation). But, unlike the obligatory mutualism between leafcutter ants and their symbiotic fungi, the association between *Aspergillus* and navel orangeworm appears to be facultative in nature. Navel orangeworm larvae can grow in the absence of fungi (Bush 2015). The facultative nature of the relationships is suggested by results of this study showing that neither the fungal extract nor conophthorin alone was attractive; conversely, a preference over almond meal extract for extract spiked with conophthorin was observed, suggesting synergism between the volatile kairomones. In contrast, there was no preference determined for almond meal extract spiked with the attractant 1-octen-3-ol over an

unaugmented extract, suggesting no synergism between a breakdown product common to many hostplants and undamaged hostplant volatiles.

Larvae also displayed no preference for the almond compounds farnesene, β-caryophyllene, α-humulene, and methyl salicylate. This lack of preference may reflect adaptive avoidance of fungicidal compounds. Farnesene is ubiquitous among plants and other studies have demonstrated its fungicidal potential. It is the primary sesquiterpene constituent of *Lippia rugosa* essential oil, which has been shown to inhibit *Aspergillus flavus* growth and aflatoxin B1 production (Tatsadjieu 2008). Similarly, β-caryophyllene is a component of cinnamon essential oil, which has been shown to inhibit both growth of the fungus and production of aflatoxin in damaged fruit (Xing et al. 2010). Several essential oils, such as those of juniper (*Juniperus communis*), lemon thyme (*Thymus citriodorus*), and goatweed (*Ageratum conyzoides*), that contain α-humulene as a minor component are additionally inhibitory of *Aspergillus* growth (Cavaleiro 2010; Juliana 2006; Pinto et al. 2006). Lastly, methyl salicylate itself is toxic to *Aspergillus* fungi (Chipley and Uraih 1980). All four compounds are associated with natural systems that antagonize the growth of *A. flavus*. While not repelled by any of the four compounds, orienting toward these compounds, given a facultative mutualism between *A. flavus* and navel orangeworm, would likely not accrue to the benefit of a larva.

In conclusion, both adults and, as I have shown here, larvae of navel orangeworm respond behaviorally to phytochemicals associated with damaged and decaying hostplant, consistent with their tendency to prefer to infest damaged fruit. Orientation toward 1-octen-3-ol may enable neonates to orient on their host to find a point of entry--for example, a hull split in an almond. Also, both adults and larvae respond behaviorally to ubiquitous phytochemicals, consistent with their ability to utilize a wide variety of taxonomically unrelated hostplants. A

more thorough characterization of the behavior of navel orangeworm larvae and the nature of the relationship between navel orangeworm and *As. flavus* may help to inform control methods. It may be possible, for example, to enhance insecticide formulations with the volatiles identified in this study, or even use a combination of chemical attractants and repellents, a kairomone disruption control strategy, to confuse larvae and prevent them from locating hull splits and gaining access to their host fruits. Current management practice includes changing out lures for navel orangeworm egg traps once they become moldy, but in view of the fact that, in this study, larvae preferentially orient toward almond meal spiked with fungal volatiles, it may prove beneficial to leave them in place after fungus begins to grow (Zalom et al. 2009). Developing new methods aimed at modifying navel orangeworm larval behavior will likely lead to a more robust integrated pest management program and, in turn, further reduce reliance on synthetic organic insecticides, with their attendant environmental impacts.

Tables and Figures

Table 2.1. Almond constituents found to be electrophysiologically active in navel orangeworm antennae. Compounds marked with * were found in damaged almonds. Table is modified from Table 1 in Beck et al. (2014).

	EAG response (μV)[a]	
Compound	**Male**	**Female**
conophthorin*	55	130
β-caryophyllene	0	190
ethyl benzoate*	0	525
α-humulene	187	250
(*E*,*E*)-α-farnesene	160	155
methyl salicylate	245	175
2-phenylethanol*	54	320
1-octen-3-ol*	0	475

Table 2.2. Proportion of navel orangeworm larvae on filter paper discs at each timepoint with individual methanolic (MeOH) extracts. Significance: $^{+}$ $p < 0.05$, * $p < 0.01$
Green = attractant, orange = repellent

