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The ability of conditioned *Microplitis croceipes* (Hymenoptera: Braconidae) to distinguish between odors of aflatoxigenic and non-aflatoxigenic fungal strains

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Summary. The parasitic wasp *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae) learns to associate odors with food resources and subsequently exhibits a characteristic food-seeking behavior when encountering the learned odor. Wasps so conditioned, learned and subsequently demonstrated an ability to distinguish among aflatoxigenic and non-aflatoxigenic *Aspergillus flavus* and *A. parasiticus* strains. The effects of fungal species, strain, age (5, 10-12, 20, and 30 d) and growth media (potato dextrose agar, peanut agar and corn agar) on the learning and recognition responses of the conditioned wasps were examined. The level of differentiation between fungal strains by conditioned wasps was lowest when working with 5-d-old fungal cultures but increased with age and generally peaked with 20-d-old fungi. Wasps responded generally stronger to the fungal strain conditioned to independent of growth media. This ability of parasitic wasps to learn and distinguish fungal odors can open new avenues in insect learning.

Key words. Fungus – odors – learning – *Microplitis croceipes* – behavior

Introduction

Many studies have demonstrated the odor learning ability of insects such as, Lepidoptera (Menzel & Bitterman 1983; Cunningham *et al*. 1998; Daly & Smith 2000) and Hymenoptera (Bhagavan & Smith 1997; Stopfer et al. 1997; Menzel & Bitterman 1983). Recent studies have shown that *Microplitis croceipes* Cresson (Hymenoptera: braconidae), a larval parasitoid of *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) and *Heliothis virescens* (F) (Lepidoptera: Noctuidae) (Lewis 1970) has the ability to learn novel chemical odors (Tertuliano *et al.* 2004; Olson *et al.* 2003; Wäckers *et al.* 2002; Takasu & Lewis 1993; 1996). Additionally, food and host-associated behaviors, such as area-restricted searching (food-associated behavior) and coiling (host-associated behavior whereby female wasps rise on their hind legs with a characteristic bending of their antennae) (Olson *et al*. 2003) exhibited by conditioned wasps are distinctly observable and suitable for use as a diagnostic method for detecting conditioning chemicals (Olson *et al*. 2003; Wäckers *et al*. 2002). This wasp has also exhibited specific yet respectively different behaviors for the same chemical odor when associated with food (food seeking) versus with host (coiling) (Olson *et al.* 2003). The particular ability of parasitic wasps to exhibit different specific behaviors in concert with food odor and host odor learning makes *M. croceipes* an ideal candidate to study insect's ability to learn novel chemicals (Wäckers *et al.* 2002). The food-associated seeking behavior (Curio 1976; Wäckers *et al*. 2002), intense antennation on substrate and the area restricted searching in the direction of greatest odor concentration, commonly referred to as osmotropotaxis was previously investigated (Krammer 1976).

Aspergillus flavus (Link) and *Aspergillus parasiticus* (Speare) are the most important fungal pathogens infecting corn and peanut from field to storage. It has been reported that they produce volatile metabolites, chemicals, and aflatoxins (aflatoxigenic strains) as an indication of fungal growth (Borjesson *et al.* 1990, Jain *et al.* 1991; Magan 1993). Aflatoxins are naturally occurring mycotoxins produced mainly by the fungal species, *A. flavus* and *A. parasiticus* (Wood 1989, 1992; FAO 1993; Wilson & Payne 1994). These toxins are common contaminants in peanuts, corn and other commodities, and are chemically resistant to degradation under normal agricultural product transformation and cooking procedures (Shane 1994). Aflatoxins are carcinogenic and the FDA (Food and Drug Administration) limits the amount that can be passed into food and feedstuff. As such, their accurate detection is a critical issue for the food and feed industry. To complicate matters, there are aflatoxigenic

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and non-aflatoxigenic producing fungal strains which cannot be visually discriminated.

Different methods for aflatoxin detection exist including High Performance Liquid Chromatography (HPLC) and Enzyme-Linked Immunoassay (ELISA). The ELISA methods are techniques relying on antibody recognition (Gourama & Bullerman 1995). HPLC uses fluorescent detection of aflatoxin under UV light (Malone *et al.* 2000; Lemke *et al.* 1989; Cotty 1989; Harra *et al.* 1974; Lemke *et al.*1988; Yabe *et al.* 1987). These techniques are time consuming, expensive, and are not suitable for field detection (Magan & Evans 2000).

