

FACULTATIVE MUTUALISM BETWEEN THE NAVEL ORANGEWORM *AMYELOIS
TRANSITELLA* (LEPIDOPTERA: PYRALIDAE) AND *ASPERGILLUS FLAVUS*

BY

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THESIS

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ABSTRACT

The navel orangeworm, *Amyelois transitella* Walker (Lepidoptera: Pyralidae), is an economic pest of considerable importance by virtue of its habit of attacking damaged or overripe tree nuts and fruits in Californian orchards. Its economic impact is increased by its common association with the highly toxigenic fungus *Aspergillus flavus* Link. Despite the capacity of this fungus-insect association to damage a wide variety of tree crops, relatively little is known about the ecology of the interaction. I examined three aspects of this association to examine the possibility that the interaction represents a facultative mutualism. These studies are here presented in three chapters. First, to determine whether associating with the fungus allows the navel orangeworm to utilize its hostplants more efficiently, I conducted a series of laboratory bioassays to test if the presence of *A. flavus* decreases the toxicity of hostplant phytochemicals. Growth rates, mortality rates, and pupal weight for navel orangeworms were measured on an artificial potato dextrose agar (PDA) diet containing almond meal, in the presence and absence of the furanocoumarins xanthotoxin and bergapten, which occur in some fruit hostplant species.

In the absence of furanocoumarins, larvae reached adulthood in 29.6 d on average in the presence of the fungus as opposed to 43.8 d in the absence of *A. flavus* ($t = 16.06$; $df = 3$; $P < 0.001$). Female and male pupae had on average five or six g more mass, respectively, when the larvae were fed with a diet containing fungus ($t = 5.56$; $df = 3$; $P = 0.011$ and $t = 7.16$; $df = 3$; $P = 0.006$, respectively). By contrast, the presence of furanocoumarins at natural concentrations in the diet (at concentrations determined by prior LC50 experiments) in the absence of the fungus extended the development time of the navel orangeworm by 14 to 22 d on average for bergapten and xanthotoxin, respectively ($F = 1013.73$; $df = 3$; $P < 0.001$). Pupal weights decreased as much as two-thirds in the presence of furanocoumarins ($F = 328.09$; $df = 5$; $P < 0.001$ for males, $F =$

83.00; $df = 5$; $P < 0.001$ for females), and mortality increased two-fold ($F = 9.61$; $df = 5$; $P < 0.001$). When navel orangeworms were raised on furanocoumarin-containing diets in the presence of the fungus, however, very little growth rate reduction was observed, and mortality returned to the levels observed on diets lacking furanocoumarins, although pupal weights remained depressed. These findings indicate that the presence of the fungus benefits navel orangeworm larvae by enhancing growth rate and survival, both directly and indirectly (by reducing the toxicity of host phytochemicals).

Independent of its association with the navel orangeworm, *A. flavus* is an important plant pathogen that contributes to millions of dollars of crop loss annually. The adult navel orangeworm moth is a known vector of *Aspergillus* species. The competition between caterpillars and fungus for resources would seem to be a drawback of this partnership, as both organisms use the same plant materials for nutrition, but previous work has shown that *A. flavus* thrives in the presence of the navel orangeworm. In a second study, I assessed the growth of *A. flavus* in the presence of navel orangeworm larvae and in or on their frass by rearing larvae on an artificial diet in the presence and absence of the fungus. I found that the fungus grew ~2-fold faster on almond PDA in the presence of navel orangeworm larvae ($t = 52.14$; $df = 19$; $P < 0.001$). Additionally, I collected frass from larvae fed on standard lepidopteran diet and almond PDA and incorporated it into agarose diets to see if these diets could support fungal growth. On both frass diets, *A. flavus* grew rapidly, at rates intermediate between PDA and almond PDA. Therefore, *A. flavus* appears to use navel orangeworm larvae as both vectors and sources of nitrogen-rich substrate in the form of frass. I then collected frass from larvae that had fed on fungus-containing diets and examined it (at 800-2000X magnification) for the presence of fungal conidia, hyphae, or other intact fungal elements. In frass from both diets, I observed intact

conidial heads, ornamented conidia, and septate hyphae. Moreover, conidia were isolated from this frass and transferred to PDA to assess viability. In some cases, the recovered conidia produced a viable colony, indicating that caterpillar excrement may be an alternative vehicle for fungal dispersal.

If the interaction between the navel orangeworm and *Aspergillus* fungi represents a mutualistic relationship, it would be highly advantageous for the insect to be able to identify and seek out fungal colonies as well as spread them. I conducted a series of behavioral assays to assess whether larval and adult navel orangeworms can preferentially orient to the fungus. In an oviposition assay, I presented gravid female moths with a choice between *Aspergillus*-infected and uninoculated oviposition sites. Mating pairs were released in arenas with two PDA dishes, one inoculated with *A. flavus*, and one sterilized. On average, females laid 44.3 eggs on inoculated dishes and 12.7 on the uninoculated sites, with no oviposition elsewhere in the arena ($t = 4.30$; $df = 2$; $P = 0.026$). In addition, 62% of eggs on the sites with fungus present were fertilized, compared to only 26% on uninoculated sites ($t = 4.30$; $df = 2$; $P = 0.048$). The differential rates of fertilization were unexpected. One possible explanation is that females preferentially lay greater numbers of unfertilized eggs on nutritionally deficient food sources to provide supplemental nutrients to hatching larvae of this cannibalistic species. To test this hypothesis, I conducted an assay comparing larval survivorship in the presence and absence of supplemental unfertilized eggs. I found that neonates provisioned with eggs survived 208.8 h on average, while starved neonates survived only 85.2 h ($t = 2.78$; $df = 4$; $P = 0.002$). Thus, navel orangeworms may be taking advantage of cannibalism to compensate for ovipositing on less nutritious hosts. These results are consistent with the hypothesis that the interaction between *A. flavus* and the navel orangeworm is a facultative mutualism.

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**CHAPTER I: NAVEL ORANGEWORM LARVAL DEVELOPMENT AND TOXICITY
OF FURANOCOUMARINS IN THE PRESENCE AND ABSENCE OF *ASPERGILLUS
FLAVUS***

Introduction

The navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), is a highly polyphagous pest in California, particularly of nut crops. This native lepidopteran has colonized and become established on a wide variety of orchard crops and causes extensive losses to almonds (*Prunus dulcis* [Mill.] D.A. Webb), pistachios (*Pistacia vera* L.), pomegranates (*Punica granatum* L.), and figs (*Ficus carica* var. *domestica* Czern. & Rav.) (Connell 2002). Larvae gain access to almonds during hull split, and, depending on almond variety, feed on the exposed kernel (Palumbo et al. 2014). In the course of feeding, navel orangeworms contaminate nuts with frass and webbing, and damaged nuts can be colonized by fungi, particularly species in the genus *Aspergillus* (Zalom et al. 2012). These species are parasites on a wide variety of crop plants, often damaging fruits or vegetative tissues exposed to adverse environmental conditions (Amaike and Keller 2011). In addition to direct spoilage, some species of *Aspergillus*, especially those in section *Flavi*, produce toxic compounds, including aflatoxins and ochratoxins (Niu et al. 2009). Aflatoxins are the most carcinogenic natural substances known, and high levels of aflatoxin B1 render crops unsaleable (Eaton and Gallagher 1994).

High concentrations of both *Aspergillus flavus* conidia and aflatoxins have frequently been found in almond orchard soil and nuts (Luo et al. 2009, Palumbo et al. 2014). Considerable effort has been expended to develop sanitation protocols to minimize *Aspergillus* infections of tree nuts (Campbell et al. 2003, Zalom et al. 2012), but toxigenic species of *Aspergillus* remain a

serious threat to orchard crops in the California Central Valley. *Aspergillus* species require mechanical damage (e.g., hull cracking) to colonize plant hosts (Widstrom 1979, Campbell et al. 2003), and *A. flavus* is closely associated with nuts damaged by navel orangeworms (Phillips et al. 1976, Doster and Michailides 1994). Navel orangeworm moths and larvae transport the conidia of *Aspergillus* species in the orchard (Palumbo et al. 2014), and the relationship between these two species may be a facultative mutualism. Such relationships are known to exist between moths and fungi (e.g., Mondy et al. 1998), although they are often poorly understood.

Consistent with a mutualistic relationship is the fact that, relative to other lepidopterans, navel orangeworms are highly resistant to aflatoxin toxicity, due in part to cytochrome P450 monooxygenase-mediated detoxification of aflatoxin B1, rather than bioactivation of aflatoxin B1 into more toxic metabolites (Niu et al. 2009). Consequently, larvae can tolerate fungus-infected substrates on which other pests cannot survive.

Little is known of the performance of navel orangeworm larvae in the presence and absence of this fungus. Fungal infection of tree nuts may provide nutritional benefits, as has been shown in other lepidopteran larvae (Rawlins 1984). However, as a highly polyphagous pest, the navel orangeworm encounters a wide variety of host plant challenges, including phytochemicals such as furanocoumarins, which are found in some of its hosts (Niu et al. 2011).

Furanocoumarins bind to DNA and other macromolecules, and they are frequently phototoxic, causing oxygen-dependent or oxygen-independent photosensitization in lepidopteran larvae (Berenbaum and Feeny 1981, Berdegué et al. 1997). Although navel orangeworm caterpillars are capable of detoxifying furanocoumarins to some degree via cytochrome P450 monooxygenases (Niu et al. 2011, 2012), their growth and survival can still be hampered somewhat by the presence of linear furanocoumarins in their diet (Bagchi, personal communication). *Aspergillus*

fungi are known to detoxify a variety of phytochemicals, including furanocoumarins (Desjardins et al. 1989). Therefore, cohabiting with an opportunistic plant pathogen such as *A. flavus* may provide benefits to the navel orangeworm in the form of fungus-mediated detoxification or sequestration of phytochemicals in addition to the insect's native P450 activity.

In this study, I conducted a bioassay to determine whether navel orangeworm larvae experience enhanced growth in the presence of fungus. I monitored the performance of the larvae using potato dextrose agar (PDA) and almond PDA diets with and without *A. flavus*. In addition, I assessed the performance of larvae with and without *A. flavus* on PDA diets containing xanthotoxin or bergapten to determine whether the fungus helps larvae overcome chemical defenses. Xanthotoxin is found in navel orangeworm host plants in the Rutaceae, while bergapten is found in hosts in the Rutaceae and Moraceae.