Treatment (per 1µL MeOH)		Proportion of larvae on treatment disk Time point (minute) 1	2	3	4	5
MeOH (solvent control)		0.60	0.47	0.38	0.49	0.51
1-octen-3-ol	0.01µL	0.69^{+}	0.65	0.66	0.68^{+}	0.75^{+}
	0.001µL	0.46	0.44	0.49	0.42	0.50
2-phenylethanol 0.01µL	0.01µL	0.50	0.53	0.56	0.66	0.73^{+}
	0.001µL	0.48	0.44	0.48	0.38	0.46
conophthorin 0.01µL	0.01µL	0.66	0.61	0.54	0.57	0.51
	0.001µL	0.51	0.49	0.53	0.51	0.55
farnesene 0.01µL	0.01µL	0.36	0.46	0.42	0.41	0.39
	0.001µL	0.63	0.52	0.52	0.48	0.58
β-caryophyllene 0.01µL	0.01µL	0.33	0.41	0.39	0.37	0.34
	0.001µL	0.48	0.66	0.66	0.67	0.60
ethyl benzoate 0.01µL	0.01µL	0.46	0.24*	0.32^{+}	0.31^{+}	0.43
	0.001µL	0.48	0.47	0.53	0.63	0.52
α-humulene 0.01µL	0.01µL	0.54	0.51	0.39	0.41	0.40
	0.001µL	0.36	0.51	0.45	0.42	0.36
methyl salicylate .01µL	0.01µL	0.44	0.54	0.52	0.52	0.54
	0.001µL	0.53	0.53	0.47	0.39	0.46

Table 2.3. Proportion of navel orangeworm larvae on filter paper discs at each timepoint with methanolic extracts. Significance: $^{+}$ $p < 0.05$, * $p < 0.01$, † $p < 0.001$
Green = attractant

Treatment (per 1µL MeOH)	Control	Proportion of larvae On Treatment Time point (minute) 1	2	3	4	5
almond meal 1mg/10mL	MeOH	0.50	0.63	0.67	0.66	0.65
fungal extract 1mg/10mL	MeOH	0.45	0.46	0.46	0.5	0.51
fungal + almond extract	MeOH	0.57	0.56	0.50	0.48	0.56
conophthorin 0.01µL + almond extract	MeOH	0.56	0.58	0.62	$0.81^{\dagger}$	$0.79^{\dagger}$
	almond	0.44	0.42	0.47	0.63	0.70^{+}
1-octen-3-ol 0.01µL + almond extract	MeOH	0.49	0.63	0.65	0.71^{+}	0.68^{+}
	almond	0.41	0.47	0.51	0.57	0.65

Figure 2.1. Example bioassay arena made from a bisected 100 mm diameter x 15 mm height plastic Petri dish with a 6.0±0.5 mm grade 1 Whatman® qualitative filter paper disc placed on each side, saturated with 2 µL of test solution**.**