Aflatoxigenic fungi are known to produce strain specific volatiles (Fischer *et al*. 1999). Additionally, the effect of fungal age on the profile of volatiles produced by the fungus *Trichoderma viride* has been investigated (Zeppa *et al*. 1990). Besides being dependent on fungal species and fungi age, volatiles produced can vary depending on the growth medium (Strachan *et al.* 1990; Norman 1971). Investigation of *M. croceipes* ability to learn volatile chemical compounds produced by toxin and non-toxin producing strains not only reveals important information about the scope and processes of their olfactory sensory system, but also contributes to potential development of a new chemical sensor.

Three studies are presented in this paper. Initially, we examined the ability of *M. croceipes* to learn, respond and differentiate between *A. flavus* and *A. parasiticus* at the species and strain level using volatiles; and determined if food seeking behavior exhibited by the conditioned wasps (Wäckers *et al.* 2002) is a suitable diagnostic measure. Once the wasp's response to the fungi was determined, we examined the effects of fungal age on the ability of *M. croceipes* to learn and distinguish between *A. flavus* and *A. parasiticus*. The third study focused on the ability of *M. croceipes* to retain their ability to detect and distinguish between fungal strains cultured on three growth media, potato dextrose agar (PDA), and peanut agar and corn agar.

Materials and methods

Media and Fungal cultures

A. flavus NRRL 3357 (aflatoxigenic), *A. flavus* NRRL 1957 (non-aflatoxigenic), *A. parasiticus,* NPL 32 (aflatoxigenic) and *A. parasiticus,* NRRL 13539 (non-aflatoxigenic) were used in our study. Individual fungi were grown for various periods of time on 20 ml of the specific media, potato dextrose agar (PDA), peanut agar (50 g of ground peanut grains +15 g agar per liter of water), and corn agar (50 g ground corn grains +15 g agar per liter of water) in 25 × 150 mm Kimax® test tubes (Fischer Scientific, Norcross, GA), according to need for each test as described below. The grains were previously tested by HPLC to ensure that there was no fungal growth activity. The media were also sterilized in autoclave at 125 °C for 30 min before use. Cultures were maintained in a growth chamber (catalog # 11-679-25C Fischer Scientific, Norcross, GA) at 28 °C.

Insect

M. croceipes was reared on *H. zea* larvae according to the method described by Lewis and Burton (1970). Because hunger state affects the wasps response to odor (Tertuliano *et al*. 2004; Takasu & Lewis 1993; Lewis & Takasu 1990), female wasps were starved for 48 h post-emergence in a cage (30 × 30 × 17 cm); they were provided with distilled water only and held in a rearing room set at 28 °C, 50-70 % RH and 16L:8D photo cycle.

Wasp conditioning procedure

Prior to conditioning of wasps, the opening of the test tube containing the conditioning fungal strain was covered with a 5 × 5 cm sheet of aluminum foil, which was held in place with a plastic clasp. Volatiles produced by the fungus were allowed to accumulate in the tube headspace for 15 min, time sufficient for the odor to equilibrate. Opening the tube and covering it with aluminum foil takes less than 15 seconds, keeping the amount of odor lost from the tube at a minimum. Seven holes (each approximately one mm diameter) separated by approximately two mm were placed in a circle near the center of the foil. A droplet (<0.5 ml) of 50 % sucrose was placed on a square piece of filter paper (1 × 1 mm) in the center of the ringlet of holes over the odors. An individual wasp was captured in a glass vial (5-ml) from a rearing cage and placed over the odor; the wasp walked outside of the vial and allowed to feed on the sugar water for 10 s, before being gently removed with the vial. The training was repeated three times with 30 to 60 s between training sessions. While feeding, the wasps were conditioned to the volatiles diffusing through the holes. After the final conditioning session, the wasps were held individually in the glass vials for 15 min before testing.

