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Effect of the entomopathogenic fungus, *Entomophthora muscae* (Zygomycetes: Entomophthoraceae), on sex pheromone and other cuticular hydrocarbons of the house fly, *Musca domestica*

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Abstract

House fly (*Musca domestica*) males are highly attracted to dead female flies infected with the entomopathogenic fungus *Entomophthora muscae*. Because males orient to the larger abdomen of infected flies, both visual and chemical cues may be responsible for the heightened attraction to infected flies. Our behavioral assays demonstrated that the attraction is sex-specific—males were attracted more to infected females than to infected males, regardless of cadaver size. We examined the effect of *E. muscae* on the main component of the house fly sex pheromone, (*Z*)-9-tricosene, and other cuticular hydrocarbons including *n*-tricosane, *n*-pentacosane, (*Z*)-9-heptacosene, and total hydrocarbons of young (7 days old) and old (18 days old) virgin females. Young *E. muscae*-infected female flies accumulated significantly less sex pheromone and other hydrocarbons on their cuticular surface than uninfected females, whereas the cuticular hydrocarbons of older flies were unaffected by fungus infection. These results suggest that chemical cues other than (*Z*)-9-tricosene, visual cues other than abdomen size, or a combination of both sets of cues might be responsible for attraction of house fly males to *E. muscae*-infected females.

Keywords: *Entomophthora muscae*; *Musca domestica*; Sex pheromone; Cuticular hydrocarbons; Behavior

1. Introduction

The entomopathogenic fungus, *Entomophthora muscae* (Cohn) Fresenius, is a well-known pathogen of house flies and other filth-dwelling flies (Mullens, 1989). The fungus enters the hemocoel by penetrating the cuticle and forms hyphal bodies that proliferate and within 28 h invade the hemocoel, especially the abdomen and fat body (Mullens et al., 1987). Death of the host usually occurs within 5–8 days after infection (Brobyn and Wilding, 1983). Within 3 h after the fly dies conidiophores emerge on the surface of the cadaver through the intersegmental membranes of the abdomen. Conidial discharge then takes place in about 10–21 h (Mullens et al., 1987; Watson and Petersen, 1993).

Typical signs and symptoms of *E. muscae* infection include behavioral and postural changes, including a swollen abdomen angled away from the substrate, outstretched legs, wings raised above the thorax, extended proboscis, and white conidiophores on the intersegmental membranes of the abdomen (Krasnoff et al., 1995; Mullens, 1990). Given a choice, house fly males are more attracted to infected than uninfected female flies; they engage in extensive courtship and mounting and consequently may become infected (Møller, 1993). Because mate recognition in the house fly is guided by visual and chemical cues (Adams et al., 1995), the swollen abdomen might serve as an attractive visual cue to males. However, Møller (1993) showed that the abdomen of infected flies was more attractive to males than the abdomen of uninfected flies, even when the size

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of the abdomens was held constant. These results demonstrated that fungus-induced features other than the size of the abdomen enhance the attractiveness of fly cadavers (Møller, 1993). Potential cues, then, are visual cues associated with the conidiophores, chemical cues, or both. Because the target flies in Møller's experiments were all females (personal communication), it was not possible to separate these two sets of cues.

The female house fly produces (*Z*)-9-tricosene (muscalure) as the main component of a sex pheromone (Carlson et al., 1971) that includes (*Z*)-14-tricosen-10-one, (*Z*)-9,10-epoxytricosane, and a series of methyl branched alkanes with chain lengths from 28 to 30 carbons (Adams and Holt, 1987; Adams et al., 1995; Uebel et al., 1976, 1978). We hypothesized that the behavioral alteration induced by *E. muscae* in the house fly is related to elevated amounts of the pheromone and that female house flies infected with *E. muscae* might produce more (*Z*)-9-tricosene than uninfected females. We therefore predicted that males would be attracted more to infected females than to infected male house flies.

2. Materials and methods

2.1. Insects and the pathogen

A colony of adult house flies originating from the colony at the Department of Entomology, Cornell University, was maintained on water and a mixture of dry milk and powdered sugar (1:1) ad libitum. The strain of *E. muscae* (Keller et al., 1999) was isolated from infected house flies collected from the Lake Wheeler Animal Experimental Station, NCSU, and maintained in the laboratory, Department of Entomology, NCSU, by direct cadaver-to-fly transmission (Mullens, 1986).

