Male *Diachasma alloeum* parasitoids from two host species of tephritid fruit flies respond equally to female-produced sex pheromone

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**Abstract.** *Diachasma alloeum* is a braconid wasp that parasitizes hawthorn-infesting *Rhagoletis pomonella* and blueberry-infesting *Rhagoletis mendax*, both tephritid fruit fly sibling species. The behavioural responses of male *D. alloeum* originating from both fruit fly host species to hexane extracts of conspecific females originating from both host species and females of various mating status are investigated in a laboratory Y-tube olfactometer. Male *D. alloeum* originating from either *R. mendax* or *R. pomonella* respond to one whole-body, female equivalent hexane extract in equal frequency as to a live, virgin female; this response is at least 7.5-fold greater than to solvent controls. Male *D. alloeum* originating from *R. mendax* or *R. pomonella* are attracted to hexane extracts of female *D. alloeum* abdomens but not to extracts of the head and thorax regions. Virgin *D. alloeum* females attract significantly more male *D. alloeum* originating from either host species compared with mated females of the same age. Copulation behaviour between same host and mixed host pairs appears to be identical. Male *D. alloeum* are equally responsive to the female-produced sex pheromone of female *D. alloeum*, irrespective of females’ host-species origin, suggesting that these host-specific populations can potentially interbreed.

**Key words.** Apple maggot, blueberry maggot, *Diachasma alloeum*, fruit flies, host race, parasitic wasps, *Rhagoletis mendax*, *Rhagoletis pomonella*, sex-attractant pheromone.

**Introduction**

*Diachasma alloeum* (Muesebeck) is a braconid parasitoid that specifically attacks two species of tephritid flies: hawthorn-infesting *Rhagoletis pomonella* (Walsh) (Glas and Vet, 1983; Stelinski and Liburd, 2005) and blueberry-infesting *Rhagoletis mendax* Curran (Liburd and Finn, 2003; Stelinski et al., 2004). Male and female wasps emerging from each species of fly respond specifically to the odour of the fruit of their larval *Rhagoletis* host species (Stelinski and Liburd, 2005). *D. alloeum* parasitizing *R. pomonella* preferentially respond to the odour of hawthorn fruit in Y-tube olfactometer assays, whereas those parasitizing *R. mendax* preferentially respond to the odour of blueberry fruit. However, no difference is found in the positive response of male *D. alloeum* reared from either *R. pomonella* or *R. mendax* to the odour of live, virgin females originating from both *Rhagoletis* species in Y-tube olfactometer assays (Stelinski and Liburd, 2005). It was postulated that populations of *D. alloeum* may exhibit ‘host fidelity’, where distinct host races of *D. alloeum* have a greater tendency to mate and reproduce among the host plants of their preferred *Rhagoletis* hosts (Stelinski and Liburd, 2005). Furthermore, such host fidelity may have resulted in the evolution of distinct host races or sibling species of *D. alloeum* that have tracked the speciation of their larval *Rhagoletis* prey.

Several studies show that female parasitic wasps produce and secrete volatile sex pheromones from abdominal glands that attract conspecific males (Weseloh, 1976; McNeil and Brodeur, 1995; Syvertsen et al., 1995; De Freitas et al., 2004). The Dufour’s gland is thought to be the specific source of sex pheromone on the abdomen of at least four
species of parasitoids: Cotesia liparidis and C. melanoscelsus (Weseloh, 1976), Cardiochiles nigriceps (Syvertsen et al., 1995) and C. flavipes (De Freitas et al., 2004). Boush and Baerwald (1967) were the first to show evidence of a female-produced sex-attractant pheromone in *Diachasma alloeum* (= Opius alloeus). Ethological assays performed in static-air 12 × 45 mm glass vials show that males respond positively and exhibit precopulatory behaviour in response to filter-paper discs that have been kept in similar vials with five virgin female *Diachasma alloeum* for 96 h. Furthermore, filter discs upon which a female *Diachasma alloeum* is macerated effect a response in 63% of tested males in larger Petri dishes (15 × 150 mm) assays (Boush and Baerwald, 1967). However, male activity has not been associated with a specific body region of females. Furthermore, water-ether extracts of whole female *Diachasma alloeum* do not elicit behavioural responses from males (Boush and Baerwald, 1967).

In the present study, the behavioural responses of male *Diachasma alloeum* are described from two host species of tephritid flies to hexane extracts of conspecific females.