Wasp testing procedure

Individually conditioned wasps were tested. The same design that was used to condition the wasps, but without the sugar water was used to test their subsequent response to fungal odors. The antennation behavior was used in our experiments to indicate a positive response to the conditioning odor (Rains *et al*. 2004). The wasps were released near the holes, so their antennae could sense the odors immediately. They responded positively by exhibiting antennation behavior and turning their body in circles over the odor source. We recorded the duration of time (sec) spent by females responding positively until they stopped or flew away. The wasps responded negatively by walking over the holes without antennation or flying away. The conditioning and testing took place under a hood to optimize ventilation. All wasps used in our experiments were tested only once, to one odor only and then discarded. The Data analyses were performed by GLM procedure and LSD using statistical software (SAS Institute 2001).

Discrimination ability between species and strains

In order to determine the capability of conditioned wasps to discriminate fungal species and strains, 12 wasps per day over 6 days (different cohort utilized each day) were conditioned individually to the odor of either a strain of *A. flavus* (NRRL 3357, aflatoxigenic; NRRL 1957, non-aflatoxigenic) or *A. parasiticus* (NPL 32, aflatoxigenic; NRRL 13539, non-aflatoxigenic) (S. Peterson, personal communication) as described earlier. Fungi were grown on PDA for 10 days. After training, 12 odor-conditioned wasps were randomly divided into 4 groups (3 wasps per group); each group was tested to one fungal odor. Individual wasps of each group were tested randomly to the odorants of the conditioning fungal strain, as well as the odorants of the other 3 strains (non-conditioning) included in the study. Eighteen wasps (3 per day for 6 days with a different cohort utilized each day) were tested to each fungus.

Effect of fungal age on discrimination ability of conditioned wasps

In order to investigate the effects of fungal age on the ability of wasps to learn and distinguish between fungal strains, individual

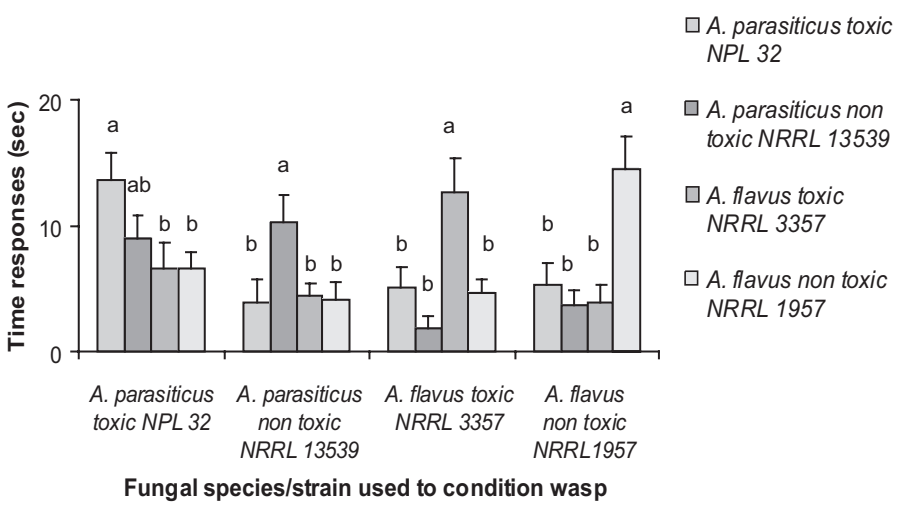


Fig. 1 Mean time response (sec) of wasps tested to each fungus strains. I-bars represent standard error. Different letters on the bars for each fungus indicate significant difference between other fungi (F-test, followed by LSD, $P < 0.05$)

molds were cultured for 5, 10, 20 and 30 d on 20-ml of PDA (Difco Inc., Sparks, MD) slants in 25 × 150 mm Kimax® test tubes (Fischer Scientific, Norcross, GA). The wasps were conditioned to each fungus of various ages and tested within the same fungus age. Two experiments were designed herein, one to distinguish *A. parasiticus* strains by age and the second to distinguish *A. flavus* strains by age.