2.2. Behavioral assays

The response of healthy male house flies to female and male cadavers (killed by *E. muscae*) of similar and different weights was assessed in behavioral assays. For each assay, laboratory reared house flies were separated by sex 24 h post-eclosion and placed in screened containers (250 ml) with food and water. Two groups (40 males, 40 females—48 h post-eclosion) were infected with *E. muscae* by exposure to conidial showers from 15 fresh cadavers placed on the mesh cover of each container. Containers were kept in the dark overnight at 20 °C and 40% R.H. After the exposure period, cadavers were removed and exposed females were maintained at 20 °C, 40% R.H., and 12/12 h light/dark regime. Flies were supplied with powdered milk and granulated sugar (1:1) and water ad libitum and monitored closely for the distinct white bands forming at the intersegmental membranes indicative of an expanding abdomen and conidiophore formation. These flies were weighed and sorted by weight. Male and female flies were paired by weight in three ways (Table 1): (1) flies of the same or similar weight, (2) heavy females and light males, and (3) heavy males and light males. Fly pairs were then placed in the dark at 20 °C until the conidiophores began to emerge from the cadavers. A group of healthy males was chilled and sorted into 8 groups of 5 flies each, placed in separate 250 ml screened cups, and used for behavioral assays. Each assay was replicated four times.

Behavioral assays were conducted under standard fluorescent lighting and room temperature, using wide-mouth 3.8 L transparent plastic containers (Klear Stor, Anchor Hocking Plastics, Eagan, MN). These cages permitted full, unobstructed views of the flies held within. Each lid was fitted with a $7 \times 5 \times 1.5$ cm foam core rectangular block into which two minutin pins were positioned horizontally 5 cm apart. Paired cadavers (one male and one female) were pinned to the foam core block. Five chilled healthy males were released into each cage and a temporary lid placed on the cage. Once the male flies had fully recovered, the lid was exchanged for one containing the pinned cadavers. The males were given 5 min to respond to the pinned cadavers. Two behavioral events were monitored—first contact and attempted copulation—defined as the sex of the first cadaver contacted by any healthy male, and the sex of the first cadaver that any healthy male attempted to copulate, respectively. Attempted copulation involved a healthy male landing on the back of the cadaver, curving the abdomen and attempting penetration (Murvosh et al., 1964). At the end of the 5-min period the healthy males were set aside for use in subsequent behavioral

Table 1
Mating response of healthy male house flies (*M. domestica*) to paired female and male fly cadavers killed by *E. muscae*

Sex and mean cadaver mass (mg ± SEM)					% First contact (*N*)			% Attempted copulation (*N*)		
Relation of paired cadavers	No. of pairs	Female mass	Male mass	*P*[a]	Female	Male	*P*[b]	Female	Male	*P*[b]
Female = Male	39	20.04 ± 0.0005	20.06 ± 0.0004	0.980	74.5 (31)	25.5 (8)	<0.001	92.3 (36)	7.6 (3)	<0.001
Female > Male	41	24.72 ± 0.0005	18.26 ± 0.0002	<0.001	80.5 (33)	19.5 (8)	<0.001	90.2 (37)	9.8 (4)	<0.001
Female < Male	10	16.39 ± 0.0006	20.81 ± 0.0002	<0.001	80.0 (8)	20.0 (2)	0.057	100.0 (10)	0.0 (0)	<0.001

[a] Student's *t* test on paired samples.
[b] The normal approximation of binomial distribution test.

assays (3–4 times) and the next cage was prepared with new males.

2.3. Hydrocarbon analysis

Young virgin female flies (2 days post-eclosion) and old virgin female flies (13 days post-eclosion) were used for the experiments. Approximately 50 virgin females in each age group were transferred to screened containers and infected with an *E. muscae* conidial shower as described above. Approximately 50 uninfected virgin female flies of each age group were kept as a control group in the same conditions. Mortality in all colonies was monitored daily.

Twenty fly cadavers from each age group were removed from the containers immediately after the emergence of conidiophores through the intersegmental membranes of the abdomen (before the conidial discharge) and used for hydrocarbon extraction. Twenty flies from each age group from the control colonies were removed at the same time and anesthetized with CO_2. Individual cadavers were submerged in 2 ml of hexane containing 1 μg of 1-docosene as internal standard and lipids were extracted for 5 min with gentle mixing (Young and Schal, 1997). The solvent was decanted into a clean vial and the fly was washed with clean hexane twice more. The hydrocarbons were eluted from 500 mg of silica gel (Selecto, Fisher) in a Pasteur pipette column with 7 ml hexane and the hexane was reduced under a gentle stream of N_2. The hydrocarbon fraction was then injected into a splitless injector leading to a 25 m × 0.32 mm × 1 μm HP-1 capillary column (Hewlett-Packard, Avondale, PA) in a HP6890 gas chromatograph (GC). The oven temperature was programmed from 70 to 150 °C at 30 °C/min and then at 5 °C/min to 320 °C, where it was held for 10 min. Helium was used as the carrier gas. The injector and detector temperatures were 300 and 310 °C, respectively. Data acquisition was on a HP Chemstation A.08.01 (Agilent, Palo Alto, CA).