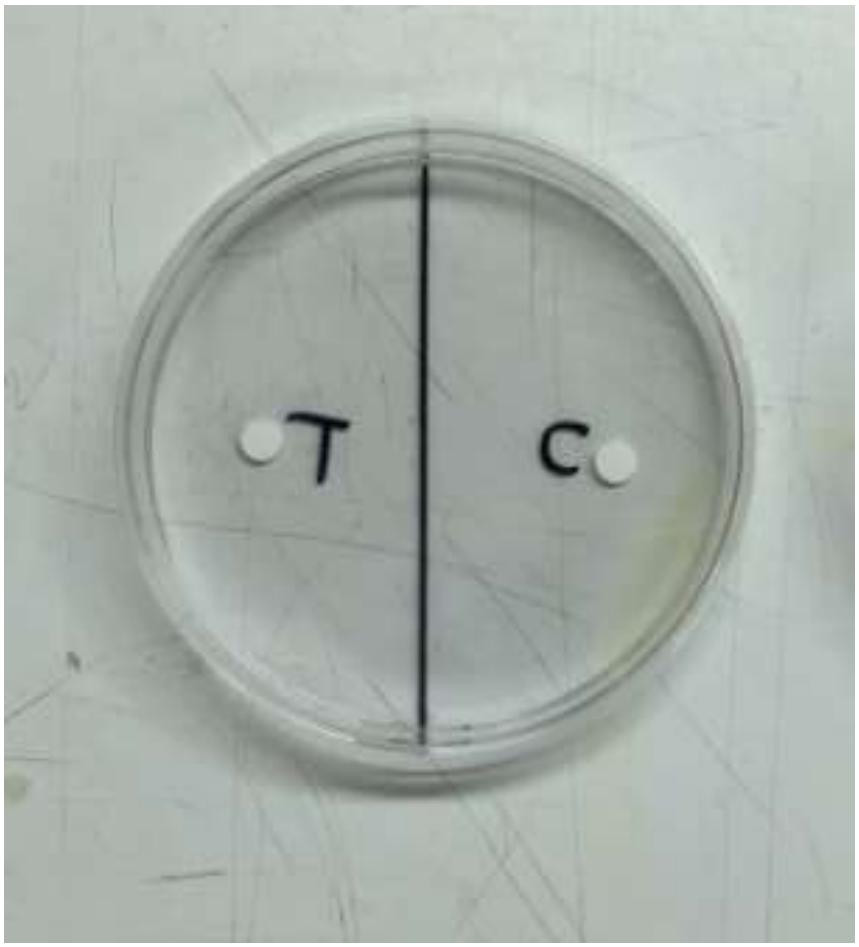


Figure 2.2. Number of larvae found on either side of the assay arena at each time point presented with filter paper discs with or without chemicals. Only compounds with significant effects are shown. Significance: * p<0.05, ** p<0.01, *** p<0.00 1