To distinguish *A. parasiticus* fungi strains, 9 wasps per day over 6-12 days (different cohort utilized each day) were conditioned individually to the odor of either *A. parasiticus,* NPL 32 (aflatoxigenic) or to *A. parasiticus,* NRRL 13539 (non-aflatoxigenic) as described earlier. After training, 9 odor conditioned wasps per day were randomly divided into 3 groups (3 wasps per group). Each group of wasps (3 wasps per day over 6-12 days, with different cohort utilized each day) was tested randomly either to control (PDA only), *A. parasiticus,* NPL 32 (aflatoxigenic) or to *A. parasiticus,* NRRL 13539 (non-aflatoxigenic).

To distinguish *A. flavus* fungi strains, 9 wasps per day over 6 days (different cohort utilized each day) were conditioned individually to the odor of either *A. flavus* NRRL 3357 (aflatoxigenic) or *A. flavus* NRRL 1957 (non-aflatoxigenic) as described earlier. After training, 9 odor conditioned wasps per day were randomly divided into 3 groups (3 wasps per group). Each group (3 wasps per day over 6 days, with different cohort utilized each day) was tested randomly either to control (PDA only), to *A. flavus* NRRL 3357 (aflatoxigenic), or to *A. flavus* NRRL 1957 (non-aflatoxigenic).

Effect of media culture on wasp response to fungal strains

In order to determine if conditioned wasps are capable of detecting and discriminating between fungal strains on various of each media resources (PDA, Corn and peanut), 9 wasps per day over 4 days (different cohort utilized each day) were conditioned individually to the odor of either *A. flavus* NRRL 3357 (aflatoxigenic) or *A. flavus* NRRL 1957 (non-aflatoxigenic) as described earlier. Both fungi were grown individually for 5 days on PDA. After training, 9 odor conditioned wasps per day were randomly divided into 3 groups (3 wasps per group). Each group was tested randomly either to control (PDA only), to *A. flavus* NRRL 3357 (aflatoxigenic), or to *A. flavus* NRRL 1957 (non-aflatoxigenic). Both fungi were grown individually for 5 days respectively on three media: PDA, peanut agar and corn agar. A total of twelve wasps (3 per day over 4 days) were tested per treatment.

Results

Discrimination ability between species and strains

When conditioned to the specific fungus with sugar water, the parasitic wasp linked the fungus odor to the food and responded significantly stronger to each conditioning fungus than to the odors of the other fungi (Fig. 1). Duration of response of conditioned wasps to the conditioning fungus was significantly greater than that recorded for non-conditioning fungi except when conditioned to aflatoxigenic *A. parasiticus* NPL 32. In this case, mean response time was significantly greater than that recorded for the *A. flavus* strains but not the non-aflatoxigenic *A. parasiticus* strain (NRRL 13539) (Figure 1), ($P < 0.05$).

Effect of fungal age on discrimination ability of conditioned wasps

Generally, conditioned wasps responded stronger to the conditioning fungus, as well as the aflatoxigenic strains, for *A. flavus* and *A. parasiticus* (Figs. 2-3), ($P < 0.05$). The wasp response level to the conditioning fungal strain was greater than to the non-conditioning fungal strain or to the control. These results were observed after 5 to 30 days when the non-aflatoxigenic strain of *A. parasiticus* was used as the conditioning fungus (Fig. 2B), and after 10 to 30 days when the aflatoxigenic strain of *A. parasiticus* (Fig. 2A) and the aflatoxigenic strain of *A. flavus* were used as the conditioning fungus (Fig. 3A & B). In contrast, when fungi were grown for 5 days, there was no difference in wasp response to the conditioning fungus versus the non-conditioning fungi (Fig. 2A & Fig. 3).

Effect of media culture on wasp response to fungi strains

The wasps responded stronger to the conditioning fungal strains than the non- conditioning fungal strains and control,