Statistical analyses of the cuticular hydrocarbons of control and treated flies within each age group as well as analysis of the differences in the body weight of paired fly cadavers in behavioral assays were conducted by Student's t test ($\alpha = 0.05$). Normal approximation of the binomial distribution test was used for analysis of mating responses of male house flies to female and male cadavers ($\alpha = 0.05$) (Snedecor and Cochran, 1989).

3. Results

3.1. Behavioral assays

Healthy male flies were not immediately responsive to the female cadaver in the cage. Once their movements brought them within about 8 cm of the pinned cadavers, the male flies exhibited more directed orientation. Males approaching female cadavers from a distance of 1–2 cm immediately attempted to copulate. Conversely, when healthy males approached the male cadaver, first contact was slow and tentative. This behavior suggests that female-specific cues, either visual or chemical, trigger the male sexual response. A prominent visual cue is the larger abdomen of infected female flies. However, when the weights of the female and male cadavers were similar, healthy male flies were still significantly more attracted to female cadavers than to male cadavers; 79.5% of the males contacted the female cadaver first and 92.3% attempted to copulate with the female cadavers (Table 1).

Normally, female house flies are larger than males. Under these conditions, healthy male flies were significantly more attracted to the heavier female cadavers than to male cadavers (Table 1). They also made first contact and attempted to copulate with heavier female cadavers 80.5% and 90.2% of the respective tests. Surprisingly, the relative attraction to female cadavers did not change when light females were paired with significantly heavier males (Table 1). Healthy males made first contact with the lighter female cadavers in 80% of the observations and they initiated copulation with the female cadaver in all tests (Table 1). These results strongly implicate female-specific chemical cues in favor of visual cues.

3.2. Hydrocarbon analysis

The hydrocarbons selected for analysis included (*Z*)-9-tricosene (a female-specific compound), (*Z*)-9-heptacosene (a compound prominent in males), and hydrocarbons that are common in both sexes (*n*-tricosane and *n*-pentacosane). All young (2 days old when infected) and old (13 days old when infected) flies died 5 days after infection. In the young infected flies, all the hydrocarbons examined, including (*Z*)-9-tricosene, *n*-tricosane, *n*-pentacosane, (*Z*)-9-heptacosene, and total hydrocarbons, were significantly diminished in comparison to those of young control flies (Fig. 1). In old flies, in contrast, there were no significant differences between infected and control flies for any of the hydrocarbons examined (Fig. 1).

4. Discussion

Many parasites and pathogens induce alterations in the behavior and physiology of their hosts. Such changes may be adaptive responses by the host, aimed at impending colonization, growth, and development of the pathogen. Often, however, it is the pathogen that induces changes in its host that facilitate growth, development, reproduction, or dissemination of the pathogen. Of particular interest to us are syndromes in which