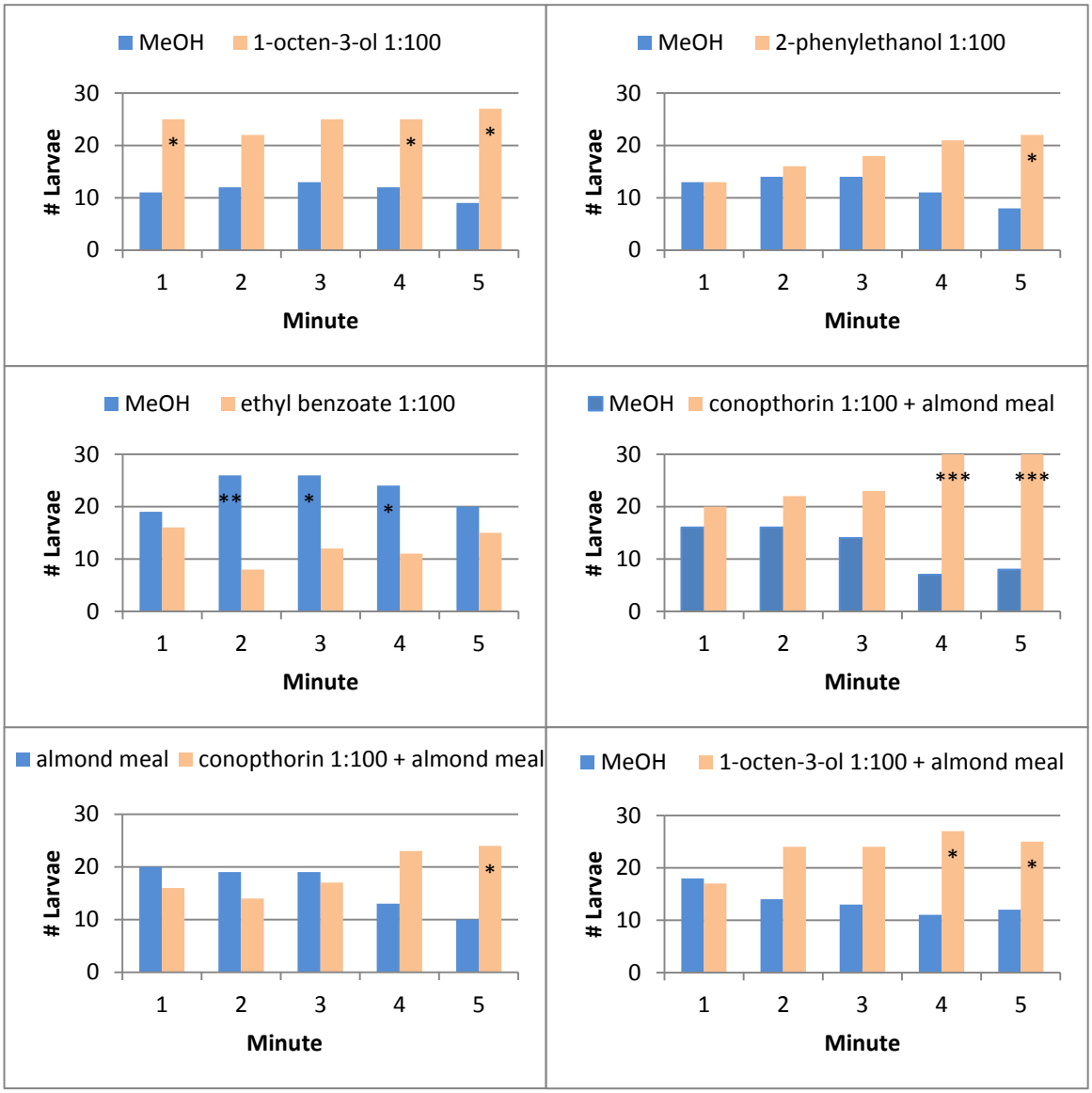


Figure 2.3. Number of larvae found on either side of the assay arena at each time point presented with filter paper discs with or without chemicals. Only compounds without significant effects are shown. Significance: * p<0.05, ** p<0.01, *** p<0.001

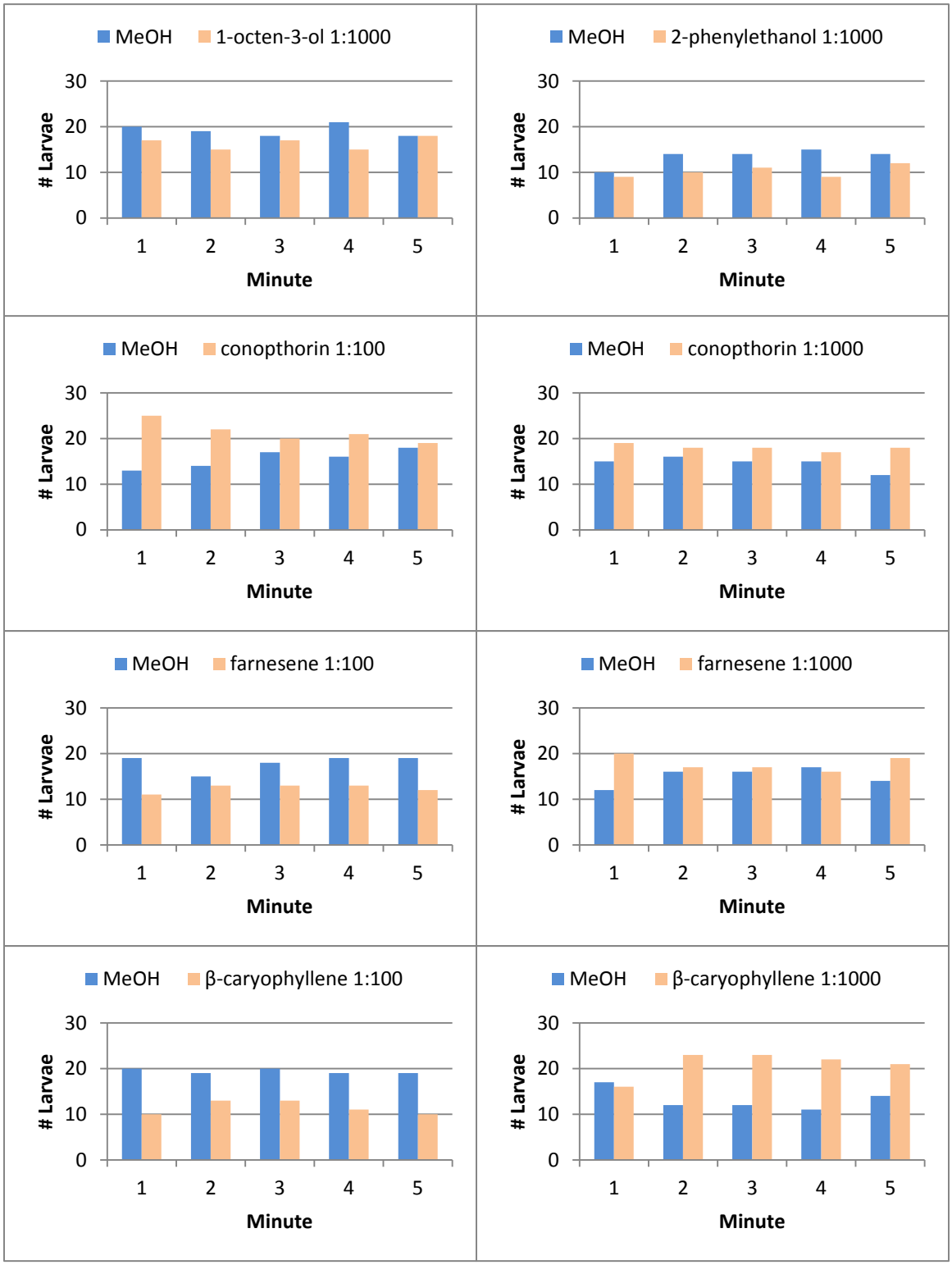


Figure 2.3 continued.