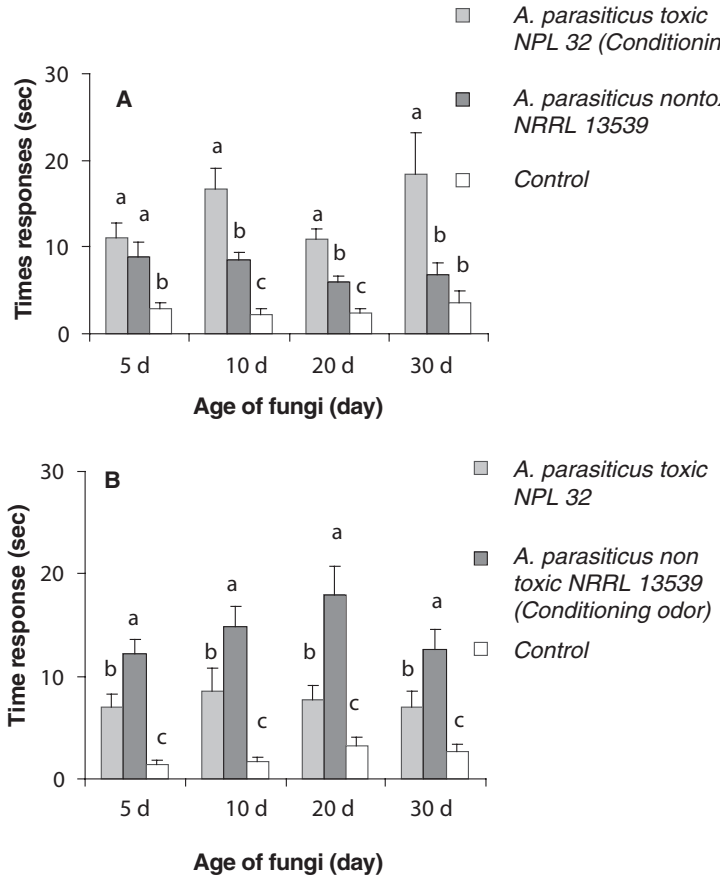


Fig. 2 Mean response time (sec) of wasps tested to each *A. Parisiticus* strain and control (PDA only; no-inoculate). I-bars represent standard error. Different letters on the bars for the same age indicate significant difference (F-test, followed by LSD, $P < 0.05$). (A) Wasps were conditioned to *A. parasiticus*, toxic NPL 32, (B) wasps were conditioned to *A. parasiticus*, non toxic NRRL 13539

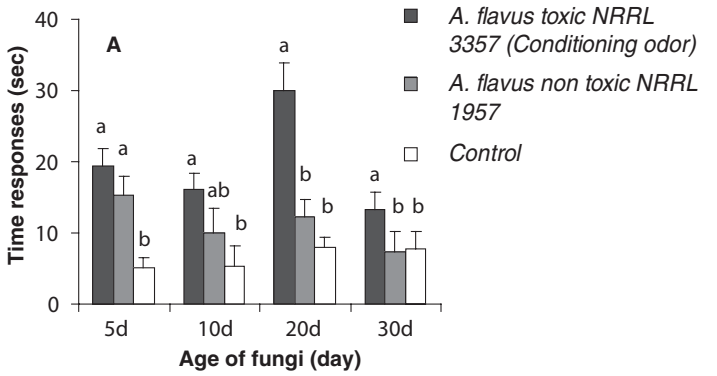
independent of media used to culture them, (Fig. 4), ($P < 0.05$), but the difference was less pronounced for the corn and peanut media (Fig. 4). The greatest responses were recorded on PDA with 19 s of time spent for aflatoxigenic *A. flavus* strain (NRRL 3357) (Fig. 4A) and 25 s of time spent for non-aflatoxigenic *A. flavus* strain (NRRL 1957), (Fig. 4B).

Discussion

We have demonstrated that *M. croceipes* can be conditioned to recognize and distinguish between different *Aspergillus* fungal species as well as between aflatoxigenic and non-aflatoxigenic strains of the same species. Additionally, these results suggest that the antenning behavior exhibited by conditioned *M. croceipes* females can be used to distinguish between aflatoxigenic and non-aflatoxigenic strains of *A. flavus* and *A. parasiticus*. Interestingly, we found that when the wasps were conditioned to the fungi's complex odor bouquets with sugar water, they successfully linked the odors to food and exhibited the antennating behavior to identify specific fungi. The trained wasp responded less to control than fungus. Previous studies have demonstrated that untrained wasps do not respond to any odor; they walk and/or fly away (Walker *et al*. 2002; Tertuliano *et al*. 2004). These results indicate that training with odor is necessary to produce a learned response and suggest that odor and sugar are associated (Menzel & Bitterman 1983).