Materials and Methods

Navel Orangeworm Rearing and Media Used. A colony was established from larvae obtained from the USDA-ARS Parlier laboratory and reared on a modified wheat bran diet under conditions of $28 \pm 4^\circ\text{C}$ with a 16:8 (L:D) h photoperiod as described by Finney and Brinkman (1967) and Demkovich et al. (2015). Newly emerged first instar individuals were placed on a semi-defined standard lepidopteran diet (Waldbauer et al. 1984) and then transferred to the experimental medium upon reaching the third instar. The experimental media included potato dextrose agar (PDA) and an almond PDA mix. Almond PDA was prepared using 400 mL of water, 15.6 g of PDA (Sigma-Aldrich, St. Louis, MO), 21.88 g of Bob's Red Mill almond meal (Milwaukee, OR), and 0.057 g of streptomycin (Sigma-Aldrich) for every ~10 (8.5 cm diameter)

plate. For treatments excluding fungus, 1 mL of 10% formaldehyde (diluted from 36.5-38.0% stock (Macron, Center Valley, PA)) was also added to prevent fungal growth.

***Aspergillus flavus* Culture.** A laboratory culture of atoxigenic *A. flavus* (AF36) was started from infected wheat seeds provided by Themis Michailides (University of California, Davis). The *A. flavus* was grown on PDA and maintained under laboratory conditions ($23 \pm 2^\circ\text{C}$). In the bioassays, PDA and almond PDA plates were inoculated with an agar plug (5 mm diameter) taken from the margins of a sporulating culture (10-15 d old). *Aspergillus flavus* development times stated in this study denote time after inoculation.

Growth and Survival Bioassay. For the purposes of this study, “performance” was measured primarily in terms of pupal weight, time to pupation and survival rates. I used third-instar larvae to test growth and survival because lepidopteran larvae add most of their body weight in the third through fifth instars. Five larvae (reared through first and second instar on standard lepidopteran diet) were transferred to each of sixteen plates containing either PDA or almond PDA. Half of the plates were then inoculated with *A. flavus* plugs. There were four replicates per treatment, for a total of twenty individuals per treatment. Larval growth and survival were monitored at 24 h intervals until pupation, and pupal weights were obtained for all survivors. Larvae on PDA plates without almond were tracked only to fourth instar due to the consistency and nutritional constraints of this diet. The plates were maintained under conditions of $28 \pm 4^\circ\text{C}$ with a 16:8 (L:D) h photoperiod.

Furanocoumarin Bioassays. The same experimental design was used to test furanocoumarin toxicity, with sixteen almond PDA plates, but this time half of the plates were mixed with xanthotoxin (Sigma-Aldrich, 1.99 g per 10 plates), and half were mixed with bergapten (Sigma-Aldrich, 2.88 g per 10 plates) by grinding up the crystals with mortar and

pestle and stirring them into the diet after autoclaving. Concentrations were selected based upon earlier lethal concentration assays (V. Bagchi et al., in preparation). Next, half of the plates for each furanocoumarin treatment were inoculated with *A. flavus* as before. Larval survival and development time were monitored as before under the same rearing conditions.

Statistical Analyses. I created stage-specific life tables tracking the time to develop to the next instar for each larva, and I also compared pupal weights for all individuals that reached pupation, using SPSS version 22 (SPSS Inc., Chicago IL). Analysis of variance (ANOVA) was used to assess differences among all treatments in development time to the fourth instar. ANOVA was also used to test for significant differences in time to pupation and mortality rate among almond PDA treatments. Paired *t*-tests or Fisher's Least Significant Difference (LSD) test (post hoc) were used to identify significant differences between pairs of treatments.

Results

Larvae on almond PDA reached adulthood 14 d faster in the presence of *A. flavus* than on uninoculated plates ($t = 16.06$; $df = 3$; $P < 0.001$) (Table 1.1). In addition, male and female pupal weights were higher on average for individuals reared on *Aspergillus*-inoculated diet than for those on uninoculated diet ($t = 5.56$; $df = 3$; $P = 0.011$ and $t = 7.16$; $df = 3$; $P = 0.006$, respectively). I also noted that larvae raised in the presence of *A. flavus* were visibly attracted to the site of inoculation during the first 48 h, while larvae raised in isolation had no tendency toward the middle of the plate. However, when I repeated the experiment in a darkened incubator, no such orientation occurred on any of the plates (data not shown).

Both diet ($F = 101.81$; $df = 1$; $P < 0.001$) and the presence of *A. flavus* ($F = 292.40$; $df = 1$; $P < 0.001$) affected development time, and a significant interaction ($F = 70.02$; $df = 1$; $P <$

0.001) indicates that development differed on the two diets (Table 1.1). Development time to fourth instar was shorter on PDA inoculated with *A. flavus* than on uninoculated PDA ($t = 10.47$; $df = 3$; $P = 0.002$). Mortality rates were also higher on PDA plates ($F = 5.88$; $df = 1$; $P = 0.032$) than on almond PDA plates, particularly in the absence of *A. flavus* ($F = 5.88$; $df = 1$; $P = 0.032$), when mortality rose from 25% on almond PDA to 55% on normal PDA. While all pupation on both almond PDA treatments occurred within 36 d, no individuals on PDA plates pupated within 40 d, and observations were terminated due to deteriorating dietary and fungal conditions.

Larvae raised on diets containing furanocoumarins experienced considerably longer (56 d for xanthotoxin, and 48 d for bergapten) development times ($F = 1013.73$; $df = 3$; $P < 0.001$). However, on plates inoculated with *A. flavus*, development time was much shorter (19 d for xanthotoxin and 25 d for bergapten), less than in the control treatment and almost as short as in the *A. flavus* treatment without furanocoumarins (Fig. 1.1). In contrast, pupal weights were significantly depressed by the presence of furanocoumarins in the diet (14 g for males and 24 g for females with xanthotoxin; 14 g for males and 28 g for females with bergapten) irrespective of whether *A. flavus* was present, although fungal growth did allow significantly greater pupal weights in bergapten treatments ($F = 328.09$; $df = 5$; $P < 0.001$ for males, $F = 83.00$; $df = 5$; $P < 0.001$ for females) (Figs. 1.2 and 1.3). Pre-pupal mortality rates were much higher for larvae raised on diet containing furanocoumarins and no *A. flavus* (80% with xanthotoxin and 75% with bergapten), but not on a diet inoculated with *A. flavus* (19% with xanthotoxin and 25% with bergapten) ($F = 9.61$; $df = 5$; $P < 0.001$) (Fig. 1.4).

Discussion

This study demonstrates that navel orangeworm performance is significantly enhanced in the presence of *Aspergillus flavus*, supporting the idea that there is a facultative mutualism between these two species. Larval survival and growth rate were better on almond PDA than on PDA with *A. flavus*, indicating that *A. flavus* alone is not a high-quality food source for larval development. Nevertheless, even on the nutritionally deficient PDA, larvae developed much faster in the presence of *A. flavus*. While these larvae developed only to fourth instar due to the constraints of our experimental design, it would be instructive to conduct a more prolonged experiment to determine if a larva could develop through pupation on this diet.

Ingestion of furanocoumarins such as xanthotoxin and bergapten is detrimental to larval performance. Although navel orangeworm cytochrome P450 monooxygenases are capable of detoxifying natural levels of some furanocoumarins, including imperatorin (Niu et al. 2011), high concentrations of furanocoumarins cause toxicity to a broad diversity of caterpillars, and some species are sensitive even to low amounts (Li et al. 2000, Mao et al. 2006). However, larval survival and development time are “rescued” to a great degree by the presence of *A. flavus*, so much so that mortality rates are not significantly different from control treatments. There is prior evidence that some ascomycetes (including *Aspergillus* species) are capable of metabolizing furanocoumarins such as xanthotoxin (Desjardins et al. 1989, Myung et al. 2008). In addition, there are examples of mutualistic associations in which an associated organism detoxifies phytochemicals with benefit accruing to the insect. For instance, gut microbiota including *Pseudomonas* species metabolize caffeine in plant matter ingested by the coffee berry borer *Hypothenemus hampei* Ferrari (Coleoptera: Curculionidae) (Ceja-Navarro et al. 2015). It is possible that *A. flavus* is capable of detoxifying xanthotoxin and bergapten, with navel

orangeworm larvae benefiting from this metabolic capability. Alternatively, the presence of *A. flavus* may improve the quality of the diet in other ways to such a degree that the navel orangeworm is able to overcome the toxicological barrier of the furanocoumarins. It is important to note that pupal weights are depressed by furanocoumarins in the diet even in the presence of *A. flavus*, so not all detrimental effects of furanocoumarins are ameliorated.

Whether the association between the navel orangeworm and *A. flavus* is a mutualism depends on demonstrating a benefit of interacting for both species. For example, in the mutualistic association between the European grapevine moth, *Lobesia botrana* (Denis and Schiffermuller, Lepidoptera: Tortricidae) and the fungus *Botrytis cinerea* (Pers.), caterpillars vector the fungus, while fungal presence improves caterpillar development through the addition of sterols in the diet (Mondy and Corio-Costet 2000). With respect to the fungus, caterpillar feeding damage facilitates *A. flavus* infection of almond through transport of conidia and the creation of infection sites (Palumbo et al. 2014). In this study I have shown nutritional benefits accruing to navel orangeworm larvae that ingest a diet containing *A. flavus*; consequently, association of the navel orangeworm with *A. flavus* is likely beneficial for both species.

In conclusion, by demonstrating that first-instar navel orangeworms develop faster and survive better in the presence of *Aspergillus flavus*, I provide additional support for the proposed mutualistic relationship between these two species. My findings also suggest that insect–fungus pest mutualisms may be more common than previously reported. Several agricultural pests are known vectors of fungal conidia (Widstrom 1979), but there is still a dearth of information on possible pest mutualisms between insects and fungi in these systems. One important and relatively well-studied example is the vectoring of black aspergilli by the European grapevine moth, *Lobesia botrana* (Cozzi et al. 2006). As in the case of navel orangeworm larvae,

caterpillars of *L. botrana* mechanically damage the fruit and allow contamination by fungal ochratoxins. However, even in the context of the navel orangeworm pest complex, there are still interactions that require further study. Although *A. flavus* is of particular interest for the toxins it produces, the navel orangeworm is associated with a wide variety of fungi and bacteria. These include the toxigenic *Aspergillus parasiticus* and other common molds that also produce known attractants (Beck 2013), as well as bacteria that synthesize some of the same volatiles (Citron et al. 2012). The relationship between the navel orangeworm and mutualist pathogens, including *A. flavus*, has significant implications for improving both pest management and basic understanding of larval and fungal ecology. Understanding the interactions between insects and fungi is important to furthering a greater understanding of plant–insect interactions as well as developing more sustainable integrated pest management programs. Ultimately, management efforts should integrate control of both pests to reduce both direct and indirect crop damage.