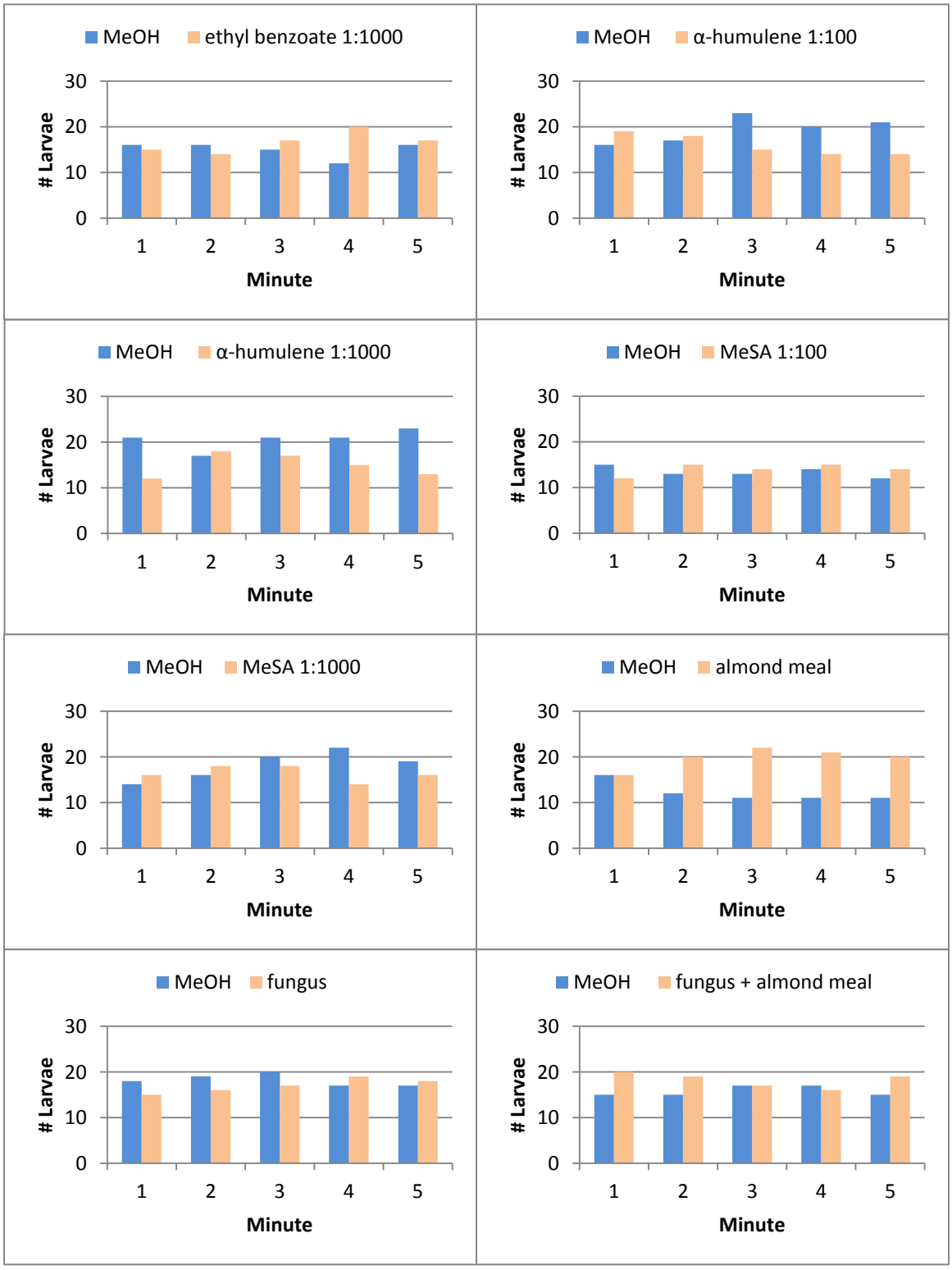
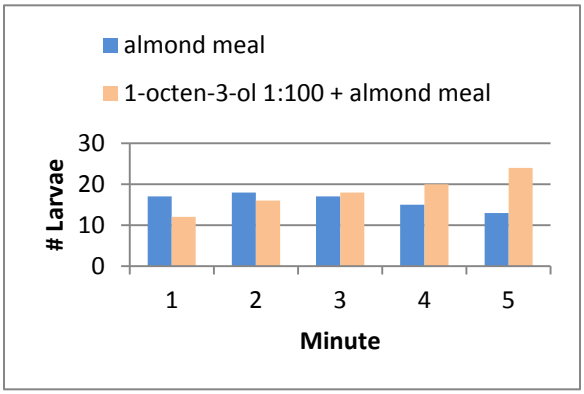