The strongest antennating time of conditioned females were recorded with the conditioning fungal strain regardless of fungus age. However, when 5-day old *A. flavus* species were used as the conditioning fungus, the wasp response between conditioning and non-conditioning fungus was not significantly different. The lack of discrimination by the wasp may be due to the low concentration of volatile chemicals as shown in honey bee studies (Bhagavan & Smith 1997). Additional research is needed to confirm this explanation and to understand if any unique volatiles produced by *A. flavus* are lacking.

Our results are supported by previous studies that examined the volatiles produced by toxigenic and non-toxigenic fungal strains of two *Fusarium* species (*F. moniliforme* and *F. proliferatum*) with an electronic nose (Keshri & Magan 2000). Sunnesson *et al.* (1995) found that the production of volatiles is highly dependent on fungal species, while another study in contrast determined that the distinctive and characteristic volatiles were produced by both non-toxigenic and toxigenic strains of *F. sambucinum* suggesting chemical production irrespective of fungal strain (Jelen *et al.* 1995). The variation of wasp response during fungus growth (5, 10, 20 and 30 days) indicated that the production of volatiles changed with time, as



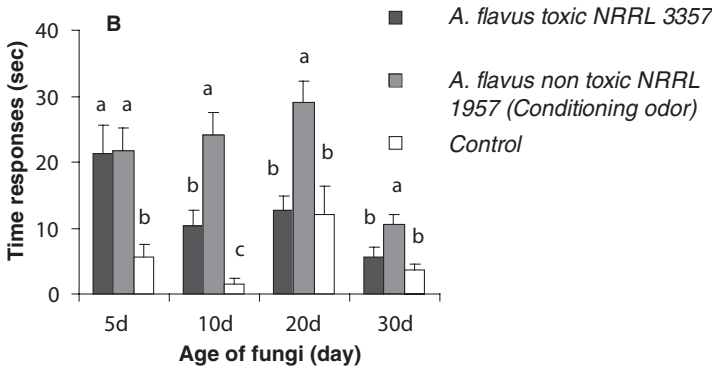
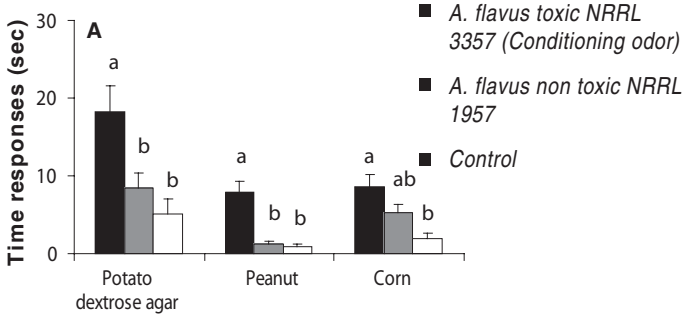


Fig. 3 Mean response time (sec) of wasps tested to each *A. flavus* strain and control (PDA only; no-inoculate). I-bars represent standard error. Different letters on the bars for the same age indicate significant difference (F-test, followed by LSD, $P < 0.05$). (A) Wasps were conditioned to *A. flavus* toxic NRRL 3357 and (B) wasps were conditioned to *A. flavus* non toxic NRRL 1957



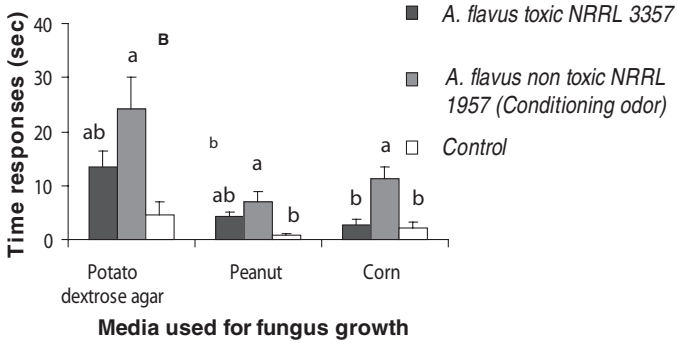


Fig. 4 Mean response time (sec) of wasps tested to each *A. flavus* strain and control (PDA no-inoculate). I-bars represent standard error. Different letters on the bars for the same medium indicate significant difference (F-test, followed by LSD, $P < 0.05$). (A) Wasps were conditioned to *A. flavus* toxic NRRL 3357; (B) wasps were conditioned to *A. flavus* non toxic NRRL 1957

found by previous studies examining the profile of volatiles for several species of mold during incubation (Borjesson *et al.* 1989; Zeringue & Mccormick 1989; Larsen & Frisvad 1995; Sunesson *et al.* 1995; Zeppa *et al.* 1990).