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Table 1.1 Development time and pupal weight for third instar navel orangeworm (*Amyelois transitella*) reared at $28 \pm 2^\circ\text{C}$ on potato dextrose agar (PDA) or almond PDA diet with and without *Aspergillus flavus* (AF36).

Treatment	Time to stage (da)				Pupal weight (mg)		Mortality (%) (pre-4th) ^a
	4th instar ^a	5th instar ^b	Pupa ^b	Adult ^b	Male ^b	Female ^b	
Almond	8.9a	26.7a	33.8a	43.8a	32.13a	43.22a	25a
Almond + AF36	7.9a	17.2b	22.9b	29.6b	38.00b	48.63b	20a
PDA	24.3c	-	-	-	-	-	55c
PDA + AF36	13.3b	-	-	-	-	-	35b

Means in the same column followed by the same letter are not significantly different, where significance is $P < 0.05$.

^a Statistically significant difference ($P < 0.05$) determined by two-way ANOVA.

^b Statistically significant difference ($P < 0.05$) determined by paired sample *t*-test.

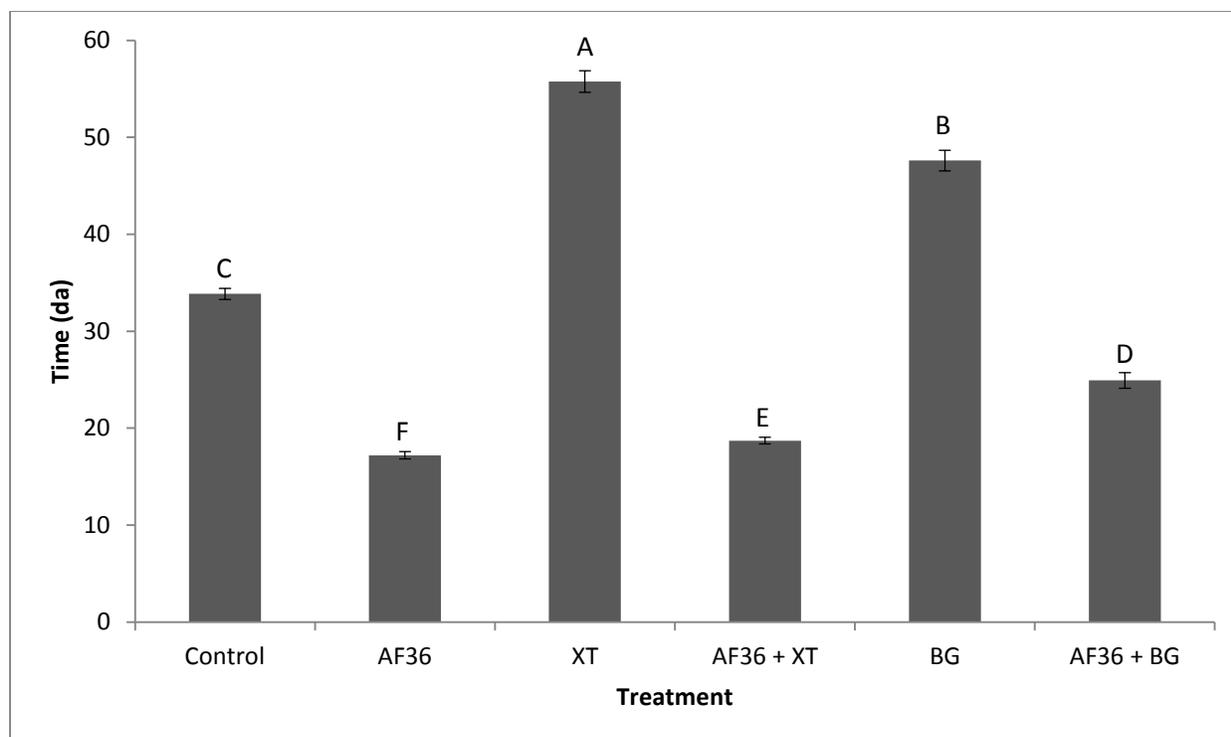


Fig. 1.1 Mean time (± 1 SE) to pupation for third-instar navel orangeworm (*Amyelois transitella*) larvae on almond PDA (Control), with *Aspergillus flavus* (AF36), on diets containing furanocoumarins (xanthotoxin, XT and bergapten, BG), and with both *A. flavus* and furanocoumarins (AF36 + XT and BG).

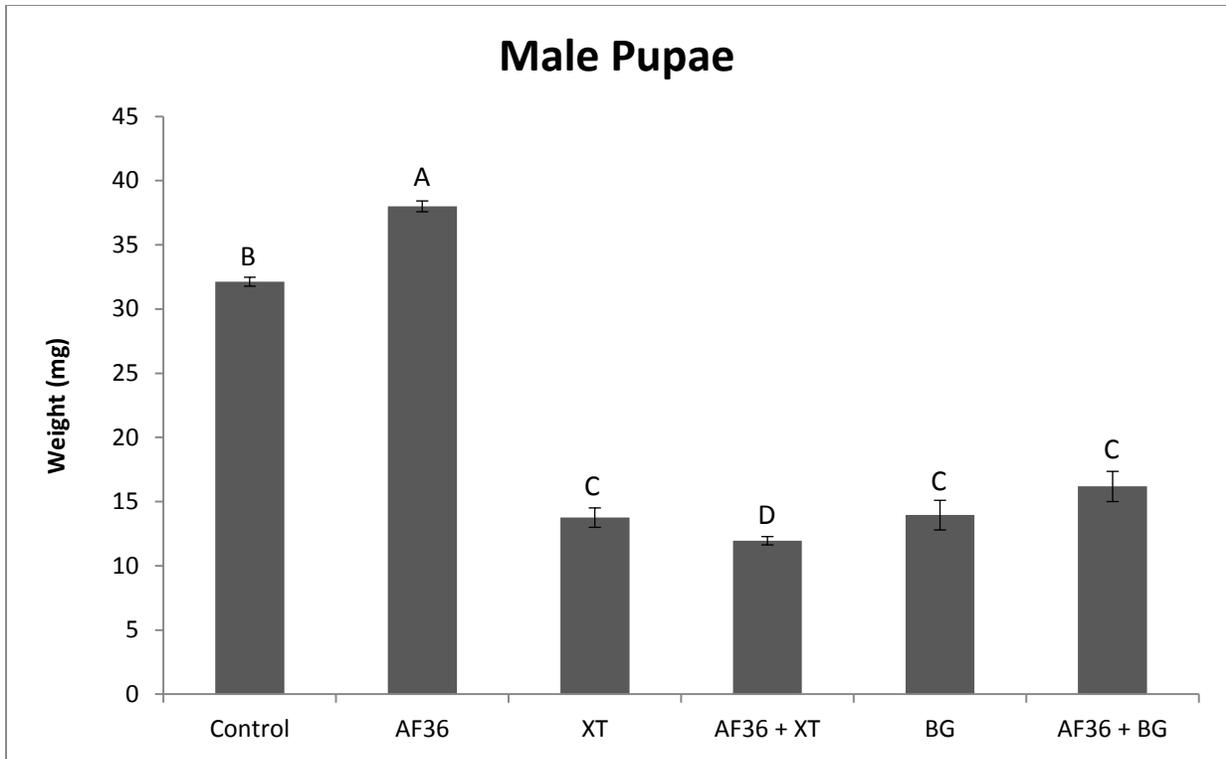


Fig. 1.2 Average pupal weights (± 1 SE) for male navel orangeworm (*Amyelois transitella*) surviving to pupation on almond PDA (Control), with *Aspergillus flavus* (AF36), on diets containing furanocoumarins (XT and BG), and with both *A. flavus* and furanocoumarins (AF36 + XT and BG).

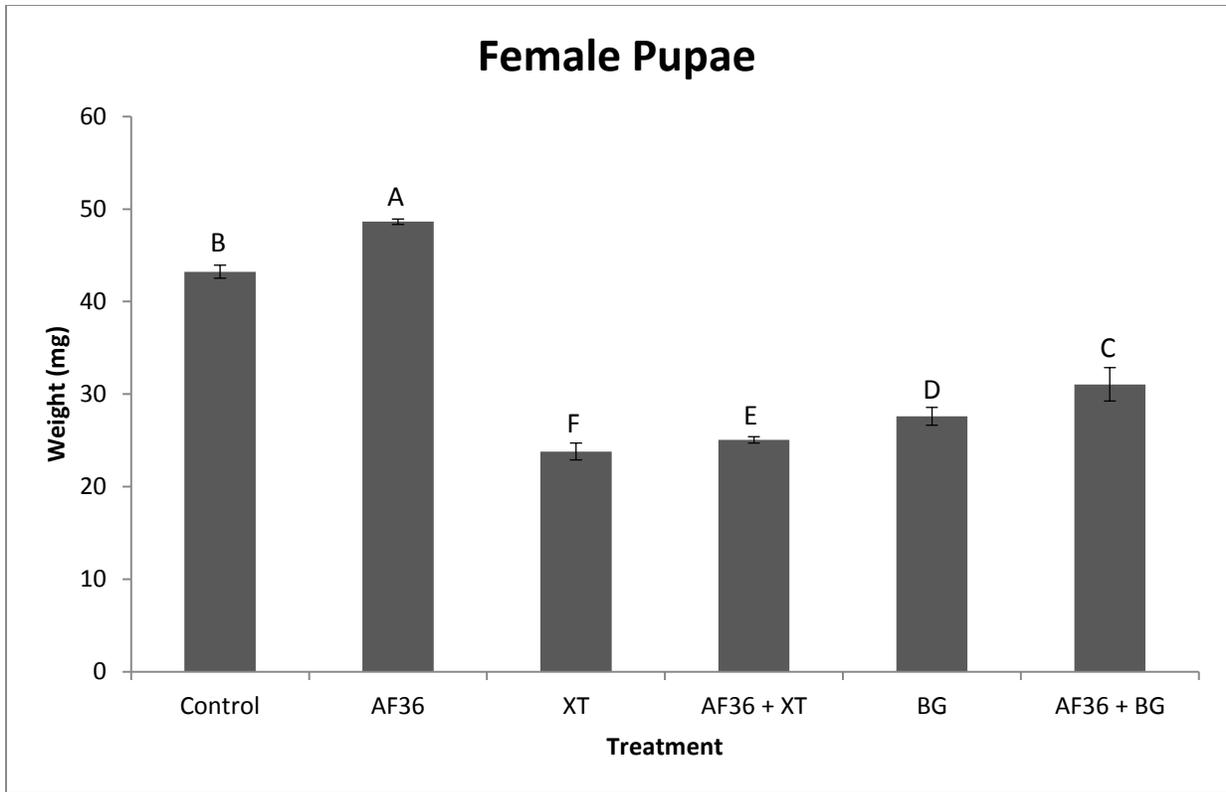


Fig. 1.3 Average pupal weights (± 1 SE) for female navel orangeworm (*Amyelois transitella*) surviving to pupation on almond PDA (Control), with *Aspergillus flavus* (AF36), on diets containing furanocoumarins (XT and Ber), and with both *A. flavus* and furanocoumarins (AF36 + XT and Ber).