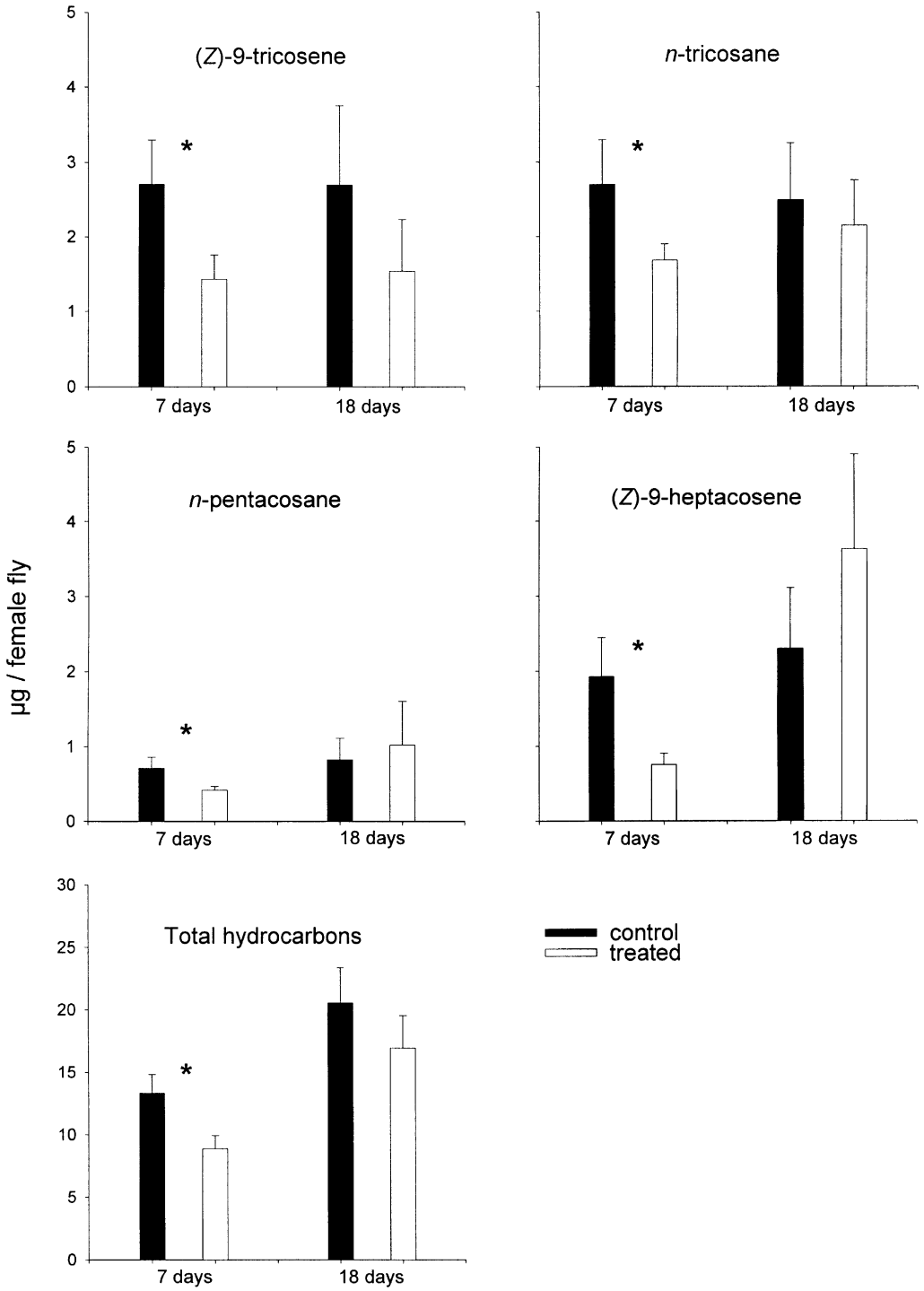

Fig. 1. The effect of *E. muscae* on (*Z*)-9-tricosene, *n*-tricosane, *n*-pentacosane, (*Z*)-9-heptacosene, and total external hydrocarbons of young (7 days old) and old (18 days old) virgin females of *M. domestica*. Error bars represent standard error of the mean. * indicates a significant difference between means within the same age group (Student's *t* test, $P < 0.05$).

the host exhibits behavior that promotes dissemination of the pathogen to conspecific hosts, thus facilitating epizootic outbreaks. An especially intriguing case is that of infected hosts becoming more attractive to members of the opposite sex which, in turn, disseminate and propagate the pathogen.

The *Musca domestica–Entomophthora muscae* interaction appears to follow this pattern. The behavior of infected flies changes in favor of promoting dissemination of the fungus. The infected fly alights on an exposed substrate and its distended abdomen becomes highly apparent visually as conidiophores form on the inter-

segmental membranes (Krasnoff et al., 1995; Mullens, 1990). Male flies prefer the hypertrophied abdomen of infected flies over normal abdomens (Møller, 1993). Interestingly, however, infected female abdomens are more attractive to males than uninfected abdomens of equal size, suggesting that the infected flies advertise more attractive visual or chemical cues (Møller, 1993). Our behavioral assays extend these observations and show that male flies prefer infected females over infected males of the same size. Even significantly smaller infected females are more attractive to males than are larger infected male flies. These results suggest that if visual cues play a dominant role in this interaction, the male flies must use very subtle visual differences between infected male and female flies. More likely, males orient to female-specific chemical cues that emanate more from infected females. We therefore hypothesized that the female sex pheromone may mediate this interaction.

The main component of the house fly sex pheromone is (*Z*)-9-tricosene. It is produced by cells associated with the abdominal integument (Dillwith et al., 1981; Dillwith and Blomquist, 1982) and then probably transported to the hemolymph before it is deposited on the surface of the cuticle (Mpuru et al., 2001) and in other tissues (Schal et al., 2001). Female house flies begin producing the sex pheromone about 2 days after emergence (Dillwith et al., 1983) and production increases until day 6 (Mpuru et al., 2001). While the amounts of (*Z*)-9-tricosene and most other hydrocarbon constituents on the female's epicuticle increase significantly, (*Z*)-9-heptacosene, a compound prominent in males, declines in sexually mature females (Mpuru et al., 2001; Schal et al., 2001). These quantitative changes in the cuticular hydrocarbon profile coincide with the production of ovarian ecdysteroids, and a clear requirement of vitellogenic ovaries for sex pheromone production has been demonstrated (Blomquist et al., 1984; Dillwith et al., 1983).