Materials and methods

**Insect source**

Collections of *Rhagoletis mendax* puparia were made from infested fruit of blueberry plants (var. Jersey) unsprayed for over 5 years. This plantation, located in Fennville, Michigan, U.S.A., has been described previously (Stelinski *et al.*, 2004). Puparia of hawthorn-race *Rhagoletis pomonella* were also obtained in Fennville from infested hawthorn located within 0.8 km of the blueberry plantation. The puparia were stored at 4 °C for 140 days. Thereafter, puparia were removed from cold storage and placed into an environmental chamber at 24 °C, 55–60% relative humidity (RH), under an LD 16 : 8 h photoperiod. *Diachasma alloeum* began to emerge from approximately 25 and 18% of *Rhagoletis mendax* and *Rhagoletis pomonella* puparia, respectively, 3 weeks after removal of puparia from 4 °C. Prior to behavioural assays, wasps were kept in aluminium-screen cages (30 × 30 × 30 cm) and supplied with 5% sugar in water. Adults were kept at 24 °C, 55–60% RH, under an LD 16 : 8 h photoperiod.

**Pheromone extracts and release devices**

Virgin female *Diachasma alloeum*, 1–2 days old, were used to prepare hexane extracts. The macerated whole wasp, macerated head plus thorax or macerated abdominal regions of five female *Diachasma alloeum* originating from either *Rhagoletis mendax* or *Rhagoletis pomonella* were soaked in 50 μL of hexane for 20 min. Subsequently, 10 μL of each hexane extract were pipetted onto Whatman No. 1 filter paper (Whatman plc, U.K.) giving a proportion of one female equivalent per test sample. Filter paper samples were left for 5 min after preparation and prior to ethological studies to allow solvent evaporation. Control treatments consisted of filter paper impregnated with 10 μL of hexane alone.

**Y-tube olfactometer studies**

Choice tests comparing behavioural responses of male *Diachasma alloeum* to olfactory stimuli were conducted in a horizontal, glass Y-tube olfactometer (stem length 25 cm, arm length 12.5 cm, internal diameter 1.5 cm) as described previously (Stelinski and Liburd, 2005). Experiments were conducted at 24 °C with a light intensity of 2.2–2.8 W m⁻² (Young *et al.*, 1987) generated by two fluorescent bulbs (Lumichrome model 1XC, 40 W, Lumiram, White Planes, NY) mounted 2.0 m above the olfactometer. Assays ran between 12.30 and 15.00 h, a time of day when *Diachasma alloeum* are highly active in the field (Stelinski *et al.*, 2004). All male wasp used in Y-tube tests were virgins and 3–4 days post emergence. The number of male *Diachasma alloeum* contacting the end of an arm of the olfactometer, as well as the number of male wasps wing fanning in responses to stimulus sources, was recorded.

**Response of male *Diachasma alloeum* from *Rhagoletis mendax* or *Rhagoletis pomonella* to females or female extracts originating from their respective host**

The responses of male *Diachasma alloeum* originating from *Rhagoletis mendax* (blueberry-origin) or *Rhagoletis pomonella* (hawthorn-origin) to one female equivalent extract of hexane (whole body) vs. a hexane control were compared with responses to live virgin females vs. a clean-air control in the Y-tube olfactometer. Virgin females and extracts of females originating from *Rhagoletis mendax* were used in behavioural assays of male *Diachasma alloeum* from *Rhagoletis mendax* and, similarly, females originating from *Rhagoletis pomonella* were used in assays of males from *Rhagoletis pomonella*. Filter-paper treatments impregnated with hexane extracts (described above) or one live, virgin female were randomly selected and inserted individually into a 1-L flask connected via Tygon tubing (United States Plastic Corporation, Lima, OH) to one arm of the Y-tube. Control filter paper was placed into a second 1-L flask connected to the other arm of the Y-tube. Immediately after test stimuli were inserted, carbon-filtered air (100 mL min⁻¹) was delivered via Tygon tubing through each flask and into each arm of the Y-tube olfactometer. Twenty replicates of two wasps, placed into the olfactometer simultaneously, were observed per treatment. Observations were conducted for 5 min on each group of wasps assayed.

**Response of male *Diachasma alloeum* from *Rhagoletis mendax* or *Rhagoletis pomonella* to extracts from head/thorax or abdomen of females originating from *Rhagoletis mendax**

Male *Diachasma alloeum* originating from *Rhagoletis mendax* or *Rhagoletis pomonella* were presented with head plus thorax or abdominal hexane extracts (described above) of *Diachasma alloeum* females.
originating from *R. mendax* vs. hexane controls. Experimental protocols were identical to those described above.