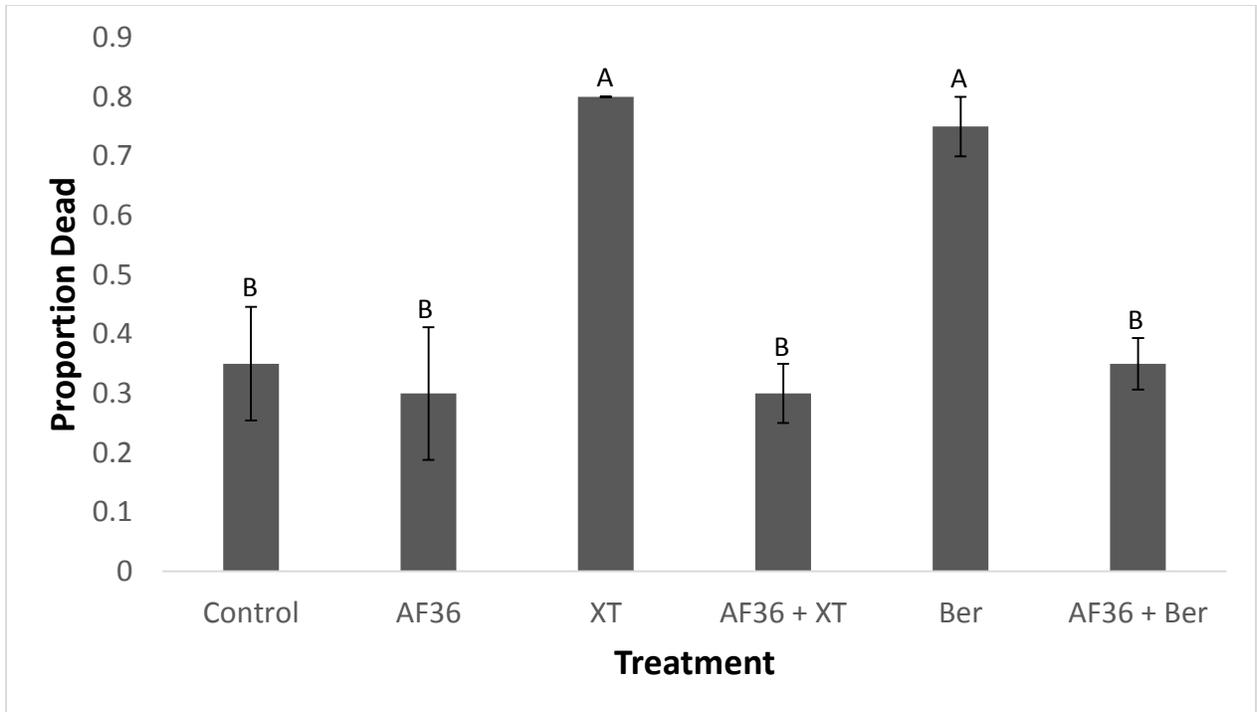


Fig. 1.4 Proportion of navel orangeworm (*Amyelois transitella*) individuals (± 1 SE) to die before reaching pupation on almond PDA (Control), with *Aspergillus flavus* (AF36), on diets containing furanocoumarins (XT and Ber), and with both *A. flavus* and furanocoumarins (AF36 + XT and Ber).

CHAPTER II: EFFECTS OF NAVEL ORANGEWORM FEEDING AND FRASS ON GROWTH OF *ASPERGILLUS FLAVUS*

Introduction

Insects and fungi are groups that contain many important plant pests and parasites. Individually, they may cause extensive damage to crops and stored food, but the two groups have a long history of association, which can lead to even greater deleterious effects on their mutual hosts (Vega and Blackwell 2005). Among the most common and economically important group of fungal plant pathogens are the molds, a polyphyletic grouping of filamentous ascomycetes and zygomycetes. Species in the genus *Aspergillus* are found as parasites on a wide variety of crop plants, often damaging fruits or vegetative tissues exposed to adverse environmental conditions (Amaiike and Keller 2011). Because they damage or destroy crops and stored food products, *Aspergillus* molds on their own are an important economic threat.

In addition, some species of *Aspergillus*, most notably *A. flavus* and *A. parasiticus*, produce toxic compounds, including aflatoxins (Cleveland et al. 2003). Aflatoxins are the most potent known natural carcinogens, and high levels of aflatoxin render crops unsaleable (Eaton and Gallagher 1994). Even when only small amounts of aflatoxins are present, the economic effect can be disproportionately large. The difficulties faced by growers and pest managers are compounded by the fact that *Aspergillus flavus* Link is often associated with insect pests. The mold is unable to penetrate some crops on its own and requires mechanical damage (e.g., hull cracking by tree nut pests) to colonize a host (Widstrom 1979, Campbell et al. 2003). In the case of tree nuts, *A. flavus* is often associated with the navel orangeworm, *Amyelois transitella* Walker (Lepidoptera: Pyralidae), which causes damage to orchard crops such as figs (*Ficus*

carica var. *domestica* Czern. & Rav.), pomegranates (*Punica granatum* L.), almonds (*Prunus dulcis* [Mill.] D.A. Webb), and pistachios (*Pistacia vera* L.). The navel orangeworm can cause direct harm via feeding damage and contamination of fruits with frass and webbing, and in conjunction with *A. flavus*, it is part of the most economically important pest complex in some parts of California (Zalom et al. 2012). Recent work indicates that there may be a facultative mutualism between the two pests. Mechanical damage by navel orangeworm caterpillars allows invasion of mold into the fruit interior, generally upon hull split. In addition, navel orangeworm adults are important in the transport of fungal conidia throughout the orchard (Palumbo et al. 2014), and *A. flavus* conidia and high levels of aflatoxins are associated with navel orangeworm presence in orchards (Luo et al. 2009). The navel orangeworm is in some ways better suited for a mutualism with *A. flavus* than are other insect pests. For example, unlike most other insects, the navel orangeworm is able to tolerate relatively high concentrations of aflatoxins due to cytochrome P450 monooxygenases that detoxify aflatoxin B1 rather than bioactivating it into more toxic metabolites (Lee and Campbell 2000; Niu et al. 2009).

A major element of this putative mutualism is nutritional—that is, *A. flavus* increases the quality of plant material as a diet for navel orangeworm larvae (Ampt et al. 2015). In turn, *A. flavus* is phoretic on the adult moth (Palumbo et al. 2014). Ostensibly, because both the fungus and the caterpillar feed on the same tissues, the potential exists for competition. In fact, I have observed that the navel orangeworm will eat the fungus itself along with substrate being colonized by *A. flavus*, and thus the surface area available for colonization is reduced, sometimes extensively if caterpillar density is high. Since the larva, like the adult moth, is a mobile life stage, it is likely that there is a phoretic benefit to the fungus in which the caterpillar spreads conidia within or between fruits. An additional potential “compensatory” substrate for the plant

matter lost to competition is insect frass, which is typically plentiful as the caterpillars develop within the plant (Zalom et al. 2012). The objectives of this study thus were to (1) determine whether *Aspergillus flavus* growth is greater in the presence of navel orangeworm larvae, (2) test the hypothesis that navel orangeworm frass is a suitable substrate for *A. flavus* growth, and (2) determine if *A. flavus* conidia survive passage through the digestive tract of navel orangeworm caterpillars, thereby providing another vehicle for dissemination of the fungus. Evidence of any of these benefits would provide support for the suggestion that the association between these two species represents a facultative mutualism.

Materials and Methods

Navel Orangeworm Colonies and Diet. The source of navel orangeworms used in this study was a laboratory strain, CPQ, which originated in the USDA-ARS laboratory in Parlier, California and has been maintained both in Parlier and at the University of Illinois at Urbana-Champaign. The colony is maintained on a wheat bran diet under conditions of $28 \pm 4^\circ\text{C}$ with a 16:8 (L:D) h photoperiod (Finney and Brinkman 1967, Demkovich et al. 2015). For phoresy assays, first-instar caterpillars were placed on a lab standard semi-defined lepidopteran diet (Waldbauer et al. 1984, hereafter referred to as “standard diet”). Once they reached third instar, the larvae were transferred to the experimental medium, either potato dextrose agar (PDA) or almond PDA (Ampt et al. 2015). For frass substrate assays, fourth and fifth instar larvae were placed on almond PDA and standard diet. Finally, in *A. flavus* consumption assays, third-instar caterpillars were placed in Petri dishes on PDA containing *A. flavus* for three days and then transferred to sterile PDA dishes.