Figure 2.3 continued.



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APPENDIX

Appendix 1. Sample annotated R code and output for statistical analysis. Code annotations are denoted by #### preceding the note.

```
> #### define the explanatory variable with five levels:
> #### 1= @1minute, 2= @2minute, 3= @3minutes, 4= @4minutes, 5=@5 minutes
>
> etbenzoate=as.factor(c(0,1,2,3,4,5))
>
> #### NOTE: if we do almondmeal=c(1,2,3,4,5) R will treat this as
> #### a numeric and not categorical variable
>
> #### need to create a response vector so that it has counts for both
"success" and "failure"
> #### success = treatment, failure = control
> #### Note: for this data need to compare odds ratios not probability
> #### use exp(cbind)to compute odds ratios
>
> response<-cbind(no=c(0,19,26,26,24,20),yes=c(0,16,8,12,11,15))
>
> response
     no yes
[1,]  0   0
[2,] 19  16
[3,] 26   8
[4,] 26  12
[5,] 24  11
[6,] 20  15
>
> #### fit the logistic regression model
>
>
> etbenz.logistic<-glm(response~etbenzoate+0, family=binomial(link=logit))
>
>
> #### OUTPUT
> exp(cbind(OR = coef(etbenz.logistic), confint(etbenz.logistic)))
Waiting for profiling to be done...
                 OR     2.5 %   97.5 %
etbenzoate0      NA        NA       NA
etbenzoate1 1.187500 0.6108058 2.338400
etbenzoate2 3.250000 1.5389794 7.681431
etbenzoate3 2.166667 1.1176828 4.451587
etbenzoate4 2.181818 1.0947220 4.635250
etbenzoate5 1.333333 0.6857057 2.649239
> etbenz.logistic

Call:  glm(formula = response ~ etbenzoate + 0, family = binomial(link =
logit))

Coefficients:
etbenzoate0  etbenzoate1  etbenzoate2  etbenzoate3  etbenzoate4  etbenzoate5
         NA       0.1719       1.1787       0.7732       0.7802       0.2877

Degrees of Freedom: 5 Total (i.e. Null);  0 Residual
Null Deviance:     21.24
Residual Deviance: 7.105e-15   AIC: 29.52
> summary(etbenz.logistic)

Call:
glm(formula = response ~ etbenzoate + 0, family = binomial(link = logit))
```

```
Deviance Residuals:
[1]  0  0  0  0  0  0

Coefficients: (1 not defined because of singularities)
            Estimate Std. Error z value Pr(>|z|)
etbenzoate0       NA         NA      NA       NA
etbenzoate1   0.1719     0.3393   0.506  0.61253
etbenzoate2   1.1787     0.4043   2.915  0.00355 **
etbenzoate3   0.7732     0.3490   2.215  0.02673 *
etbenzoate4   0.7802     0.3641   2.143  0.03214 *
etbenzoate5   0.2877     0.3416   0.842  0.39965
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 2.1235e+01  on 5  degrees of freedom
Residual deviance: 7.1054e-15  on 0  degrees of freedom
AIC: 29.521

Number of Fisher Scoring iterations: 3

> anova(etbenz.logistic)
Analysis of Deviance Table

Model: binomial, link: logit

Response: response

Terms added sequentially (first to last)


           Df Deviance Resid. Df Resid. Dev
NULL                           5     21.235
etbenzoate  5   21.235         0      0.000
```