Response of male *D. alloeum* from *R. pomonella* or *R. mendax* to extracts from head/thorax or abdomen of females originating from *R. pomonella*

Male *D. alloeum* originating from *R. pomonella* or *R. mendax* were presented with head plus thorax or abdominal hexane extracts of *D. alloeum* females originating from *R. pomonella* vs. hexane controls. Experimental protocols were identical to those described for above.

Effect of female mating status on behavioural response of males

Male *D. alloeum* originating from *R. pomonella* or *R. mendax* were presented with a live virgin female *D. alloeum*, of their respective origin, vs. similar females that had been mated 24 h prior to the assay. Paired females of each mating status were 2–3 days old. Mated females had been held in cages with males for 4 h and subsequently transferred to holding cages without males for 20 h prior to assays under the temperature and photocycle conditions described above.

Mating assay

The hypothesis tested in this experiment was that male and female *D. alloeum* emerging from the same host fruit would copulate in equal frequency to males and females from different host fruit. One male and female wasp were introduced into a 9 × 2 cm Petri dish arena under the same light and temperature conditions described above for Y-tube experiments. Introduced pairs had either emerged from *R. pomonella* or *R. mendax* or were mixed such that a male from *R. pomonella* was introduced with a female from *R. mendax* and vice versa. Wasp behaviour was observed for 4 min after introductions.

Statistical analysis

A logistic model was used to measure the probability that a particular behavioural response from wasps in the Y-tube was nonrandom among treatments. Numbers of wasps responding to treatments within the Y-tube olfactometer were analysed using the G statistic (Sokal and Rohlf, 1981) with the *PROC GENMOD* procedure in *SAS* (SAS, 2000). Data in tables are presented as proportions of the total number of wasps responding. In all cases, the significance level was *α* < 0.05.

Results

Response of male *D. alloeum* from *R. mendax* or *R. pomonella* to females or female extracts originating from their respective host

Significantly more (*P* < 0.05) *R. mendax*-origin male *D. alloeum* moved into the air stream containing the odour from one female-body equivalent extract of hexane or to a live, virgin female compared with the control (Table 1). There was no significant difference between the number of males responding to the female hexane extract vs. the number responding to the source of one live virgin female (Table 1). Male *D. alloeum* originating from *R. mendax* wing fanned in response to inserted hexane extracts and live, virgin females in 100% and 95% of the trials conducted, respectively.

The results obtained with *R. pomonella*-origin male *D. alloeum* were similar. As before, significantly more (*P* < 0.05) male wasps responded to one female-body equivalent extract of hexane or to a live, virgin female compared with the control (Table 1). In addition, the number of males responding to the female hexane extract vs. the number responding to the source of one live virgin female was not significantly different. *Diachasma alloeum* males originating from *R. pomonella* wing fanned in response to inserted hexane extracts and live, virgin females in 100% and 92% of the trials conducted, respectively.

Significantly more (*P* < 0.05) *R. mendax*-origin and *R. pomonella*-origin male *D. alloeum* responded to the odour source of one *R. mendax* female abdomen equivalent

<table>
<thead>
<tr>
<th>Extract source</th>
<th>Percentage of male <em>D. alloeum</em> from <em>R. mendax</em> responding</th>
<th>Percentage of male <em>D. alloeum</em> from <em>R. pomonella</em> responding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment</td>
<td>Control</td>
</tr>
<tr>
<td><em>D. alloeum</em> virgin female hexane extract</td>
<td>80.0 ± 4.0***</td>
<td>5.0 ± 1.0a</td>
</tr>
<tr>
<td><em>D. alloeum</em> live, virgin female</td>
<td>72.5 ± 2.0***</td>
<td>7.5 ± 4.0a</td>
</tr>
</tbody>
</table>

Pairs of means in the same column followed by the same superscript letter are not significantly different and paired values within rows for treatment and control wasps marked with an asterisk are significantly different (*P* < 0.05, *G*² test of homogeneity). (n = 40 males, 20 replicates).
Table 2. Percentage (mean ± SE) of virgin male Diachasma alloeum, originating from Rhagoletis mendax or Rhagoletis pomonella, responding to female D. alloeum body-part extracts of hexane originating from R. mendax in Y-tube olfactometer.