Frass Collection and Processing. Frass was collected from rearing cups or dishes with sterilized paintbrushes and carefully separated from exuviae, silk, and uneaten diet. Because formaldehyde is used in the original diet and passes through the insect, it must be removed before fungus can grow on the frass. This can be accomplished via Fenton's reaction by using ferrous sulfate (Iron (II) sulfate heptahydrate, Sigma-Aldrich, St. Louis, MO) and 3% hydrogen peroxide (or Fenton's reagent, diluted from 30% solution, Sigma-Aldrich) at 40° C, which oxidizes formaldehyde to CO₂ (Do and Chen 1993). The pH of the frass can then be re-adjusted to its normal level, as described in Do and Chen (1993). Afterwards, the frass was autoclaved at 121° C to further purify it and re-liquefy the agar remaining in it. The resulting liquid was cooled, stirred, and poured into Petri dishes (~8.5 cm diameter) as with any other agarose medium. Streptomycin (Sigma-Aldrich) was also added to prevent bacterial growth, as in Ampt et al. (2015). This protocol was followed for frass from larvae fed on both almond PDA and standard diet.

***Aspergillus flavus* Colonies.** Wheat seeds infected with *A. flavus* were provided by Themis Michailides (University of California, Davis). The fungus was isolated and kept in colonies on PDA at 23 ± 2°C. For *A. flavus* growth assays, a 5-mm diameter plug of agar taken from the margin of a sporulating culture (10-15 d old) was placed in the center of the plate.

***Aspergillus flavus* Growth Assays.** A plug of *Aspergillus flavus* was placed in the center of eight almond PDA dishes, and five third-instar caterpillars were added to four of them. I then inoculated twenty dishes each of standard diet frass and almond PDA frass, along with PDA and almond PDA diets, which served as controls. Fungal growth was assessed by averaging colony diameter in two perpendicular axes (Brancato and Golding 1953). After 72 h, fungal growth was compared across all treatments.

***Aspergillus flavus* Consumption.** Third-instar caterpillars fed with a diet containing *Aspergillus flavus* were placed on sterile PDA and allowed to defecate for 48 h. Frass was collected and plated on new PDA, and the inoculated plates were monitored for fungal growth as described. Additional frass was observed at 800-2000X magnification with an Olympus BX51 microscope with differential interference contrast (DIC) and equipped with an Olympus QColor 3 digital camera. Images were viewed in Adobe Photoshop 7.0 at the Miller Mycology laboratory (University of Illinois at Urbana-Champaign) to visually check for conidia, hyphae, and other evidence of fungal presence.

Statistical Analyses. Paired sample *t*-tests (using SPSS version 22, SPSS Inc., Chicago IL) were used to test for significant differences in *A. flavus* growth with and without the navel orangeworm. In the frass culture assays, analysis of variance (ANOVA) was used to find significant differences among all fungal growth treatments. Fisher's Least Significant Difference (LSD) test was used to find significant differences between pairs of treatments after ANOVA.

Results

Aspergillus flavus growth in the presence of navel orangeworm larvae outpaced its growth in isolation, especially after the 24-h mark (Fig. 2.1), and there was an increase in 72-h growth from in the presence of caterpillars ($t = 52.14$; $df = 19$; $P < 0.001$). The fungus grew on all experimental substrates, with the lowest rate on PDA and the highest on almond PDA (Fig. 2.2). The two frass treatments were intermediate between the two controls. There were significant differences between the means of the various treatments ($F = 105.16$; $df = 3$; $P < 0.001$), and each pair was significantly different, with *A. flavus* growth on almond PDA frass 3 mm greater than on standard diet frass.

Microscopic examination of frass collected from larvae revealed multiple fungal elements. I identified a complete conidial head, septate hyphae, and intact ornamented conidia (Fig. 2.3) in samples of frass deposited on a sterile substrate. When additional samples were plated on PDA, one inoculation resulted in the establishment of *Aspergillus flavus* (Fig. 2.4). Three other attempts at *A. flavus* establishment via frass were unsuccessful.

Discussion

Aspergillus flavus grew on agarose diet more than twice as rapidly in the presence of navel orangeworm larvae as when no larvae were present, which suggests that the larvae aid fungal dissemination in multiple ways. For instance, our observations comport with the findings of Palumbo et al. (2014), who showed that mobile life stages of the navel orangeworm spread fungal conidia throughout the orchard or across the dietary substrate. In addition, a phoretic association would explain the increased levels of aflatoxins in nuts damaged by the navel orangeworm (Doster and Michailides 1994). Phoretic relationships have also been observed or hypothesized to exist in other lepidopteran-fungal associations, including the corn earworm, *Helicoverpa zea*, which acts as a vector for *Aspergillus flavus*, among other fungal species (Lillehoj et al. 1984).

In addition to phoretic benefits, caterpillar frass appears to be a viable substrate for the growth of *A. flavus*. Fungal growth rates on each of the frass treatments fall between the PDA and almond PDA diets, with almond PDA frass providing a somewhat better substrate than standard diet frass. That frass from a more “natural” food source (i.e., almond meal) is more suitable than that from the standard diet may be due to the fact that the standard diet is made

specifically for lepidopteran nutrition, and the caterpillars are more efficient at digesting it, thereby producing frass with fewer undigested nutrients for the fungus.

The survival of intact fungal elements, including conidia, after traveling through the larval digestive system suggests another means by which caterpillars could spread *A. flavus*. Consumption of fungus and subsequent defecation at a distance from the origin of the meal could be a case of time-dependent phoresy, in which further spread occurs after the caterpillar ultimately passes the meal. However, I was able to grow *A. flavus* from only one of the inoculations, indicating that some physical properties of the frass might inhibit growth. A study of the pH and other properties of the frass might be helpful in determining whether fungal growth is restricted even with apparently healthy conidia. In addition, the fact that I used strain AF36, an experimental strain of *A. flavus* isolated from wheat, may be important. This strain might not be adapted to association with the navel orangeworm in all respects as well as strains from navel orangeworm hosts.

This study illustrates that there may be circumstances in which frass production by the caterpillar may offset the negative effects of competition for plant tissue between insect herbivore and fungal pathogen, possibly strengthening a mutualistic relationship. The navel orangeworm tends to burrow into the reproductive tissues of its hosts (Palumbo et al. 2014); this concealed feeding habit and the production of silk webbing helps to keep frass within the feeding site and allows the fungus to remain closely associated with the caterpillars throughout their respective development periods. This hypothesis is supported by the fact that frass and webbing are common sources of crop contamination and markers of navel orangeworm damage (Palumbo et al. 2014).

In spite of several studies describing fungal spore isolation from frass, very little work has been done on fungal colonies growing on frass or their success in doing so. Most of the existing work focuses on attine ants, which use their frass as an important component of “manure” for their associated fungi (de Fine Licht and Boomsma 2010). However, viable fungal conidia have been discovered in the frass of many insects (Chen et al. 2014). Shore flies and fungus gnats, for instance, spread fungal propagules by smearing their frass onto the stems and roots of crop plants (Gillespie and Menzies 2008, Hyder et al. 2009). Ambrosia beetles and other wood-boring beetles have well-characterized mutualisms with many fungal species; for instance, fungal conidia of *Fusarium* species have been isolated from the frass of the ambrosia beetle *Euplatypus parallelus* Fabricius (Coleoptera: Curculionidae) (Bumrungsri et al. 2008). Among other factors, frass is probably important in the spread of fungal infection of trees by these beetles. Similarly, several species of mold, including the aflatoxigenic *Aspergillus parasiticus* Speare, have been found in the frass of the larger grain beetle *Prostephanus truncates* Horn (Coleoptera: Bostrichidae), a coleopteran grain pest (Osipitan et al. 2011). Other lepidopteran caterpillars are associated with *Aspergillus flavus*. For instance, *Aspergillus* conidia have been found in the frass of wax moth larvae (*Galleria mellonella* L., Lepidoptera: Pyralidae) infesting honey bee colonies (Gilliam et al. 1989). However, it should be noted that *Aspergillus flavus* is already present at high levels in beebread (Gilliam et al. 1974), so the caterpillars may not be important vectors of the fungus.

This study demonstrates that navel orangeworm larvae contribute to the spread of *Aspergillus flavus* by transporting the fungus around the host both internally and externally and by producing frass that can serve as suitable substrate for fungal growth. Because these benefits to the fungus likely strengthen the pest complex as a whole, these findings bear some importance

for agriculture and food production. Economic losses in many agriculturally significant plant species are compounded by the dual threat of herbivorous insects and fungi (Zalom et al. 2012, Palumbo et al. 2014). Managing both organisms simultaneously has proven challenging, but such efforts may be vital to preventing major crop losses to insect-fungus mutualists.

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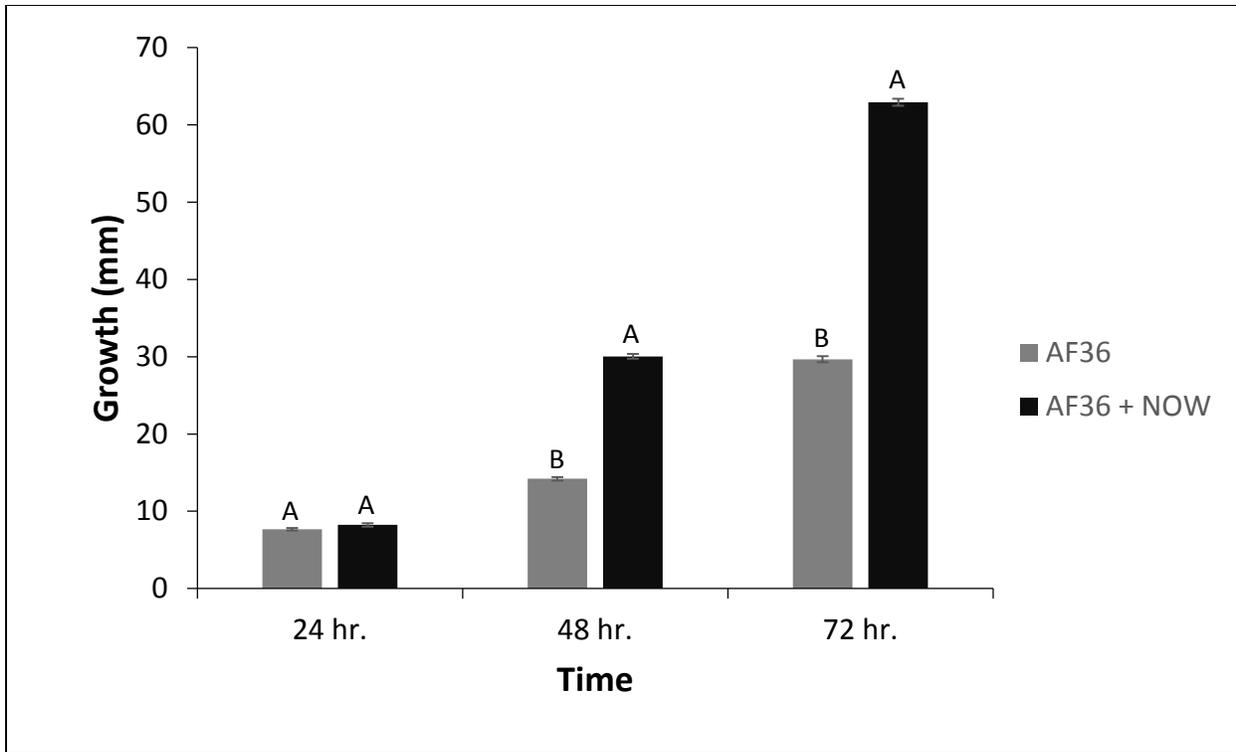


Fig. 2.1 *Aspergillus flavus* growth (± 1 SE) with and without navel orangeworm (*Amyelois transitella*) larvae at 24 h, 48 h, and 72 h after inoculation.