Generally this wasp can detect the conditioning fungus regardless of the media (PDA, corn and peanut) on which they were cultured. The wasps responded generally stronger to conditioning fungus than the other fungus independent of

media. Additional chemical analysis should help to understand if there are any unique volatiles produced by fungus grown on different media. However, the strongest responses observed for fungus grown on PDA suggested the richness of this media vs. peanut and corn may play a factor in volatile production by fungi. Previous research indicates that although media can influence volatile production (Borjesson *et al.* 1990; Sunesson *et al.* 1995; Zeppa *et al.* 1990), there are some compounds that are independent of the growth media, suggesting that the presence of a compound(s) may serve as a signature of a fungal strain (Colling & Halin 1972; Borjesson *et al.* 1992). The choice of media was based on the desire to investigate different artificial substrates (commonly used for fungus growth experiments) as Borjesson *et al*. 1990, and later investigate on real grains as Borjesson *et al*. 1992. Later experiments in our laboratory have investigated the chemical compounds associated with fungal strains on corn and peanuts (Z. Jurjevic, unpublished data).

We have successfully conditioned *M. croceipes* to learn and discriminate bouquets of fungal odors as previous studies have successfully shown this wasp's ability to learn and recognize single chemicals associated with food and/or host (Tertuliano *et al*. 2004; Olson *et al*. 2003; Takasu & Lewis 2003). These results are in accordance with other studies that demonstrate a wide range of learning ability in insects (Daly & Smith 2000; Bhagavan & Smith 1997; Gunningham *et al*. 1998). For example, Park *et al*. (2001) used different species of insects including *M. croceipes* (insect used in our experiment) to discriminate successfully 20 different compounds. However, some chemicals with similar structures were very difficult to discriminate such as nonanol and decanol (Meiner *et al*. 2002; Park *et al*. 2001), 1-heptanol and 1-octanol (Park *et al*. 2001), 3-octanone and 2-octanone (D. Olson, unpublished data). This is because generalization responses to some odorants depend on their molecular structures. Moreover, generalization effects may also be due to the concentration used to condition the organisms. The honey bee *Apis mellifera* generalizes to the concentration of odors tested when conditioned with a low concentration of odor, but discriminate clearly when conditioned with a higher concentration; the bees generalized low to high, but not high to low (Bhagavan & Smith 1997). The effect of generalization found in the bees may also be due to the procedure used for odor presentation; the first odor presentation affects the bees' response in the next odor presentation (Daly & Smith 2000). Unlike with the bee study, we tested the wasps only once, there is no successive presentation of fungus odor first, and then to control or vise versa.

The findings from this study of a parasitic wasp and selected fungi species provide not only key information about chemical sensory ability of insects and other invertebrates, but opens potential valuable application of this knowledge. Chemical volatiles could serve as bioindicators of fungal presence in the prevention of food contamination. They could be used to detect chemical production as an indicator of toxin production prior to any visible signs of mold growth (Borjesson *et al.* 1989; Borjesson *et al.* 1990, Jain *et al.* 1991; Magan 1993). Detection of fungal chemicals with the parasitoid wasp may serve as a potential resource for a sensor to monitor other species of mold as well. The demonstrated abilities by *M. croceipes* and the understanding of what chemicals are used by the wasp to discriminate fungi can open avenues of research to develop a new non-biological and/or biological volatile chemical sensor. A flexible and fast responding portable device using the olfactory sensory capabilities of *M. croceipes* wasps has been developed and tested successfully to detect chemical compounds in corn (S. Utley, unpublished data). However, research still is needed to improve techniques for use.

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