<table>
<thead>
<tr>
<th>Extract source</th>
<th>Percentage of male D. alloeum from R. mendax responding</th>
<th>Percentage of male D. alloeum from R. pomonella responding</th>
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<tbody>
<tr>
<td></td>
<td>Treatment</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
</tr>
<tr>
<td>D. alloeum female head + thorax from R. mendax</td>
<td>15.0 ± 1.0b</td>
<td>10.0 ± 1.0b</td>
</tr>
<tr>
<td>D. alloeum female abdomen from R. mendax</td>
<td>80.0 ± 4.0**</td>
<td>85.0 ± 3.0**</td>
</tr>
</tbody>
</table>

Pairs of means in the same column followed by the same superscript letter are not significantly different and paired values within rows for treatment and control wasps marked with an asterisk are significantly different (P < 0.05, G² test of homogeneity). (n = 40 males, 20 replicates).

effect of hexane than to extracts of head and thorax regions (Table 2). Similar numbers of male wasps responded to hexane extracts of the head and thorax from R. mendax-origin females and to the control treatment (Table 2). Male D. alloeum originating from R. mendax and R. pomonella wing fanned in response to inserted abdominal hexane extracts in 95 and 100% of the trials conducted, respectively. No wing fanning was observed in assays that compared extracts of head and thorax regions compared with the solvent control.

Response of male D. alloeum from R. pomonella or R. mendax to extracts from head/thorax or abdomen of females originating from R. pomonella

Significantly more (P < 0.05) R. pomonella-origin and R. mendax-origin male D. alloeum responded to the source of one R. pomonella female abdomen equivalent extract of hexane than to extracts of head and thorax regions (Table 3). As with females from R. mendax, hexane extracts of the head and thorax from R. pomonella-origin females did not affect more male wasps of either host origin compared with the control treatment (Table 3). Slightly more male R. pomonella-origin D. alloeum males responded to the abdominal extracts from R. pomonella-origin females compared with R. mendax-origin males, but this difference was not statistically significant (P > 0.05). As before, a high percentage of males tested from R. pomonella and R. mendax (90 and 100%, respectively) wing-fanned in response to insertion of abdominal hexane extracts of R. pomonella-origin females. No wing fanning was observed in trials that compared extracts of the head and thorax vs. the control.

Effect of female mating status on behavioural response of males

Significantly more (P < 0.05) R. pomonella-origin and R. mendax-origin male D. alloeum responded to live, virgin females, originating from their respective host species, compared with mated females of similar age (Table 4).

Mating assay

Observed copulation behaviour of D. alloeum was similar to that described by Boush and Baerwald (1967). Males typically became activated within 30 s of introduction into the Petri dish arena and approached females within 2 min; copulations persisted for 5–20 s. The frequencies of observed copulations between same host pairs (70% from R. mendax; 80% from R. pomonella; n = 10 pairs) were nearly identical to the frequencies of observed copulations for the two mixed-host pairs (70% R. mendax male-R. pomonella female; 90% R. pomonella male-R. mendax female). No differences in copulation behaviour were observed between same host vs. mixed host pairs.

Discussion

The results of the current study confirm that the female-produced sex pheromone of D. alloeum, which parasitizes

Table 3. Percentage (mean ± SE) of virgin male Diachasma alloeum, originating from Rhagoletis mendax or Rhagoletis pomonella, responding to female D. alloeum body-part extracts of hexane originating from R. pomonella in Y-tube olfactometer.

<table>
<thead>
<tr>
<th>Extract source</th>
<th>Percentage of male D. alloeum from R. pomonella responding</th>
<th>Percentage of male D. alloeum from R. mendax responding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
</tr>
<tr>
<td>D. alloeum female head + thorax from R. pomonella</td>
<td>10.0 ± 1.0b</td>
<td>10.0 ± 2.0b</td>
</tr>
<tr>
<td>D. alloeum female abdomen from R. pomonella</td>
<td>85.0 ± 5.0**</td>
<td>75.0 ± 0.0**</td>
</tr>
</tbody>
</table>

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Table 4. Percentage (mean ± SE) of virgin male Diachasma alloeum, originating from Rhagoletis mendax or Rhagoletis pomonella, responding to one live virgin, female D. alloeum or one live, mated female in Y-tube olfactometer.

<table>
<thead>
<tr>
<th></th>
<th>Percentage of male D. alloeum from R. mendax responding</th>
<th>Percentage of male D. alloeum from R. pomonella responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. alloeum virgin female</td>
<td>67.5 ± 3.0a</td>
<td>72.5 ± 4.0a</td>
</tr>
<tr>
<td>D. alloeum mated female</td>
<td>25.0 ± 2.0b</td>
<td>22.5 ± 1.0b</td>
</tr>
</tbody>
</table>

Pairs of means in the same column followed by the same superscript letter are not significantly different ($P < 0.05$, $G^2$ test of homogeneity). ($n = 40$ males, 20 replicates).