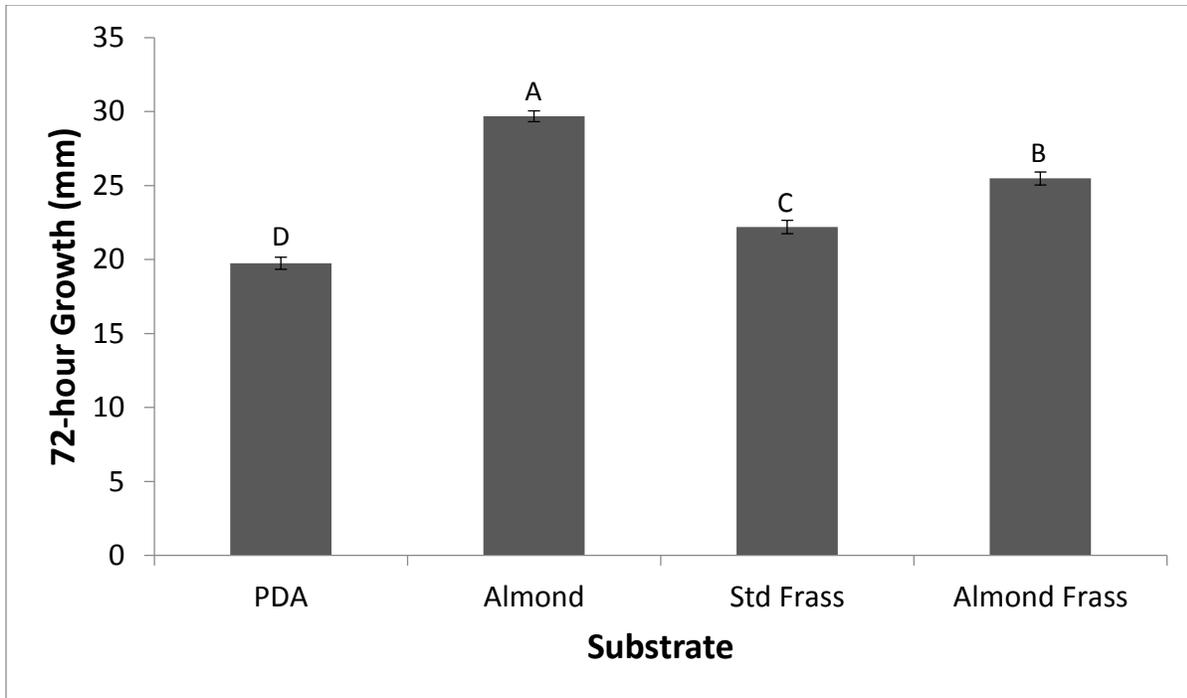


Fig. 2.2 *Aspergillus flavus* growth (± 1 SE) 72 hours after inoculation on PDA, almond PDA, and frass from navel orangeworm (*Amyelois transitella*) larvae fed on standard diet and almond PDA.

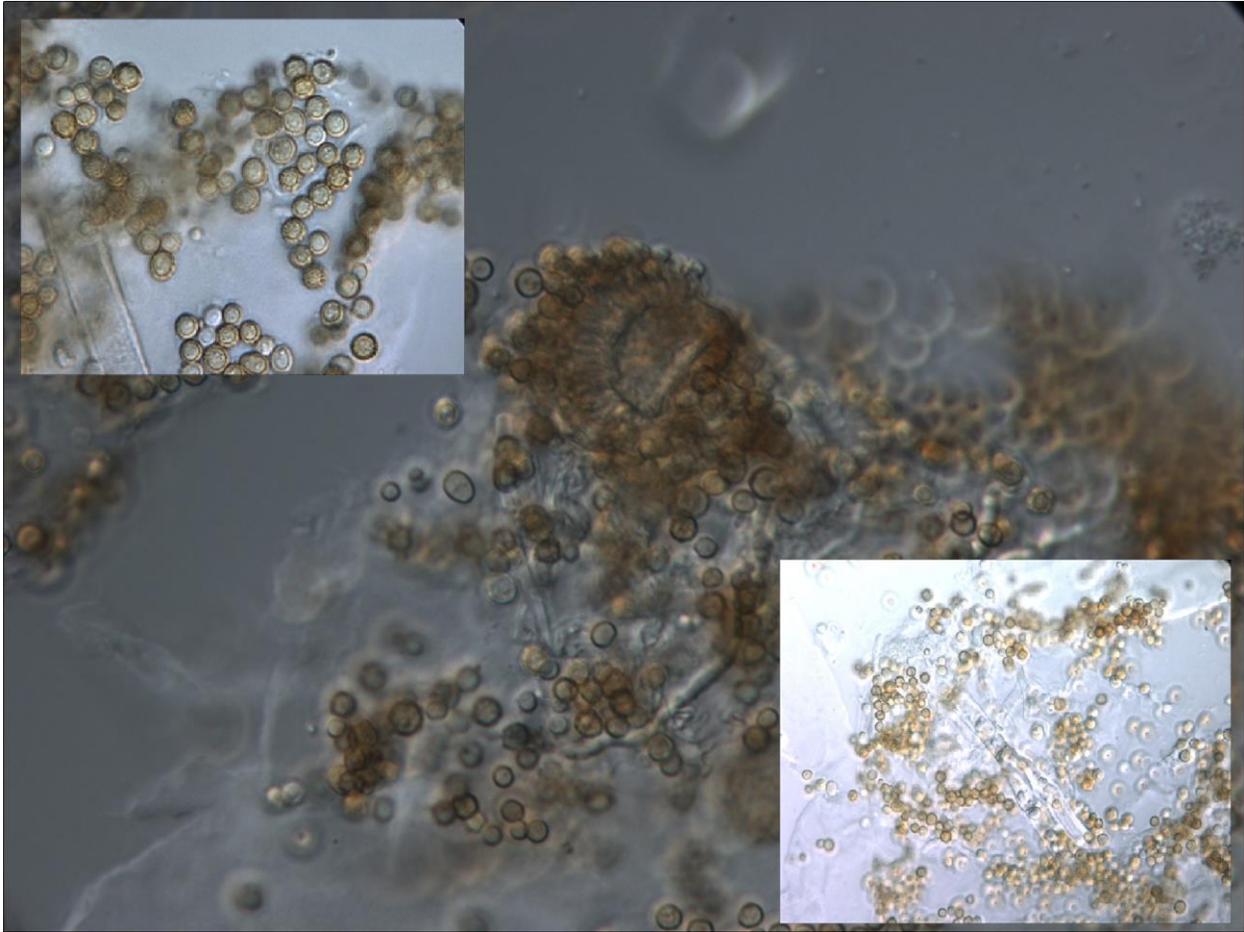


Fig. 2.3 Main: Complete conidial head of *Aspergillus flavus* in a sample of navel orangeworm (*Amyelois transitella*) frass (800X magnification). **Insets:** ornamented conidia (1200X and septate hyphae (800X).