Because large amounts of internal hydrocarbons, including (*Z*)-9-tricosene, are found on surface-extracted females (Ahmad et al., 1989; Mpuru et al., 2001), we originally hypothesized that the emerging conidiophores in infected females rapidly transfer a large amount of the sex pheromone from the internal tissues and hemolymph to the epicuticular surface and thus enhance the chemical appearance of female cadavers to males. Clearly, this idea has not been confirmed. Rather, it appears that the fungus interferes with both pheromone production and pheromone transport, as evidenced by the decline in (*Z*)-9-tricosene in young diseased flies (Fig. 1).

Disruption of ovary development in young infected flies may account for the lower amount of (*Z*)-9-tricosene on their cuticular surface. Mullens (1990) infected young (<36 h post-eclosion) and old (2–5 days post-eclosion) female house flies with *E. muscae* and monitored ovarian development, fecundity, and mortality. Development of the ovaries in young infected females was greatly delayed, follicles were resorbed by day 4–5 of the infection, and no eggs were oviposited. All the infected flies died on day 5 of the infection with the hemocoel packed with hyphal bodies. Old infected flies, on the other hand, fully developed their ovaries and laid eggs before they died by day 7 of the infection, although fecundity was significantly reduced in comparison to that of control, uninfected flies (Mullens, 1990, 1985).

Our results on cuticular hydrocarbons are consistent with these observations. Flies in both groups (2 and 13 days old when infected) produced sex pheromone, indicating that their ovaries developed. However, in young flies cuticular (*Z*)-9-tricosene was significantly reduced by day 7, suggesting that the fungus destroyed internal tissues, including the ovaries, as shown by Mullens (1990). In addition, it is possible that the proliferating hyphal bodies and conidiophores in the hemocoel of young flies destroy or disrupt lipid transport. In house flies, as in other insects, hemolymph lipophorin shuttles hydrocarbons from biosynthetic sites to the cuticle and other tissues (Schal et al., 2001). Interference with the function of this lipoprotein would severely disrupt pheromone transport to the cuticle.

Conversely, the cuticular hydrocarbons, including (*Z*)-9-tricosene, of old infected flies were not significantly altered. However, the amount of cuticular hydrocarbons varied greatly among individual flies within both the fungus-treated and control groups. Such variation in external hydrocarbons of old (>8 days post-eclosion) healthy virgin female flies has been observed previously (Schal, unpublished) and is probably related to senescence and asynchrony of reproductive development among flies. Nevertheless, in older flies, ovarian development and pheromone production precede fungus infection and the fungus also appears to interfere less with ovary development in older flies (Mullens, 1990), thus allowing hydrocarbon and pheromone production to proceed with less disruption.

What chemical cues, then, might males use to alight on *E. muscae*-infected flies? In the present investigation we considered only (*Z*)-9-tricosene, one pheromone component of a complex chemical blend that includes many other cuticular lipids (Mpuru et al., 2001). It is possible, though unlikely, that other pheromone constituents, namely (*Z*)-14-tricosen-10-one and (*Z*)-9,10-epoxytricosane, may be specifically elevated in infected flies. Indeed, because these compounds are metabolites of (*Z*)-9-tricosene, a decline in the latter may be due to its conversion to ketones and epoxides. Alternatively, *E. muscae*-produced compounds might mimic the house fly pheromone, serving a role in "aggressive chemical mimicry." However, this explanation would require that the fungus use female-specific compounds because infected female flies are more attractive than infected males. If so, it is tempting to speculate that one or more of the pheromone components is modified by the fun-

gus. It is also conceivable that healthy males are at first attracted visually to fungus-infected flies, possibly to the white stripes formed by conidia on the abdomen, and then use chemical cues to differentiate female from male cadavers.

In conclusion, healthy males are more attracted to female house fly cadavers infected with *E. muscae* than to infected male cadavers irrespective of their size. The fungus *E. muscae* reduced the amount of sex pheromone in young virgin female flies and had no significant effect on the sex pheromone of old virgin females. The same effect was observed for other cuticular hydrocarbons and also for total hydrocarbons. Therefore, features other than elevated levels of (*Z*)-9-tricosene are responsible for the greater attractiveness of female flies infected with *E. muscae*.

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