Both blueberry-infesting R. mendax and hawthorn-infesting R. pomonella, is equally ‘attractive’ to male wasps originating from both fly hosts. Males emerging from R. pomonella could potentially respond to R. mendax-origin females and vice versa, resulting in copulation and gene flow between these two populations. Recent studies have shown that both male and female D. alloeum are specifically attracted to the odour of the fruit that is parasitized by their tephritid fly host (Stelinski and Liburd, 2005). Diachasma alloeum emerging from R. mendax respond positively to the odour of blueberries but not hawthorn, whereas those emerging from R. pomonella are attracted to the odour of hawthorn but not blueberries. It has been suggested that this behavioural preference for the odour of the fruit that is infested by their larval host may be indicative of ‘host fidelity’ (Feder, 1998), where populations of D. alloeum exhibit a genetically based behavioural tendency to mate and reproduce in the vicinity of the fruit that harbours their larval host (Stelinski and Liburd, 2005). Thus, there may be at least two distinct host races of D. alloeum; one associated with blueberries and specifically attacking R. mendax and a second associated with hawthorn and specifically attacking R. pomonella. Host races are thought to be an incipient stage of species formation in sympathy whereby host-associated adaptations eventually lead to the evolution of distinct and noninterbreeding species (Feder et al., 1994). Although populations of D. alloeum exhibit a behavioural association with the specific fruit infested by their larval host, the current data strongly suggest that genetic introgression amongst populations may occur when males are attracted to females associated with a different host fly species. Furthermore, the current results indicate that males are capable of copulating with females of the opposite host. However, other pre- and postzygotic factors may also be acting to keep these populations separate. For example, Linn et al. (2005) recently demonstrated that sympatrically occurring Rhagoletis fly host races are not only attracted to their natal host volatiles, but also are antagonized by volatiles emitted by non-natal fruit of their sister race. Furthermore, postzygotic barriers can impart isolation amongst host races. For example, hybrids of Rhagoletis fly host races exhibit reduced responses to the odour of their natal host-fruit odours compared with parental lines (Linn et al., 2004). The degree of host-race isolation between R. mendax- and R. pomonella-infesting D. alloeum is yet to be determined, but Feder et al. (1994) estimated that gene flow between hawthorn- and apple-infesting host races of R. pomonella is 6% per generation.

Boush and Baerwald (1967) were unable to associate a behavioural response in male D. alloeum with a particular site of pheromone production on conspecific females in their original ethological studies of the copulation behaviour of this wasp species. The current results demonstrate that the female-produced sex pheromone originates from the abdomen. Not only did abdominal hexane extracts attract 75–85% of males compared with solvent controls, but also nearly 100% of males exposed to abdominal hexane extracts fanned their wings in response to this treatment. Wing fanning is a stereotypical initial response of male D. alloeum to the female-produced sex pheromone (Boush and Baerwald, 1967). In addition, not a single male wasp in the current investigation wing fanned in response to extracts of the head and thorax of female D. alloeum. Sex pheromones of female parasitic wasps are typically produced and secreted from abdominal glands (Weseloh, 1976; McNeil and Brodeur, 1995; Syvertsen et al., 1995; De Freitas et al., 2004). The Dufour’s gland is typically the specific site of origin. This gland is yet to be identified and described for female D. alloeum; however, it is likely to be the specific site of pheromone origin on the abdomen.

Female D. alloeum mate only once, whereas males can and do mate multiple times (unpublished data). The current results reveal that mated female D. alloeum are less attractive to males compared with virgin females, suggesting that female wasps cease producing or actively emitting pheromone after mating. These results are in contrast to a recently reported study on Cotesia flavipes, where virgin and mated females are equally attractive to conspecific males (De Freitas et al., 2004). Females also mate only once in this species; however, they appear to continue actively emitting pheromone post mating (De Freitas et al., 2004).

Diachasma alloeum can be an important biological control agent of R. mendax in unmanaged blueberry plantations not receiving broad-spectrum insecticide sprays in the U.S.A., and parasitization rates can exceed 50% (Stelinski et al., 2004). However, rates decrease to 2% in commercially managed blueberry plantations sprayed with broad-spectrum insecticides (Stelinski et al., 2004). Identification of the female-produced sex pheromone of D. alloeum could be an important first step in the development of a biorational management strategy for controlling Rhagoletis fly pests given that a synthetic pheromone could be used as a monitoring tool for detecting the presence of D. alloeum.
This may allow growers to avoid applying insecticides during peak activity of *D. alloeum* females to conserve the effectiveness of this natural enemy in suppressing *Rhagoletis* fly populations. The results of the current study confirm the presence of a common sex pheromone produced and secreted in the abdomens of *D. alloeum* infesting both *R. mendax* and *R. pomonella*.

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**References**


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