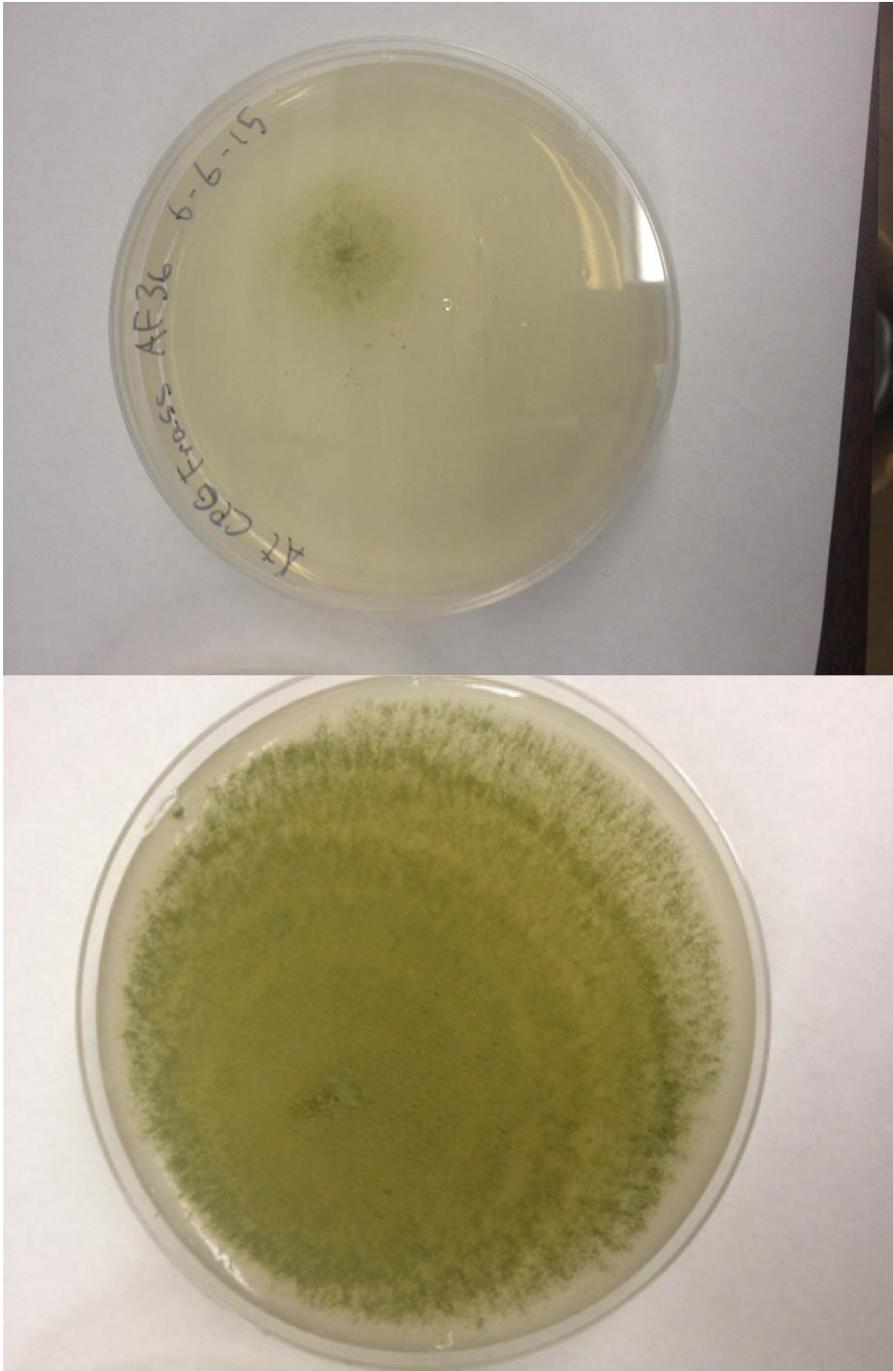


Fig. 2.4 *Aspergillus flavus* at 48 hours (top) and 96 hours (bottom) after inoculation from frass of navel orangeworm (*Amyelois transitella*) larvae fed on diet containing *A. flavus*.

CHAPTER III: PREFERENCE OF NAVEL ORANGEWORM MOTHS FOR OVIPOSITION SITES INFECTED BY *ASPERGILLUS FLAVUS*

Introduction

The navel orangeworm, *Amyelois transitella* (Lepidoptera: Pyralidae) and the associated fungus *Aspergillus flavus* comprise the most economically damaging pest complex in California tree nut orchards. Both are broad generalists, and their host ranges overlap to a significant degree. Much of the crop loss occurs in fig (*Ficus carica* var. *domestica* Czern. & Rav.), tree nut, and pomegranate (*Punica granatum* L.) orchards, where both the insect and the fungus can be abundant (Connell 2002, Higbee and Siegel 2012). The navel orangeworm causes damage in almonds (*Prunus dulcis* [Mill.] D.A. Webb) and pistachios (*Pistacia vera* L.) by burrowing into the fruit, damaging the kernel and contaminating it with frass and webbing (Palumbo et al. 2014). *Aspergillus flavus* damages crops directly (Amaike and Keller 2011), and it can also produce aflatoxins, the most carcinogenic natural products known (Niu et al. 2009). Because nuts and fruits contaminated with even low levels of aflatoxins cannot be sold (Eaton and Gallagher 1994) and the orchard host plants of *A. flavus* are valuable crops, infection by this fungus causes significant economic damage. The combined threat of the navel orangeworm and *A. flavus* has resulted in widespread attempts to manage both of these pests and prevent aflatoxin contamination in orchards (Campbell et al. 2003; Zalom et al. 2012).

There is mounting evidence that these two species comprise a facultative mutualism. Mobile life stages of the insect transport *A. flavus* conidia throughout the orchard and across the host plant (Palumbo et al. 2014; See Chapter 2). In return, navel orangeworm larvae experience faster growth and lower mortality in the presence of *A. flavus* (See Chapter 1). One important

element of this putative mutualism is the ability of the navel orangeworm to orient toward substrates infected with *A. flavus*, in both larval and adult stages. Female adults are attracted to volatiles from *A. flavus* and almond meal (Beck et al. 2012b, Beck 2013), while larvae orient toward fungal masses and volatiles associated with *Aspergillus* infection and away from volatiles associated with fungicidal activity (Ampt et al. 2015, A. Lawrance, unpublished).

Although navel orangeworm larvae perform better in the presence of *A. flavus*, it is unclear why adults should be attracted to the fungus. They are not known to feed on either the hostplant tissues or the fungus. Relationships between oviposition preference and larval performance do occur in Lepidoptera, whereby females oviposit preferentially on host plant tissues that support superior larval growth (Bonebrake et al. 2010). Accordingly, I hypothesized that navel orangeworm adults are evolutionarily adapted to prefer *Aspergillus*-infected substrates due to the fact that larvae exhibit increased performance in the presence of the fungus. In this study, I used two-choice oviposition assays in mating arenas to determine orientation behavior of ovipositing moths.

In the process of characterizing oviposition preferences, it became apparent that ovipositing moths laid different proportions of unfertilized eggs on infected and uninfected substrates. I could find no precedent for this behavior (among Lepidoptera) in the literature, and I hypothesized that females ovipositing on suboptimal substrates lay higher proportions of unfertilized eggs to provide supplemental nutrition for cannibalistic sibling larvae. In order to assess the nutritional benefit of nearby eggs to recently emerged caterpillars, I used presence and absence of unfertilized eggs to test the propensity of navel orangeworm larvae for cannibalism and the effects of cannibalism on survival.

Materials and Methods

Navel Orangeworm Rearing. Navel orangeworm colonies were maintained at $28 \pm 4^{\circ}\text{C}$ with a 16:8 (L:D) h photoperiod on honey wheat bran diet (Finney and Brinkman 1967, Demkovich et al. 2015). They were derived from a laboratory strain originating at the USDA-ARS laboratory in Parlier, California and maintained in continuous culture in Parlier and at the University of Illinois at Urbana-Champaign. Newly emerged adults were removed from the rearing jars, sexed, and placed in the experimental arena (see below). For cannibalism experiments, newly hatched first instar larvae were selected from egg masses laid on paper towels (Wausau Paper Corporation, Harrodsburg, KY). Unfertilized eggs for the cannibalism experiments were obtained by cutting out sections of these paper towels where the eggs were colorless. Shortly after oviposition, fertilized eggs appear orange due to the developed larvae contained within. Colorless eggs are therefore unfertilized.

***Aspergillus flavus* Colonies.** Themis Michailides (University of California, Davis) provided wheat seeds infected with *Aspergillus flavus*. I isolated *A. flavus* by surface sterilizing the seeds, splitting them, and placing halves on potato dextrose agar (PDA). I then maintained continuously sporulating colonies (10-15 d old) on PDA for use in experiments. An atoxigenic strain designated AF36 was used to avoid complications arising from the production of mycotoxins. In the oviposition experiment, the plates inoculated with *A. flavus* were smeared with a cotton swab covered in conidia from the fungal culture.

Oviposition Experiment. I created experimental arenas out of clear plastic boxes (25 X 18 X 12 cm) (Figure 3.1). Two plates containing almond potato dextrose agar (PDA) (Ampt et al. 2015) were placed in opposite corners, and one plate was inoculated with *A. flavus*, while the other one was treated with formaldehyde to prevent fungal growth. Autoclaved paper towels

were wrapped around each plate to provide a surface for oviposition, and they were then perforated to allow volatiles to escape the plates. Finally, two mating perches (plastic spatulas, VWR, Radnor, PA) were placed in the remaining corners of each arena and four mating pairs of navel orangeworm adults, collected *in copula*, were released into it. The moths were allowed to mate and lay eggs for five days, and the eggs were then removed and counted.

Cannibalism Experiment. I placed eight navel orangeworm neonates individually in empty diet cups (28 g soufflé cups, Solo Cup Company, Lake Forest, IL). Into four of the cups I placed a small (~5 X 5 cm) section of paper towel (the oviposition substrate) with 10-20 unfertilized eggs. This experiment was repeated five times, for a total of 40 individual assays. The larvae were checked every 24 h for mortality.

Statistical Analyses. Differences in egg number and egg fertilization rate were analyzed using paired sample *t*-test (SPSS version 22, SPSS Inc., Chicago IL). Paired sample *t*-tests were also used to find significant differences in mortality rates and survival time for larvae in the cannibalism experiment.

Results and Discussion

On average, 44.3 eggs were laid on the fungus-containing surfaces, of which the majority (62%) were fertilized (Fig. 3.2). However, only 12.7 eggs on average were laid on uninoculated surfaces, of which only 26% were fertilized. Ovipositing females showed a significant preference for surfaces inoculated with *A. flavus* ($t = 4.30$; $df = 2$; $P = 0.026$). Similarly, a significantly higher proportion of eggs laid on the fungus-containing surfaces were fertilized ($t = 4.30$; $df = 2$; $P = 0.048$).

Navel orangeworm neonates provided with unfertilized eggs as their only food source experienced significantly lower mortality than larvae provided with no food ($t = 2.78$; $df = 4$; $P < 0.001$). After 168 h, all larvae that had been provided with no food had died; by comparison, 70% of the larvae provisioned with unfertilized eggs were still alive at the end of 168 h. Larvae provided with eggs survived 208.8 h on average, while their unfed counterparts survived only 85.2 h ($t = 2.78$; $df = 4$; $P = 0.002$).

I found that navel orangeworm moths prefer to oviposit on surfaces inoculated with *A. flavus* over uninoculated surfaces, a behavior suggestive of a coevolutionary relationship between the fungus and the moth. The almond meal used in our assays consists of ground skinless blanched almond kernels, which contain the polyunsaturated fatty acids linoleic acid and linolenic acid (Sathe et al. 2008). Beck et al. (2012a) showed that *A. flavus* conidia placed on these fatty acids produce two spiroketals, conophthorin and chalcogran, both of which have been identified by Beck et al. (2012b) as attractant semiochemicals for adult navel orangeworms. Behavioral bioassays conducted by A. Lawrance (personal communication) suggest that these compounds may also serve this function for larvae. However, conophthorin and chalcogran are probably produced only by germinating conidia, because Mahoney et al. (2014) noted that conophthorin production halted as soon as fungal growth became visible to the naked eye. I posit that these volatiles are involved in attraction of navel orangeworm females to appropriate oviposition sites.

Because they can have a large effect on the health of the hostplant and may even be directly harmful or beneficial to the insect itself, fungi are frequently an important part of herbivore preference-performance relationships. The presence of fungal pathogens can be a deterrent to oviposition or herbivory (Röder et al. 2007, Tasin et al. 2012). Similarly, fungal

symbionts such as endophytes and arbuscular mycorrhizae may be deterrents or attractants to insect oviposition. Endophytes frequently protect the host plant from insect attack (Crawford et al. 2010), while arbuscular mycorrhizae increase nitrogen content in the host plant, which actually increases both preference and performance in insects such as rice water weevil (*Lissorhoptrus oryzophilus* Kuschel, Coleoptera: Curculionidae) (Cosme et al. 2011). There are relatively few previously described cases of preference-performance relationships involving a lepidopteran and a plant pathogen. Among these (much like the navel orangeworm), the light brown apple moth *Epiphyas postvittana* Walker (Lepidoptera: Tortricidae) displays a preference for ovipositing on host tissues (grape leaves) infected by a fungal pathogen (*Botrytis cinerea* Pers.), and larvae preferentially feed on slightly or moderately infected leaves over uninfected ones (Rizvi et al. 2015).

The differential fertilization rates of eggs on substrates with and without *A. flavus* was unexpected and apparently without precedent in the literature. Maternal control of egg fertilization does occur among insects, for example, among haplodiploid hymenopterans (Verhulst et al. 2010), but there are no such examples in Lepidoptera, to my knowledge. Navel orangeworm moths may simply have exhausted their supply of fertilized eggs on the inoculated substrate and then moved on to the less desirable surface afterwards. However, the improved survival of the larvae consuming unfertilized eggs suggests that these eggs are provided as a form of dietary supplement. Along with others (M. Demkovich and J. Siegel, personal communications), I have observed that cannibalism of eggs and other larvae is common among navel orangeworm caterpillars (despite reports that they “are not cannibalistic,” Bentley et al. 2008). Other studies of cannibalism have demonstrated that larval cannibalism of eggs is common across many noncarnivorous insect taxa, including the family Pyralidae (Richardson et

al. 2010). The results of this experiment indicate that there is some nutritional value to eating unfertilized eggs, enough to extend larval survival for several more days. The possibility exists that ovipositing navel orangeworm moths lay a disproportionate number of unfertilized eggs on suboptimal substrates deliberately, to provide hatching larvae sufficient nutrients to survive in the absence of the fungus.

There are examples of non-social insects with plastic trophic egg production. For instance, the multicolored Asian ladybird beetle (*Harmonia axyridis* Pallas, Coleoptera: Coccinellidae) alter the proportion of trophic eggs laid based upon availability of food, apparently informed by prey encounter rates (Perry and Roitberg 2005). Larvae of *H. axyridis* have been observed to cannibalize eggs, and the nutritional benefits of this behavior suggest a possible parallel with the navel orangeworm. Similarly, the subsocial burrower bug *Adomerus triguttulus* Motschulsky (Hemiptera: Cydnidae) exhibits varying trophic egg production based upon maternal diet (Kudo and Nakahira 2005). Females with poor diet produce more inviable eggs, which act as a food source for emerging larvae. If the navel orangeworm is indeed capable of some form of maternal control of fertilization or intentional supplementation of larval diet via egg-laying, there may be exciting new topics of study in lepidopteran physiology and oviposition behavior. The presence or absence of *Aspergillus* on the host substrate could be an important cue in plastic trophic egg production, further highlighting the importance of mutualistic relationships between these two pests. Insect-fungus relationships are an underappreciated area of ecology, but they are nonetheless important in pest management and tritrophic interactions.

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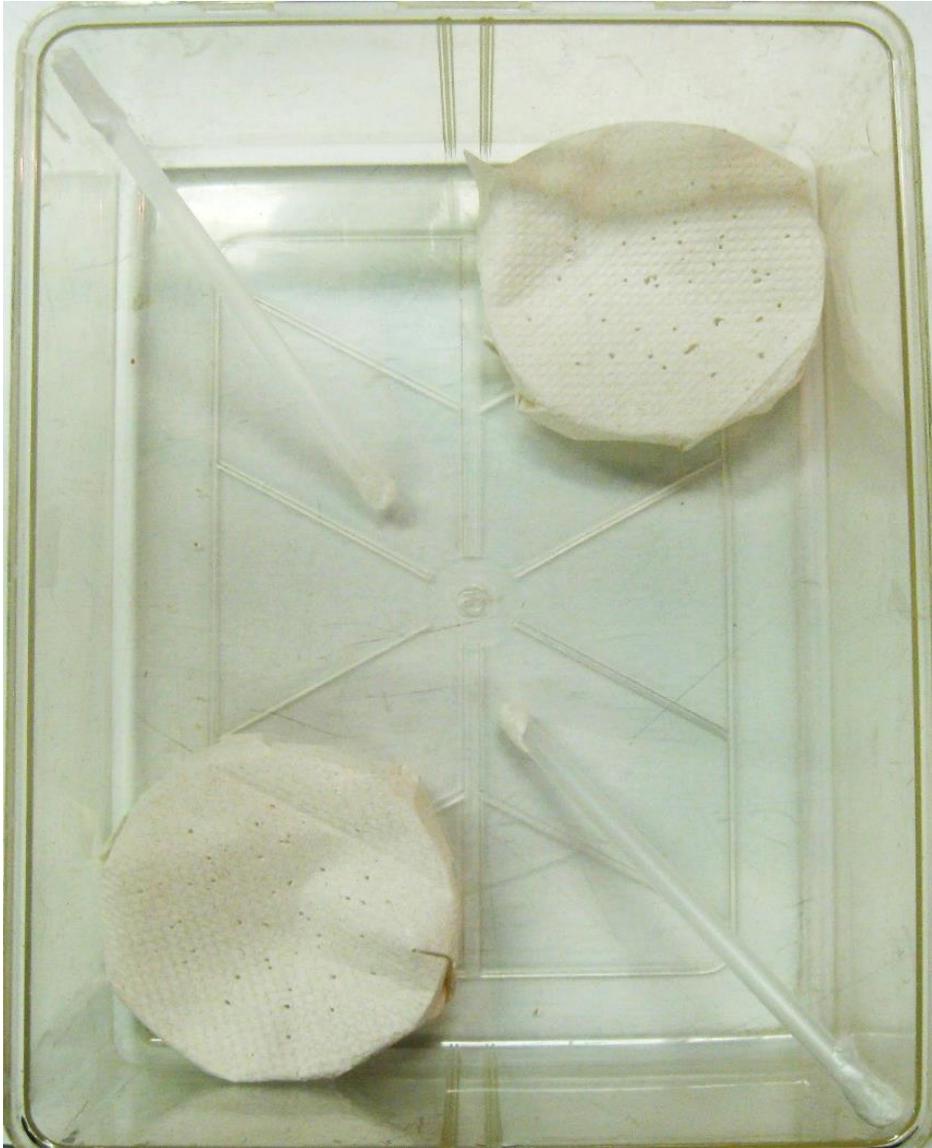


Fig. 3.1 Mating arena for navel orangeworm (*Amyelois transitella*) oviposition experiments. The arena consists of a plastic box, two plastic spatula mating perches, and two agarose diet plates (one inoculated with *Aspergillus flavus*) wrapped in perforated paper towels.

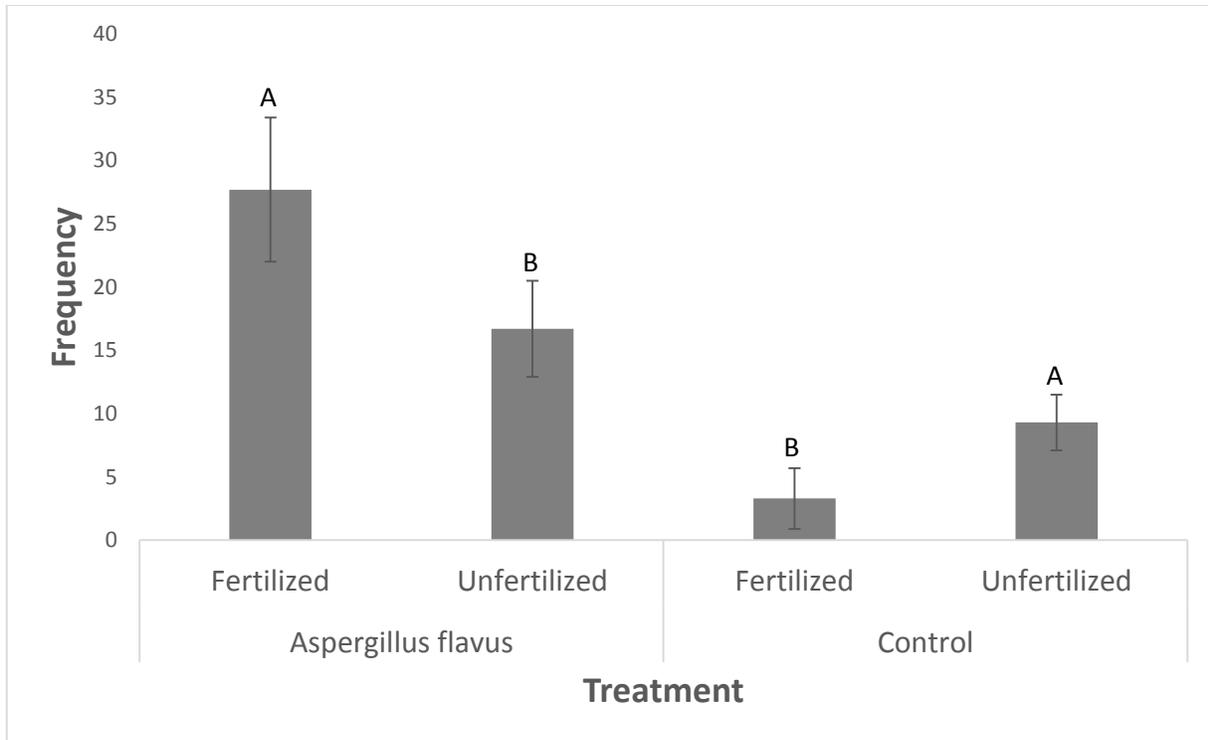


Fig. 3.2 Mean navel orangeworm (*Amyelois transitella*) preference and egg fertilization rate for different oviposition sites (± 1 SE); the choices provided were a surface inoculated with *Aspergillus flavus* and a sterile agarose substrate (Control).